

**Liver-bone relationships: integrative
pathophysiology, diagnostic, prognostic,
preventive and therapeutic considerations**

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Doctor of Medical Science

December 2019

Faculty of Medicine, Dentistry and Health Sciences

**Submitted in total fulfilment for the degree of Doctor of
Medical Science**

Abstract

Over the last 10 years we examined complex multidirectional interactions between the digestive system and osteoporotic fractures focusing on pathophysiological and clinical issues of liver-bone relationships. These included: vitamin D, vitamin K and parathyroid hormone in chronic liver and pancreatic diseases (2 papers: [1, 2]); associations between liver function, bone-mineral biomarkers, indices of iron metabolism and adipokines (leptin, adiponectin, resistin) in orthogeriatric patients (3 papers: [3-5]); diagnostic and prognostic indicators of osteoporosis, fractures, and in-hospital outcomes (5 papers: [6-10]). A shortened overview of our main findings is presented.

Declaration

I, Leon Fisher, declare that:

- this thesis comprises my original work towards the degree of Doctor of Medical Science except where indicated in the preface
- due acknowledgement has been made in the text to all other material used; and
- the thesis is fewer than the maximum word limit in length.

Leon Fisher

Declaration

Dr Leon Fisher initiated, designed and conducted the projects, collected socio-demographic, clinical and laboratory data, created databases, performed data analyses (statistical multivariate logistic regression together with *Dr Wichat Srikusalanukul, MD, PhD*), prepared literature reviews, and drafted all manuscripts.

All the co-authors of the presented papers recognized his initiatives and contribution to the research; the proportion of his personal work was estimated as 75-85%. In some papers the order of authors follows their seniority.

The roles and intellectual input of each of the co-authors are summarized below.

Prof P Smith, Director of the Department of Orthopaedic Surgery, The Canberra Hospital, supervised projects on hip fracture and other orthopaedic patients; he was involved in discussing and editing surgical-related issues in the manuscripts.

Prof P Pavli, Director of the Department of Gastroenterology, The Canberra Hospital, supervised the project on “Perioperative acute upper gastrointestinal haemorrhage in older patients with hip fracture” and was involved in interpreting the results and editing the manuscript.

Dr M Davis, Director of the Department of Geriatric Medicine, The Canberra Hospital, supervised together with Prof P Pavli the project on “Perioperative acute upper gastrointestinal haemorrhage in older patients with hip fracture”. He organised important meetings with the junior medical staff and nurses carrying after these patients; he participated in interpretation issues related to main geriatric syndromes and final editing of the paper.

A/Prof A Fisher, Senior Consultant in Orthogeriatric Service, The Canberra Hospital, participated as a physician-geriatrician in design and conduction of the projects on elderly individuals (including consenting), results interpretation and discussion of main homeostatic mechanisms, integrative pathophysiological topics and prognostic approaches; he was also involved in the final editing.

Dr W Srikusalanukul, MD PhD, Study Coordinator (Medical) & Research Fellow^[1]_{SEP} Clinical Trials Unit, The Canberra Hospital, was involved in statistical analyses, specifically the multivariate logistic regressions.

Ms E Byrnes, Clinical Scientist, Clinical Biochemistry Research and Development PathWest Laboratory Medicine WA, Queen Elizabeth II Medical Centre, Perth, performed analyses of biochemical parameters, in particular measurements of serum vitamin D and vitamin K levels.

No part of this thesis has ever been submitted for other qualifications.

Most of the projects and related papers included in the thesis had been done within 10-year period prior to enrolment in the degree.

No third party editorial assistance was provided in preparation of any of the papers.

Contents

Page

Abstract	2
Key Findings	8
1. Introduction	9
2. Aims	10
3. Patients and Methods	
3.1. Patients	10
3.2. Laboratory Analyses	11
3.3. Statistical Analyses	13
4. Results and Comments	
4.1. Vitamin D, vitamin K and parathyroid hormone in patients with chronic liver and pancreatic diseases [1, 2]	14
Main Findings	16
Comments	16
4.2. Relationships between liver function, adipokines, parameters of bone-mineral and iron metabolism, fractures and inflammation in orthogeriatric patients	
4.2.1. Pathophysiological aspects [3-5]	19
Main Findings	20
Comments	21
4.2.2. Clinical implications: diagnostic, prognostic and therapeutic aspects [5-10]	
Main Findings	30
Comments	
4.2.2.1. Hepatic markers as indicators of the mineral-bone metabolism	32
4.2.2.2. Biomarkers of osteoporotic fractures in the context of liver function status	33
4.2.2.3. Liver-related prognostic indicators of hospital outcomes in orthogeriatric patients	36
4.2.3. Practical therapeutic considerations	42
5. Limitations and strengths	44
6. Conclusions	44

Abbreviations

AF, atrial fibrillation;
ALP, alkaline phosphatase;
ALT, alanine aminotransferase;
AUC, area under curve;
BAP, [bone specific] alkaline phosphatase, a bone formation marker;
 β CTX, β - isomerised C-terminal cross-linking telopeptide of type I collagen, a serum bone resorption marker;
BMD, bone mineral density;
BTM, bone turnover marker;
CAD, coronary artery disease;
CHF, chronic heart failure;
CKD, chronic kidney disease ≥ 3 stage (GFR < 60 ml/min/1.73m²)
CLD, chronic liver disease;
DM, diabetes mellitus;
DPD/Cr, deoxypyridinoline corrected for urinary creatinine concentration, a bone resorption marker;
ERCP, endoscopic retrograde cholangiopancreatography;
GFR, glomerular filtration rate;
GGT, gamma-glutamyltransferase;
HF, hip fracture;
HPIR, high postoperative inflammatory response;
LOS, length of hospital stay;
MI, myocardial infarction;
NLR, neutrophil to lymphocyte ratio;
NTx/Cr, cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration, a bone resorption marker;
OC, osteocalcin, an osteoblast-specific noncollagen protein, a serum bone formation marker;
OC/BAP ratio, an index of osteoblast differentiation;
OP, osteoporosis;
P1NP, amino-terminal propeptide of type 1 procollagen, a serum bone formation marker;
P1NP/OC ratio, an index of osteoblast differentiation;
P1NP/ β CTX ratio, an index reflecting the balance between bone formation and bone resorption;
PO₄, phosphate;
PTH: parathyroid hormone;
RCF, residential care facility (permanent);
RF, risk factor;
ROC, receiver operating characteristic;
TSAT, transferrin saturation;
25(OH) D, 25-hydroxy vitamin D;
SHPT, secondary hyperparathyroidism;
ucOC/cOC ratio, undercarboxylated osteocalcin to carboxylated osteocalcin, a surrogate marker for vitamin K of bone status.

Included Publications

1. Fisher L, Fisher A. Vitamin D and parathyroid hormone in outpatients with non-cholestatic chronic liver disease *Clin Gastroenterol Hepatol* 2007; 5 (4): 513-520.
2. Fisher L, Byrnes E, Fisher A. Prevalence of vitamin K and vitamin D deficiency in patients with hepatobiliary and pancreatic disorders *Nutr Res* 2009; 29(9): 676-83.
3. Fisher L, Fisher A. Associations Between Liver Function, Bone Turnover Biomarkers and Adipokines in Older Patients With Hip Fracture. *Journal of Endocrinology and Metabolism* 2014; 4(5-6):121-135.
4. Fisher L, Srikusalanukul W, Fisher A, Smith P. Liver function parameters in hip fracture patients: relations to age, adipokines, comorbidities and outcomes. *Int J Med Sci* 2015; 12(2): 100-115.
5. Fisher L, Fisher A. Relationship between Serum Gamma-Glutamyltransferase (GGT) Activity and Indices of Iron Metabolism with Bone-Mineral Parameters in Orthogeriatric Patients. *Journal of Biomedical Sciences* 2016; 5 (4): 26-37.
6. Fisher A, Srikusalanukul W, Fisher L, Smith P. The Neutrophil to Lymphocyte Ratio on Admission and Short-Term Outcomes in Orthogeriatric Patients. *Int J Med Sci* 2016; 13(8): 588-602.
7. Fisher A, Srikusalanukul W, Fisher L, Smith P. Lower serum P1NP/ β CTX ratio and hypoalbuminemia are independently associated with osteoporotic nonvertebral fractures in older adults. *Clin Interv Aging* 2017; 12:1131-1140.
8. Fisher A, Fisher L, Srikusalanukul W, Smith P. Bone Turnover Status: Classification Model and Clinical Implications. *Int J Med Sci* 2018; 15(4): 323-338.
9. Fisher A, Fisher L, Srikusalanukul W and Smith PN. Usefulness of simple biomarkers at admission as independent indicators and predictors of in-hospital mortality in older hip fracture patients. *Injury* 2018; 49(4): 829-840.
10. Fisher A, Fisher L, Srikusalanukul W and Smith PN. Prognostic Significance of serum urea concentration at admission in older patients with hip fracture. *The Open Orthopaedics Journal* 2018; 12(1):536-553.

Other publications by the candidate

1. Fisher L, Fisher A, Thomson A. Cardiopulmonary complications of ERCP in older patients *Gastrointest Endosc* 2006; 63(7): 948-955.
2. Fisher L, Fisher A, Pavli P, Davis M. Perioperative acute upper gastrointestinal haemorrhage in older patients with hip fracture: incidence, risk factors and prevention *Aliment Pharmacol Ther* 2007; 25 (3): 297-308.
3. Fisher L, Fisher A. Acid-suppressive therapy and risk of infections: pros and cons. *Clin Drug Investig*. 2017; 37(7): 587-624.
4. Fisher L, Segarajasingam D, Stewart C, Deboer W, Yusoff I. Endoscopic ultrasound guided fine needle aspiration of solid pancreatic lesions: performance and outcomes *J Gastroenterol Hepatol* 2008; 24 (1): 90-96.
5. Fisher M, Fisher L, Waxman B, Fisher A. Changing trends in colorectal cancer: possible cause and clinical implications *Health* 2010; 2(8): 842-848.

Key findings

- Inadequate vitamin D and/or vitamin K status is common in CLD (noncholestatic and cholestatic), and correlates with the severity of the disease, indicating the need for appropriate supplementation. Liver dysfunction is predictive for vitamin K deficiency of bone, and cholestasis is associated with depressed OC production.
- In older HF patients, liver functions (within normal range in the vast majority) are associated with indices of bone metabolism, comorbidities and outcomes. The complex liver-bone-adipokine interactions include bidirectional links between GGT and markers of bone formation (OC, BAP), differentiation (OC/BAP ratio) and resorption (NTx/Cr), as well as between ALT and OC. Adiponectin (but not leptin or resistin) is an independent contributor to GGT, whereas leptin is a determinant of OC and bone resorption markers; these relationships are modulated by nutritional status and gender. GGT > 20 U/L is associated with a two times higher prevalence of low OC levels and 2.7 times higher prevalence of low OC/BAP ratio; it may be used as a marker of impaired bone metabolism. Serum GGT (>30 IU/L) and albumin (<33g/L) levels on admission may be helpful in predicting prolonged hospital stay and in-hospital death, respectively.
- Multidirectional links between serum GGT activity, indices of iron and bone metabolism have been identified, and the role of GGT and iron homeostasis in maintaining bone health and developing osteoporotic fractures presented/described.
- In orthogeriatric patients, both serum P1NP/βCTX ratio and albumin levels demonstrate an inverse dose–effect relationship with the prevalence of nonvertebral fractures and independently indicate fracture presence with acceptable discriminatory power. Lower P1NP/βCTX (<100) and hypoalbuminemia (<33 g/L) could be useful simple additive prognostic tools for fracture risk stratification and in selection of the most suitable type of antiosteoporotic treatment in the elderly; high NLR on admission is also an independent indicator of fracture presence.
- The following laboratory variables were found as independent predictors of poor hospital outcomes:

for ***in-hospital mortality***: albumin<33 g/L, GGT>30U/L, GGT/ALT ratio >2.5, NLR≥5.1, urea>7.5mmol/L, vitamin D deficiency and hyperparathyroidism;

for ***prolonged LOS***: GGT>30U/L, albumin<33g/L and adiponectin >17.14 ng/ml;

for ***myocardial injury*** (identified by cTnI rise): albumin<33g/L and NLR≥5.1;

for ***inflammatory complications*** (with high CRP levels: >100 mg/L): albumin<33g/L, NLR≥5.1 and urea>7.5mmol/L;

for ***being discharged to a RCF***: NLR≥5.1 and vitamin D deficiency.

These easily identifiable at admission characteristics can be used for risk stratification and individualized management. The predictive performance of these markers increases significantly when used in combination with other laboratory or clinical prognostic indicators.

1. Introduction

In recent years, the integrative physiology paradigm (a whole organism approach) has gained popularity. Accumulating evidence suggests that in health and disease liver and gut functions and bone metabolism are closely related with each other. The liver and gut produce a large number of biologically active molecules that exert substantial direct and indirect systemic effects, coordinate key metabolic pathways including those that are involved in bone homeostasis.

On the other hand, increasing evidence suggests that the skeleton, in addition to its structural role, is an endocrine organ which cooperates with different systems and organs, including the digestive system, and its hormonal functions [“osteokines”] play a key role in a number of homeostatic metabolic processes and regulation of energy balance.¹⁻⁴

Furthermore, both the digestive system and bones share multiple common physiological factors of the neuro-endocrine regulation, as well as risk factors for diseases.

The importance of liver-bone interactions under both normal and disease conditions is supported by a growing number of evidence. 1) Liver plays a fundamental role in metabolism of vitamins D and K, parathyroid hormone (PTH) and minerals, essential regulators of bone homeostasis ([3]:1-5). 2) Osteopenia/osteoporosis (hepatic osteodystrophy) is present in 20-50% of patients with chronic liver disease ([3]:6-10). 3) Osteocalcin (OC), an osteoblast-derived hormone, is recognized as a critical determinant of energy and glucose homeostasis ([3]:11-16). 4) Dysregulation and dysfunction of adipokines, adipose tissue-derived hormones, in particular, adiponectin, leptin and resistin, which have receptors expressed in both hepatocytes and bone cells and control a vast diversity of physiological functions, are involved in initiation and progression of many diseases including liver and bone (osteoporosis) disorders ([3]:17-23]. 5) Iron homeostasis, which is regulated by the liver, is recognized as an important determinant of bone metabolism ([5]: 8-10)

However, the mechanisms of the liver-bone links remain incompletely understood, and the concept of bi- (multi-) directional links still has a very limited effect on the real-world clinical practice. To date, despite the intense research carried out in recent years, neither clinical, nor the animal models provide a uniform explanation of the liver-bone link(s).

Although both liver and skeletal diseases are prevalent, interrelated (share common risk and pathophysiological factors) and associated with significant morbidity and mortality, a large number of patients still remains without adequate prevention and treatment, especially in regard to undiagnosed and undertreated osteoporosis. Osteoporotic fracture is well known as a frequent complication in patients with chronic liver diseases,⁵ but the complex interplay between hepatic functions, vitamin

D, PTH, vitamin K status, circulating adipokine levels, iron and bone metabolism in subjects without overt liver diseases has not been investigated.

We hypothesise that an integrative approach to the mechanisms of the liver-bone links may help better understand the pathogenesis of the related diseases, develop new diagnostic and prognostic biomarkers and improve preventive and treatment therapies. In other words, we hoped that further insights and knowledge about main molecular basis of the abovementioned links, and new biomarkers capable of patient stratification and prognosis will offer important changes in clinical management and the possibility of improved outcomes in patients with CLD and/or OP.

2. Aims

To review our studies on selected aspects of complex multidirectional liver-bone relationships (1) in patients with non-cholestatic and cholestatic CLD focussing on vitamin D, vitamin K and parathyroid hormone status and (2) in older orthopaedic patients without overt liver diseases focusing on links between liver functions, adipokines, indices of bone-mineral and iron metabolism, highlighting their pathophysiological and clinical (diagnostic, prognostic, preventive and therapeutic) importance.

3. Patients and Methods

3.1. Patients

In total 4036 patients have been included in our studies. The following is a short description of patients and methods used; our original referred study is marked by square brackets [] in bold.

[1]. One hundred consecutive patients (63 men and 37 women, mean age 49.0 ± 12.1 (SD) years) attending the outpatient clinic of the Gastroenterology Department at Canberra Hospital in whom there was a confirmed diagnosis of noncholestatic CLD. The cause of CLD was alcohol use ($n = 40$), viral hepatitis C ($n = 38$), viral hepatitis B ($n = 12$), autoimmune hepatitis ($n = 4$), hemochromatosis ($n = 4$), and nonalcoholic steatohepatitis ($n = 2$). None of the patients received vitamin D or calcium supplements, bisphosphonates, calcitonin, or hormone replacement therapy.

[2]. The study population comprised 90 consecutive consenting patients (45 men and 45 women, mean age of 65.5 ± 17.7 (SD) years) who underwent ERCP. There were 68 patients with choledocholithiasis, 8 with cholangiopancreatic malignancies (cholangiocarcinoma, 3; pancreatic cancer, 3; ampullary cancer, 1; and metastatic squamous cell carcinoma, 1), 6 with benign biliary strictures, 3 with chronic pancreatitis, 2 with pancreas divisum, 2 with bile leak, and 1 with choledochal cyst. Thirty five (38.9%) patients were postcholecystectomy. The diagnosis was based on consistent clinical, laboratory, imaging, and histologic findings.

[3] and [4]. Prospective observational study included 294 consecutive older (≥ 60 years of age) patients (212 women and 82 men, mean age 82.0 ± 7.9 (SD) years) with low-trauma osteoporotic HF.

[5] and [6]. Cross-sectional study of 493 consecutive older patients (>60 years) admitted to the orthopaedic unit. After excluding patients with high-trauma fracture,

acute or known chronic hepatobiliary diseases, iron metabolism-related diseases (hemochromatosis, thalassemia), acute infection, primary hyperparathyroidism, and Paget's disease, or who lacked adequate laboratory data, 416 patients (282 women, 134 men, mean age 78.9 ± 8.7 (SD) years) were evaluated: with a non-vertebral fracture (n=256), including with hip fracture (HF, n=168), and without a fracture (n=160).

[7] and [8]. In 1,239 orthogeriatric patients (69.1% women, mean age 78.1 ± 9.52 [SD] years), including 854 (68.9%) with osteoporotic non-vertebral fractures, 455 (36.7%) with hip fracture [HF]) and 385 (31.1%) without fractures, markers of bone formation (P1NP, OC), and bone resorption (β CTX), indices of mineral metabolism, and parameters of liver and renal functions were assessed; data on clinical and laboratory characteristics were collected prospectively.

[9] and [10]. In 1,820 consecutive patients with low-trauma osteoporotic HF aged >60 years (76.4% women; 65% community-dwelling; mean age 82.8 ± 8.1 [SD] years) 35 laboratory variables along with 22 clinical and socio-demographic characteristics at admission were analyzed. The validation cohort included data on 455 older (≥ 60 years of age) HF patients (mean age 82.1 ± 8.0 years, 72.1% women).

In all patients a complete medical history, including current drug regimen, daily alcohol intake and smoking history, was obtained; physical examination was performed; anthropometric measurements were taken; and body mass index (BMI; weight in kilograms/height in meters squared) was calculated. The diagnosis was based on consistent clinical, laboratory, imaging, and histologic (were appropriate) findings.

All studies were conducted according to the standards of the Declaration of Helsinki and were approved by the local Health Human Research Ethical Committees. Informed consent from each patient or carer was obtained.

3.2. Laboratory Analyses

Samples of venous blood were obtained in the morning after an overnight fast and each sample [where appropriate] was divided into 2 portions. In one portion, hematologic and biochemical parameters of liver and kidney function, mineral, bone and iron metabolism, fasting blood glucose (and HbA1C in diabetic patients), lipase, and thyroid function tests (TSH, T4) were determined. All routine hematologic and biochemical assessments were performed by standardized methods.

In the other blood portion, sera were separated by centrifugation at 4°C, frozen, and stored at -80°C until analysis for osteocalcin (OC), cOC and undercarboxylated OC (ucOC), three adipokines (adiponectin, leptin and resistin).

Biochemical indicators of liver function

The following variables were measured as the blood samples were collected: alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), bilirubin, albumin and prothrombin time. These markers were evaluated by using commercially available standard enzymatic reagents and diagnostic kits by spectrophotometry on the biochemical autoanalyzer Abbott Architect CI16200 (Abbott Laboratories, IL, USA). ALT, GGT and ALP were measured with enzymatic

methods, total bilirubin was analysed using diazonium salt, albumin was measured using bromocresol green, and total protein was tested by a Biuret method. The mean inter-assay and intra-assay coefficients of variations (CVs) for these tests were within 1.1-6.6%. For liver enzymes two times the upper normal limit (UNL), cut-off levels were used to define abnormal tests. The international normalized ratio (INR) for prothrombin time was calculated.

Vitamin K status assessment

Both ucOC and cOC were measured with recently developed enzyme immunoassays, which use specific monoclonal antibodies highly reactive to each type (glutamyl or glutamate) of OC (Takara Biomedical Inc, Tokyo, Japan). According to the manufacture, the intraassay and interassay coefficients of variations (CVs) for ucOC were 4.4% to 6.7% and 5.7% to 9.9%, respectively, and for cOC, they were 3.0% to 4.8% and 0.7% to 2.4%, respectively. In our laboratory, all CVs were less than 10%. Based on the manufacturer's instructions, the suggested reference range for ucOC is 0.1 to 2.37 ng/mL, and for cOC, it is 1.47 to 10.9 ng/mL. Of note, serum vitamin K concentrations fluctuate with recent dietary vitamin K intake and, therefore, are not reliable markers of tissue vitamin K status. Because bones have a high susceptibility to vitamin K deficiency, measurement of circulating ucOC levels and especially the ratio between ucOC and cOC (ucOC/cOC) are used widely.

Markers related to mineral and bone metabolism

These included serum concentrations of 25(OH)D, intact PTH, total calcium, phosphate, magnesium, and markers of bone turnover - osteocalcin (OC), amino-terminal propeptide of type 1 procollagen (PINP) and bone-specific alkaline phosphatase (BAP) as markers of bone formation and serum β -isomerised C-terminal cross-linking telopeptide of type I collagen (β CTX) levels or urinary concentrations of deoxypyridinoline (DPD/Cr), and cross-linked N-telopeptide of type 1 collagen (NTx/Cr) as markers of bone resorption (both corrected for urinary creatinine concentrations in the same sample). The serum calcium level was corrected for albumin concentration. Glomerular filtration rate (GFR) was calculated using a standardized serum creatinine based formula normalized to a body surface area of 1.73 m². Chronic kidney disease (CKD) was defined as GFR < 60 mL/min/1.73 m² (CKD stage $\geq$ 3).

Serum 25(OH)D was measured by radioimmunoassay kit (Dia Sorin, Stillwater, MN, USA) and intact PTH by two-site chemiluminescent enzyme-linked immunoassay on DPC Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA, USA). Serum OC was determined by electrochemiluminescent immunoassay (Elecsys 1010, Roche Diagnostics, Ltd Corp., IN, USA), serum BAP by Metra BAP ELISA (Quidel Corp., San Diego, CA, USA), urinary NTx by enzyme-linked immunosorbent assay (ELISA) (Wampole Labs, Princeton, NJ, USA), and urinary DPD by two-site chemiluminescent enzyme-labelled immunoassay (DPC Immulite 2000, Diagnostic Products, Los Angeles, CA, USA). All samples were analysed with commercially available kits of the same lot number according to the manufacturer's protocol and blind to any clinical information. In these methods both the intra- and inter-assay CVs ranged from 2.1% to 12.7%. On the basis of data reported in the literature, the serum 25(OH)D concentration was defined as deficient when it was less than 50 nmol/L (severe deficiency, <12.5 nmol/L; moderate deficiency, 12.5–25 nmol/L; mild deficiency, 25–49 nmol/L), insufficient when it was 50–80 nmol/L, and sufficient

(adequate, desirable, normal) when it exceeded 80 nmol/L; secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (> 6.8 pmol/L, the upper limit of the laboratory reference range). For levels of bone turnover markers, we used the standard laboratory reference ranges and data provided by the manufacturer.

Parameters of iron metabolism

Serum iron was assessed by direct colorimetric determination, serum ferritin concentration was measured by a two-step chemiluminiscent microparticle immunoassay and transferrin was measured using an immunoturbidimetric procedure. The mean inter-assay and intra-assay CV for these tests were between 1.6% and 3.7%. Transferrin saturation (TSAT) was calculated using the IFCC protein standards (1 mg of transferrin carries 1.49 µg of iron) as follows: TSAT (%) = iron (µmol/L) × 3.8 / transferrin (g/L); reference range 18-46%.

Measurements of adipokines

Serum levels of leptin were determined by ELISA method (Diagnostic System Laboratories, Webster, TX, USA), and total adiponectin and resistin by human ELISA kits (B-Bridge International, Mountain View, CA, USA). All assays were performed according to the manufactures' instructions with kits of the same lot number. Intra- and inter-assay CVs were less than 7% for these three tests. Malnutrition was defined as serum leptin concentration < 4 ng/mL in males and < 6.5 ng/mL in females ([3]:26).

3.3. Statistical Analyses

Data were presented as mean values and SDs. For differences between groups, significance was assessed using an unpaired 2-tailed Student *t* test for continuous variables and the Pearson χ^2 test with the Fisher exact test for categorical variables.

The relations between variables were also analysed by Pearson's correlation coefficient with log-transformed data (to achieve normal distribution); Bonferroni and Sidak adjustments for multiplicity have been performed. Univariate and multivariate linear regression analyses were performed to determine the associations between liver markers and different parameters related to mineral, bone and iron metabolism and studied adipokines. All potential confounding variables (demographic, clinical and biochemical) with statistical significance ≤ 0.10 on univariate analyses were included in multivariate analyses. Manual backwards and forwards stepwise logistic regressions were performed to determine the best-fitting final model. Goodness of fit was determined by the likelihood ratio χ^2 statistic and by the pseudo- R^2 . The appropriateness of the regression models were assessed by Jack-knife residuals, Cook's D, and Mallow's Cp. The significance of multicollinearity phenomena in regression analyses was evaluated by the variance inflation factor.

The predictive abilities of the laboratory and clinical parameters of interest were evaluated by the receiver operating characteristic (ROC) analyses and the accuracy was expressed as area under curve (AUC); the Hosmer-Lemeshow goodness of fit test was used to assess model performance. Data were presented with 95% confidence intervals (CI). The cut-off values for the prediction of risk were calculated based on ROC analyses (Youden Index).

In all statistical analyses, two-sided $P < 0.05$ values were considered statistically significant. Statistical analyses were performed with a statistical software package (Stata versions 7 or 10; Stata Press, College Station, TX).

4. Results and Comments

[For full data please find attached our full-length articles (with references) in the “Contents” order].

4.1. Vitamin D, vitamin K and parathyroid hormone in patients with chronic liver and pancreatic diseases [1, 2]

Fat-soluble vitamins D and K are important physiologic factors in liver, bone, and vascular metabolism and are powerful regulators of cell proliferation and differentiation. The liver is a major organ in the vitamin D endocrine system. To function physiologically vitamin D must first be converted in the liver to 25-hydroxyvitamin D [25(OH)D], the main circulating form of vitamin D, and this in turn is converted in the kidney (and other tissues) into 1,25-dihydroxyvitamin D, the active metabolite. Liver cells along with parathyroid glands and kidneys express a calcium-sensing receptor that plays a critical role in regulating systemic calcium homeostasis. The normal liver is a target organ for the vitamin D endocrine system and parathyroid hormone (PTH).

In higher organisms, vitamin K is an essential cofactor for γ -glutamyl carboxylase, an enzyme that catalyses the posttranslational conversion of glutamyl residues into γ -carboxyglutamate residues in target proteins. In the liver, vitamin K is responsible for the synthesis of functional forms (e.g., carboxylation) of procoagulant factors II, VII, IX, and X as well as anticoagulant proteins C, S, and Z. In bone metabolism, the hydroxyapatite binding capacity of osteocalcin (OC), the main noncollagen protein in human bone synthesized mainly by osteoblasts, is dependent on the degree of vitamin K-mediated carboxylation. In case of vitamin K inadequacy, undercarboxylated OC (ucOC) is produced. Vitamin K deficiency of the bone can exist in the absence of vitamin K deficiency of the liver. Although the full complexity of liver-bone (patho) physiology is beyond our scope, a simplified paradigm is useful to understanding the relationship between multiple components involved [Figure 1].

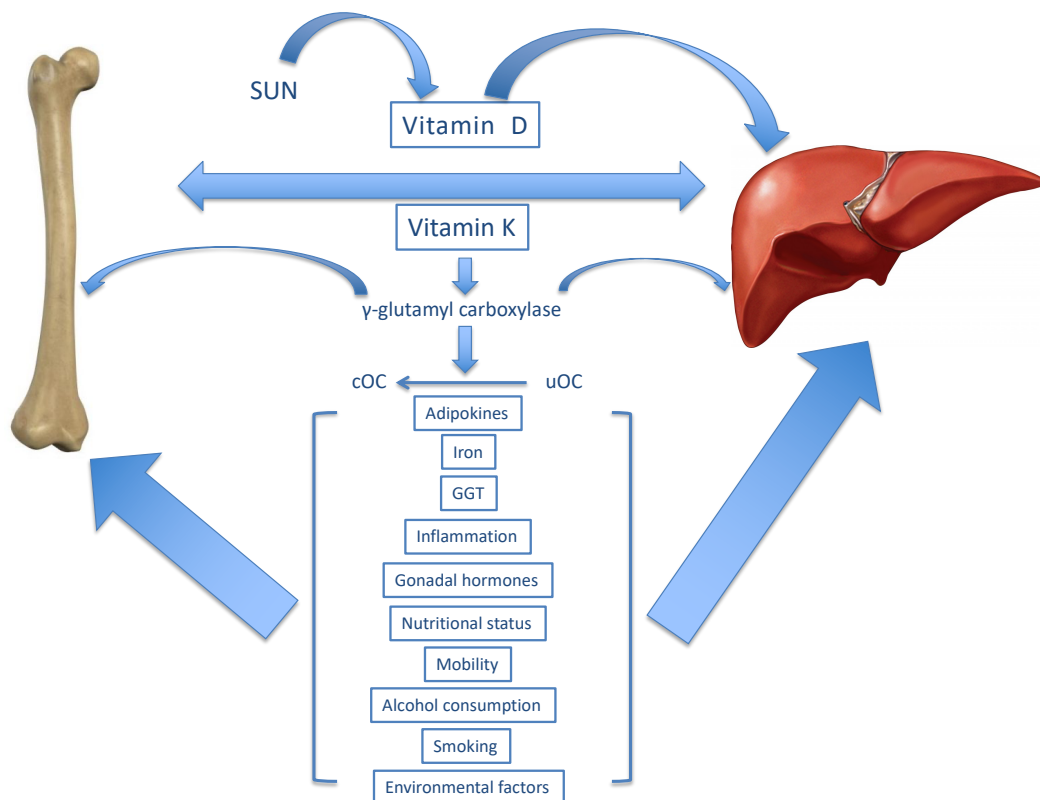


Figure 1. Schematic representation of the main factors contributing to the liver-bone relationship.

ucOC, undercarboxylated osteocalcin; cOC, carboxylated osteocalcin; GGT, gamma-glutamyltransferase.

Despite accumulating evidence that vitamins D and K have a number of actions that may be relevant to liver function and CLD, currently the evaluation and correction of vitamin D and vitamin K status is not part of the routine management of these patients. Moreover, patients with CLD and cirrhosis are known to develop OP⁶⁻¹⁰ and to have an increased risk of falling¹¹ and fractures. However, the clinical relevance of disturbances in vitamin D–PTH–calcium axis and vitamin K status in hepatic osteodystrophy is still unclear, and the need for vitamin D and vitamin K supplementation in adults with hepatobiliary and pancreatic disorders has not been fully established.

Therefore, we investigated vitamin D, vitamin K and PTH status in two groups of ambulant patients: (1) with non-cholestatic CLD and (2) with biliary and pancreatic disorders undergoing endoscopic retrograde cholangiopancreatography (ERCP). Our aims were to determine the prevalence, extent, and type of disturbances in calcium–vitamin D–PTH and vitamin K status in relation to the severity of disease and liver function injury. To our knowledge, at the time of publication of our papers these were the first studies to determine the prevalence and severity of vitamin K deficiency of bone, vitamin D, and PTH abnormalities in ambulatory patients with non-cholestatic chronic liver disease (CLD) and with cholestatic disease undergoing ERCP, and to identify those who may require nutritional supplementation.

Main Findings

[1]. In 91 of 100 ambulatory patients (63 men, 37 women; mean age 49.0 ± 12.1 [SD] years) with non-cholestatic chronic liver disease (CLD) serum 25(OH)D levels were inadequate: vitamin D deficiency (<50 nmol/L) was found in 68 patients and vitamin D insufficiency ($50\text{--}80$ nmol/L) was found in 23 patients, and secondary hyperparathyroidism (serum PTH >6.8 pmol/L) was present in 16 patients. The prevalence of vitamin D deficiency was significantly higher in cirrhotic vs. noncirrhotic patients (86.3% vs 49.0% ; $P < 0.0001$). In Child–Pugh class C patients, 25(OH)D levels were significantly lower than in class A patients (22.7 ± 10.0 nmol/L vs 45.8 ± 16.8 nmol/L; $P < 0.001$). Serum 25(OH)D independently correlated with international normalized ratio (INR, negatively; $P < 0.018$) and serum albumin (positively; $P < 0.007$). Serum 25(OH)D levels of less than 25 nmol/L predicted coagulopathy, hyperbilirubinaemia, hypoalbuminaemia, increased alkaline phosphatase, and anaemia and thrombocytopaenia. [2]. In 90 consecutive patients (45 females, mean age 65.5 ± 17.7 [SD] years) undergoing endoscopic retrograde cholangiopancreatography (68 with choledocholithiasis, 14 with other benign condition, and 8 with cholangiopancreatic cancers) vitamin D insufficiency/deficiency (25(OH)D <50 nmol/L) was found in 45.6% and elevated PTH levels (<6.8 pmol/L) in 27.8%. The ratio ucOC/cOC (index of vitamin K deficiency) was above 20% in 50.6% of patients, above 30% in 31%, and above 50% in 18.4%. Hyperbilirubinemia was a significant independent predictor of low cOC (odds ratio [OR], 11.6; 95% confidence interval [CI], 1.9-59.4; $P = 0.07$). The ratio ucOC/cOC positively correlated with alanine aminotransferase levels ($r = 0.410$; $P < 0.001$). Elevated γ -glutamyltransferase (>180 U/L) and international normalized ratio (>1.1) levels were significant independent predictors of ucOC/cOC greater than 30% after adjustment for other covariant (OR, 5.5; 95% CI, 1.2-25.2; $P = 0.027$, and OR, 3.1; 95% CI, 1.1-8.8; $P = 0.036$, respectively). The study demonstrates that vitamin K and vitamin D deficiencies are common in patients undergoing endoscopic retrograde cholangiopancreatography. Liver dysfunction is associated with and predictive of vitamin K deficiency of bone and decreased production of osteocalcin, indicating the need for appropriate supplementation.

Comments

Serum 25(OH)D. Our data indicated the significance of low vitamin D status both as a common complication of and a contributing factor to the pathogenesis of CLD. The strong relationship between both the prevalence and degree of vitamin D insufficiency and the severity of CLD, especially Child–Pugh class, may indicate specific impairment of vitamin D metabolism in the liver.

Our findings are consistent with many, but not all^{12, 13} studies published after ours and reporting high prevalence of hypovitaminosis D among patients with severe CLD.¹⁴⁻¹⁸ These observations are in line with wide presence of vitamin D receptors in the liver, the inverse correlation between vitamin D status and the severity of inflammation in patients with viral or metabolic hepatitis,¹⁹ and inhibition of stellate cell activation by vitamin D receptor ligands.²⁰

On the other hand, it is worth noting that reduced vitamin D hydroxylation in the liver could not be considered as the only or universal mechanism of low serum 25(OH)D levels in CLD. Other possible factors contributing to vitamin D insufficiency in CLD

include: (1) reduced exposure to sunlight (patients with CLD possibly spend less time outdoors), (2) dietary insufficiency (particularly in alcohol-related CLD), (3) malabsorption, (4) low levels of serum proteins that bind with vitamin D, (5) GC gene polymorphism affecting vitamin D production, (6) vitamin D receptor gene polymorphism, (7) effects of medications (antiviral drugs, glucocorticoids, drugs affecting hepatic cytochrome P450 enzymes involved in vitamin D metabolism, (8) impaired cutaneous synthesis of vitamin D in jaundiced patients, and (9) reduced physical activity and altered body composition.

There are a number of ways in which vitamin D and its derivatives, potent regulators of cell proliferation, differentiation, and immunomodulation, may influence hepatic injury, fibrosis, and tissue remodelling. These effects include inhibition of certain matrix metalloproteinases (MMPs) and induction of their inhibitors ([1]:30), suppression of proliferation of fibroblasts, and increased collagen production ([1]:29). Vitamin D insufficiency is associated with increased circulating MMP-2 and -9, which is correctable by supplementation ([1]:51). Hepatocytes produce the major MMPs and tissue inhibitors involved in liver extracellular matrix remodelling ([1]:52). MMP-2 and -9 are of particular relevance to the liver because they are critically involved in the degradation of components of the basement membrane such as collagen IV and fibronectin ([1]: 2), main components of the space of Disse. Inhibition of MMPs protects from hepatic ischemic injury ([1]:53, 54). Vitamin D deficiency also increases production of proinflammatory cytokines and acute-phase proteins. Therefore, low serum 25(OH)D may, at least in part, contribute to the progression of liver disturbance in CLD. Secondary hyperparathyroidism also significantly promotes acute-phase responses. It is possible that PTH elevation observed in a proportion of our patients was also among factors that determined the severity of the inflammatory and malignant processes.

PTH status. The cause of normal to low PTH levels in 52% of patients with vitamin D insufficiency observed in our and in other studies ([1]:23–25) is unclear. In our study the absence of compensatory increases in PTH level cannot be explained either by the use of antiviral drugs, glucocorticoids, spironolactone, or by disturbances in calcium, phosphate, and magnesium levels. Possible explanations may include vitamin D–receptor gene polymorphism, which is associated with hypoparathyroidism in chronic renal failure ([1]:58) and suppression of PTH secretion by L-amino acids that activate calcium-sensing receptor ([1]:59). Pathophysiologic mechanisms contributing to the vitamin D–PTH paradox in CLD require further study.

Vitamin K. We used ucOC/cOC ratio as a surrogate marker for vitamin K of bone status. The rationale behind this approach is that this ratio (an estimate of the degree of carboxylation of circulating OC) is independent of bone turnover and, therefore, is a more reliable marker of tissue stores of vitamin K than serum ucOC, which was often used as an indicator of vitamin status of the bone.

The possible causes of vitamin K deficiency in patients with hepatopancreatobiliary disease, in addition to poor dietary intake, include decreased vitamin K absorption from intestine, disturbance of vitamin K cycle, and the decrease in vitamin K storage.

In this study, there was no association found between inadequate vitamin K status and diagnosis, age, or alcohol consumption; male sex demonstrated a protective effect; and ex-smokers had a lower mean ucOC/cOC ratio than did nonsmokers. These data are consistent with studies that have shown no relationship between specific diagnosis

and osteodystrophy in CLD ([2]:15), between plasma phylloquinone concentrations and alcohol consumption in the general population ([2]:9), and higher plasma phylloquinone levels in men than in women ([2]:30). There are reports of age related increase in vitamin K requirements, especially in postmenopausal women ([2]:27), and the majority of our patients were in the fifth to seventh decades of life (mean age was 65 years). Patients who quit smoking possibly increased the dietary intake of vitamin K as a result of changed lifestyle.

Liver-bone links. The accelerated development of osteoporosis (OP) and fractures in CLD is influenced by abnormal bone metabolism and mineral ion homeostasis due to vitamin D and vitamin K imbalance, two multifunctional molecules which play critical roles in bone turnover.²¹⁻²⁴ In our study, hyperbilirubinemia and vitamin K inadequacy (ucOC/cOC >30%) were the only 2 variables associated independently with low serum cOC concentrations, and the latter could be predicted from these variables with an accuracy of more than 81%. These data suggest that cholestasis, along with vitamin K deficiency, may be a major pathophysiologic factor affecting OC production, an informative marker of osteoblast activity.

Low OC production, high ucOC/cOC ratio, and vitamin D insufficiency, all three are recognized markers of altered bone homeostasis and independent risk factors for fracture. It should also be noted that skeleton through secretion of OC exerts a profound and complex influence on glucose and fat metabolism ([2]:42). Our results highlight the relevance of vitamin D and vitamin K insufficiencies in CLD, support screening and surveillance of skeletal system regarding OP in patients with CLD, pointing to the need of prophylactic measures and treatment.

Practical recommendations. In individual CLD patients, inadequacy in vitamin D, vitamin K and PTH status may be determined by different pathogenic factors. However, irrespective of factors involved in the development of these abnormalities, which are likely to be multifactorial, they should not be ignored. The high prevalence of poor vitamin D and vitamin K status, two factors with pleiotropic actions and of vital importance for liver, bone, and vascular health, indicate that corrective strategies should be considered as a part of “standard of care” in patients with CLD, including those undergoing ERCP. Unfortunately, this is often ignored by practising physicians. Correction of vitamin D and vitamin K insufficiencies may represent important therapeutic targets in anti-cirrhotic and anti-osteoporotic strategies for CLD. On practical ground, in CLD an evaluation of the 25(OH)D serum level as well as the ucOC/cOC ratio is necessary and oral vitamin D and / or vitamin K should be administered were appropriate to maintain a 25(OH)D level of >50 nmol/L and the ucOC/cOC ratio <20%. However, it should be recognised that the literature data on the effects of vitamin D and vitamin K supplementation, especially on bone health, are still controversial,^{13, 24, 25} and large well controlled intervention studies in CLD patients with inadequate vitamin D and/or vitamin K status are needed to find whether supplementation with these vitamins will reduce the decrease in liver function and prevent osteoporotic fractures over time. Nevertheless, our findings highlight the importance of clinicians to keep a high index of suspicion in identification of patients with vitamin D and/or vitamin K insufficiency among individuals with both CLD and OP.

4.2. Relationships between liver function, adipokines, parameters of bone-mineral and iron metabolism, fractures and inflammation in orthogeriatric patients

4.2.1. Pathophysiological aspects [3-5]

Although liver is critically involved in metabolism of many factors contributing to bone health, and hepatic osteodystrophy is a common well-documented complication in patients with CLD ([4]:51-58), surprisingly limited research has examined the liver function in older orthopaedic patients including those with HF ([4]:59, 60). The potential pathogenic role of factors affecting both liver and bone, such as low vitamin D status, elevated PTH levels, dysregulation in iron metabolism and secretion of adipokines (especially, adiponectin, leptin and resistin), conditions all of which are common in the elderly, virtually have not been investigated systematically in orthogeriatric patients, despite growing evidence that the liver, the bone and adipose tissue are functionally interrelated organs ([4]:70). The majority of research on adipokine-liver-bone interactions has focused primarily on obesity-related metabolic syndrome, diabetes mellitus (DM) and non-alcoholic fatty liver disease (NAFLD). There has been limited evaluation (with considerable controversy in the reports) of liver function, bone metabolism and adipokines in patients with HF, the most devastating consequence of osteoporosis. There are no data concerning the association of serum hepatic biomarkers with indices of mineral and bone metabolism and the mechanisms involved, nor are there any data showing whether liver function indicators predict clinically important markers of bone turnover in orthogeriatric patients.

Recently in osteology there has been considerably increased interest in gamma-glutamyltransferase (GGT; E.C.2.3.2.2), the heterodimeric glycosylated protein embedded into outer surface of the plasma membrane of numerous cells and strongly associated with many major non-skeletal diseases ([5]:1). It has been reported that GGT-deficient (GGT^{-/-}) mice exhibit severe osteoporosis caused by decreased osteoblast activity and increased osteoclast number and activity ([5]:2). GGT induces osteoclast formation in vitro ([5]:3), accelerates bone resorption causing osteopenia/osteoporosis in transgenic mice ([5]:4,5), and increases in urinary excretion of GGT correlate with urinary bone resorption markers in osteoprotegerin (OPG)-deficient osteoporotic mice as well as in postmenopausal osteoporotic women ([5]:6). In older Korean men (>50 years), higher serum GGT levels were associated with incident osteoporotic fractures over a mean 3-year follow-up period ([5]:7).

Iron, an essential trace element in all three kingdoms of living organisms, is recognized as an important determinant of bone metabolism ([5]:8-10). However, there is disagreement as to whether iron overload or iron deficiency lead to osteopenia/osteoporosis and whether the iron-related bone loss is due to reduced bone formation or to increased resorption of bone.

Studies in humans without haematological disorders on relationship between markers of iron metabolism and bone status are scarce and controversial. A significant association between higher serum ferritin level and lower bone mineral density (BMD) was reported in healthy Koreans aged >40 years ([5]:11), especially in women ≥45 years of age ([5]:12), in postmenopausal Chinese women with fragility fractures ([5]:13), and in astronauts during space flights ([5]:14), whereas in other studies, increased serum ferritin levels were inversely correlated with BMD (of lumbar spine)

only in premenopausal women, but not in postmenopausal women (either at the femur or lumbar spine) ([5]:15). No significant association between ferritin levels and osteoporosis has been observed in Turkish postmenopausal women ([5]:16). Italian postmenopausal women with osteoporotic fractures were found to have higher transferrin levels and lower ferritin levels than controls ([5]:17) and a positive association between serum ferritin and BMD (in both femoral neck and lumbar spine). A positive ferritin-BMD correlation was also observed in adolescent girls ([5]:18) and in elderly Korean men (>65 years) ([5]:19). Furthermore, numerous nutritional studies suggested that iron deficiency, rather than excess, may lead to osteoporosis ([5]:10).

Both GGT ([5]:20) and iron ([5]:21] are implicated in generating oxidative stress, which plays a significant role in bone pathophysiology ([5]:22, 23), but these relationships remain unclear. Despite accumulating data suggesting importance of both GGT and iron in bone health, and the central role of liver in iron storage and regulation, no analysis of the potential links between serum GGT activity, markers of iron and bone-mineral metabolism in humans has been reported to date.

Moreover, the aforementioned metabolic mechanisms are important for optimal function of many organs and tissues throughout the body and involved in numerous age-related comorbidities which may substantially contribute to falls and fractures as well as to poor outcomes in orthogeriatric patients.

To our knowledge, no published study has evaluated simultaneously the relationship between liver function parameters and age, comorbidities, adipokines, indices of bone and iron metabolism, vitamin D and PTH status, as well as their influence on short-term outcomes in HF patients.

The aim of our prospective observational studies in elderly patients with and without osteoporotic bone fractures and in the absence of overt liver diseases [3-5] was three-fold: 1) to determine associations of serum liver markers with age and comorbidities, 2) to evaluate the relationship between serum liver markers, on one hand, and serum concentrations of three adipokines (leptin, adiponectin and resistin), biomarkers of mineral – bone and iron metabolism, on the other, and 3) to assess the value of liver function tests and other biomarkers as indicators of osteoporotic fractures and/or as predictors of short-term outcomes. We simultaneously measured serum levels of liver function parameters, bone formation markers (OC, PINP and BAP), bone resorption markers (β CTX, NTx/Cr, DPD/Cr), and their ratios (PINP/OC, PINP/ β CTX, OC/ β CTX), 25(OH)D, intact PTH, calcium, phosphate, magnesium, three adipokines (adiponectin, leptin and resistin), indices of iron metabolism (serum ferritin, iron, transferrin and transferrin saturation) and routine haematological and biochemical variables.

Main Findings

[3]. In the cohort of 294 consecutive older patients with low-trauma osteoporotic HF, in fully adjusted multivariate linear regression analyses, lower OC was an independent predictor of higher GGT, ALT and bilirubin, whereas higher BAP was positively associated with GGT and ALP; NTx/Cr, haemoglobin (both inversely), adiponectin, coronary artery disease (CAD) and alcohol overuse (all three positively)

were also independently associated with GGT activity. However, in malnourished women, OC was not an independent predictor of GGT or ALT and NTx/Cr predicted ALP activity. OC was independently predicted by GGT, ALT (both negatively), ALP, leptin and age (all three positively), BAP by GGT and ALP, OC/BAP ratio by GGT (inversely) and leptin (positively) and both elevated NTx/Cr and DPD/Cr by higher ALP and lower leptin levels. The GGT > 20 U/L indicated increased prevalence of low OC levels (two-fold) and low OC/BAP ratio (2.6-fold) with a positive predictive value above 75%.

[4]. Elevated ALT, GGT, ALP or bilirubin levels on admission were observed in 1.7% - 9.9% of 294 older HF patients. With age GGT, ALT and leptin decrease, while PTH and adiponectin concentrations increase. Higher GGT (>30U/L, median level) was associated with coronary artery disease (CAD), diabetes mellitus (DM), and alcohol overuse; lower ALT ($\leq$ 20U/L, median level) with dementia; total bilirubin >20 μ mol/L with CAD and alcohol overuse; and albumin >33g/L with CAD. Multivariate adjusted regression analyses revealed ALT, ALP, adiponectin, alcohol overuse and DM as independent and significant determinants of GGT (as continuous or categorical variable); GGT for each other liver marker; and PTH for adiponectin. The risk of prolonged hospital stay (>20 days) was about two times higher in patients with GGT>30U/L or adiponectin >17.14 ng/L (median level) and 4.7 times higher if both conditions coexisted. The risk of in-hospital death was 3 times higher if albumin was <33g/L.

[5]. In a cross-sectional study of 416 older orthogeriatric patients multivariate regression analyses demonstrated significant bidirectional links between P1NP and GGT, P1NP/OC ratio and GGT, P1NP and TSAT, OC and ferritin, GGT and ferritin. GGT, ferritin and TSAT were independent indicators of β CTX, while ferritin and TSAT were also independent predictors of the P1NP/OC ratio, and TSAT was an independent predictor of the P1NP/ β CTX ratio. In multivariate regression, P1NP, β CTX, P1NP/ β CTX ratio, ferritin, magnesium and age were independent indicators of fracture.

Comments

In these studies we focused on integrating across LFTs, adipokines, iron, bone-mineral metabolism, demographic and multiple clinical characteristics to derive a bigger picture on the liver-bone interactions and their clinical significance in orthogeriatric patients.

Liver-adipokines-bone links. Our results demonstrated that in older HF patients: (i) the prevalence of abnormal liver function tests is relatively low, (ii) with ageing GGT and ALT activities and leptin concentrations decrease, while serum PTH, adiponectin and bone turnover (OC and NTx/Cr) levels increase; (iii) circulating adiponectin, leptin, PTH levels and urinary bone resorption markers are significantly higher in females than in males; (iv) GGT activity and other hepatic markers exhibit bidirectional relationships: a positive with ALT, ALP and bilirubin and a negative with albumin; (v) adiponectin (but not leptin or resistin) is an independent contributor to higher GGT, whereas resistin is a determinant for albumin and bilirubin, (vi) hepatic functions (even within normal range) are associated with common age-related disorders: higher GGT levels (>30U/L) with CAD, DM, and excess alcohol consumption; lower ALT levels ($\leq$ 20U/L) with dementia; total bilirubin levels

(>20 μ mol/L) with CAD, cervical (vs. trochanteric) HF type and alcohol overuse; and albumin concentrations > 33g/L with CAD.

A main novelty of our study is a significant positive correlation between serum adiponectin, the most abundant adipocytokine, and GGT activity. Why adiponectin is more strongly linked to GGT than to ALT and other liver markers is not entirely clear. Although serum GGT is predominantly secreted by the liver, it is present and active on the surface of most cell types where it plays an important role in glutathione metabolism; GGT may also capture extra-hepatic processes relevant to ageing. The age-related increase in adiponectin and its positive association with GGT were independent of other liver parameters (ALT, ALP, bilirubin and albumin), leptin (a sensitive marker of adiposity negatively associated with adiponectin) and resistin (a biomarker strongly associated with an inflammatory response) levels, 25(OH)D, PTH, GFR, alcohol consumption, presence of DM or CVD, although some of these factors were also independent contributors to higher GGT levels. In other words, the origin of higher serum GGT activity is multifactorial (eight factors accounted for 54.3% of GGT variance), and the elevated adiponectin concentration and its relation to GGT are part of and reflect the complex homeostatic dysregulation (-s) that accompany ageing. Consistent with this hypothesis, are our findings demonstrating that PTH, which also increases with age, correlated positively with adiponectin and in the multivariate analysis was an independent determinant of adiponectin (but not of leptin or resistin). The positive correlation between PTH and adiponectin has not been previously documented in HF patients, and it may explain, at least partially, the adiponectin-GGT “paradox”: although in general with ageing GGT activity decreases, adiponectin levels, mediated in part by elevated PTH, rise resulting in higher GGT levels. We hypothesise that PTH elevation may partly contribute to higher adiponectin concentration, which is clearly related to higher GGT. These complex interactions are illustrated in Figure 2.

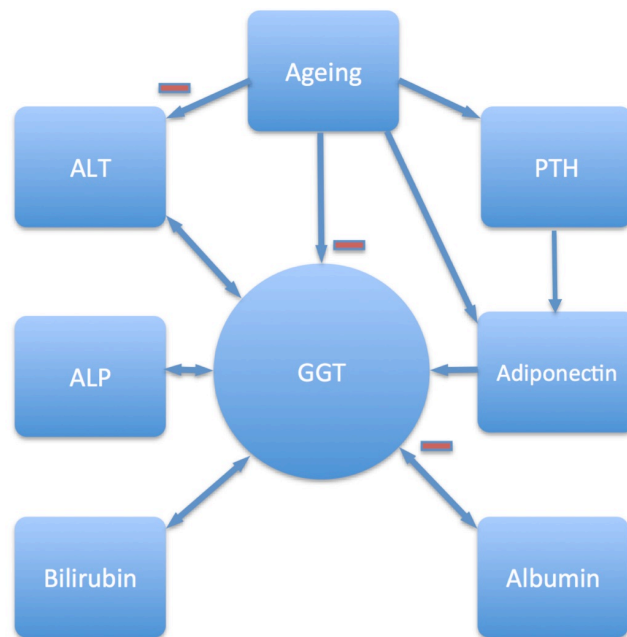


Figure 2. Relationships between age, liver function parameters, circulating levels of adiponectin and PTH (independent and significant associations shown).

GGT, gamma-glutamyltransferase; ALT alanine aminotranferase; ALP, alkaline phosphatase; PTH, parathyroid hormone; - negative correlation.

Ageing is associated with decreased GGT and ALT activities and higher serum PTH and adiponectin levels. PTH correlates with adiponectin and adiponectin is associated with higher GGT levels. A bidirectional relationship links GGT with ALT, ALP and bilirubin (positively) and albumin (negatively). [4]: [Figure 1]

Integrating multidirectional interrelationship between adipokines, hepatic and bone metabolism, factors acting on and/or being influenced by each other, it was possible to propose a model that unifies a number of functions and observations that have been considered contradictory in the past. Our linear regression analyses revealed the following bidirectional significant and independent associations between liver markers, adipokines and indices of bone metabolism: (i) GGT and OC, NTx/Cr (both inverse) and BAP (positive), (ii) ALT and OC (inverse), (iii) ALP and BAP (positive), and (iv) leptin (but not adiponectin or resistin) and OC, OC/BAP ratio, NTx/Cr and DPD/Cr levels (inversely for both resorption markers). These interactions are depicted in Figure 3.

We found that bone formation and resorption depends, at least partially, on the regulatory interaction between adiponectin and leptin which may exert opposite effects on bone metabolism through different pathways. Our observations are in agreement with a recent animal study, which concluded that adiponectin has the ability to regulate the same function in two opposite manners depending on where it

acts and it opposes leptin's influence ([3]:101). Our results show that adiponectin may exert a negative net effect on bone metabolism by increasing GGT and decreasing circulating leptin levels (inverse adiponectin-leptin relationship); on the other hand, OC and adiponectin have opposite effects on GGT. However, these relationships are not consistently maintained, but expressed differently under various pathophysiological conditions indicating the level of complexity of the homeostatic system(s). Specifically, in malnourished women increases in serum adiponectin, which is produced almost exclusively by adipocytes, are associated with higher OC levels and do not correlate with GGT. Our model based on the results of multivariate regression analyses takes into account that homeostasis necessitates reciprocal signalling between hepatic function, components of mineral-bone metabolism and adipokines. It should be viewed as a part of a complex network that integrates and orchestrates the liver-bone-adipose tissue axis in health and diseases. Of major pathophysiological interest is the feedback loop between GGT and OC, in which adiponectin and leptin appear as two important counter-players: the former acts as a positive regulator of GGT, while the latter is a positive regulator of OC (Figure 3, upper part). Importantly, these relationships are not invariant, but depend on multiple underlying conditions, among which the nutritional status and gender may be the key contributors. The stronger (compared to other liver indices) and bidirectional association of GGT, an indicator of oxidative stress ([3]:28,104) with bone turnover markers suggests that the liver-bone links might reflect systemic rather than solely hepatic processes.

Vitamin D-PTH status. Although vitamin D insufficiency and elevated PTH levels were recorded in 79.7% and 38.2% of our patients, respectively, in multivariate analyses no significant correlations were detected between GGT and ALT activities, on the one hand, and 25(OH)D and PTH concentrations, on the other, suggesting that the vitamin D-PTH axis is not an independent determinant of the transaminase activities. Also, 25(OH)D and PTH were not independent predictors of bone turnover markers, indicating that vitamin D and PTH are not involved in the relationship of these liver enzymes with bone remodelling. We found positive correlations between 25(OH)D and ALP, and between PTH and bilirubin, which indicate that vitamin D insufficiency and SHPT may affect specific liver functions, as it was previously reported for the PTH-bilirubin association ([3]:102,103).

Towards an integrated and unifying hypothesis. Our data in line with numerous previous publications showed that decline in liver functions, increase in adiponectin and PTH are interconnected universal phenomena associated with human ageing. Age-related rise in serum adiponectin and PTH concentrations are positively correlated, but age and adiponectin are paradoxically compatible with hepatic function, especially with GGT activity. In parallel with adiponectin elevation GGT activity increases indicating that the hormonal effect of adiponectin takes precedence over age-related suppression in the enzyme activity. The serum GGT activity reflects the integrated response of these opposite effects.

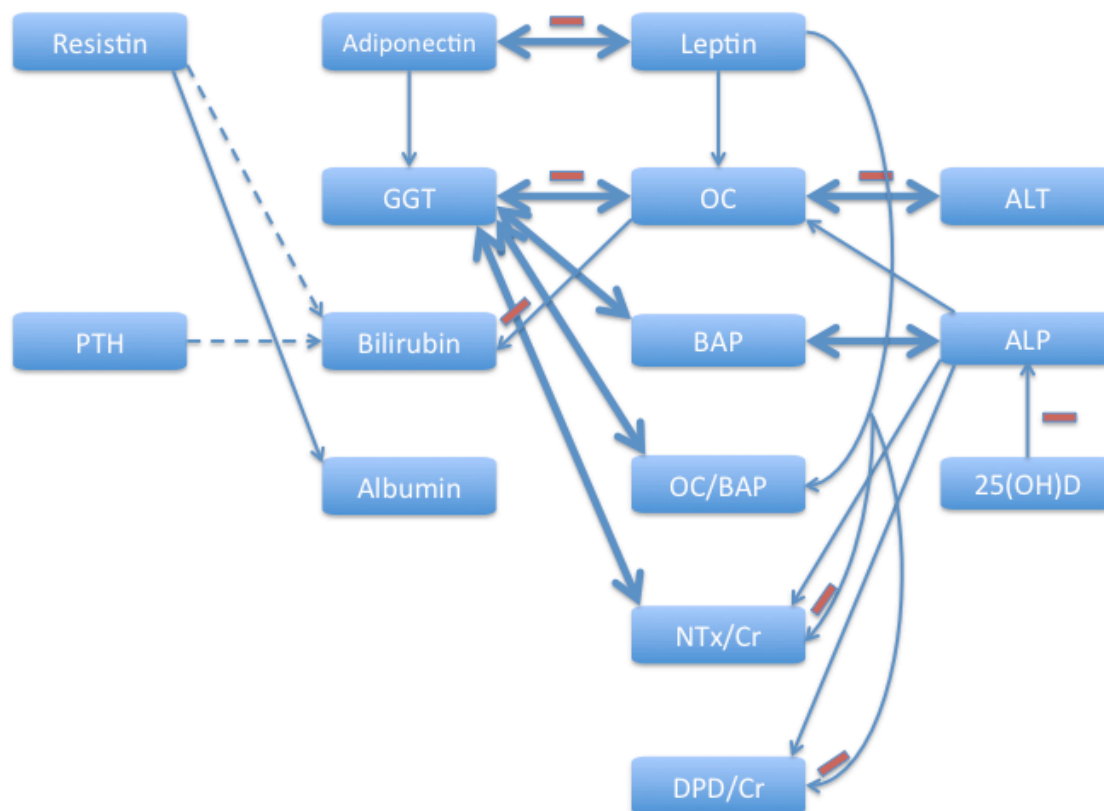


Figure 3. Links between liver markers, indices of bone metabolism and adipokines in older patients with hip fracture.

GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; OC, osteocalcin; BAP, bone specific alkaline phosphatase; NTx/Cr, cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration; DPD/ Cr, deoxypyridinoline corrected for urinary creatinine concentration; PTH, parathyroid hormone; Hb, haemoglobin. Bidirectional links are shown in bold arrows; -- indicates inhibitory effect.

The interactions between GGT and OC, BAP, and NTx/Cr, as well as between ALT and OC, and between ALP and BAP are bidirectional: the activity of hepatic enzymes contributes to synthesis and release of molecules reflecting bone formation and bone resorption, and these, in turn, influence the circulating levels of liver enzymes. ALP activity correlates with levels of serum OC and urinary resorption markers. There is a strong reciprocal association between adiponectin and leptin. Adiponectin is positively associated with GGT activity, while leptin is positively associated with OC and OC/BAP ratio and inversely with both resorption markers; therefore, adiponectin and leptin may exert opposite effects on bone metabolism; resistin is associated with serum albumin concentration. In multiple logistic regression analyses adjusted for age, sex and confounding laboratory (but not clinical) variables both resistin and PTH were independently associated with bilirubin levels (shown in dashed lines). All other data are based on fully adjusted model. Of note, in malnourished patients circulating adiponectin and OC levels are positively associated, while adiponectin does not correlate with GGT activity (not shown). [3]: [Figure 1]

These observations raised two key questions: 1) is there a special common cause that underlies the metabolic changes occurring with advancing age, although each change results from an interplay of numerous independent mechanisms, and 2) is higher serum adiponectin concentration associated with GGT elevation, as in our case, an adoptive/compensatory response or a harmful effect. The exact answers remain largely unknown, and any attempt to adequately explain the observations should

include at least two fundamental mechanisms: homeostasis and oxidative stress. Several lines of evidence suggest close but complex interactions between oxidative stress and GGT, albumin, bilirubin, adiponectin and PTH (these factors may act as causes and consequences of oxidative stress). Oxidative stress, an imbalance between the production and inactivation of reactive oxygen species in favour of oxidants accumulation, is widely accepted as an important mechanism associated with human ageing and its adverse effects ([4]:195-199). Hepatic aging is associated with greater oxidative stress and cell apoptosis ([4]:200-202). GGT, a membrane-bound enzyme, plays a pivotal role in the intracellular antioxidant defence being involved in the gamma-glutamyl cycle by which extracellular glutathione is transported into cells. Depletion of intracellular glutathione, a principal intracellular antioxidant ([4]:203) in response to oxidative stress results in an increase in GGT so that the metabolic homeostasis are maintained. Serum GGT activity is inversely associated with the concentration of serum antioxidants ([4]:204). Serum GGT within its normal range is recognized as a sensitive marker of oxidative stress ([4]:30,141,203-207). However, in physiological conditions, GGT may also act as a pro-oxidant ([4]:203,204,208) generating reactive oxygen species ([4]:209,210) which could exceed the capacity of the antioxidant system and induce cellular oxidative stress damage.

The oxidative stress responses involve also other potent antioxidants, namely albumin ([4]:211) the major protein in plasma, which accounts 80% of thiol's antioxidant effect in the body ([4]:212,213), bilirubin, which protects cells from a 10 000-fold excess of oxidants through rapid regeneration of bilirubin by biliverdin reductase ([4]:119,214), and adiponectin. In animal models ([4]:215,216) and in humans ([4]:217,218), including the elderly ([4]:219), adiponectin inhibits oxidative stress, but oxidative stress suppresses adiponectin production and its powerful protective antioxidant properties ([4]:220). Importantly, adiponectin is also involved in apoptosis, an evolutionary conserved controlled-death program, which ensures proper regulation of the size and quality of cell populations in tissues ([4]:221). PTH may affect oxidative stress directly or by intracellular calcium accumulation ([4]:222,223). In other words, GGT, albumin, bilirubin, adiponectin and PTH, all of which play a critical role in homeostasis, may be elicited by oxidative stress and/or may have both protective and promoting effects on oxidative stress. The interplay and high degree of complexity of aforementioned and other factors involved in oxidative stress indicate the role of regulatory feedback mechanisms in different conditions. Normally, the oxidative stress responses maintain metabolic homeostasis and are beneficial for adaptation and survival, while dysregulation in the feedback processes may cause the vicious cycle(-s) of oxidants overproduction resulting in aging and aging- related diseases ([4]:224). Despite considerable gaps in our knowledge, emerging data suggests that adiponectin and oxidative stress can function in both a defence and harmful manner.

In regard to our observations, age-related decrease of GGT activity may reflect the decline not only in liver function, but in the whole antioxidant defence system (including the decrease in the antioxidant effects of albumin as one of its components), and the increase in serum GGT level may be a compensatory response to oxidative stress, a recognised hallmark of ageing and chronic diseases. This response includes increases in PTH and adiponectin. The former is at least in part responsible for the adiponectin elevation, while the later enhances the anti-oxidant potential in the cells by increasing GGT and promotes apoptosis ([4]:225). In this way, it can be hypothesized that in our patients, higher GGT activity which is

positively associated with both adiponectin and bilirubin (also a potent antioxidant) levels, reflects a compensatory response to oxidative stress. However, in the setting of advanced age and co-morbidities this response is unable fully counter-regulate the oxidative damage and prevent its progression if oxidative stress increases, as in the perioperative period. Indeed, in our cohort, both GGT and adiponectin above the median levels were independently and synergistically associated with prolonged LOS, but not with in-hospital mortality, whereas even mild decrease in albumin concentration, an important extracellular antioxidant, demonstrated a strong relation to fatal outcome. These complex interactions are summarised in Figure 4.

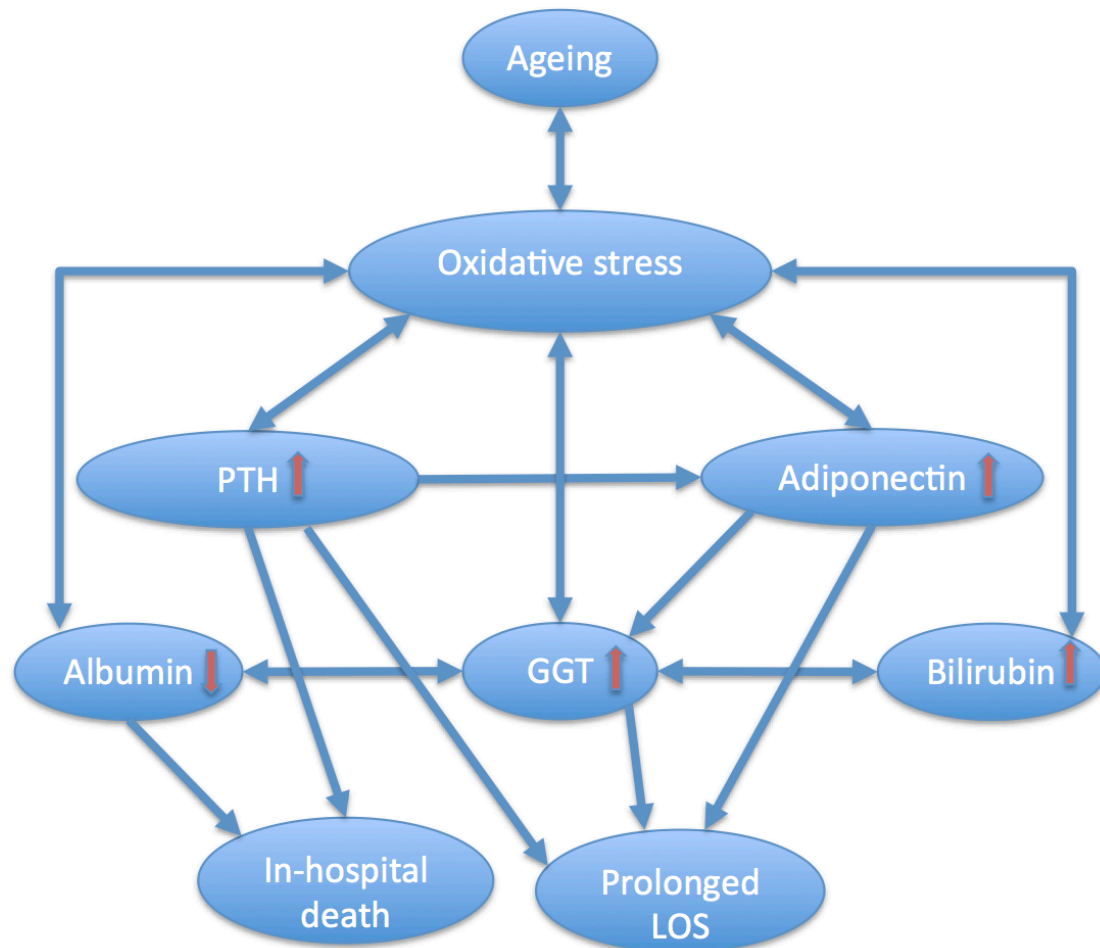


Figure 4. Diagram illustrating complex interplay between ageing, oxidative stress, parathyroid hormone (PTH), adiponectin and gamma-glutamyltransferase (GGT) and other hepatic markers and their relation to short-term outcomes in older patients with hip fracture.

LOS, length of hospital stay.

In advanced age, GGT, albumin, bilirubin, adiponectin and PTH, all of which are influenced by oxidative stress, may exert both protective and promoting effects on oxidant formation, affect each other, and contribute to poor outcomes. Excessive PTH along with different other age-related factors drives adiponectin levels higher, and such elevations increase GGT activity which reflects an integrative adaptive/compensatory or pathological response (homeostatic regulation or dysregulation). In HF patients, both GGT and adiponectin concentrations above the median levels are independent markers of prolonged LOS and contribute synergistically to this outcome, hypoalbuminaemia is associated with in-hospital death, and hyperparathyroidism is an independent predictor of both fatal outcome and prolonged hospital stay. [4]: [Figure 3]

Liver-iron metabolism-bone links. The complex interplay between serum GGT activity, iron status and bone metabolism in orthogeriatric patients is shown in Figure 5. In brief, bone remodelling is regulated by GGT and iron status, and specific bone-derived molecules (P1NP and OC) affect both the GGT activity and iron homeostasis, while GGT and ferritin are also interrelated. The bidirectional links between P1NP and GGT (1), P1NP/OC ratio and GGT (2), P1NP and TSAT (3), OC and ferritin (4), GGT and ferritin (5) suggest that these molecules and factors act as feedback loop signals participating in the complex homeostatic, adaptive and compensatory mechanisms. Serum GGT activity, ferritin and TSAT are independent indicators of β CTX, while ferritin and TSAT are also independent predictors of the P1NP/OC ratio, and TSAT is an independent predictor of the P1NP/ β CTX ratio. The study provides evidence that GGT and iron status indices (even within normal or mildly-moderately abnormal range) act as independent determinants of different aspects of bone metabolism, and are, in turn, regulated by bone-derived peptides. Remarkably, both osteoblast-specific secreted molecules - OC (well documented previously ([5]:24, 25) and P1NP (as described, for the first time, in this study) – exert multiple extra-skeletal metabolic functions.

Our findings suggest that:

- Higher iron status (as indicated by higher TSAT and/or ferritin levels) is associated with suppression of osteoclast function and bone resorption (lower β CTX).
- Bone formation (P1NP) increases when TSAT is higher, but higher ferritin suppresses the process of bone differentiation (lower OC and higher P1NP/OC ratio).
- Iron-related markers have different effects on specific aspects of osteoblast function; feedback signals between molecules involved in iron and bone metabolism generate a positive loop with osteoblast formation (TSAT – P1NP) and a negative loop with osteoblast differentiation (ferritin – OC). Thus, the bidirectional links TSAT- P1NP and ferritin - OC, as well as the effects of both ferritin and TSAT on β CTX, of TSAT on P1NP/OC and P1NP/ β CTX ratios should be considered as components of homeostatic systems that maintain structural and functional integrity in the bone and in different other tissues. Notably, in our study, three iron indices - TSAT, ferritin and transferrin - were independently and inversely associated with β CTX, suggesting that iron deficiency results not only in decreased bone formation but also in increased bone resorption. An important point to note is that ferritin demonstrated a bidirectional inverse link with OC, whereas TSAT showed a bidirectional positive association with P1NP, despite the fact that P1NP is correlated with OC. It appears, therefore, that the contribution of ferritin to bone metabolism may reflect an effect mediated by mechanism (-s) other than increased iron stores. Furthermore, despite the strong inverse bidirectional link between ferritin and OC, neither serum iron, nor TSAT (additional indicators of body iron stores, transport and availability) independently predicted OC in our and other studies ([5]:28). These observations are in keeping with the existing evidence that elevated serum ferritin is a multifactorial metabolic marker which reflects in addition to body iron stores other conditions such as inflammation, oxidative stress, chronic kidney, liver and autoimmune disorders; elevated ferritin levels do not exclude underlying iron deficiency. Indeed, 78.9% of our patients had low TSAT, and 26.0% demonstrated a “paradoxical” combination of elevated ferritin levels with low TSAT, indicating functional iron deficiency ([5]:40),

a condition typical for anaemia of chronic disease (anaemia of inflammation) and chronic kidney disease. In our study ferritin was independently predicted by CRP (positively) and GFR (negatively).

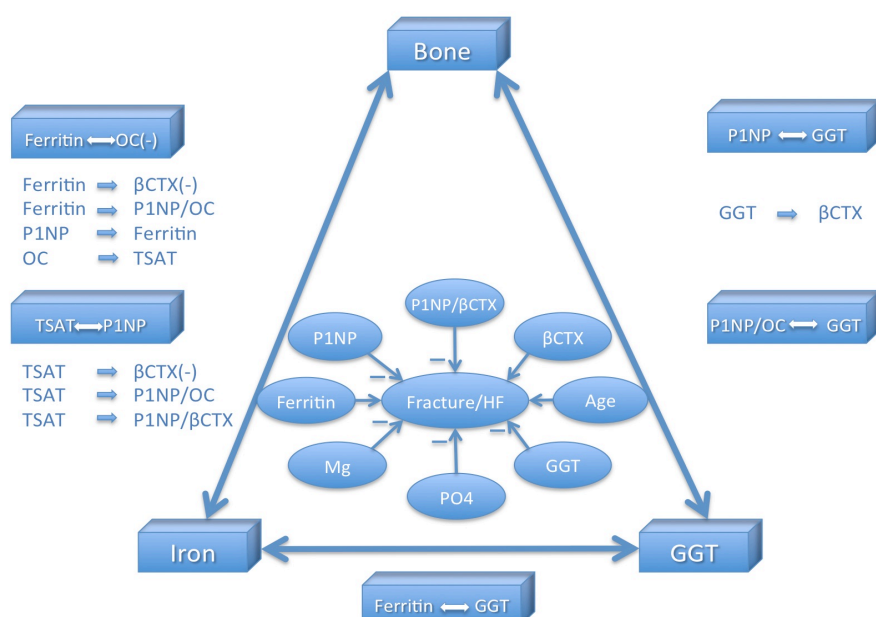


Figure 5. Schematic representation of the complex interactions between serum gamma-glutamyl transferase (GGT) activity, markers of iron and bone-mineral metabolism in the elderly.

Factors independently associated with bone fractures are shown in ovals.

GGT, gamma-glutamyl transferase; TSAT, transferrin saturation; P1NP: amino-terminal propeptide of type 1 procollagen; OC, osteocalcin; β CTX, crosslinked carboxy-terminal telopeptide of type 1 collagen; PO₄, phosphate. Bidirectional links are underlined; (-) indicates an inverse association; arrows indicate the direction of affect.

Serum GGT activity is an independent determinant of P1NP, β CTX, P1NP/OC ratio and ferritin, which, in turn, is an independent determinant of OC, β CTX, and P1NP/OC ratio, while TSAT is an independent predictor of P1NP, β CTX, P1NP/ β CTX and P1NP/OC ratios. The bidirectional links between ferritin and GGT, ferritin and OC, GGT and P1NP, GGT and P1NP/OC ratio, TSAT and P1NP indicate feedback signals involved in the complex homeostatic mechanisms. Higher serum concentrations of β CTX and ferritin, lower P1NP, P1NP/ β CTX ratio, PO₄ levels and advanced age are independent indicators of any fracture; lower GGT activity is an additional independent indicator of HF. [5]: [Figure 1]

Although hydroxylation of 25(OH)D to 1,25(OH)D requires iron ([5]:36) and low vitamin D levels were linked with iron deficient anaemia in females ([5]:10), in our study, 25(OH)D was not an independent determinant of TSAT or ferritin.

Finally, it should be emphasised that the complexity of the described homeostatic mechanisms including the positive-feedback loops between GGT and both P1NP and P1NP/OC ratio, TSAT – P1NP and a negative-feedback loop ferritin – OC, supports the hypothesis that oxidative stress is an integrative (patho)physiologic mechanism that links both GGT activity and iron status with bone integrity. Higher level of GGT,

the main determinant of extracellular hydrolysis of glutathione, and elevated serum ferritin concentrations leading to release of more free iron, could facilitate glutathione catabolism, increase production of reactive oxygen species and subsequent oxidative stress, which in turn, affects bone metabolism and causes bone loss ([5]:22,23,41). The hypothesis that oxidative stress is involved in the pathogenesis of bone loss is supported by animal studies showing that treatment with the antioxidant N-acetyl-L-cysteine, precursor of glutathione, reversed osteopenia in GGT-deficient mice ([5]:2) and prevented the development of bone abnormalities in iron-overloaded mice ([5]:33). In ours and other studies, GGT and ferritin correlated with each other. On the other hand, these variables demonstrated associations with β CTX and presence of HF, which is in line with the dual role of reactive oxygen species (positive at moderate levels and damaging at elevated levels) in bone metabolism, as in a number of other pathological conditions. Interrelated serum GGT and ferritin levels appear as an important homeostatic bridge between metabolic, oxidative and bone status.

Taken together, our observations in line with the existing literature (although results are mixed) provide evidence that regulation of both osteoblastic and osteoclastic differentiation and function requires an optimal iron status: iron levels should be sufficiently high to facilitate bone formation and suppress bone resorption, but not so high that they affect bone differentiation and increase GGT activity. Importantly, these factors are in a state of mutual influence: feedback mechanisms involving both osteoblast-specific proteins (P1NP and OC) participate in maintaining iron homeostasis and GGT activity.

4.2.2. Clinical implications: diagnostic, prognostic and therapeutic aspects [3, 6-10]

Currently the ability to predict and prevent fragility fractures is limited and the prognosis of clinical outcome in the orthogeriatric patients is extremely difficult. Due to practical importance of these topics we conducted studies [3, 6-10] aimed to (1) to assess the prognostic impact of liver markers (alone or in combination) as indicators of bone turnover, (2) to identify additional prognostic fracture risk factors not included in current guidelines, and (3) to examine the predictive value of liver-related biomarkers at admission as indicators of short-term outcomes in orthogeriatric patients, especially with HF.

Main Findings

[6]. Multivariate regression analysis of clinical and laboratory characteristics in 415 consecutive orthogeriatric patients revealed four variables - presence of HF, hypoalbuminaemia ($<33\text{g/L}$), anaemia ($<120\text{g/L}$) and hyperparathyroidism ($\text{PTH}>6.8\text{ pmol/L}$) - as independent determinants of elevated neutrophil to lymphocyte ratio (NLR) at admission ($\text{NLR}\geq 5.1$). There was a dose-graded relationship between presence of fracture, especially HF, postoperative complications and levels of NLR categorized as tertiles. Compared to patients with $\text{NLR}<5.1$ (first tertile), patients with NLR 5.1-8.5 (second tertile) had a 1.8-, 3.1-, 2.6-, and 2.5-fold higher risk for presence of any fracture, HF, developing postoperative myocardial injury (troponin I rise) and a high inflammatory response/infection ($\text{CRP}>100\text{mg/L}$ after the 3rd postoperative day), respectively, while in subjects with $\text{NLR}>8.5$ (third tertile) these

risks were 2.6-, 4.9-, 5.9- and 4.5-times higher, respectively; subjects with $\text{NLR} > 8.5$ had a 9.7 times higher chance of dying in the hospital compared to patients with $\text{NLR} 5.1-8.5$; the NLR retained its significance on multivariate analyses. The $\text{NLR} \geq 5.1$ predicted postoperative myocardial injury with an area under the curve (AUC) of 0.626, $\text{CRP} > 100\text{mg/L}$ with AUC of 0.631 and the $\text{NLR} > 8.5$ predicted in-hospital mortality with an AUC of 0.793, showing moderately high sensitivity (86.7%, 80% and 90%, respectively) and negative predictive value (92.9%, 71.2%, 99.6%, respectively), but low specificity. Admission NLR was superior to other, except hypoalbuminaemia, prognostic markers; combined use of both $\text{NLR} \geq 5.1$ and $\text{albumin} < 33\text{g/L}$ only moderately increased the accuracy of prediction. The validation study confirmed the prognostic value of the admission NLR .

[7] Analysis of 1,239 hospitalized orthogeriatric patients with and without nonvertebral fractures showed that both lower serum $\text{P1NP}/\beta\text{CTX}$ ratio and albumin concentration (as continuous or categorical variables) were independently associated with fracture presence in multivariate logistic regressions. Compared with the highest $\text{P1NP}/\beta\text{CTX}$ tertile, the prevalence of HF, after adjustment for multiple covariates, was 3-fold higher in the lowest tertile and 1.5 times higher in the middle tertile; presence of any fracture was 2.3- and 1.6-fold higher, respectively; patients with albumin levels in the lowest tertile had multivariate odds ratio (OR) of 4.6 for HF and 2.8 for any fracture, in the middle tertile the ORs were 2.2 and 1.3, respectively. The $\text{P1NP}/\beta\text{CTX} < 100.0$ (median) and hypoalbuminemia ($< 33\text{ g/L}$) demonstrated area under the curve values for HF of 0.802 and 0.806, respectively, and for any fractures of 0.711 and 0.706, respectively. When both characteristics were combined, the ORs for HF or any fracture, compared with the nonfractured group, were 7.8 and 3.2, respectively, with an accuracy of 79.6% and 71.6%, respectively.

[8] We develop a practical model for classification bone turnover status and evaluate its clinical usefulness. Our classification of bone turnover status is based on internationally recommended biomarkers of both bone formation (N-terminal propeptide of type I procollagen, P1NP) and bone resorption (beta C-terminal cross-linked telopeptide of type I collagen, βCTX), using the cutoffs proposed as therapeutic targets. Six subtypes of bone turnover status were identified: 1- normal turnover ($\text{P1NP} > 32\text{ }\mu\text{g/L}$, $\beta\text{CTX} \leq 0.250\text{ }\mu\text{g/L}$ and $\text{P1NP}/\beta\text{CTX} > 100.0$ [(median value)]); 2- low bone formation ($\text{P1NP} \leq 32\text{ }\mu\text{g/L}$), normal bone resorption ($\beta\text{CTX} \leq 0.250\text{ }\mu\text{g/L}$) and $\text{P1NP}/\beta\text{CTX} > 100.0$ (subtype 2A) or $\text{P1NP}/\beta\text{CTX} < 100.0$ (subtype 2B); 3- low bone formation, high bone resorption ($\beta\text{CTX} > 0.250\text{ }\mu\text{g/L}$) and $\text{P1NP}/\beta\text{CTX} < 100.0$; 4- high bone turnover (both markers elevated) and $\text{P1NP}/\beta\text{CTX} > 100.0$ (subtype 4A) or $\text{P1NP}/\beta\text{CTX} < 100.0$ (subtype 4B). The relationships between turnover subtypes and clinical characteristic were assessed in 1223 hospitalised orthogeriatric patients. Compared to subtypes 1 and 2A, subtype 2B was strongly associated with nonvertebral fractures (odds ratio [OR] 2.0), especially HF (OR 3.2), age > 75 years and hyperparathyroidism. Hypoalbuminaemia and not using osteoporotic therapy were two independent indicators common for subtypes 3, 4A and 4B; these three subtypes were associated with in-hospital mortality. Subtype 3 was associated with fractures (OR 1.7, for HF OR 2.4), age > 75 years, chronic heart failure (CHF), anaemia, and history of malignancy, and predicted post-operative myocardial injury, high inflammatory response and length of hospital stay (LOS) above 10 days. Subtype 4A was associated with chronic kidney disease (CKD), anaemia, history of malignancy and walking aids use and predicted LOS > 20 days, but

was not discriminative for fractures. Subtype 4B was associated with fractures (OR 2.1, for HF OR 2.5), age>75 years, CKD and indicated risks of myocardial injury, high inflammatory response and LOS>10 days.

[9]. In a cohort of 1820 consecutive HF patients the mortality rate was 6% (n = 109). On univariate analysis 14 laboratory and 8 clinical parameters have been associated with in-hospital mortality. Multiple regression analyses determined 7 variables at admission as independent indicators of a fatal outcome: 4 biomarkers (albumin <33 g/L; gamma-glutamyl transferase / alanine aminotransferase ratio [GGT/ALT] >2.5; parathyroid hormone [PTH] >6.8 pmol/L; 25(OH)vitamin D < 25 nmol/L) and 3 pre-fracture clinical conditions (history of myocardial infarction, chronic kidney disease [GFR <60 ml/min/1.73 m²] and chronic obstructive pulmonary disease); the area under the receiver operating characteristic curve (AUC) was 0.75 (95%CI 0.70-0.80). The risk of in-hospital death was 1.6-2.6 times higher in subjects with any of these risk factors (RFs), and increased by 2.6-6.0-fold in patients with any two RFs (versus no RFs). The mortality rate increased stepwise as the number of RFs increased (from 0.43% -none RF to 16.8%- ≥4RF). The prognostic value of a single RF was low (AUC ≤0.635) but combination of 2 or more RFs improved the prediction significantly; AUC reached 0.84(95%CI 0.77-0.90) when ≥4 RFs (versus 0-1RF) were present. In the validated and main cohorts the number of predicted by 1, 2, 3 or ≥4 RFs and observed deaths were practically similar.

[10]. Elevated serum urea levels (>7.5mmol/L) at admission were prevalent (44%), independently determined by chronic kidney disease, history of myocardial infarction, anaemia, hyperparathyroidism, advanced age and male gender, and significantly associated with higher mortality (9.4% vs. 3.3%, p<0.001), developing a high postoperative inflammatory response (HPIR, 22.1% vs.12.1%, p=0.009) and prolonged hospital stay (>20 days: 31.2% vs. 26.2%, p=0.021). The predictive value of urea was superior to other risk factors, most of which lost their discriminative ability when urea levels were normal. Patients with two abnormal parameters at admission, compared to subjects with the normal ones, had 3.6-5.6 -fold higher risk for hospital mortality, 2.7-7.8-fold increase in risk for HPIR and 1.3-1.7-fold higher risk for prolonged hospital stay. Patients with increased admission urea and a high inflammatory response had 9.7 times greater mortality odds compared to patients without such characteristics.

Comments

4.2.2.1. Hepatic markers as indicators of the bone turnover status [3]

Our results indicate that GGT, even within the normal range, is linked to osteoblast dysfunction. Among HF patients with the GGT > 20 U/L (above the cut-off level of the first tertile), there was a 2.3-fold increase of subjects with low circulating OC levels (< 14 ng/ mL, lower limit of normal range), about a 4.8-fold increase of subjects with elevated of BAP activity (> 43 U/L, upper limit of normal range) and, consequently, a 2.6-fold increase of subjects with low OC/BAP ratio (< 0.6, median level). These associations remained significant after adjustments for age, sex, serum adiponectin, leptin and resistin levels, alcohol overuse, presence of CAD or DM. Diagnostic value of GGT > 20 U/L was as follows: for presence of low OC (< 14 ng/mL) sensitivity 59.9%, specificity 60.2%, positive predictive value (PPV) 75.7%,

negative predictive value (NPV) 42.1%, positive likelihood ratio (LR) 1.51; for high BAP (> 43 U/L) 9.5%, 97.8%, 90.0%, 34.6%, 4.41, respectively; for low OC/ BAP ratio (< 0.6) 57.9%, 65.6%, 77.5%, 43.3%, 1.68, respectively. Although the diagnostic value was relatively low, this still would have translated in identifying about 76% of patients with abnormal bone formation status (PPV = 75.7% for low OC and PPV = 77.5% for low OC/BAP ratio) indicating that GGT > 20 U/L in the elderly can be used as a simple first step in detecting bone disease. Interestingly, GGT > 20 U/L was also associated with a two-fold increase of presence of CAD (OR: 1.94, 95% CI: 1.01 - 3.65, $P = 0.050$) (sensitivity 25.0%; specificity 85.6%, PPV 76.7%, NPV 37.6%, LR 1.73), a condition known to be associated with osteoporosis, falls and fractures..

These data suggests that older patients with GGT > 20 U/L need an examination of their bone status and consideration of an anti-osteoporotic medication with anabolic properties. Currently, osteoporosis is predominantly treated with anti-resorptive medications, despite the fact that in a significant proportion of elder patients bone loss is primarily attributed to the impaired osteoblastic activity ([3]:124). Serum GGT > 20 U/L may be a promising indirect marker useful as the first and easy step in diagnostic evaluation of impaired bone metabolism in the elderly and pointing to the need of individualized therapy; its PPV for low OC and for low OC/BAP ratio is above 75%. The clinical significance of this marker should be confirmed and validated by longitudinal studies.

4.2.2.2. Biomarkers of osteoporotic fractures in the context of liver function status [5-8]

The predicting of osteoporotic fractures is largely based on bone mineral density (BMD) testing and several clinical risk factors (the World Health Organization's fracture risk assessment tool FRAX, Garvan and QFracture) ([7]: 1). However, BMD indicated osteoporosis only in 30%–50% of patients with major fragility fracture ([7]:6) and in 4% of women with a distal radial fracture ([7]:7). The prognostic value of clinical risk factors alone in FRAX is comparable to that of BMD alone ([7]: 8). Although P1NP and β CTX were recommended as osteoporotic markers by the Bone Marker Standards Working Group ([5]: 45), the P1NP/ β CTX ratio (that combines two different indices reflecting both bone formation and bone resorption) has not been evaluated previously. No study examined LFTs as possible indicators/predictors of an osteoporotic fracture. There is an obvious need of identifying additional fracture risk factors not included in currently available strategies.

In our initial multivariate regression [5], P1NP, β CTX, P1NP/ β CTX ratio, ferritin, magnesium and age were independent indicators of HF or any non-vertebral fracture; GGT and phosphate levels were additional independent negative indicators of HF. The P1NP/ β CTX ratio was an independent and better indicator of fracture than absolute P1NP and β CTX values.

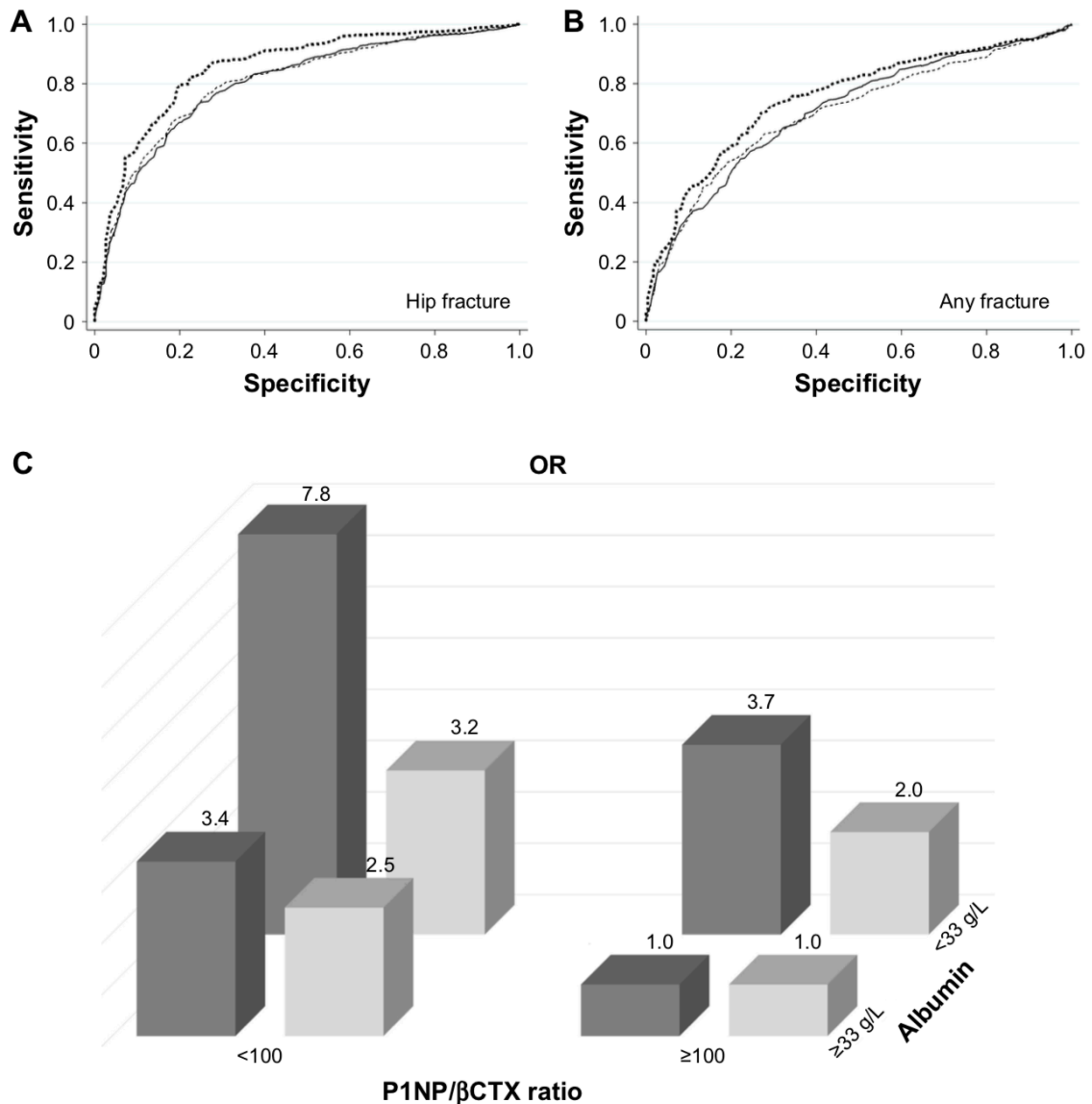


Figure 6. Discriminative information on non-vertebral fracture presence according to serum P1NP/βCTX ratio and albumin concentrations in orthogeriatric patients.

βCTX, cross-linked carboxy-terminal telopeptide of type 1 collagen; HF, heart failure; P1NP, amino-terminalpropeptide of type 1 procollagen.

Notes: (A, B) Receiver operating characteristic curves (ROC) adjusted for age and gender for P1NP/βCTX <100 (solid line), albumin <33 g/L (thin dashed line), and their combination (thick dashed line) as prognostic tests for HF (A) or for any fracture (B). (C) Odds ratios (ORs) adjusted for age and gender for the presence of an HF or of any nonvertebral fracture. The group with P1NP/βCTX >100.0 and albumin >33 g/L on admission used as the reference one. Among patients with only P1NP/βCTX <100.0, the ORs are 3.4- and 2.5-fold higher in subjects with an HF or any non-vertebral fracture, respectively, and among patients with only albumin <33 g/L, the ORs are 3.7- and 2.0-fold higher, respectively; if both conditions are present (combined), the ORs are 7.8- and 3.2-fold higher, respectively. [7]: [Figure 1]

Next, in a large cohort of consecutive hospitalized orthogeriatric patients [7,8], we found that both lower levels of serum albumin concentration and P1NP/βCTX ratio (imbalance between total bone formation and resorption in favour of the latter) were 1) strong independent indicators of HF or any non-vertebral fracture, 2) showed a

dose-dependent relationship with the prevalence of fractures, and 3) demonstrated a discrimination ability of acceptable precision that exceeded the discrimination ability of other studied laboratory parameters (Figure 6). To the best of our knowledge, this is the first study to demonstrate the clinical utility of hypoalbuminemia and lower serum P1NP/ β CTX ratio as promising biomarkers for predicting osteoporotic fractures in older adults.

It appears that two characteristics – serum albumin and P1NP/ β CTX ratio–accumulate key determinants of fracture risk incorporating the effects of multiple clinical and metabolic abnormalities reported in the literature and observed in our univariate analysis.

Taken together, in older patients, serum BTMs and albumin may perhaps help distinguish subgroups with different prognoses for osteoporotic fracture: 1) high risk if P1NP/ β CTX <100.0 and albumin <33 g/L (OR 7.8 for HF and 3.2 for any fracture), 2) intermediate risk if albumin <33 g/L (OR 3.7 and 2.0, respectively) or P1NP/ β CTX <100.0 (OR 3.4 and 2.5, respectively), and 3) low risk (<0.5%) in older adults with β CTX <0.250 μ g/L and P1NP >62.0 μ g/L. Although the discriminative ability of these markers is only moderate (but higher when compared with other currently available indices), they may be particularly useful in persons who have negative BMD test. It should be, however, emphasized that the fracture risk remains substantial in subjects with P1NP/ β CTX >100.0 and albumin >33 g/L; such characteristics demonstrated 58.1% of orthogeriatric patients without fracture but also 18.7% of all fracture patients including 9.0% with HF, indicating that in near 1/5 of subjects with fragility fractures other factors rather than the albumin homeostasis and/or the total balance between bone formation and resorption are important in the development of non-vertebral fractures.

Indeed, we found [6] a dose-graded relationship between presence of fracture, especially HF, and levels of neutrophil to lymphocyte ratio (NLR), a marker of dysregulated immune system and chronic inflammation. Independent determinants of $\text{NLR} \geq 5.1$ were lower levels of albumin and haemoglobin and elevated serum PTH concentration. Adjusted ORs (multivariate regression analyses), demonstrated a 3.11- and 1.74-fold increases in presence of HF or any fracture, respectively, in patients with $\text{NLR} \geq 5.1$. These observations are in line with several recent studies which demonstrated that NLR levels are significantly elevated in the elderly with osteoporosis and inversely correlated with BMD ([6]:79-81). Our findings showed that in orthogeriatric patients higher NLR which represents age-related changes in the immune system (immunosenescence, ([6]:125, 126) and chronic inflammation, a phenomenon known as “inflamm-ageing” ([6]:127, 128), may be an important contributor to the pathogenesis of osteoporotic fractures (as well as other age-related diseases). Therefore, it appears that $\text{NLR} \geq 5.1$, an easily obtained clinical test, as a global index of inflammatory–immunological status may be a useful marker for screening and preventing multimorbidity including risk of osteoporotic fracture in older adults.

Predicting osteoporotic fracture is not trivial because the biochemical cascade of the osteoporotic process is highly complex and causes for falls are multifactorial. Because low BMD, currently the main diagnostic criterion of fracture risk, has a poor sensitivity (about half of low-trauma fractures occur in subjects with non-osteoporotic

BMD), our findings that simple laboratory parameters such as GGT, albumin, P1NP/ β CTX ratio and NLR may be useful independent indicators of increased risk of osteoporotic fracture are of practical significance. An imbalance in bone turnover favouring an increase in bone resorption, hypoalbuminaemia, GGT>20 IU/L and NLR $\geq$ 5.1 – each of these factors demonstrated a good/moderate discriminative ability in regard to non-vertebral fracture presence. Moreover, these markers may also be helpful to select in a given patient the most suitable treatment (i.e., nutritional, anti-inflammatory), including the type of anti-osteoporotic therapy (anabolic vs. antiresorptive medications).

4.2.2.3. Liver-related prognostic indicators of hospital outcomes in orthogeriatric patients [6-9]

The high incidence of concurrent medical comorbidities and frailty amongst orthogeriatric patients emphasises the importance of identification of vulnerable persons, recognition of potentially reversible risk factors and preoperative stabilisation. The reported one-year mortality rates of HF patients range between 12%–37% ([9]:4–9), and 3.3%–19.5% of them die during hospitalization or in the first month following injury ([9]:4,6,10,11–17). Among patients experiencing HF, 30-day mortality is two times higher than in the general population (matched by age and gender) without fracture.²⁶ Early prediction of outcome and the ability to identify at admission patients at a higher risk of morbidity and mortality can help optimise their management and reduce the burden of this disease. Most of the previous studies on predictors of survival in the HF population centred predominantly on clinical characteristics ([9]:4,6,15,18–24) and rarely took into consideration laboratory, especially biochemical, data ([9]:25–28). Among hepatic-related markers only low serum albumin concentration has been found to be a prognostic factor for mortality ([9]:6,26,29–36), and one study documented a positive association between alanine aminotransferase (ALT) activity and mortality within 3 months ([9]:37). The prognostic input of the available on admission spectrum of biochemical markers has not been studied, and systematic characterization of metabolic, specifically liver-related factors, in regard to survival in a large cohort of patients with HF has not been performed.

However, there are numerous reports linking all-cause mortality in community-dwelling older adults and various groups of hospitalised patients with serum activities (even in the normal range) of GGT, ALT and ALP ([9]:38–46). Recently, combined indices (ratios and scores) – serum GGT/ALT ([9]:47], albumin/bilirubin ([9]:48–51), albumin/GGT ([9]:52), and ALT/ALP ([9]: 53,54), were proposed as simple and objective tools for evaluation of hepatic reserve function and prediction survival and therapeutic outcomes in different settings including non-liver disease-related mortality, especially in various malignancies. None of these ratios which represent simultaneous changes of two liver function indices have been intended for HF patients.

Although liver is the main ureagenic organ and urea, the terminal product of protein and amino acid metabolism, plays multiple roles in different homeostatic mechanisms, in clinical studies until now, urea is often interpreted as a biochemical marker of renal function and its relation with other liver-specific functions and protein/nitrogen metabolism remains largely neglected. No study was performed on

the relationship between serum urea, liver function characteristics at admission and outcomes in orthogeriatric patients.

Given the potential effects of metabolic characteristics on outcomes, the aims of our studies were: (1) to examine in a large, well-characterised cohort of older orthopaedic patients, especially with HF, the relationships between a broad set of routine biochemical, mainly liver-related, parameters at admission and in-hospital outcomes (mortality, length of hospital stay, myocardial injury, inflammatory complications, need to be discharge to a permanent residential care facility) and (2) to evaluate the prognostic value such biomarkers provide alone or in combination to predict the outcome. We analysed in total 35 laboratory variables along with 20 clinical and socio-demographic characteristics at admission to ascertain their feasibility to predict in-hospital death.

Our data showed that in older HF patients admission liver and related markers are associated with a continuum of risk for poor in-hospital outcomes. Namely, the following variables were found as independent predictors:

for ***in-hospital mortality***: albumin<33 g/L, GGT>30U/L, GGT/ALT ratio >2.5, NLR≥5.1, urea>7.5mmol/L, vitamin D deficiency and hyperparathyroidism and three clinical characteristics– history of MI, CKD and COPD);

for ***prolonged LOS***: GGT>30U/L, albumin<33g/L and adiponectin >17.14 ng/ml;

for ***myocardial injury*** (identified by cTnI rise): albumin<33g/L and NLR≥5.1;

for ***inflammatory complications*** (with high CRP levels: >100 mg/L): albumin<33 g/L, NLR≥5.1 and urea>7.5mmol/L;

for ***being discharged to a RCF***: NLR≥5.1 and vitamin D deficiency.

In HF patients, GGT, albumin and adiponectin levels analysed as categorical variables independently of known risk factors may help to identify subjects at increased risk of prolonged hospital stay (LOS>20 days) and in-hospital death [4]. Patients with GGT>30U/L and albumin<33g/L were near two times as likely to have a LOS>20 days, and more than 3 times as likely to have died during hospitalisation, respectively, compared to those without such conditions (Figure 7). The calculated sensitivity and specificity of serum GGT>30U/L for discriminating those with LOS>20 days were 48.9% and 65.8%, respectively, and of albumin <33g/L for prediction in-hospital death were 42.9% and 78.9%, respectively; serum albumin<33g/L on admission had a NPV of 96.5%. The risk of LOS>20 days also doubled in subjects with adiponectin>17.14 ng/L (median level). Furthermore, joint effects of GGT>30U/L and adiponectin >17.14 ng/ml raised the odds of LOS>20 days by 4.7-fold, demonstrating synergism; the specificity of two markers in combination for predicting LOS>20 days was 83.2%. These two biomarkers, although interrelated, are associated with different pathogenetic processes, and, not surprisingly, when measured in parallel are more informative for predicting LOS (Figure 7). Our observations are in line with previous reports that low albumin is of prognostic value on mortality in HF patients ([4]:61,62,65,66,69,137,226-228). The prognostic value of GGT and/or adiponectin in HF patients has not been reported previously.

Next, we evaluated and compared the predictions of LFTs with that of twelve other most widely recommended and used predictor markers for determining outcomes

among orthogeriatric patients [6]: age >75 years, dementia, presence of CVDs, AF, high white blood cell (WBC) count, low lymphocyte count, NLR, anaemia (Hb<120g/L), vitamin D deficiency (25(OH)D<25nmol/L) and insufficiency (25(OH)D<50nmol/L), elevated PTH (>6.8 pmol/L), and CKD≥3stage. The performance of these markers, which have been suggested as independent prognostic factors for unfavourable outcomes in HF patients, was variable. In our study [6], the predictive value examined using ROC curve analysis revealed that among orthogeriatric patients the highest discriminative ability for predicting in-hospital death has NLR>8.5 (AUC 0.847) and albumin<33g/L (AUC 0.765). Low admission albumin and elevated NLR were also the two variables which demonstrated the highest prognostic values for postoperative high inflammatory responses.

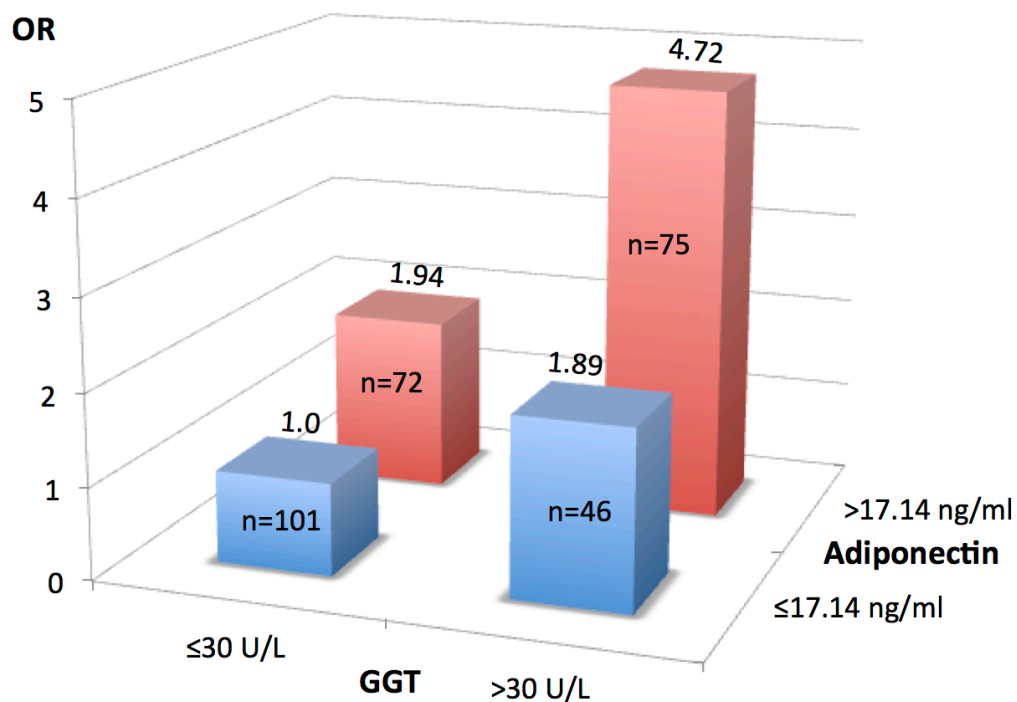


Figure 7. Association between prolonged hospital stay (>20 days) and gamma-glutamyltransferase (GGT) and adiponectin profiles.

Study patients were classified into four groups according to serum levels of GGT (≤30U/L or >30U/L) and adiponectin (≤17.14 ng/ml and >17.14 ng/ml). Values are odds ratio (OR) adjusted for age, sex, alcohol consumption, presence of type 2 diabetes and cardiovascular disease. Number of subjects in each group is shown in the column. Group 1(n=101)-reference (OR=1.0), group 2(n=46)-high GGT and low adiponectin (OR=1.89, 95%CI 1.29 – 4.49, p=0.026), group 3 (n=72)- low GGT and high adiponectin (OR= 1.94, 95%CI 1.64 -3.22, p=0.004), group 4 (n= 75)- high GGT and high adiponectin (OR=4.72, 95%CI 2.58 – 8.65, p=0.001). Of note, both higher serum GGT (>30U/L) and adiponectin (>17.14 ng/L) levels are synergistically associated with prolonged LOS.

[4]: [Figure 2]

Among 35 evaluated laboratory variables and 20 clinical characteristics at admission (n=1820 consecutive older HF patients) multivariate analysis revealed seven factors (four metabolic – hypoalbuminaemia, GGT/ALT ratio >2.5, vitamin D deficiency and hyperparathyroidism and three clinical – history of MI, CKD and COPD) as strong and independent indicators of in-hospital mortality [9]. The mortality rate increased stepwise as the number of these RFs increased (from 0.43% – none RF to 16.8% ≥ 4 RF); the prognostic value of any single RF was low (AUCs of ≤ 0.635) but combination of 2 or more RFs improved the prediction significantly, and AUC reached 0.839 when ≥ 4 RFs (vs. 0–1RF) were present.

Of note, to evaluate the prognostic and prediction value of metabolic status/liver function reserve we assessed 10 ratios (combined indices) regarding the outcomes [9]. Univariate analysis showed that 6 of 10 ratios were associated with in-hospital death. In non-survivors the GGT/ALT, ALT/ALP and GGT/ bilirubin ratios were significantly higher, while albumin/ALP and albumin/bilirubin ratios were significantly lower than in survivors. However, in multivariate analysis only the GGT/ALT ratio remained as a significant and independent determinant of in-hospital mortality. The GGT/ALT ratio was more closely related to survival than the serum activities each of the two enzymes. The multivariate analyses of GGT/ALT ratio both as a continuous and as a categorical (GGT/ALT >2.5) variable demonstrated its significant association with in-hospital mortality.

With regard to presence of two RFs (vs. no such RFs, Figure 8), the highest AUC for a fatal outcome showed the combination of CKD with albumin <33 g/L (AUC 0.698), 25(OH)D <25 nmol/L (AUC 0.698), PTH >6.8 pmol/L (AUC 0.695) or GGT/ALT >2.5 (AUC 0.694), followed by combination of elevated PTH with low albumin (AUC 0.677) or history of MI (AUC 0.674) or GGT/ALT >2.5 (AUC 0.673). Importantly, the specificity was 98.1% - 91.1% in five combinations and 84.1%–89.2% in five other combinations (Figure 9).

These easily identifiable RFs appeared as a useful objective tool for clinical risk stratification of HF patients at admission and, when combined, provided prognostic information more accurate than most of currently proposed models.

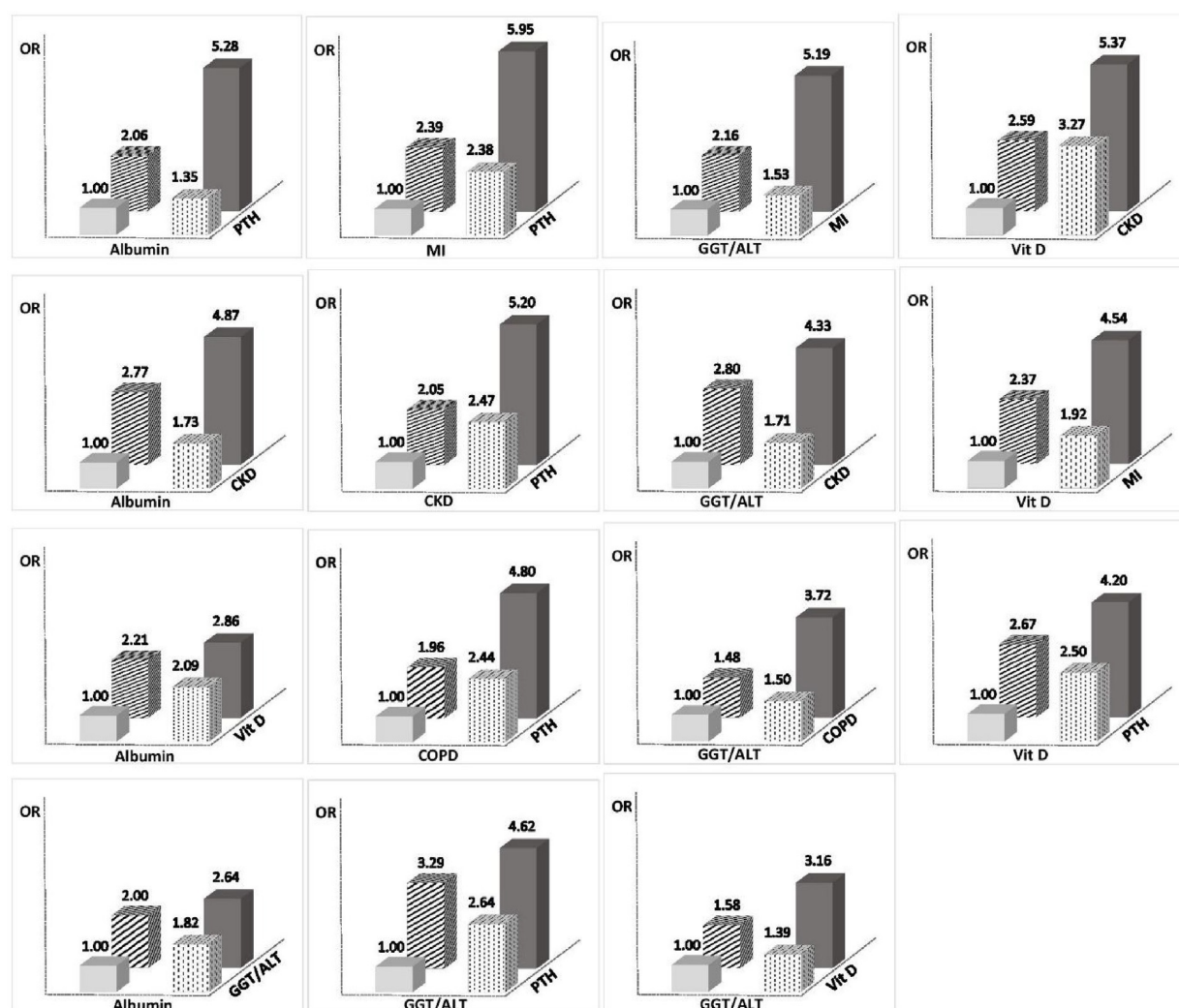


Figure 8. Prognostic performance of the 2-marker combinations (comparing patients with and without indicated factors) for predicting in-hospital mortality in older hip fracture patients.

The laboratory parameters were dichotomised as follows: albumin <33 g/l vs. ≥ 33 g/L; PTH >6.8 pmol/L vs. ≤ 6.8 pmol/L; GGT/ALT >2.5 vs. ≤ 2.5 ; 25-hydroxyvitamin D < 25nmol/L vs. ≥ 25 nmol/L. All clinical characteristics were dichotomised as yes vs. no. The abnormal conditions are depicted on the right hand side. Abbreviations: PTH, parathyroid hormone; CKD, chronic kidney disease; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; Vit D, 25- hydroxyvitamin D. [9]: [Figure 1]

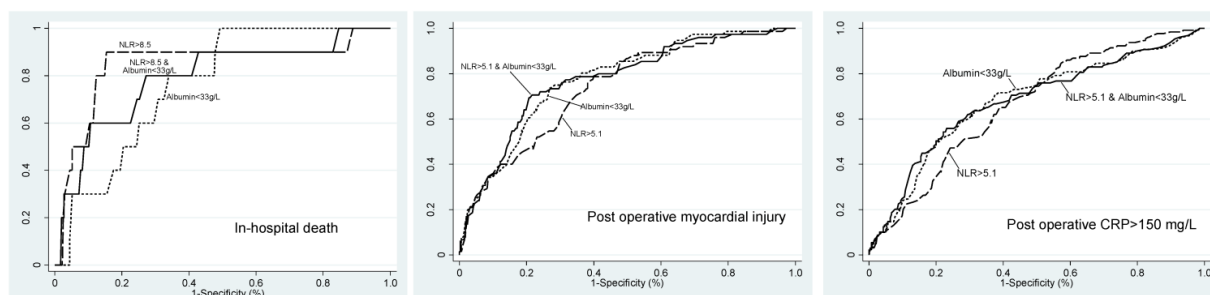


Figure 9. Receiver operating characteristics curves for predicting in-hospital mortality, postoperative myocardial injury with troponin I rise and high inflammatory response (with CRP>150 mg/L) using low albumin, elevated NLR and combination of both parameters.

NLR, neutrophil to lymphocyte ratio; CRP, C-reactive protein.

[6]: [Figure 3]

Next, we assessed the predictive value of serum urea levels at the time of admission (n=1819 older HF patients) [10]. Our results demonstrated that elevated serum urea levels (>7.5mmol/L) have a substantial impact on prognostication hospital outcomes, in particular, mortality and developing a high postoperative inflammatory response (HPIR), being associated with 2.6-fold and 2.2-fold greater odds, respectively. Patients with increased admission urea and HPIR demonstrated almost 10 times greater mortality odds compared to patients without such characteristics.

Urea level expressed both as a continuous and categorical variable demonstrated a significant association with poor outcomes in HF patients; it appears to be an integrative measure reflecting the most severe changes in protein metabolism, nutritional and hydration status, and, therefore, its quantification should not be ignored in predictive models. For fatal outcome urea level >10mmol/L was a stronger contributor than any other biochemical or clinical variable at admission.

Integration of two characteristics (urea>7.5mmol/L plus one traditional risk factor) materially improves the prediction of mortality and HPIR, as evidenced by increases in AUCs. Among combined parameters at admission highest AUC for prediction mortality yielded elevated urea (>7.5mmol/L) plus low albumin (AUC 0.7731) or GGT/ALT>2.5 (AUC 0.7698) (Figure 10) and for prediction HPIR elevated urea plus anaemia (AUC 0.6928) or increased creatinine (AUC 0.6561) or GGT/ALT>2.5 (AUC 0.6418).

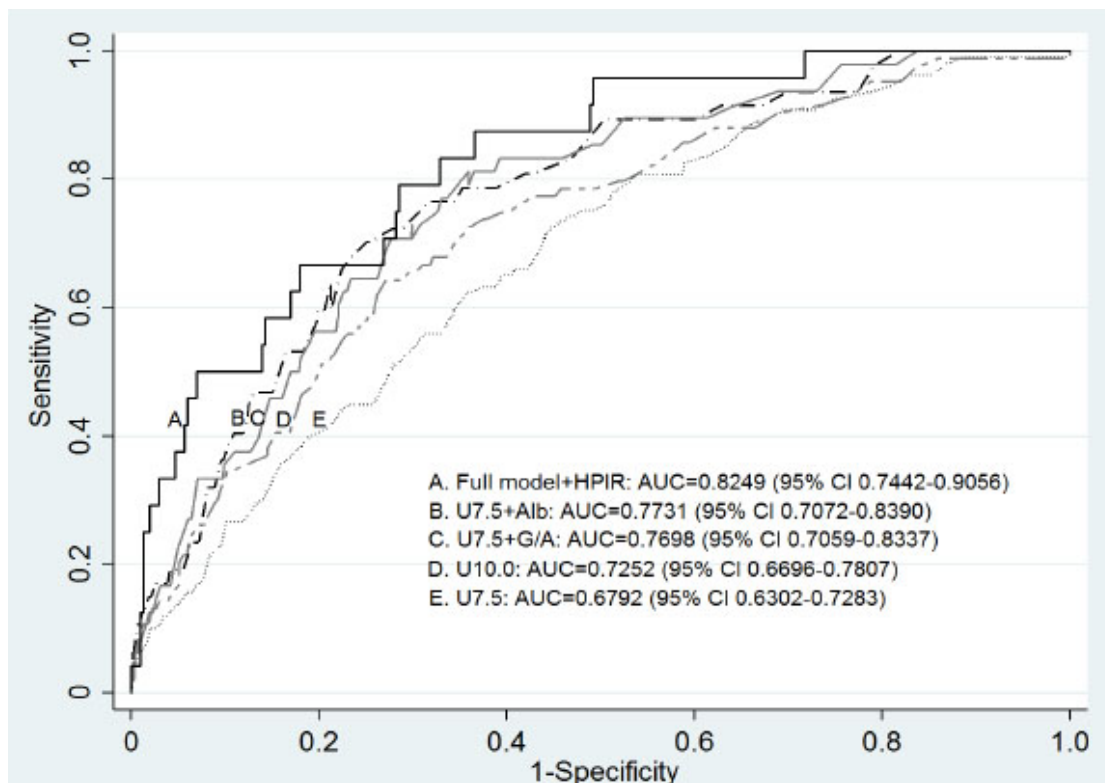


Figure 10. Receiver-operating characteristic curves (ROC) of elevated serum urea levels at admission (alone and in combination with other factors) for predicting the all-cause in-hospital mortality in older patients with hip fracture.

A (solid line), ROC curve based on data from the full model (8 on admission independent risk factors plus HPIR vs. 0 risk factors); B, ROC curve based on combination of urea>7.5mmol/L and albumin <33g/L; C, ROC curve based on combination of urea>7.5mmol/L and GGT/ALT>2.5; D, ROC curve based on urea>10.0mmol/L and E, ROC curve based on urea>7.5mmol/L.

AUC, area under receiver operating characteristic curve; CI, confidence interval; HPIR, high postoperative inflammatory response; U7.5, serum urea>7.5mmol/L; Alb, serum albumin<33g/L; G/A, gamma-glutamyl transferase/alanine aminotransferase ratio (GGT/ALT>2.5); U10.0, serum urea>10.0mmol/L. [10];[Figure 1]

Our validation studies [6, 7, 9, and 10] confirmed the prognostic value of the abovementioned variables.

Taking together, our results indicate that in orthogeriatric patients, particularly with HF, specific liver (albumin<33g/L, GGT>30U/L, GGT/ALT >2.5) and related (urea >7.5mmol/L, adiponectin >17.14 ng/ml, NLR>5.1) markers at admission give reasonable prognostic information for short-term outcomes. These novel predictors of postoperative adverse outcomes are based on routinely collected objective data, simple, easy to use and have a fair precision rate, especially when two parameters are measured in parallel.

4.2.3. Practical therapeutic considerations

Firstly, our observations indicate the importance of screening for and treating the modifiable metabolic alterations such as vitamin D and vitamin K insufficiency, hypoalbuminaemia, hyperparathyroidism and iron deficiency in the pre-fracture management of elderly persons. These factors are prevalent, exert calciotropic and noncalciotropic effects, and contribute to liver functions, bone metabolism, occurrence of osteoporotic fractures and in-hospital outcomes in orthogeriatric

patients. Therefore, the pre-fracture preventive strategies should focus on treatments that integrate these targets. Such approach may have numerous beneficial effects on falls, fractures and a wide range of common chronic inflammatory, metabolic and autoimmune diseases prevention and treatment, including CLDs.

Although routine measurement of serum 25(OH)D concentrations does not provide additional information for the diagnosis of CLD or osteoporosis, it appear to have a role in the management of these patients. Inverse associations of circulating 25(OH)D with progression, severity of CLD, and many other diseases as well as beneficial effects of vitamin D supplementation on certain health outcomes has been documented in the literature.^{24, 27-30} In regard to abnormal iron status, also a preventable and curable factor, it should be realised that ferritin levels are not specific for iron overload, and this parameter has to be interpreted with caution. Moderately elevated ferritin levels, as in our study, especially if combined with a low TSAT (an index of the amount of iron molecules available in the serum that are bound to transferrin), may reflect multiple systemic processes, especially with an inflammatory component. Therefore, the recommendation of lowering “iron overload” (based on serum ferritin levels only) by chelation therapy ([5]:9) or by a biologically active form of hepcidin ([5]:8) for the treatment of osteoporosis seems premature if not inappropriate, specifically in the elderly, in whom osteoporosis, iron-deficiency, chronic inflammation and renal impairment are common. Therapeutic manipulation of iron balance in this population is challenging; to be effective and safe it should be based on detailed assessment of pathogenic mechanisms and individualised.

Secondly, our results showing the prognostic value of specific liver-related biomarkers at admission as predictors of short-term outcomes suggest that these factors may be useful additional indicators for risk stratification, therapeutic selection and preventive intervention in hospitalised orthogeriatric patients. The importance of identification of individuals in whom poor outcome should be suspected and, if possible, prevented is obvious; more than 50% of hospital deaths are classified as “at least possibly preventable” ([10]:70).

Markers discriminatory for survival may be (in conjunction with a comprehensive clinical assessment) helpful in preoperative predicting a fatal outcome and aid decision making with regard to operative plan.

The clinical relevance of preoperative identification of subjects with a high risk of myocardial injury/infarction, a serious and often silent (asymptomatic in >80% of patients) complication associated with short- and long-term morbidity/mortality is emphasized by the reports that beta-blockers, alfa-2 agonists, calcium channel blockers, statins, and aspirin can prevent postoperative myocardial injury and reduce the risk of mortality ([6]:121-124).

Similarly, prediction of perioperative infectious complications might be useful when considering empirical antibiotic therapy. Prognostication, prevention and appropriate treatment of these conditions in the frail orthogeriatric population may improve outcomes, especially prolonged LOS. Currently, HFs represent about two-thirds of all hospital days due to fracture and account for more hospital days than any other musculoskeletal injury ([4]:228).

To conclude, our results showed that metabolic alterations reflecting the liver-bone interactions should be integrated in pre-fracture preventive strategies. In orthogeriatric patients, liver-related biomarkers at admission can significantly decrease the

ambiguity regarding the outcomes and help to timely initiate individualized appropriate management.

5. Limitations and strengths

Several limitations of our studies should be considered. First, because of its cross-sectional design the causative nature of the associations cannot be established. Second, a single-point measurement of the biomarkers may be subject to within-individual variation, although the majority of biochemical tests, including serum GGT, ferritin, iron, OC, P1NP, indices of mineral metabolism are relatively stable and have no circadian and between-day variations. Third, we measured serum GGT activity and total adiponectin, but the GGT fractions and high-molecular weight adiponectin, shown in some, but not all ([4]:185) studies to be more informative, were not assessed. Forth, potential effects of confounding from variables not represented in our models (e.g. some medication used) cannot be excluded. Finally, as the cohorts were mainly Caucasian our findings may not be generalizable to other ethnic and racial groups.

The strengths of our studies are their prospective design, comprehensive clinical and laboratory evaluations, simultaneously measuring multiple parameters of liver, iron and bone metabolism in a moderately large sample size and validating the results in separate cohorts, whereas most of previous clinical studies assessed a limited number of variables and often did not control for important factors. Although multiple comparisons may potentiate the significance of multicollinearity phenomena in multivariate regression analysis, the variance inflation factor in all presented models was less than 1.3, indicating that the amount of multicollinearity was not significant.

6. Conclusions

Over the last decade we have undertaken ten studies in different pathophysiological and clinical aspects of liver - bone interactions. Our findings increased the evidence surrounding the idea that the liver and skeleton in health and disease share common molecular mechanisms related to their physiology, as well as disease development, pathogenesis, progression and complications. We highlighted in our papers the concept that liver-bone links are critical for maintaining homeostasis in health and disease, provided a framework for understanding the associations between these conditions and presented practical applications of an integrative approach, particularly for orthogeriatric patients. We proposed and evaluated new/non-traditional previously undescribed diagnostic and prognostic biomarkers for different clinical conditions. We discussed the basis and clinical need for an integrative management of adults with CLD and bone diseases. Comprehensive understanding of the mechanisms underlying the liver-bone interaction may lead to management based on a more holistic approach. We concluded that physicians should be aware of the effects of even mild liver dysfunction (often age-related) on bone status and *vice versa*, and, therefore, should consider strategies to manage both conditions simultaneously. The additional (albeit moderate) prognostic information to in-hospital outcomes provided by liver-related markers, accurate and not expensive laboratory tests, could be helpful in early identification and appropriate treatment of frail orthogeriatric patients. However, confirmation in other cohorts is needed to further support the applicability of these prognostic parameters to the total geriatric population.

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Vitamin D and Parathyroid Hormone in Outpatients With Noncholestatic Chronic Liver Disease

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Background & Aims: The liver plays a central role in vitamin D metabolism. Our aim was to determine the prevalence and type of vitamin D–parathyroid hormone (PTH) disturbance in ambulatory patients with noncholestatic chronic liver disease (CLD) and its relationship with disease severity and liver function. **Methods:** We studied 100 consecutive outpatients (63 men, 37 women; mean age, 49.0 ± 12.1 [SD] y) with noncholestatic CLD caused by alcohol (n = 40), hepatitis C (n = 38), hepatitis B (n = 12), autoimmune hepatitis (n = 4), hemochromatosis (n = 4), and nonalcoholic steatohepatitis (n = 2); 51 patients had cirrhosis. Serum concentrations of 25-hydroxyvitamin D (25[OH]D), PTH, calcium, phosphate, magnesium, creatinine, and liver function tests were determined. **Results:** Serum 25(OH)D levels were inadequate in 91 patients: vitamin D deficiency (<50 nmol/L) was found in 68 patients and vitamin D insufficiency (50–80 nmol/L) was found in 23 patients. Secondary hyperparathyroidism (serum PTH, >6.8 pmol/L) was present in 16 patients. The prevalence of vitamin D deficiency was significantly higher in cirrhotic vs noncirrhotic patients (86.3% vs 49.0%; $P = .0001$). In Child–Pugh class C patients, 25(OH)D levels were significantly lower than in class A patients (22.7 ± 10.0 nmol/L vs 45.8 ± 16.8 nmol/L; $P < .001$). Serum 25(OH)D independently correlated with international normalized ratio (negatively; $P = .018$) and serum albumin (positively; $P = .007$). Serum 25(OH)D levels of less than 25 nmol/L predicted coagulopathy, hyperbilirubinemia, hypoalbuminemia, increased alkaline phosphatase, and anemia and thrombocytopenia. **Conclusions:** Vitamin D inadequacy is common in noncholestatic CLD and correlates with disease severity, but secondary hyperparathyroidism is relatively infrequent. Management of CLD should include assessment of vitamin D status in all patients and replacement when necessary.

The liver is a major organ in the vitamin D endocrine system. To function physiologically vitamin D must first be converted in the liver to 25-hydroxyvitamin D (25[OH]D), the main circulating form of vitamin D, and this in turn is converted in the kidney into 1, 25-dihydroxyvitamin D, the active metabolite.^{1–3} Liver cells along with parathyroid glands and kidneys express a calcium-sensing receptor⁴ that plays a critical role in regulating systemic calcium homeostasis.

In recent years it has been recognized that the vitamin D endocrine system is not only the principal regulator of calcium and phosphate homeostasis and bone metabolism, but it also exerts potent noncalcitropic functions including antiproliferative, prodifferentiative, and immunomodulatory activities.³ Vitamin D insufficiency has been linked, apart from osteoporosis,

to a wide range of inflammatory, autoimmune, and metabolic disorders and malignancies.⁵ On the other hand, the normal liver is a target organ for the vitamin D endocrine system⁶ and parathyroid hormone (PTH).^{7,8}

Although chronic liver disease (CLD), especially cholestatic, alcoholic, and in advanced stages from any causes, often (20%–60%) is complicated by bone disease,^{9–11} the clinical relevance of vitamin D–PTH disturbances in hepatic osteodystrophy still is unclear.^{9,12–22}

Vitamin D deficiency traditionally is considered to cause secondary hyperparathyroidism and this has been observed in up to 42% of patients with CLD in some studies,^{16,22} whereas in other studies the PTH levels were normal²³ or even low.^{24–26}

The few studies correlating 25(OH)D–PTH status and severity of the liver injury reported conflicting results. Some investigators^{13,18,25–28} have suggested that 25(OH)D levels decrease with disease progression, but others did not find differences between cirrhotic and noncirrhotic patients²² or between Child–Pugh groups.²⁴

Despite accumulating evidence that vitamin D has a number of actions that may be relevant to liver function and CLD, including the regulation of secretion of metalloproteinases and their inhibitors, fibroblast proliferation, and collagen synthesis,^{29,30} currently the evaluation and correction of vitamin D status is not part of the routine management of these patients.

It also should be noted that most work has been performed in patients with chronic cholestatic liver disease (particularly primary biliary cirrhosis) and studies often were limited by small numbers. In the present study we investigated vitamin D and PTH status in a diverse ambulant group of patients with noncholestatic CLD and a range of disease activity. Our aims were to determine the prevalence, extent, and type of disturbances in calcium–vitamin D–PTH status and its relationship with the severity of disease and liver function injury.

Materials and Methods

Patients

One hundred consecutive patients attending the outpatient clinic of the Gastroenterology Department at Canberra

Abbreviations used in this paper: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLD, chronic liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus; GGT, γ -glutamyltransferase; INR, international normalized ratio; MMP, matrix metalloproteinase; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.

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1542-3565/07/\$32.00
doi:10.1016/j.cgh.2006.10.015

Hospital in whom there was a confirmed diagnosis of noncholestatic CLD were included in this study. The group consisted of 63 men and 37 women, with a mean age of 49.0 ± 12.1 (SD) years. The cause of CLD was alcohol use ($n = 40$), viral hepatitis C ($n = 38$), viral hepatitis B ($n = 12$), autoimmune hepatitis ($n = 4$), hemochromatosis ($n = 4$), and nonalcoholic steatohepatitis ($n = 2$). The diagnosis of CLD was based on consistent clinical findings, serologic markers (antibodies to hepatitis C virus [anti-HCV], hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B core antibody, hepatitis B e antigen, hepatitis B e antibody, hepatitis B virus DNA, and HCV RNA measurements by polymerase chain reaction, autoantibodies [antinuclear antibody, anti-smooth muscle antibody]), biochemical features (including iron studies and hemochromatosis gene test), endoscopic ($n = 50$) and imaging (including ultrasound, $n = 100$) evidence, and histologic examinations (liver biopsy examination, $n = 37$). Of 38 patients with CLD as a result of HCV, 8 subjects (including 4 with cirrhosis) had a history of alcohol overuse; the HCV cause was confirmed by liver biopsy examination in 2 of these patients. None of the patients had steatorrhea. The severity of cirrhosis was graded using the Child-Pugh score and patients were grouped into 3 categories: class A (scores 5–6; $n = 15$), class B (scores 7–9; $n = 16$), or class C (scores 10–15; $n = 20$). The Model for End-Stage Liver Disease score also was calculated according to the United Network for Organ Sharing formula.³¹ None of the patients received vitamin D or calcium supplements, bisphosphonates, calcitonin, or hormone replacement therapy. Twenty-one patients were treated with spironolactone, 14 with furosemide, 6 with lamivudine (one of whom was also on adefovir), 5 with combination peginterferon alfa-2a or 2b and ribavirin, and 4 were on corticosteroids. Seven other patients had previously received either standard or pegylated interferon alone or in combination with ribavirin for hepatitis C treatment.

All patients were residents of the Canberra region (latitude, $33^\circ 15'$ S). All patients gave their informed consent to participate in the study, which was approved by the local ethics review committee.

Laboratory Analysis

Samples of venous blood were obtained in the morning after an overnight fast and were kept frozen at -70°C for the assay of 25(OH)D and intact PTH. The tests were performed using commercially available kits according to the manufacturers' instructions. 25(OH)D was measured with ^{125}I radioimmunoassay kit (DiaSorin, Stillwater, MN); the intra-assay and interassay coefficient of variation were 8.6% and 9.4%, respectively. The laboratory reference range was 31–107 nmol/L. Intact PTH was measured by 2-site chemiluminescent enzyme-labeled immunoassay for the 1–84 amino acid chain on the Immunolite 2000 auto-analyzer (Diagnostics Products Corporation, Los Angeles, CA); intra-assay and interassay coefficients of variation were 5.2% and 6.3%, respectively. The laboratory reference range was 1.3–6.8 pmol/L.

All subjects had serum total calcium, phosphate, magnesium, albumin, total bilirubin, aminotransferases, alkaline phosphatase, prothrombin time, creatinine, urea, sodium, potassium, glucose, hemoglobin, and full blood count determined by routine laboratory techniques. The serum calcium level was corrected for albumin concentration; the international normalized ratio (INR) for prothrombin time was calculated.

On the basis of data reported in the literature,^{32,33} the serum 25(OH)D concentration was defined as deficient when it was less than 50 nmol/L (severe deficiency, <12.5 nmol/L; moderate deficiency, 12.5–25 nmol/L; mild deficiency, 25–49 nmol/L), insufficient when it was 50–80 nmol/L, and sufficient (adequate, desirable, normal) when it exceeded 80 nmol/L.

Statistical Analysis

Statistical analyses were performed with a statistical software package (Stata version 7; Stata Press, College Station, TX). Data are presented as mean values and SDs. For differences between groups, significance was assessed using an unpaired 2-tailed Student *t* test for continuous variables and the Pearson χ^2 test with the Fisher exact test for categorical variables. Correlations between 25(OH)D, PTH, biochemical markers of liver function, and demographic parameters were examined using linear regression analysis and the Pearson correlation coefficient. Multiple linear regression analysis was performed to identify independent variables associated with a low 25(OH)D level. The appropriateness of the regression model was assessed by Jack-knife residuals, Cook's *d*, and Mallow's *C_p*. A *P* value of less than .05 was considered statistically significant.

Results

Patient Characteristics

The patients were diverse in age, nature, and severity of their noncholestatic CLD. Table 1 summarizes the demographic, etiologic, and main biochemical and hematologic data on all subjects studied. The age of the patients ranged from 22 to 76 years. The cirrhotic patients were significantly older than the noncirrhotic patients, but there was no difference between the 2 groups regarding sex, with a male predominance in both groups. The main causative factor for cirrhosis was alcohol (72.5%), whereas in the noncirrhotic group it was viral hepatitis C (57.1%) and B (22.4%). As expected, the biochemical parameters of liver function and hematologic characteristics differed significantly between the cirrhotic and noncirrhotic patients. The former group had higher mean INR values, increased concentrations of serum bilirubin, alkaline phosphatase (ALP), γ -glutamyl transferase, and lower serum albumin, alanine aminotransferase, hemoglobin, and platelets. These differences were not related to sex and were more pronounced in advanced stages of cirrhosis. No differences were seen between the groups for creatinine and urea concentrations.

Vitamin D Status

In total, inadequate vitamin D status, defined as a serum 25(OH)D level lower than 80 nmol/L, was present in 91 patients, vitamin D deficiency (<50 nmol/L) was seen in 68 patients, and insufficiency (50–80 nmol/L) was seen in 23 patients. Only 9 noncirrhotic patients showed a normal (>80 nmol/L) serum 25(OH)D concentration (Table 2). There was no difference in serum 25(OH)D levels between the sexes (42.6 ± 28.0 nmol/L in men vs 43.3 ± 21.9 nmol/L in women).

The severity of CLD and the stage of cirrhosis according to Child-Pugh classification and Model for End-Stage Liver Disease score showed significant correlation with the serum 25(OH)D concentration (Table 2). The mean serum concentration of 25(OH)D was significantly lower in patients with cirrhosis compared with noncirrhotic patients. When patients

Table 1. Demographic, Clinical, Hematologic, and Serum Biochemical Characteristics of Studied Patients With Noncholestatic CLD

Characteristics	Cirrhotic patients (n = 51)	Noncirrhotic patients (n = 49)	P value
Age, y			
Range	37–76	22–66	
Mean $\pm$ SD	55.0 $\pm$ 9.6	42.8 $\pm$ 11.2	<.001
Men, n (%)	36 (70.6%)	27 (55.1%)	.163
Disease cause			
Alcohol, n (%)	37 (72.5%)	3 (6.1%)	<.001
HCV, n (%)	10 (19.6%)	28 (57.1%)	<.001
HBV, n (%)	1 (2.0%)	11 (22.4%)	.005
Nonalcoholic steatohepatitis	2 (3.9%)	0	.493
Autoimmune	1 (2.0%)	3 (6.1%)	.581
Hemochromatosis	0	4 (8.2%)	.116
Total bilirubin level, $\mu\text{mol/L}$	59.7 $\pm$ 63.5	12.6 $\pm$ 12.6	<.001
Albumin level, g/L	31.8 $\pm$ 8.6	41.7 $\pm$ 3.4	<.001
ALT level, U/L	49.4 $\pm$ 38.9	122.8 $\pm$ 189.8	.010
ALP level, U/L	175.4 $\pm$ 118.9	93.0 $\pm$ 38.7	<.001
GGT level, U/L	231.8 $\pm$ 302.3	103.2 $\pm$ 113.6	.006
INR, U/L	1.5 $\pm$ 0.36	1.1 $\pm$ 0.24	<.001
Hemoglobin level, g/L	116.1 $\pm$ 24.7	146.6 $\pm$ 15.9	<.001
Platelet level, $\times 10^9/\text{L}$	151.5 $\pm$ 87.9	235.9 $\pm$ 50.0	<.001
Creatinine count, $\mu\text{mol/L}$	80.6 $\pm$ 28.1	75.4 $\pm$ 13.2	.236
Urea level, mmol/L	6.0 $\pm$ 6.1	4.9 $\pm$ 1.5	.218

HCV, hepatitis C virus; HBV, hepatitis B virus; ALT, alanine aminotransferase; GGT, γ -glutamyltransferease.

were classified according to their severity of liver disease there was a consistent trend for lower 25(OH)D levels with increasing severity of cirrhosis. None of the cirrhotic patients had a desirable level of serum 25(OH)D. The percentages of subjects with severe to moderate vitamin D deficiency (<25 nmol/L) were as follows: Child–Pugh class A, 6.7%; class B, 43.8%; and in class C, 65%. The mean 25(OH)D concentration in class C patients was 2 times lower than in class A patients ($P = .001$).

Calcium, Phosphate, and Magnesium

The mean concentrations of corrected calcium, inorganic phosphate, and magnesium showed no difference between cirrhotic and noncirrhotic patients, nor among cirrhotic subgroups. However, a low serum magnesium level (<.70 mmol/L) was found more often in subjects with cirrhosis than in noncirrhotic patients (16 of 51 vs 2 of 49; $P = .001$). Of 16

Table 2. Serum Concentrations of 25(OH)D, PTH, Calcium, Phosphate, and Magnesium in Patients With Cirrhosis Classified According to Child–Pugh Score and Noncirrhotic Patients With CLD

	Cirrhotic patients				Noncirrhotic patients (n = 49)	<i>P</i> value ^a
	Child–Pugh class			Total (n = 51)		
	A (n = 15)	B (n = 16)	C (n = 20)			
Age, y	53.4 ± 9.1	53.9 ± 10.0	57.1 ± 9.8	55.0 ± 9.6	42.8 ± 11.2	<.001
Men, n (%)	8 (53.3%)	12 (75.0%)	16 (80%)	36 (70.6%)	27 (55.1%)	.163
25(OH)D, nmol/L	45.8 ± 16.8	32.4 ± 20.1	22.7 ± 10.0	32.6 ± 18.2	53.6 ± 28.3	<.001
<25 nmol/L, n (%)	1 (6.7%)	7 (43.8%)	13 (65.0%)	21 (41.2%)	7 (14.3%)	.006
25–49 nmol/L, n (%)	9 (60.0%)	7 (43.8%)	7 (35%)	23 (45.1%)	17 (34.7%)	.005
50–80 nmol/L, n (%)	5 (33.3%)	2 (12.5%)	0	7 (13.7%)	16 (32.6%)	.010
>80 nmol/L, n (%)	0	0	0	0	9 (18.4%)	.005
Calcium level, mmol/L	2.33 ± 0.10	2.37 ± 0.12	2.37 ± 0.14	2.36 ± 0.12	2.35 ± 0.09	.639
Phosphate level, mmol/L	1.17 ± 0.20	1.13 ± 0.31	1.06 ± 0.28	1.11 ± 0.27	1.15 ± 0.20	.401
Magnesium level, mmol/L	0.76 ± 0.11	0.76 ± 0.11	0.77 ± 0.18	0.76 ± 0.14	0.84 ± 0.09	.085
PTH level, pmol/L	4.7 ± 2.3	3.8 ± 2.4	4.7 ± 3.0	4.4 ± 2.6	4.7 ± 2.7	.572
>6.8 pmol/L, n (%)	3 (20.0%)	3 (18.8%)	2 (10.0%)	8 (15.7%)	8 (16.3%)	1.000
<1.3 pmol/L, n (%)	0	2 (12.5%)	1 (5.0%)	3 (5.9%)	3 (6.1%)	1.000
Child–Pugh score	5.4 ± 0.51	8.0 ± 0.89	11.4 ± 1.35	8.6 ± 2.69		
MELD score ^b	9.0 ± 1.88	12.5 ± 3.80	19.8 ± 4.26	11.0 ± 5.61	7.58 ± 2.25	<.001

^aComparing cirrhotic and noncirrhotic patients.

^bModel for End-Stage Liver Disease (MELD) score was calculated according to the United Network for Organ Sharing formula.³¹

Table 3. Demographic, Biochemical, and Hematologic Data in Patients With CLD by Serum 25(OH)D Concentration

	Serum 25(OH)D, nmol/L				P value ^a
	<25 (n = 28)	25–49 (n = 40)	50–80 (n = 23)	>80 (n = 9)	
Age, y	51.4 ± 11.6	48.6 ± 11.5	48.9 ± 13.8	43.7 ± 11.4	.412
Men, n (%)	19 (67.9%)	26 (65.0%)	12 (52.2%)	6 (66.7%)	.529
25(OH)D, nmol/L	16.9 ± 6.0	37.7 ± 6.5	60.6 ± 7.7	101.3 ± 22.7	<.001
INR	1.5 ± 0.4	1.3 ± 0.4	1.1 ± 0.1	1.0 ± 0.1	<.001
Bilirubin level, μ mol/L	62.8 ± 63.1	37.0 ± 54.7	15.6 ± 11.9	7.3 ± 2.4	.002
Albumin level, g/L	29.4 ± 7.5	38.1 ± 7.9	41.0 ± 4.7	41.6 ± 3.0	<.001
ALT level, U/L	109.4 ± 240.6	68.2 ± 75.8	77.3 ± 57.4	107.7 ± 75.8	.637
ALP level, U/L	184.2 ± 105.4	126.3 ± 108.2	108.3 ± 61.4	89.0 ± 19.5	.094
GGT level, U/L	197.2 ± 178.9	196.6 ± 328.4	115.2 ± 119.8	94.0 ± 74.2	.396
Creatinine level, μ mol/L	85.3 ± 30.6	76.6 ± 14.5	73.3 ± 23.6	74.2 ± 8.7	.211
Urea level, mmol/L	6.3 ± 7.9	5.1 ± 2.0	5.4 ± 1.9	4.7 ± 1.0	.714
Calcium corrected, mmol/L	2.35 ± .10	2.35 ± .12	2.37 ± .10	2.35 ± .09	.828
Phosphate level, mmol/L	1.11 ± .23	1.15 ± .29	1.15 ± .18	1.09 ± .18	.803
Magnesium level, mmol/L	.76 ± .15	.82 ± .13	.80 ± .08	.83 ± .04	.168
PTH level, pmol/L	4.8 ± 3.0	4.2 ± 2.7	4.6 ± 2.2	5.5 ± 2.2	.527
>6.8 pmol/L, n (%)	4 (14.3%)	5 (12.5%)	4 (17.4%)	3 (33.3%)	.451
Hemoglobin level, g/L	116.5 ± 24.8	132.3 ± 25.2	138.7 ± 24.5	150.9 ± 7.9	<.001
Platelet count, $\times 10^9$ /L	166.0 ± 97.6	192.8 ± 80.6	207.0 ± 66.9	240.6 ± 61.5	.086

ALT, alanine aminotransferase; ALD, alkaline phosphatase; GGT, γ -glutamyltransferase; PTH, parathyroid hormone.

^aTest for linear trend.

cirrhotic patients with hypomagnesaemia, 6 had renal impairment and 1 was receiving corticosteroids.

Parathyroid Hormone Status

There was no difference in serum PTH concentrations between cirrhotic and noncirrhotic patients, nor between the Child-Pugh groups (Table 2). The percentage of patients with increased PTH levels (>6.8 pmol/L) was equal in both groups (15.7% and 16.3% in cirrhotic and noncirrhotic patients, respectively). Three patients in each group had PTH levels lower than the lower level of reference interval (<1.3 pmol/L). Of 16 patients with increased PTH levels, 3 had mildly increased serum creatinine concentrations (>90 μ mol/L, upper limit of reference range). Four of these 16 patients were taking spironolactone, 3 were taking furosemide, 1 was taking corticosteroids, and 1 was taking combination treatment with peginterferon alfa-2a and ribavirin. Of 6 patients with low PTH levels, 2 received spironolactone, 1 received furosemide, and 1 received corticosteroids. The chemistry test results showed no significant differences for albumin, calcium, phosphate, and magnesium among patients with increased or suppressed PTH levels compared with those with normal serum PTH concentrations. There were no differences in serum PTH levels between men and women.

Relationship Between Serum 25-Hydroxyvitamin D Level and Markers of Liver Disease, Parathyroid Hormone, Calcium, Phosphate, and Magnesium

Table 3 shows that when categorized by level of serum 25(OH)D, laboratory markers of CLD differed significantly and the severity of vitamin D inadequacy paralleled the changes. Patients with vitamin D deficiency (<50 nmol/L) had significantly higher values of INR, serum concentrations of bilirubin, ALP, and γ -glutamyl transferase, and lower levels of albumin,

hemoglobin, and platelets compared with subjects with vitamin D insufficiency (50–80 nmol/L) or normal (>80 nmol/L) 25(OH)D levels. The mean difference between the groups of patients with lowest and highest serum 25(OH)D levels were as follows: for INR, 0.5; for albumin, 12.2 g/L; for bilirubin, 55.5 μ mol/L; for ALP, 95.2 mmol/L; for γ -glutamyl transferase, 103.2 mmol/L; for hemoglobin, 34.4 g/L; and for platelets, 74.6×10^9 g/L. In contrast, the mean values of serum PTH, calcium corrected, and phosphate as well as age and sex showed no association with vitamin D status and no difference between groups with the lowest and highest 25(OH)D levels were seen.

The results of linear regression analysis relating serum 25(OH)D concentrations to laboratory indices of liver function, serum levels of PTH, calcium, phosphate, magnesium, creatinine, urea, hemoglobin, platelet count, age, and sex are shown in Table 4. Significant positive correlations were found between serum 25(OH)D levels and albumin, hemoglobin, and platelet count. There were significant negative correlations between the 25(OH)D concentration and INR, bilirubin, and ALP. There was no correlation between 25(OH)D and PTH, as well as calcium (corrected for albumin), phosphate, magnesium, parameters of renal function, age, or sex. Likewise, there was no correlation between serum PTH levels and any of the earlier-mentioned variables (not shown).

When multiple linear regression analysis was performed with 25(OH)D as the dependent variable and all other parameters with a P value of .15 or less as independent variables, the serum 25(OH)D level significantly and independently correlated only with INR (coefficient, -19.2 ; 95% CI, -35.1 to -3.4 ; $P = .018$) and albumin (coefficient, 1.06 ; 95% CI, 0.3 – 1.8 ; $P = .007$). This is not surprising because significant correlations existed between the indices of liver function. Pearson correlation coefficients were highly significant for albumin and bilirubin ($r = 0.524$; $P = .0001$), ALP ($r = 0.494$; $P = .001$), hemoglobin ($r = 0.700$; $P = .001$), and platelet count ($r = 0.461$; $P =$

Table 4. Results of Linear Regression Analysis in Patients With Noncholestatic CLD (n = 100) With Serum 25(OH)D as Dependent and Biochemical Markers of Liver and Renal Function, Serum PTH, Calcium, Hemoglobin, Platelet Count, Age, and Sex as Independent Variables

	Coefficient	95% CI	P value
Age	-0.3094	-0.733 to 0.115	.151
Sex	-0.6782	-11.340 to 9.984	.900
INR	-31.553	-44.120 to -18.987	<.001
Albumin level	1.520	0.971-2.069	<.001
ALT level	-0.003	-0.040 to 0.337	.862
AST level	-0.016	-0.193 to 0.160	.851
GGT	-0.017	-0.038 to 0.005	.128
Bilirubin level	-0.185	-0.277 to 0.092	<.001
PTH level	0.553	-1.462 to 2.569	.587
Calcium level	7.089	-42.481 to 56.659	.770
ALP level	-0.084	-0.134 to -0.034	<.001
Creatinine level	-0.208	-0.433 to 0.022	.076
Urea level	-0.339	-1.496 to 0.817	.562
Hemoglobin level	0.357	0.169-0.544	<.001
Platelet count	0.0826	0.003-0.126	.007

CI, confidence interval.

.001), as well as for INR and bilirubin ($r = 0.551$; $P = .0001$), ALP ($r = 0.334$; $P = .0007$), hemoglobin ($r = -0.563$; $P = .0001$), and platelet count ($r = -0.551$; $P = .0001$).

Table 5 summarizes the results of the logistic regression analysis regarding the degree of vitamin D deficiency (patients with serum 25[OH]D level >50 nmol/L used as a reference group). In noncholestatic CLD patients after adjustment for age and sex, moderate to severe vitamin D deficiency (serum 25[OH]D <25 nmol/L) predicts coagulopathy, hyperbilirubinemia, hypoalbuminemia, increased ALP concentration, anemia, and thrombocytopenia. Mild vitamin D deficiency (serum 25[OH]D 25-49 nmol/L) is predictive for the first 2 abnormalities whereas hypoalbuminemia and thrombocytopenia show borderline significance. In other words, there is a dose-response relationship between the serum 25(OH)D concentration and the presence of biochemical and hematologic abnormalities of CLD.

Taken together, these findings show that in CLD, serum 25(OH)D status is a significant predictor of liver injury, in particular for INR value, albumin level, bilirubin and hemoglobin concentrations, ALP activity, and platelet count.

Discussion

This study of 100 consecutive ambulatory noncholestatic CLD patients with a wide range of disease severity and diverse causes showed that the majority of these subjects (91%) had an inadequate vitamin D status. We showed that in CLD the prevalence and degree of vitamin D deficiency correlates with the severity and progression of liver disease, but secondary hyperparathyroidism is relatively uncommon and occurs in only 13% of those with vitamin D deficiency. Because the vitamin D endocrine system has important calciotropic and noncalciotropic functions, and vitamin D deficiency is easily preventable, heightened awareness is needed to ensure adequate vitamin D status in CLD patients.

Our data may indicate the significance of low vitamin D status both as a common complication of and a contributing factor to the pathogenesis of CLD. In our study, which describes a large diverse group of ambulatory noncholestatic CLD patients, the prevalence of vitamin D inadequacy (<80 nmol/L) was 100% in cirrhotic and 81.6% in noncirrhotic patients. The prevalence of vitamin D deficiency (<50 nmol/L) varied from 49% in noncirrhotic patients to 86.3% in cirrhotic patients. It was observed in all Child-Pugh class C patients, reflecting the severity of liver disease.

Low serum 25(OH)D concentrations have been reported in a variety of CLDs, especially in primary biliary cirrhosis^{34,35} and before orthotopic liver transplantation,^{15,23} but also in alcoholic and viral cirrhosis,^{12,13,16,17,26,27,36} noncirrhotic CLD,^{37,38} and hemochromatosis.³⁹ However, some researchers found no evidence of vitamin D insufficiency in cirrhosis,^{20-22,28} noncirrhotic viral liver disease,^{18,22} and hemochromatosis.⁴⁰ Furthermore, although in some studies serum 25(OH)D levels were reduced significantly in patients with decompensated cirrhosis,^{17,25,27} in others no differences between Child-Pugh groups were observed.²⁴ Because of heterogeneity of diseases and patients, as well as variable methods and definitions used in the assessment of vitamin D status, direct comparison of our data and previous studies cannot be performed. However, our find-

Table 5. Predicting Liver Insufficiency and Hematologic Abnormalities in Patients With CLD From Degree of Vitamin D Deficiency: Logistical Regression Analysis, Adjusted for Age and Sex

	25(OH)D < 25 nmol/L (n = 28)			25(OH)D 25-49 nmol/L (n = 40)		
	OR	95% CI	P value	OR	95% CI	P value
INR > 1.1	20.1	4.9-81.7	.000	8.4	2.5-27.7	<.001
Bilirubin level ≥ 21 $\mu\text{mol/L}$	18.2	4.3-76.2	.000	3.7	1.0-12.8	.042
Albumin level ≤ 34 g/L	24.7	9.6-93.3	.000	4.0	0.79-16.7	.060
ALP level > 110 U/L	9.4	2.5-34.5	.001	2.1	0.7-6.4	.191
Hemoglobin level < 120 g/L	10.4	2.2-50.6	.004	3.4	0.8-14.0	.092
Platelet level $< 150 \times 10^9$ /L	6.8	1.9-24.4	.003	3.2	0.9-11.0	.067
PTH level > 6.8 pmol/L	0.72	0.20-2.6	.017	0.44	0.13-1.5	.201

NOTE. Subjects with serum 25(OH)D level greater than 50 nmol/L constitute the reference group.

OR, odds ratio; CI, confidence interval.

ing of the high prevalence of vitamin D insufficiency/deficiency in noncholestatic CLD and its association with the severity of liver disease is in line with many previous studies.

Our study also showed that in noncholestatic CLD, vitamin D status correlates with and predicts in a dose-response manner liver function insufficiency such as increased prothrombin time, hypoalbuminemia, increased ALP activity, hyperbilirubinemia, and hematologic abnormalities (thrombocytopenia and anemia). This is an important A significant negative correlation between total bilirubin levels and bone mineral density in CLD was reported.^{11,41}

The strong relationship between both the prevalence and degree of vitamin D insufficiency and the severity of CLD, especially Child-Pugh class, may indicate specific impairment of vitamin D metabolism in the liver. Indeed, impaired 25-hydroxylation of vitamin D related to the degree of hepatic dysfunction has been reported in patients with alcoholic cirrhosis.^{12,34,42} In rats, bile duct ligation resulted in a 64% decrease in hepatic 25-hydroxylation of vitamin D.⁴³ In some studies, impairment of this enzymatic function was observed only in the advanced stages of CLD.⁴⁴ However, other studies claimed an adequate production of 25(OH)D even in advanced stages of CLD because after administration of oral or parental ergocalciferol or radiolabeled vitamin D to patients with cirrhosis and cholestasis, serum 25(OH)D concentrations become normal.^{45–47} It appears that reduced vitamin D hydroxylation in the liver could not be considered as the only or universal mechanism of low serum 25(OH)D levels in CLD.

Although we found a strong association between serum 25(OH)D concentration and liver injury, this does not establish the relationship as causal. One would expect older patients to have lower 25(OH)D levels. However, there was no age difference in our series. Other possible factors contributing to vitamin D insufficiency in CLD may include the following: (1) reduced exposure to sunlight (patients with CLD and greater liver function abnormalities possibly spend less time outdoors), (2) dietary insufficiency (particularly in alcohol-related CLD), (3) malabsorption, (4) low levels of serum proteins that bind with vitamin D, (5) effects of medications (antiviral drugs, glucocorticoids, drugs affecting hepatic cytochrome P450 enzymes involved in vitamin D metabolism,⁴⁸ and (6) impaired cutaneous synthesis of vitamin D in jaundiced patients. One could speculate that in individual CLD patients, inadequacy in vitamin D status is determined by different pathogenic factors.

However, irrespective of factors involved in the development of vitamin D insufficiency, which is likely to be multifactorial in CLD, this abnormality should not be ignored. Vitamin D insufficiency is a well-recognized risk factor for osteoporosis in the general population^{49,50} and increasingly is being recognized as a significant risk factor in a wide range of chronic inflammatory and autoimmune diseases (inflammatory bowel disease, rheumatoid arthritis, psoriasis, multiple sclerosis, diabetes mellitus), cancers (colon, prostate, breast), and metabolic disorders (metabolic syndrome, hypertension).^{2,3,5} There are a number of ways in which vitamin D may influence hepatic injury, fibrosis, and tissue remodeling. Vitamin D and its derivatives are potent regulators of cell proliferation, differentiation, and immunomodulation.³ These effects include inhibition of certain matrix metalloproteinases (MMPs) and induction of their inhibitors,³⁰ suppression of proliferation of fibroblasts, and increased collagen production.²⁹ Vitamin D insufficiency is associated with

increased circulating MMP-2 and -9, which is correctable by supplementation.⁵¹ Hepatocytes produce the major MMPs and tissue inhibitors involved in liver extracellular matrix remodeling.⁵² MMP-2 and -9 are of particular relevance to the liver because they are critically involved in the degradation of components of the basement membrane such as collagen IV and fibronectin, 2 main components of the space of Disse. Inhibition of MMPs protects from hepatic ischemic injury.^{53,54} Therefore, low serum 25(OH)D may, at least in part, contribute to the progression of liver disturbance in CLD, and correction of vitamin D insufficiency may represent an important therapeutic target in anticirrhotic strategies for CLD. On practical ground, in CLD an evaluation of the 25(OH)D serum level is necessary and oral vitamin D should be administered to maintain a 25(OH)D level of 80 nmol/L or more. However, a large intervention study in CLD patients with inadequate vitamin D status is needed to find whether supplementation with vitamin D will reduce the decrease in liver function over time.

Our data that severity of CLD, especially progression of cirrhosis, parallels the reduction in serum levels of 25(OH)D but not PTH changes are similar to the observations of others.^{15,23–25} No significant correlation was found between serum PTH and 25(OH)D levels in patients with end-stage liver disease.⁵⁵ Normal, low, or even undetectable levels of PTH were reported in primary biliary cirrhosis, other cirrhosis,²⁴ and before liver transplantation,²³ although some investigators found increased PTH levels in 3%–42%²² of cirrhotic and noncirrhotic patients. Interestingly, after liver transplantation an early transient increase in serum PTH levels without significant changes in serum 25(OH)D concentrations was reported.²³ Taken together, these observations do not support the view that reduced clearance capacity for PTH metabolites in the liver causes the PTH increase in advanced CLD.²²

PTH secretion is controlled by vitamin D and calcium via the vitamin D receptor and calcium-sensing receptor, respectively. A negative relationship between serum 25(OH)D and serum PTH is a well-known physiologic phenomenon. The threshold of serum 25(OH)D when serum PTH starts to increase is about 75–80 nmol/L.^{32,56,57} In our series 91 of 100 patients with CLD had 25(OH)D levels less than 80 nmol/L but only in 13 (14.3%) patients was the serum PTH concentration increased (>6.8 pmol/L). Moreover, of 68 subjects with vitamin D deficiency (25[OH]D, <50 nmol/L), secondary hyperparathyroidism was found in only 9 (13.2%) patients, although it was present in 3 of 9 patients with serum 25(OH)D levels higher than 80 nmol/L. The cause of normal to low PTH levels in the presence of vitamin D insufficiency and even severe deficiency observed in our study and other studies^{23–25} is unclear.

In our study the absence of compensatory increases in PTH level cannot be explained either by the use of antiviral drugs, glucocorticoids, spironolactone, or by disturbances in calcium, phosphate, and magnesium levels. Possible explanations may include vitamin D-receptor gene polymorphism, which is associated with hypoparathyroidism in chronic renal failure,⁵⁸ and suppression of PTH secretion by L-amino acids that activate calcium-sensing receptor.⁵⁹ Pathophysiologic mechanisms contributing to the vitamin D-PTH paradox in CLD require further study.

The present study had some limitations because of its cross-sectional design. A prospective study with administration of vitamin D, correction of serum 25(OH)D levels, and reassess-

ment of liver function tests is highly desirable. Another limitation of this study was that vitamin D, calcium, and protein intake were not assessed and the results may be applicable only to white populations. Finally, none of our patients had overt steatorrhea, however, it should be noted that stool fat was not measured.

In conclusion, vitamin D inadequacy is very common in noncholestatic CLD patients and correlates with the severity of the disease. Therefore, we recommend that clinical guidelines for managing CLD should include the assessment of vitamin D status (by measuring serum 25[OH]D concentrations) in all patients and initiating vitamin D replacement when necessary.

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Prevalence of vitamin K and vitamin D deficiency in patients with hepatobiliary and pancreatic disorders

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Received 17 August 2009; revised 8 September 2009; accepted 9 September 2009

Abstract

Little is known about the role of fat-soluble vitamins K and D in liver function and bone metabolism in biliary and pancreatic diseases associated with cholestasis and/or fat malabsorption. The aim of this study was to determine vitamin K of bone, vitamin D and parathyroid hormone status in patients with biliary and pancreatic disorders. In 90 consecutive patients (mean $\pm$ SD age, 65.5 ± 17.7 years; 45 females) undergoing endoscopic retrograde cholangiopancreatography (68 with choledocholithiasis, 14 with other benign condition, and 8 with cholangiopancreatic cancers) fasting concentrations of carboxylated (cOC) and undercarboxylated osteocalcin (ucOC), 25-hydroxyvitamin D, calcium, phosphorus, magnesium, prothrombin time, liver function tests, lipase, and creatinine were measured. Vitamin D deficiency (25-hydroxyvitamin D <50 nmol/L) was found in 45.6% of patients and elevated parathyroid hormone levels in 27.8%. The ratio ucOC/cOC (index of vitamin K deficiency) was above 20% in 50.6% of patients, above 30% in 31%, and above 50% in 18.4%. Hyperbilirubinemia was a significant independent predictor of low cOC (odds ratio [OR], 11.6; 95% confidence interval [CI], 1.9–59.4; $P = .07$). The ratio ucOC/cOC positively correlated with alanine aminotransferase levels ($r = 0.410$; $P < .001$). Elevated γ -glutamyltransferase (>180 U/L) and international normalized ratio (>1.1) levels were significant independent predictors of ucOC/cOC greater than 30% after adjustment for other covariants (OR, 5.5; 95% CI, 1.2–25.2; $P = .027$, and OR, 3.1; 95% CI, 1.1–8.8; $P = .036$, respectively). This study demonstrates that vitamin K and vitamin D deficiencies are common in patients undergoing endoscopic retrograde cholangiopancreatography. Liver dysfunction is associated with and predictive of vitamin K deficiency of bone and decreased production of osteocalcin, indicating the need for appropriate supplementation. Crown Copyright © 2009 Published by Elsevier Inc. All rights reserved.

Keywords:

ERCP; Humans; Osteocalcin; Parathyroid hormone; Vitamin D; Vitamin K

Abbreviations:

25(OH)D, 25-hydroxyvitamin D; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BMI, body mass index; CLD, chronic liver disease; cOC, carboxylated osteocalcin; CI, confidence interval; CV, coefficient of variation; ERCP, endoscopic retrograde cholangiopancreatography; γ GT, γ -glutamyltransferase; INR, international normalized ratio; LR, likelihood ratio; OR, odds ratio; PTH, parathyroid hormone; ROC, receiver operating characteristic (ROC); ucOC, undercarboxylated osteocalcin.

1. Introduction

Fat-soluble vitamins K and D are important physiologic factors in liver, bone, and vascular metabolism and are powerful regulators of cell proliferation and differentiation [1–4].

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In higher organisms, vitamin K is an essential cofactor for γ -glutamyl carboxylase, an enzyme that catalyses the posttranslational conversion of glutamyl residues into γ -carboxyglutamate residues in target proteins. In the liver, vitamin K is responsible for the synthesis of functional forms (eg, carboxylation) of procoagulant factors II, VII, IX, and X as well as anticoagulant proteins C, S, and Z [5]. In bone metabolism, the hydroxyapatite binding capacity of osteocalcin (OC), the main noncollagen protein in human bone synthesized exclusively by osteoblasts, is dependent on the degree of vitamin K-mediated carboxylation [6,7]. In case of vitamin K inadequacy, undercarboxylated OC (ucOC) is produced. Vitamin K deficiency of the bone can exist in the absence of vitamin K deficiency of the liver [8]. Serum vitamin K concentrations fluctuate with recent dietary vitamin K intake [9] and therefore are not reliable markers of tissue vitamin K status [10]. Because bones have a high susceptibility to vitamin K deficiency [8,11], measurement of circulating ucOC levels and especially the ratio between ucOC and cOC (ucOC/cOC) are used widely as sensitive indicators of vitamin K status of bone [12].

Vitamin D is metabolized in the liver to 25-hydroxyvitamin D [25(OH)D], the main determinant and the best marker of overall vitamin D status [4]. Disorders in vitamin K and Ca–parathyroid hormone (PTH)–vitamin D status in association with hepatic osteodystrophy have been found in patients with chronic liver diseases (CLDs) [13–19]. However, little is known about these factors in biliary and pancreatic disorders, often associated with transient cholestasis and/or fat malabsorption. Hemorrhagic coagulopathy due to vitamin K-deficient state is recognized in patients with obstructive jaundice, but the skeletal effects of vitamin K inadequacy are far less clear. The need for vitamin K and vitamin D supplementation in adults with hepatobiliary and pancreatic disorders has not been fully established.

In this context, the purpose of the present study was to test the hypothesis that patients with biliary and pancreatic disorders undergoing endoscopic retrograde cholangiopancreatography (ERCP) have inadequacy in the vitamin K, vitamin D, and PTH status, might be associated with hyperbilirubinemia and other liver dysfunctions. To our knowledge, this is the first study to determine the prevalence and severity of vitamin K deficiency of bone, vitamin D, and PTH abnormalities in patients undergoing ERCP and to identify those who may require nutritional supplementation.

2. Methods and materials

2.1. Patient population

The study population comprised 90 consecutive consenting patients who underwent ERCP at our institution between August 2006 and January 2007. The group consisted of 45 men and 45 women with the mean (SD) age of 65.5 (17.7) years (range, 20–90 years). Complete medical history, including current drug regimen and daily alcohol intake

was obtained; physical examination was performed; anthropometric measurements were taken; and body mass index (BMI; weight in kilograms/height in meters squared) was calculated. There were 68 patients with cholelithiasis, 8 with cholangiopancreatic malignancies (cholangiocarcinoma, 3; pancreatic cancer, 3; ampullary cancer, 1; and metastatic squamous cell carcinoma, 1), 6 with benign biliary strictures, 3 with chronic pancreatitis, 2 with pancreas divisum, 2 with bile leak, and 1 with choledochal cyst. Thirty-five (38.9%) patients were postcholecystectomy. The diagnosis was based on consistent clinical, laboratory, imaging, and histologic findings. At the time of presentation, 2 of the study patients received vitamin D, 2 received calcium supplements, 2 received bisphosphonates, 1 was on hormone replacement therapy, 1 took prednisolone, 7 took thyroxine, and 3 took ursodeoxycholic acid. Fifteen patients received 5 to 10 mg of vitamin K before ERCP: in 5 subjects because they were taking warfarin and in 10 subjects because of coagulopathy. Statistical analysis was performed before and after exclusion of data from these 15 patients. Most patients (70, or 77.8%) were abstainers or drank minimally (less than 20 g/d). The alcohol consumption before presentation was defined according to Australian alcohol guidelines [20] as moderate if up to 40 g/d or 280 g/wk in males and up to 20 g/d or 140 g/wk in females, and as heavy if above these quantities were consumed. Individuals who regularly smoked at least 1 cigarette per day in the last year were classified as current smokers.

This study conformed to the ethical guidelines of Declaration of Helsinki and was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee. Written informed consent was obtained from all participants.

2.2. Specimen collection and measurements

On the day of ERCP fasting, venous samples were obtained and each sample was divided into 2 portions. In 1 portion, hematologic and biochemical parameters including complete blood count, prothrombin time, serum bilirubin, albumin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyltransferase (γ GT), 25(OH)D, intact PTH, calcium, phosphorus, magnesium, lipase, creatinine, and urea nitrogen were determined immediately. Calcium levels were corrected for albumin concentration, and international normalized ratio (INR) and glomerular filtration rate were calculated.

In the other blood portion, sera were separated by centrifugation at 4°C, frozen, and stored at –80°C until analysis for cOC and ucOC. Both ucOC and cOC were measured with recently developed enzyme immunoassays, which use specific monoclonal antibodies highly reactive to each type (glutamyl or glutamate) of OC (Takara Biomedical Inc, Tokyo, Japan). According to the manufacture, the intra-assay and interassay coefficients of variations (CVs) for ucOC were 4.4% to 6.7% and 5.7% to 9.9%, respectively, and for cOC, they were 3.0% to 4.8% and 0.7% to 2.4%,

respectively. In our laboratory, all CVs were less than 10%. Based on the manufacturer's instructions, the suggested reference range for ucOC is 0.1 to 2.37 ng/mL, and for cOC, it is 1.47 to 10.9 ng/mL.

Serum 25(OH)D concentrations were measured using automated chemiluminescence immunoassay (Dia Sorin Liaison, Stillwater, Minn), and intact serum PTH concentrations were measured by a solid-phase, 2-site chemiluminescent enzyme-labeled immunoassay (Diagnostic Products, Los Angeles, Calif). The intra-assay and interassay CVs were 7% or less for 25(OH)D and 8% or less for PTH.

In the present study, serum 25(OH)D concentrations were defined as deficient when less than 50 nmol/L, severely moderate deficient when less than 25 nmol/L, insufficient when 50 to 80 nmol/L, and sufficient (adequate) when more than 80 nmol/L [21]. Based on the manufacturer's data on reference range for PTH, values above 6.8 pmol/L were defined as hyperparathyroidism.

2.3. Statistical analyses

Data are presented as mean values and SDs for continuous normally distributed variables or percentages (with 95% confidence interval [CI]) for categorical variables. Student unpaired *t* test was used when appropriate (normal data distribution). The difference between more than 2 groups was calculated using 1-way analysis of variance. Statistical analyses included also χ^2 test (for categorical variables), Pearson correlation coefficients (for continuous variables), univariate linear correlations, and multivariate logistic regression analyses. Parameters with $P \leq 0.20$ in univariate

analysis were included in multivariate model, and manual backwards stepwise logistic regression was performed to determine the best-fitting final model. Goodness of fit was determined by the likelihood ratio χ^2 statistic and by the pseudo- R^2 . Receiver operating characteristic (ROC) curves were applied to find the best sensitivity and specificity cutoff value of the variable for the prediction of risk group. In all statistical analyses, 2-tailed tests of significance were used and *P* values less than .05 were considered statistically significant. For all analyses, Stata, version 10 (Stata Corp, College Station, Tex) was used.

3. Results

3.1. Patient characteristics and prevalence of abnormalities in vitamin K, vitamin D, and PTH status

Demographic and clinical features of the patient population are summarized in Table 1. Of 90 patients, 50 (55.6%) were 65 years or older. The subgroup of 75 patients who did not receive warfarin and/or vitamin K supplementation did not differ from the total group in any of demographic characteristics.

The proportion of all abnormalities in vitamin K, vitamin D, and PTH status is summarized in Table 2. Vitamin D inadequacy (25(OH)D <80nmol/L) was present in 72 (80.0%) of patients. These included 41 (45.6%) patients with vitamin D deficiency, which was severe or moderate in 11 (12.2%) and 31 (34.4%) subjects with vitamin D insufficiency. Secondary hyperparathyroidism occurred in

Table 1
Demographic and clinical characteristics of study patients (total group and by serum bilirubin level)

Characteristics	All patients (n = 90)	Serum bilirubin, $\mu\text{mol/L}$		<i>P</i>
		<20 (n = 46)	≥ 20 (n = 44)	
Age (y), mean $\pm$ SD	65.5 $\pm$ 17.7	63.8 $\pm$ 19.0	67.3 $\pm$ 16.2	.354
Sex: M/F	45/45	16/30	29/15	.007
Cholelithiasis, n (%)	68 (75.6)	35 (76.1)	33 (75.0)	1.000
Benign stricture, n (%)	6 (6.7)	3 (6.5)	3 (6.8)	1.000
Chronic pancreatitis, n (%)	3 (3.3)	3 (6.5)	0	.257
Other benign conditions, n (%)	5 (5.6)	5 (10.9)	0	.730
Cholangiopancreatic cancer, n (%)	8 (8.9)	0	8 (18.2)	.008
Ethanol consumption				
Moderate, n (%),	12 (13.3)	5 (10.9)	(15.9)	.694
Heavy, n (%)	8 (8.9)	3 (6.5)	5 (11.4)	.662
Current smokers, n (%)	13 (14.4)	6 (13.0)	7 (15.9)	.931
Ex-smokers, n (%)	27 (30.0)	15 (32.6)	12 (27.3)	.747
History of				
Cholecystectomy, n (%)	35 (38.9)	23 (50.0)	12 (27.3)	.046
Cancer, n (%)	12 (13.3)	6 (13.0)	6 (13.6)	1.000
Hypertension, n (%)	28 (31.1)	16 (34.8)	12 (27.3)	.588
Coronary artery disease, n (%)	17 (18.9)	8 (17.4)	9 (20.5)	.919
Chronic obstructive pulmonary disease, n (%)	14 (15.6)	4 (8.7)	10 (22.7)	.122
Diabetes mellitus, n (%)	8 (8.9)	6 (13.0)	2 (4.5)	.296
Warfarin use, n (%)	5 (5.6)	2 (4.3)	3 (6.8)	.960
BMI (kg/m^2), mean $\pm$ SD	26.9 $\pm$ 5.6	26.6 $\pm$ 5.7	27.1 $\pm$ 5.6	.980

Data presented as mean $\pm$ SD or n (%).

Table 2

Prevalence (%) of inadequate indices of vitamin D, PTH, and vitamin K of bone status in the total study group and in patients who did not received warfarin and/or vitamin K (subgroup)

Variables	Total group (n = 90)		Subgroup (n = 75)	
	Mean	95% CI	Mean	95% CI
25(OH)D (nmol/L)				
<25	12.2	6.3–20.8	13.3	6.6
25–50	33.4	24.4–43.6	33.4	23.7
50–80	34.4	24.7–45.2	34.7	24.0
>80	20.0	12.3–29.8	18.7	10.6
PTH >6.8 pmol/L	27.8	18.9–38.2	29.7	19.7–41.5
ucOC/cOC (%)				
>20	50.6	39.6	56.2	44.1
>30	31.0	21.5	34.2	23.5
>50	18.4	10.9	19.2	10.9
25(OH)D <50 nmol/L and ucOC/cOC >30%	16.1	9.1–25.5	17.8	9.8–28.5

25 (27.8%) patients, of whom 16 (64%) had vitamin D deficiency and 5 (20%) vitamin D insufficiency.

The vitamin K status of bone was defined according to the ratio ucOC/cOC, expressed as percentage of ucOC concentration to cOC. Increased ucOC/cOC ratio ($\geq 20\%$) suggests a poor vitamin K status. [9,22] The ratio ucOC/cOC was above 20% in 50.6% of our patients, above 30% in 31.0%, and above 50% in 18.4%, indicating impairment of γ -carboxylation due to vitamin K inadequacy in a significant proportion of the study population.

Approximately 16% of patients demonstrated both vitamin D (25(OH)D <50nmol/L) and vitamin K (ucOC/cOC >30%) deficiency. Vitamin D deficiency was present in 51.9% of patients with vitamin K deficiency, and vitamin K deficiency was detected in 35% of subjects with vitamin D deficiency. When analysis was reperformed after exclusion of data from 15 patients who received vitamin K before ERCP (including 5 patients who were taking warfarin), there were no significant differences in prevalence of inadequacy in vitamin K, vitamin D, or PTH status (Table 2).

3.2. Effects of hyperbilirubinemia, alcohol, and smoking

To investigate whether the apparent inadequacy of vitamin K of bone status and vitamin D and PTH serum concentrations were associated with liver dysfunction, we subdivided the patients according to their bilirubin level (Tables 1 and 3). All patients with biliary or pancreatic cancer had hyperbilirubinemia ($>20 \mu\text{mol/L}$), but there were no significant differences between 2 groups in other diagnoses, age, alcohol consumption, smoking habits, comorbid diseases, and BMI. Among patients with hyperbilirubinemia, there was a significant prevalence of males and a lower proportion of subjects who underwent cholecystectomy. Patients with hyperbilirubinemia compared with those with normal serum bilirubin had a marked decrease in both cOC and ucOC, an increase in INR, lower serum phosphorus

and, as might be expected, significantly (2.7–4.3 fold) elevated γGT , ALP, and ALT. The groups did not differ in serum ucOC/cOC ratio, concentrations of albumin, 25(OH)D, PTH, calcium (corrected), magnesium, lipase, creatinine, hemoglobin levels, or platelet count. Nor were there significant differences between 2 patient groups in the prevalence of abnormalities in the ratio ucOC/cOC, 25(OH)D, or PTH levels.

Degree of reduction in cOC and ucOC was related to severity of cholestasis. In patients with serum bilirubin greater than $60 \mu\text{mol/L}$ compared with those with mild hyperbilirubinemia (20–60 $\mu\text{mol/L}$), cOC (4.9 ± 5.67 versus $8.6 \pm 5.04 \text{ ng/mL}$; $P = .01$) and ucOC (0.9 ± 0.63 versus $1.3 \pm 1.04 \text{ ng/mL}$; $P = .01$), levels were significantly lower. When data for the 75 patients who did not receive warfarin and/or vitamin K were analyzed, these differences in cOC (3.8 ± 3.1 versus $7.6 \pm 4.2 \text{ ng/mL}$; $P = .001$) and ucOC (1.0 ± 0.7 versus $1.5 \pm 1.1 \text{ ng/mL}$; $P = .006$) were also evident.

Multivariate regression analysis with adjustment for age, sex, BMI, 25(OH)D, PTH, INR, and liver function tests revealed that low serum cOC concentration ($<4.2 \text{ ng/mL}$, below the 25th percentile of the distribution in the study group) was significantly and independently associated with hyperbilirubinemia ($\geq 20 \mu\text{mol/L}$; odds ratio [OR], 7.1; 95% CI, 1.2–41.0; $P = .029$). Other significant and independent predictors of hyperbilirubinemia were elevated ($\times 3$) γGT levels ($>180 \text{ U/L}$) (OR, 23.3; 95% CI, 4.4–122.7; $P < .001$),

Table 3

Vitamin K of bone status, serum 25(OH), vitamin D, and PTH concentrations and liver function markers in patients with and without hyperbilirubinemia

Parameters	Serum bilirubin ($\mu\text{mol/L}$)		<i>P</i>
	<20 (n = 46)	≥ 20 (n = 44)	
cOC (ng/mL)	10.8 ± 7.66	6.9 ± 6.45	.012
cOC ^a (ng/mL)	11.1 ± 7.80	5.6 ± 4.72	.001
ucOC, ng/mL	2.5 ± 1.93	1.4 ± 1.68	.007
ucOC ^a (ng/mL)	2.6 ± 1.95	1.2 ± 0.88	.001
ucOC/cOC (%)	16.1 ± 19.00	9.6 ± 6.90	.075
25(OH) vitamin D (nmol/L)	57.3 ± 28.17	53.7 ± 24.39	.522
PTH (pmol/L)	5.6 ± 2.94	5.6 ± 3.89	.987
Calcium ^b (mmol/L)	2.4 ± 0.12	2.4 ± 0.17	.599
Phosphorus (mmol/L)	1.1 ± 0.20	1.0 ± 0.37	.049
Magnesium (mmol/L)	0.82 ± 0.09	0.81 ± 0.09	.732
Bilirubin ($\mu\text{mol/L}$)	10.2 ± 4.62	108.4 ± 100.58	.000
INR	1.03 ± 0.11	1.12 ± 0.18	.005
ALT (U/L)	74.0 ± 98.91	321.7 ± 308.62	.000
ALP (U/L)	145.9 ± 84.44	389.8 ± 323.06	.000
γGT (U/L)	167.4 ± 257.30	622.4 ± 517.52	.000
Albumin (g/L)	40.7 ± 4.81	38.7 ± 5.39	.068
Lipase (g/L)	61.7 ± 114.8	232.3 ± 619.7	.076
Creatinine ($\mu\text{mol/L}$)	82.1 ± 26.78	90.3 ± 29.70	.170
Haemoglobin (g/L)	129.9 ± 18.6	133.9 ± 17.9	.303
Platelets ($\times 10^9/\text{L}$)	300.9 ± 139.8	265 ± 92.8	.167

Data presented as mean $\pm$ SD.

^a Patients who did not receive warfarin and/or vitamin K with serum bilirubin greater than $20 \mu\text{mol/L}$ (n = 43) and $20 \mu\text{mol/L}$ or greater (n = 32).

^b Corrected for serum albumin concentration.

ALT levels (>120 U/L; OR, 4.4; 95% CI, 1.1–19.0; $P = .046$), and male sex (OR, 8.9; 95% CI, 1.8–43.7; $P = .007$).

When we compared patients with moderate or heavy alcohol consumption ($n = 20$) and subjects with no or mild alcohol intake ($n = 70$), no significant differences were found in demographic and clinical characteristics nor in biochemical parameters, including serum cOC, ucOC, ucOC/cOC ratio, 25(OH)D, and PTH concentrations (data not shown). Comparison of current smokers ($n = 13$), ex-smokers ($n = 27$), and nonsmokers ($n = 50$) revealed only that ex-smokers had a significantly lower ucOC/cOC ratio than non-smokers ($7.9 \pm 5.9\%$ versus $18.0 \pm 18.5\%$; $P = .022$).

3.3. Correlations between cOC, ucOC, 25(OH) D, PTH, and biochemical markers of liver function

We investigated these interrelationships in 75 patients who did not receive warfarin or vitamin K (Table 4). Of 18 variables (age, BMI, cOC, ucOC, ucOC/cOC ratio, bilirubin, γ GT, ALP, ALT, albumin, INR, 25(OH)D, PTH, calcium, phosphorus, magnesium, creatinine, lipase), serum cOC concentration correlated positively with ucOC and negatively with ratio ucOC/cOC, bilirubin, γ GT, and ALT levels. Serum ucOC concentration correlated negatively with bilirubin, γ GT, and ALT levels. The ucOC/cOC ratio correlated significantly with ALT levels. As expected, there was a significant positive correlation between bilirubin and γ GT, ALP, and ALT levels as well as INR. Serum 25(OH)D concentration was inversely correlated with PTH and BMI, whereas PTH levels correlated positively with serum creatinine, BMI, and age. There was no statistical association between serum 25(OH)D and either OC, biochemical markers of liver function, nor between PTH and these variables.

3.4. Indicators of vitamin K of bone deficiency, vitamin D deficiency, and low serum cOC

Using a linear regression model, we also examined the relation between ucOC/cOC more than 30% (as dependent variable) and liver function markers, serum 25(OH)D and PTH levels, BMI, age, and sex (as independent variables). By multiple regression analysis, the only significant and independent determinants of the ratio ucOC/cOC more than 30% were elevated γ GT (>180 U/L) and increased INR (>1.1) as well as cOC values in the lowest quartile and ucOC values in the highest quartile (Table 5). This model correctly classified 81.4% of patients in the total study group and had sensitivity of 70.3%, specificity of 86.4%, positive predictive value of 70.4%, and negative predictive value of 86.1%. Although sex was not initially significant in multivariate analysis, this became an independent indicator of ucOC/cOC more than 30% when ucOC and cOC were excluded from the model. In the latter model, male sex had a protective effect (OR, 0.34; 95% CI, 0.12–0.97; $P = .044$) indicating that the effect of sex is captured by absolute levels of cOC and ucOC when these 3 variables compete in the model.

Table 4
Correlation matrix for indices of vitamin K of bone status, 25(OH)D, PTH, biochemical markers of liver function, BMI, and age

Variables	ucOC	ucOC/cOC	Bilirubin	25(OH)D	PTH	γ GT	ALP	ALT	Albumin	INR	Ca	P	Mg	Cr	BMI	Age
cOC																
ucOC	0.599 ***	–0.396 ***	–0.339 **	0.003	0.095	–0.236 *	–0.144	–0.383 ***	0.056	–0.154	0.137	0.235	0.125	0.093	–0.115	0.185
ucOC/cOC		0.275 **	–0.342 **	–0.084	0.132	–0.236 *	–0.179	–0.252 *	0.123	–0.200	0.031	0.158	0.065	–0.029	0.069	–0.053
Bilirubin			0.059	–0.130	–0.098	0.089	0.068	0.410 ***	0.197	0.053	–0.119	–0.086	0.026	–0.186	0.046	–0.244
25(OH)D				0.006	–0.076	0.456 ***	0.575 ***	0.310 **	–0.130	0.368 ***	0.060	–0.053	0.129	0.085	–0.092	0.030
PTH					–0.298 **	0.004	–0.030	0.010	0.221	0.078	0.111	–0.009	0.161	0.101	–0.285 **	–0.087
						–0.088	–0.081	–0.185	–0.183	–0.095	–0.015	0.199	0.076	0.337 **	0.243 *	0.371 ***

None of the 75 patients received warfarin or vitamin K.

Ca indicates calcium corrected for albumin; P, phosphorus; Mg, magnesium; Cr, creatinine.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

Table 5

Independent predictors of vitamin K of bone deficiency, vitamin D deficiency, and low serum cOC (multivariate analyses^a)

Dependent variable	Independent variables	OR	95%CI	P
ucOC/cOC >30%	γ GT >180U/L	5.5	1.2–25.2	.027
	INR >1.1	3.1	1.1	.036
	cOC <4.1 ng/mL	10.9	2.3	.002
	ucOC >2.5 ng/mL	5.2	1.1–24.3	.036
25(OH)D <50 nmol/L	PTH >6.8 pmol/L	3.8	1.1–11.4	.019
cOC <4.1 ng/mL	Bilirubin >20 μ mol/L	11.6	1.9–59.4	.007
	ucOC/cOC >30%	10.7	1.5–90.5	.019

^a Adjusted for age, sex, liver function markers, BMI, serum 25(OH)D, and PTH levels.

A similar multivariate regression analysis showed that vitamin D deficiency [25(OH)D <50nmol/L] was significantly associated only with elevated PTH (>6.8 pmol/L).

The multivariate regression analysis after adjusting for the above listed covariates also demonstrated that hyperbilirubinemia and ucOC/cOC more than 30% are the only 2 variables independently predictive of low serum cOC (Table 5). The model correctly classified 81.9% of patients, had a sensitivity of 61.1% and a specificity of 88.9%, and had a positive predictive value of 64.7% and a negative predictive value of 87.3%.

An ROC curve was constructed to identify the discriminating value of hyperbilirubinemia in predicting low cOC (Fig. 1). Area under the ROC curve was 0.73. A serum bilirubin level of 20 μ mol/L or higher had a sensitivity of 76.2%, a specificity of 61.2%, a positive likelihood ratio (LR+) of 1.96, and a negative likelihood ratio (LR–) of 0.39 and correctly classified 64.8% of cases. The predictive value of hyperbilirubinemia was almost optimized when bilirubin level was 37 μ mol/L or higher: sensitivity, 71.4%; specificity, 77.6%; LR+, 3.19; and LR–, 0.37; this threshold correctly classified 76.2% of cases.

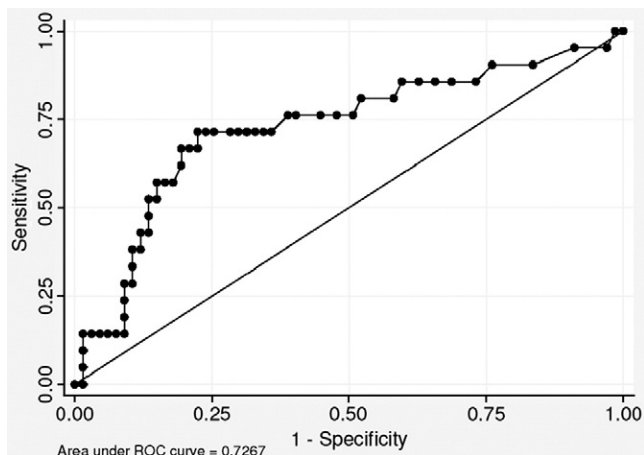


Fig. 1. Receiver operating characteristic curve of serum bilirubin for prediction of low serum cOC (<4.1 ng/mL).

4. Discussion

The results of this study revealed that a significant proportion of unselected patients with biliary and pancreatic disorders undergoing ERCP has an inadequate vitamin K, vitamin D, and PTH status and that liver dysfunction is associated with and predictive of vitamin K deficiency of bone and decreased production of OC.

We found subnormal serum levels of 25(OH)D in 80% of study patients, vitamin D deficiency in 45.6%, and vitamin D insufficiency in 34.4%. Elevated serum PTH levels were present in 27.8%. The pandemic of vitamin D deficiency is now well recognized [23], and our study population likely reflects this trend. In addition to the common inadequate sunlight exposure and absence of supplementation, in subjects with biliary and pancreatic disorders, malabsorption of vitamin D may also contribute to vitamin D insufficiency or deficiency.

Vitamin D deficiency affects the immune system, cell growth, and differentiation; increases production of proinflammatory cytokines and acute-phase proteins; and increases the risk of cancer [24]. Secondary hyperparathyroidism also significantly promotes acute-phase responses. It is possible that vitamin D inadequacy and PTH elevation observed in our patients were among factors that determined the severity of the inflammatory and malignant processes.

We used ucOC/cOC ratio as a surrogate marker for vitamin K of bone status. The rationale behind this approach is that this ratio (an estimate of the degree of carboxylation of circulating OC) is independent of bone turnover and, therefore, is a more reliable marker of tissue stores of vitamin K than serum ucOC, which was often used as an indicator of vitamin status of the bone. In our study group, ucOC/cOC was above 20% in 50.6% of patients, above 30% in 31.0%, and above 50% in 18.4%. In young healthy persons, 92% of circulating OC is carboxylated, and this increases to 98% with dietary phylloquinone supplementation [25]. The median percentage of ucOC in healthy girls was 13.6% in one study [10] and 21.9% in another [12]. A cutoff point of 20% or higher for ucOC/cOC ratio was selected as suggestive of vitamin K deficiency in adults [9,22]. Taking into account that the percentage of ucOC measured by immunosorbent assays is higher compared with its measurement by hydroxyapatite assays [26], we used ucOC/cOC more than 30% as an indicator of vitamin K inadequacy.

In our cohort, vitamin D deficiency was present in 45.6% of patients and vitamin K inadequacy in 31.0%, but both deficiencies were seen only in 16%, suggesting a different cause in individual patients. Low serum 25(OH)D concentrations negatively correlated with elevated ucOC and ucOC/cOC ratio in older women with hip fracture [22] and in adolescent girls [12]. However, we found no significant relationship between serum 25(OH)D nor PTH levels and indicators of vitamin K status. This shows that vitamin K status was not directly influenced by the vitamin D–PTH

axis. The possible causes of vitamin K deficiency in patients with hepatopancreatobiliary disease, in addition to poor dietary intake, include decreased vitamin K absorption from intestine, disturbance of vitamin K cycle, and the decrease in vitamin K storage. In agreement with previous reports [27,28], our study did not show a significant influence of long-term therapeutic anticoagulation with warfarin and/or short-term vitamin K supplementation on markers of vitamin K status of bone. In line with other studies [29], we also found that more patients were vitamin K insufficient in bone than in liver as ucOC/cOC more than 30% was found in 34.2%, whereas INR more than 1.1 (in patients not receiving warfarin) was found only in 8.9%.

In this study, there was no association found between inadequate vitamin K status and diagnosis, age, or alcohol consumption; male sex demonstrated a protective effect; and ex-smokers had a lower mean ucOC/cOC ratio than did nonsmokers. These data are consistent with studies that have shown no relationship between specific diagnosis and osteodystrophy in CLD [15], between plasma phyloquinone concentrations and alcohol consumption in the general population [9], and higher plasma phyloquinone levels in men than in women [30]. There are reports of age-related increase in vitamin K requirements, especially in postmenopausal women [27], and the majority of our patients were in the fifth to seventh decades of life (mean age was 65 years). Patients who quit smoking possibly increased the dietary intake of vitamin K as a result of changed lifestyle.

In the present study, the ratio ucOC/cOC positively correlated with ALT levels, a sensitive marker of hepatocellular damage. Furthermore, elevated γ GT (>180 U/L) and INR (>1.1) levels were significant and independent predictors of ucOC/cOC more than 30% after adjustment for other covariants, indicating a significant relationship between liver dysfunction and vitamin K status. It is well known that absorption of vitamin K from the proximal intestine is dependent on bile and pancreatic juice secretion. In patients with obstructive jaundice, approximately 80% of radioisotopic vitamin K is excreted unaltered compared with 20% in healthy subjects [31,32]. It seems, therefore, that extrahepatic cholestasis, as in our patients, is a very important contributor to vitamin K insufficiency, which in turn causes further progression of liver dysfunction manifesting in elevated INR, ALT, and γ GT. A defect in vitamin K-dependent prothrombin carboxylation affects the coagulation mechanism, and the presence of undercarboxylated prothrombin is also a well-recognized marker for hepatocellular carcinoma with tumor growth promoting properties [33].

An important finding in our study was the strong association of hyperbilirubinemia and other markers of liver dysfunction with depressed osteoblastic function as reflected in the synthesis of OC and, consequently, low serum cOC and ucOC levels. Patients with hyperbilirubinemia had significantly lower mean values of cOC and ucOC than patients with normal serum bilirubin, and these

variables demonstrated a dose-response relation. There was also a significant inverse correlation between serum cOC as well as ucOC concentrations and bilirubin, γ GT, and ALT levels. Moreover, hyperbilirubinemia and vitamin K inadequacy (ucOC/cOC $>30\%$) were the only 2 variables associated independently with low serum cOC concentrations, and the latter could be predicted from these variables with an accuracy of more than 81%. These data suggest that cholestasis, along with vitamin K deficiency, may be a major pathophysiologic factor affecting OC production, an informative marker of osteoblast activity. Our findings are consistent with several but not all previous studies. In patients with various CLD, serum bilirubin correlated with OC [34], bone mineral density [35], and bone loss [36,37]. Serum OC levels in cirrhotic patients compared with controls were reported to be lower [34,38,39]. However, some studies did not confirm the association between serum bilirubin and bone mineral density in CLD patients [17,40]. A dose-related toxic effect of bilirubin on osteoblast proliferation was shown in vitro studies with plasma taken from patients with jaundice due to different causes [41].

Most published studies like ours suggest that cholestasis contributes substantially to OC synthesis. The findings of this study emphasize the link between extrahepatic cholestasis, suboptimal vitamin K status, and OC production. Low OC production, high ucOC/cOC ratio, and vitamin D insufficiency, all three are recognized markers of altered bone homeostasis and independent risk factors for fracture. It should also be noted that skeleton through secretion of OC exerts a profound and complex influence on glucose and fat metabolism [42]. The high prevalence of poor vitamin K and vitamin D status, 2 factors with pleiotropic actions and of vital importance for liver, bone, and vascular health, indicate that corrective strategies should be considered as a part of “standard of care” in patients undergoing ERCP. Unfortunately, this is often ignored by practicing physicians.

This study has several limitations. First, because of its cross-sectional design, the causative nature of the associations cannot be established. Second, to determine the beneficial effects of supplementation with vitamin K, vitamin D, and calcium and the optimal doses needed, it will be necessary to perform a randomized controlled trial.

In conclusion, our study suggests that inadequate vitamin K and vitamin D status should be anticipated in patients undergoing ERCP. Liver dysfunction is predictive for vitamin K deficiency of bone, and cholestasis is associated with depressed OC production. Further studies are needed to elucidate therapeutic benefits of vitamin D and vitamin K supplementation in these patients.

Acknowledgment

We would like to thank the Research Fund of Sir Charles Gairdner Hospital, Perth, Australia, for providing a grant for this study.

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Associations Between Liver Function, Bone Turnover Biomarkers and Adipokines in Older Patients With Hip Fracture

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Abstract

Background: To examine the associations of serum liver markers with parameters of mineral and bone metabolism and their relationship with leptin, adiponectin and resistin in patients with hip fracture (HF).

Methods: In 294 older patients (mean age 82.2 ± 7.9 years, 72.1% women) with osteoporotic HF, we measured serum levels of alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), bilirubin, albumin, adiponectin, leptin, resistin, 25(OH) vitamin D (25(OH)D), intact parathyroid hormone (PTH), calcium, phosphate, magnesium, osteocalcin (OC), bone-specific alkaline phosphatase (BAP), urinary concentrations of deoxypyridinoline (DPD/Cr) and cross-linked N-telopeptide of type 1 collagen (NTx/Cr) (both corrected for urinary creatinine concentrations), as well as routine blood parameters.

Results: In the total cohort, in fully adjusted multivariate linear regression analyses, lower OC was an independent predictor of higher GGT, ALT and bilirubin, whereas higher BAP was positively associated with GGT and ALP; NTx/Cr, hemoglobin (both inversely), adiponectin, coronary artery disease (CAD) and alcohol overuse (all three positively) were also independently associated with GGT activity. However, in malnourished women, OC was not an independent predictor of GGT or ALT and NTx/Cr predicted ALP activity. OC was independently predicted by GGT, ALT (both negatively), ALP, leptin and age (all three positively), BAP by GGT and ALP, OC/BAP ratio by GGT (inversely) and leptin (positively) and both elevated NTx/Cr and DPD/Cr by higher ALP and lower leptin levels. The $\text{GGT} > 20$ U/L indicated increased prevalence of low OC levels (two-fold) and low OC/BAP ratio (2.6-fold) with a positive predictive value above 75%.

Conclusions: In older HF patients, bidirectional links exist between liver function (within normal range in the vast majority) and parameters of bone metabolism. Adiponectin is an independent predictor of GGT, whereas leptin is a determinant of OC and bone resorption; these relationships are modulated by nutritional status. $\text{GGT} > 20$ U/L may be used as a marker of impaired bone metabolism.

Keywords: Liver function; Adipokines; Bone turnover; Osteocalcin

Introduction

The importance of liver-bone interactions under both normal and disease conditions is supported by a growing number of evidence. 1) Liver plays a fundamental role in metabolism of vitamins D and K, parathyroid hormone (PTH) and minerals, essential regulators of bone homeostasis [1-5]. 2) Osteopenia/osteoporosis (hepatic osteodystrophy) is present in 20-50% of patients with chronic liver disease [6-10]. 3) Osteocalcin (OC), an osteoblast-derived hormone, is recognized as a critical determinant of energy and glucose homeostasis [11-16]. 4) Dysregulation and dysfunction of adipokines, adipose tissue-derived hormones, in particular, adiponectin, leptin and resistin, which have receptors expressed in both hepatocytes and bone cells and control a vast diversity of physiological functions, are involved in initiation and progression of many diseases including liver and bone (osteoporosis) disorders [17-23].

However, despite the intense research carried out in recent years, the relationship between liver and bone and the underlying mechanisms, including the role of adipokines, are still poorly characterized. To date, neither clinical, nor the animal models provide a uniform explanation of the liver-bone link(s). The majority of research on adipokine-liver-bone interactions has focused primarily on obesity-related metabolic syndrome, diabetes mellitus (DM) and non-alcoholic fatty liver disease (NAFLD). There has been limited evaluation (with considerable controversy in the reports) of liver function, bone metabolism and adipokines in patients with hip fracture (HF), the most devastating consequence of osteoporosis. There are no data concerning the association of serum hepatic biomarkers with indices of mineral and bone metabolism and the mechanisms involved, nor are there any data showing whether liver

Manuscript accepted for publication December 01, 2014

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doi: <http://dx.doi.org/10.14740/jem250w>

function indicators predict clinically important markers of bone turnover in HF patients. Thus, the aims of this study were to evaluate 1) the cross-sectional associations of serum liver markers with parameters of mineral and bone metabolism, and 2) the relationship between serum levels of three adipokines (leptin, adiponectin and resistin) and both liver and mineral-bone indicators in older patients with HF.

Methods

Study cohort

This prospective observational study included 294 consecutive older (≥ 60 years of age) patients (212 women and 82 men) with low-trauma osteoporotic HF admitted to the Canberra Hospital who did not have the following exclusion criteria: age < 60 years, high trauma, femur shaft fracture, pathological HF due to primary or metastatic bone cancer, multiple myeloma, Paget disease, or primary hyperparathyroidism. Data on sociodemographic, clinical and laboratory characteristics were collected as previously reported [24].

The study was conducted in compliance with the Declaration of Helsinki (as revised in 2008).

Informed consent was obtained from all patients or their carers. The study has approval of the local Health Human Research Ethical Committee.

Laboratory analyses

In each patient venous blood and second morning urine samples were collected between 07:00 and 11:00 after at least 12-h overnight fast, usually within 24 h after arrival at the emergency department. After centrifugation of blood, one serum sample as well as the urine sample was frozen and stored at -70°C for later analyses of bone turnover markers and adipokines.

For all patients, the following parameters were measured: complete blood count, liver function tests, urea, creatinine and electrolytes, fasting blood glucose (and $\text{HbA}_{1\text{C}}$ in diabetic patients), thyroid function tests (TSH, T_4), 25(OH) vitamin D (25(OH)D), intact PTH, total calcium, phosphate, magnesium, markers of bone turnover, and three adipokines (adiponectin, leptin and resistin). All routine hematological and biochemical assessments were performed by standardized methods on autoanalyzers at the day of collection. Glomerular filtration rate (eGFR) was calculated using a standardized serum creatinine-based formula normalized to a body surface area of 1.73 m^2 [25]. Chronic kidney disease (CKD) was defined as $\text{eGFR} < 60 \text{ mL/min/1.73 m}^2$ (CKD stage ≥ 3).

Assessment of liver parameters

The following biochemical indicators of liver function were measured as the blood samples were collected: alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), al-

kaline phosphatase (ALP), bilirubin and albumin. These markers were evaluated by using commercially available standard enzymatic reagents and diagnostic kits by spectrophotometry on the biochemical autoanalyzer Abbott Architect CI16200 (Abbott Laboratories, IL, USA). ALT, GGT and ALP were measured with enzymatic methods, total bilirubin was analyzed using diazonium salt, albumin was measured using bromocresol green, and total protein was tested by a Biuret method. The mean inter-assay and intra-assay coefficients of variations (CVs) for these tests were within 1.1-6.6%. For liver enzymes two times the upper normal limit (UNL), cut-off levels were used to define abnormal tests.

Markers related to mineral and bone metabolism

These included serum concentrations of 25(OH)D, intact PTH, total calcium, phosphate, magnesium, and markers of bone turnover - OC, and bone-specific alkaline phosphatase (BAP) as markers of bone formation and urinary concentrations of deoxypyridinoline (DPD/Cr), and cross-linked N-telopeptide of type 1 collagen (NTx/Cr) as markers of bone resorption (both corrected for urinary creatinine concentrations in the same sample). Serum calcium concentrations were corrected for serum albumin. Serum 25(OH)D was measured by radioimmunoassay kit (Dia Sorin, Stillwater, MN, USA) and intact PTH by two-site chemiluminescent enzyme-linked immunoassay on DPC Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA, USA). Serum OC was determined by electrochemiluminescent immunoassay (Elecsys 1010, Roche Diagnostics, Ltd Corp., IN, USA), serum BAP by Metra BAP ELISA (Quidel Corp., San Diego, CA, USA), urinary NTx by enzyme-linked immunosorbent assay (ELISA) (Wampole Labs, Princeton, NJ, USA), and urinary DPD by two-site chemiluminescent enzyme-labeled immunoassay (DPC Immulite 2000, Diagnostic Products, Los Angeles, CA, USA). All samples were analyzed with commercially available kits of the same lot number according to the manufacturer's protocol and blind to any clinical information. In these methods both the intra- and inter-assay CVs ranged from 2.1% to 12.7%. Insufficiency of vitamin D was defined as 25(OH)D $< 50 \text{ nmol/L}$ and secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH ($> 6.8 \text{ pmol/L}$, the upper limit of the laboratory reference range). For levels of bone turnover markers, we used the standard laboratory reference ranges and data provided by the manufacturer.

Measurements of adipokines

Serum levels of leptin were determined by ELISA method (Diagnostic System Laboratories, Webster, TX, USA), and total adiponectin and resistin by human ELISA kits (B-Bridge International, Mountain View, CA, USA). All assays were performed according to the manufactures' instructions with kits of the same lot number. Intra- and inter-assay CVs were less than 7% for these three tests. Malnutrition was defined as serum leptin concentration $< 4 \text{ ng/mL}$ in males and $< 6.5 \text{ ng/mL}$ in

Table 1. Demographic, Clinical and Laboratory Characteristics of the Studied Patients With Hip Fracture (n = 294)

Clinical characteristic		Laboratory characteristic	
Age, years (mean $\pm$ SD)	82.2 $\pm$ 7.9	ALT, U/L	23.0 $\pm$ 42.6
Females, %	72.1	GGT, U/L	54.1 $\pm$ 95.6
Cervical/trochanteric fracture, n	152/142	ALP, U/L	105.4 $\pm$ 80.1
Hypertension, %	54.0	Albumin, g/L	37.1 $\pm$ 6.3
CAD, %	21.2	Bilirubin, μ mol/L	12.4 $\pm$ 7.4
Atrial fibrillation, %	13.2	OC, ng/mL	17.2 $\pm$ 15.2
History of stroke or TIA, %	21.7	BAP, U/L	26.4 $\pm$ 14.2
Type 2 DM, %	18.8	NTx/Cr, nmol/ μ mol	159.4 $\pm$ 169.0
Dementia, %	27.8	DPD/Cr, nmol/ μ mol	12.6 $\pm$ 7.2
Parkinson's disease, %	4.4	25(OH)D, nmol/L	37.3 $\pm$ 18.0
COPD, %	11.8	PTH, pmol/L	6.9 $\pm$ 5.6
CKD stage $\geq$ 3, %	42.6	Adiponectin, ng/mL	17.5 $\pm$ 7.4
Malnutrition*, %	33.8	Leptin, ng/mL	18.5 $\pm$ 23.2
Alcohol ($\geq$ 3 times a week), %	9.5	Resistin, ng/mL	18.7 $\pm$ 10.5
Ex-smokers, %	10.0	Hb, g/L	124.9 $\pm$ 17.1
Current smokers, %	5.4	TSH, mU/L	1.55 $\pm$ 2.17
Vitamin D insufficiency**, %	79.7	T4, pmol/L	15.9 $\pm$ 3.54
SHPT, %	38.2	eGFR, mL/min/1.73 m ²	65.1 $\pm$ 23.7

CAD: coronary artery disease; TIA: transient ischemic attack; DM: diabetes mellitus; CKD: chronic kidney disease; COPD: chronic obstructive pulmonary disease; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; ALP: alkaline phosphatase; 25(OH)D: 25-hydroxyvitamin D; PTH: parathyroid hormone; TSH: thyroid-stimulating hormone; T4: thyroxine; eGFR: estimated glomerular filtration rate; SHPT: secondary hyperparathyroidism (PTH > 6.8 pmol/L); NTx/Cr: cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration; DPD/Cr: deoxypyridinoline corrected for urinary creatinine concentration. *Malnutrition was defined as serum leptin concentration < 4 ng/mL in males and < 6.5 ng/mL in females. **Vitamin D insufficiency was defined as 25(OH)D < 50 nmol/L.

females [26].

Statistical analyses

Data are presented as mean values $\pm$ standard deviations (SDs) for continuous variables or percentages for categorical variables. Comparisons between groups were performed using analysis of variance and Student's *t*-test (for continuous normally distributed variables) and χ^2 test (for categorical variables). The relations between variables were also analyzed by Pearson's correlation coefficient with log-transformed data (to achieve normal distribution); Bonferroni and Sidak adjustments for multiplicity have been performed. Univariate and multivariate linear regression analyses were performed to determine the associations between liver markers and different parameters related to mineral and bone metabolism; all potential confounding variables (demographic, biochemical and clinical) with statistical significance $\leq$ 0.10 on univariate analyses were included in multivariate analysis. The significance of multicollinearity phenomena in regression analyses was evaluated by the variance inflation factor. Two-sided *P* < 0.05 values were considered statistically significant. All statistical calculations were carried out using the Stata software version 10 (StataCorp, College Station, TX, USA).

Results

Clinical characteristics of the study patients

The clinical features and laboratory characteristics in the patients are summarized in Table 1. The prevalence of abnormal liver enzyme activities defined as two times over their respective ULNs was low or moderate: for ALT (> 80 U/L) in five (1.7%) patients, for GGT (> 128 U/L) in 23 (7.8%), and for ALP (> 120 U/L) in 26 (8.8%) subjects. Elevated bilirubin (> ULN, 20 μ mol/L) demonstrated 29 (9.9%) patients and low albumin levels (< 33 g/L) were found in 64 (21.8%) subjects. There were no gender differences in mean values of liver markers, OC, BAP, calcium (corrected for albumin), resistin, TSH and hemoglobin. Women, compared to men, had significantly lower mean serum 25(OH)D (35.3 $\pm$ 17.6 vs. 42.4 $\pm$ 18.2 nmol/L, *P* = 0.003) and eGFR (62.7 $\pm$ 22.2 vs. 71.2 $\pm$ 26.4 mL/min/1.73 m², *P* = 0.006) levels, but higher concentrations of PTH (7.4 $\pm$ 6.1 vs. 5.5 $\pm$ 3.5 pmol/L, *P* = 0.009), NTx/Cr (178.0 $\pm$ 90.5 vs. 112.3 $\pm$ 78.0 nmol/ μ mol, *P* = 0.005), DPD/Cr (13.3 $\pm$ 7.9 vs. 10.8 $\pm$ 4.6 nmol/ μ mol, *P* = 0.015), leptin (21.1 $\pm$ 24.3 vs. 11.7 $\pm$ 18.6 ng/mL, *P* = 0.002), adiponectin (18.3 $\pm$ 7.1 vs. 15.6 $\pm$ 7.6 ng/mL, *P* = 0.007) and free T4 (16.2 $\pm$ 3.50 vs. 15.2 $\pm$ 3.53 pmol/L, *P* = 0.021). Vitamin D insufficiency

Table 2. Correlation of Serum Liver Markers With Indices of Bone-Mineral Metabolism, Leptin, Adiponectin, and Resistin Levels, Thyroid Markers and Hemoglobin

	GGT	ALT	ALP	Albumin	Bilirubin
OC	-0.182**	-0.158**	0.104	-0.034	-0.147*
BAP	0.307***	0.050	0.462***	0.014	0.005
OC/BAP	-0.350***	-0.178**	-0.183**	-0.035	-0.137*
DPD/Cr	0.018	-0.071	0.191**	-0.037	-0.021
NTx/Cr	-0.134*	-0.041	0.150*	-0.069	-0.035
25(OH)D	0.014	-0.022	-0.165**	0.012	-0.021
PTH	0.053	0.058	0.111	0.036	0.139*
Cac	-0.002	0.086	0.133*	-0.111	-0.039
PO4	-0.016	-0.049	0.092	0.058	-0.075
Mg	-0.087	-0.068	0.017	0.105	-0.065
Leptin	-0.082	-0.025	-0.045	0.108	-0.029
Adiponectin	0.146*	0.087	0.059	0.010	0.128*
Resistin	0.088	0.001	0.054	0.124*	0.153*
L/A	-0.116	-0.070	-0.044	0.109	-0.055
L/R	-0.109	-0.038	-0.054	0.059	-0.077
A/R	0.022	0.053	-0.011	-0.090	-0.034
TSH	0.132*	0.057	-0.037	-0.014	0.043
T4	-0.186**	0.121*	-0.144*	0.027	0.007
Hb	-0.146*	0.071	-0.129*	0.281***	0.047

Pearson's correlation coefficients for log-transformed variables after adjustment for age and sex shown. GGT: gamma-glutamyltransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; eGFR: estimated glomerular filtration rate; OC: osteocalcin; BAP: bone specific alkaline phosphatase; DPD/Cr: deoxypyridinoline corrected for urinary creatinine concentration; NTx/Cr: cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration; 25(OH)D: 25-hydroxyvitamin D; PTH: parathyroid hormone; TSH: thyroid-stimulating hormone; T4: thyroxine; Cac: calcium corrected for albumin; PO4: phosphate; Mg: magnesium. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.001$.

(25(OH)D < 50 nmol/L) was found in 84.6% of females and 67.5% of males ($P < 0.008$) and SHPT (PTH > 6.8 pmol/L) in 43.2% and 25.3%, respectively ($P = 0.016$).

Associations between liver markers and parameters of mineral-bone metabolism, adiponectin, leptin and resistin, and clinical characteristics

In Pearson's analyses (all biochemical variables log-transformed), after adjusting for age and sex, both serum GGT and ALT activities correlated negatively with serum OC levels (Table 2). GGT was associated positively with BAP, adiponectin and TSH and negatively with NTx/Cr, T4 and hemoglobin concentrations, while ALT correlated positively with T4. Serum ALP activity, as could be expected, demonstrated a strong correlation with BAP, and a significant positive association with both urinary resorption markers (DPD/Cr and NTx/Cr) and a negative correlation with 25(OH)D, T4 and hemoglobin. Albumin correlated with resistin and hemoglobin. Bilirubin was inversely associated with OC and positively with PTH, adiponectin and resistin. We calculated also the OC/BAP ratio as an index reflecting osteoblast differentiation/maturation [27]. Results showed significant inverse correlations between

the OC/BAP ratio and all liver markers (GGT, ALT, ALP and bilirubin) except albumin.

Similar partial correlation analyses (after adjusting for age and sex) also demonstrated that serum GGT activity correlated with presence of CAD ($\beta = 0.178$, $P = 0.003$) and alcohol over-use defined as ≥ 3 times a week ($\beta = 0.211$, $P < 0.001$), ALT correlated negatively with Parkinson's disease (PD, $\beta = -0.173$, $P = 0.004$), ALP with dementia ($\beta = 0.118$, $P = 0.047$), albumin was inversely associated with DM ($\beta = -0.126$, $P = 0.032$) and positively with hemoglobin ($\beta = 0.281$, $P < 0.001$), and bilirubin correlated with CAD ($\beta = 0.142$, $P = 0.018$) and atrial fibrillation (AF, $\beta = 0.242$, $P < 0.001$). There was a statistically significant inverse correlation between adiponectin and leptin ($\beta = -0.196$, $P = 0.002$), but there was no association between adiponectin and resistin ($\beta = 0.043$, $P = 0.493$), or between leptin and resistin ($\beta = 0.009$, $P = 0.885$).

When comparing patients with GGT < 30 U/L (median level) and GGT > 30 U/L, the latter group had, as would be expected, higher mean ALT (31.9 vs. 17.2 U/L, $P = 0.004$), BAP (29.6 vs. 24.2 U/L, $P = 0.002$), ALP (129.5 vs. 89.3 U/L, $P < 0.001$) and bilirubin (13.7 vs. 11.5 $\mu\text{mol/L}$, $P = 0.011$) levels, but also higher adiponectin (18.8 vs. 16.6 ng/mL, $P = 0.015$) and resistin concentrations (20.3 vs. 17.5 ng/mL, $P = 0.038$) and lower NTx/Cr urinary excretion (133.5 vs. 176.6 nmol/

Table 3. Multiple Linear Regression Analyses for Liver Markers in Older Patients With Hip Fracture

	Log GGT		Log ALT		Log ALP		Log albumin		Log bilirubin	
	A	B	A	B	A	B	A	B	A	B
	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)
Age	0.264 (0.027)	-0.012 (0.017)								
Sex (m)	-0.219 (0.011)	-0.176 (0.029)	-0.145 (0.016)	-0.155 (0.006)						
Log OC	0.595 (< 0.001)	0.570 (< 0.001)			0.490 (< 0.001)	0.496 (< 0.001)				
Log BAP	-0.185 (0.002)	-0.172 (0.005)			-0.134 (0.015)	-0.131 (0.017)				
Log NTx/Cr										
Log 25(OH)D										
Log PTH										
Log Hb	-0.897 (0.013)						0.393 (< 0.001)	0.390 (< 0.001)		
Log adiponectin	0.228 (0.037)	0.215 (0.050)					0.043 (0.026)	0.439 (0.023)	0.119 (0.038)	0.130 (0.054)
Log resistin										
Alcohol +	0.883 (< 0.001)									
CAD	0.309 (0.023)									
AF										
PD	0.2500	0.2701	0.0497	-0.516 (0.007)	0.2380	0.2334	0.1104	0.1177	0.0678	0.1328
R ²										

Parameters identified as significant and independent predictors of GGT, ALT, albumin and bilirubin are shown. β : β -standardized regression coefficient; GGT: gamma-glutamyltransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; OC: osteocalcin; BAP: bone specific alkaline phosphatase; DPD/Cr: deoxypyridinoline corrected for urinary creatinine concentration; NTx/Cr: cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration; 25(OH)D: 25-hydroxyvitamin D; PTH: parathyroid hormone; CAD: coronary artery disease; AF: atrial fibrillation; PD: Parkinson's disease; R²: adjusted R-squared. A, model 1: adjusted for age, sex, OC, BAP, NTx/Cr, DPD/Cr, 25(OH)D, PTH, leptin, adiponectin, resistin, TSH, T4 and hemoglobin. B, model 2: further adjusted for alcohol use ≥ 3 drinks/week (yes/no), presence of CAD (yes/no), hypertension (yes/no), AF (yes/no), type 2 diabetes mellitus (yes/no), PD (yes/no) and chronic kidney disease (estimated glomerular filtration rate < 60 mL/min/1.73 m², yes/no).

μmol , $P = 0.048$). Similar analyses for ALT < 20 U/L (median level) vs. > 20 U/L, bilirubin (< 20 $\mu\text{mol/L}$ vs. > 20 $\mu\text{mol/L}$) and albumin (< 33 g/L vs. > 33 g/L) did not show significant associations either with any of the analyzed bone turnover markers, or with any of the adipokines (data not shown).

The patients were also stratified into two groups according to nutritional status: with ($n = 99$) and without malnutrition ($n = 195$). The first group showed significantly higher mean levels of urinary NTx/Cr (in women: 258.2 vs. 136.8 nmol/ μmol , $P < 0.001$, in men: 134.5 vs. 102.6 nmol/ μmol , $P < 0.05$), and serum adiponectin (19.9 vs. 17.3 ng/mL, $P = 0.020$, and 17.8 vs. 14.5 ng/mL, $P = 0.05$ in women and men, respectively). Liver markers, other mineral-bone parameters and resistin concentrations did not differ by nutritional status, in spite of the highly significant difference in mean leptin levels (3.1 vs. 30.4 ng/mL in women, 2.4 vs. 16.5 ng/mL in men, $P < 0.001$ for both groups). Malnourished men demonstrated the highest partial correlation between GGT and OC ($r = -0.497$, $P = 0.010$), while the inverse correlation between GGT and OC/BAP ratio was similar and significant in all groups regardless of the nutritional status and gender ($r = -0.310$ or higher, $P < 0.004$). Only in the malnourished women OC significantly correlated with adiponectin ($r = 0.354$, $P = 0.006$).

To further evaluate the association between OC and liver markers, adipokines and other indices of mineral-bone metabolism, we compared these parameters in HF patients, grouped according to serum OC levels. Patients with low OC levels (< 14 ng/mL, $n = 156$, or 53.2%), compared to the rest of the cohort ($n = 138$), were younger (80.7 ± 8.4 vs. 83.5 ± 7.7 years, $P = 0.003$), had higher mean serum GGT (67.3 ± 28.5 vs. 40.3 ± 21.2 U/L, $P = 0.001$) and 25(OH)D levels (40.8 ± 18.3 vs. 33.6 ± 17.1 nmol/L, $P = 0.001$), lower PTH (6.3 ± 4.3 vs. 7.7 ± 6.8 pmol/L, $P = 0.034$), calcium (2.26 ± 0.13 vs. 2.30 ± 0.11 mmol/L, $P = 0.033$), phosphate (0.88 ± 0.29 vs. 0.97 ± 0.28 mmol/L, $P = 0.007$), BAP (24.6 ± 12.6 vs. 28.4 ± 15.6 U/L, $P = 0.027$), and leptin (15.3 ± 18 vs. 22.1 ± 27.5 ng/mL, $P = 0.013$) levels, as well as lower both urinary bone resorption markers (DPD/Cr: 11.6 ± 5.7 vs. 13.7 ± 8.8 nmol/ μmol , $P = 0.024$; NTx/Cr: 122.9 ± 119.6 vs. 204.2 ± 202.8 nmol/ μmol , $P < 0.001$). No differences in mean values of ALT, ALP, bilirubin, magnesium, adiponectin, resistin and hemoglobin were observed.

Predictors of GGT, ALT, albumin and bilirubin (multiple regression analyses)

Multiple linear regression analyses with stepwise method were used to examine significant independent associations of liver markers with laboratory and clinical characteristics. In these models, each liver marker was set as dependent variable, while age, gender, OC, BAP, NTx/Cr, DPD/Cr, 25(OH)D, PTH, leptin, adiponectin, resistin, TSH, T4 and hemoglobin were set as independent variables (model 1). In model 2 further adjustment for alcohol use ≥ 3 drinks per week (yes/no), presence of CAD (yes/no), hypertension (yes/no), AF (yes/no), type 2 DM (yes/no), PD (yes/no) and CKD stage ≥ 3 (yes/no) was made. As presented in Table 3, reduced OC was an independent

Table 4. Multiple Linear Regression Analyses for Bone Turnover Markers in Older Patients With Hip Fracture

	Log OC	Log BAP	Log NTx/Cr	Log DPD/Cr	OC/BAP ratio
	β (P value)	β (P value)	β (P value)	β (P value)	β (P value)
Age	0.017 (0.001)		0.033 (< 0.001)		
Log GGT	-0.141 (0.012)	0.111 (0.003)	-0.315 (< 0.001)		-0.113 (0.043)
Log ALT	-0.132 (0.049)				
Log ALP	0.175 (0.038)	0.246 (< 0.001)	0.362 (0.008)	0.208 (0.008)	
Log leptin	0.084 (0.014)		-0.118 (0.027)	-0.069 (0.075)	0.013 (0.032)
R ²	0.1498	0.2043	0.2225	0.1350	0.1155

Parameters identified as significant and independent predictors of each bone turnover marker are shown. Adjusted for age, sex, GGT, ALT, ALP, albumin, bilirubin, 25(OH)D, PTH, leptin, adiponectin and resistin (all variables were log-transformed). β : β -standardized regression coefficient; GGT: gamma-glutamyltransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; OC: osteocalcin; BAP: bone specific alkaline phosphatase; NTx/Cr: cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration; DPD/Cr: deoxypyridinoline corrected for urinary creatinine concentration.

predictor of higher GGT, ALT and bilirubin, whereas higher BAP was associated with GGT and ALP. These analyses also showed that NTx/Cr, hemoglobin (both inversely), adiponectin, alcohol overuse and CAD (all three positively) were independent predictors of GGT activity. Higher ALP was predicted by elevated BAP and lower 25(OH)D levels. The independent predictors of serum albumin were hemoglobin and resistin. In the fully adjusted model, higher bilirubin levels were predicted by lower OC, higher adiponectin and presence of AF, although male gender, PTH and resistin were independent predictors in model 1 (included only laboratory parameters), suggesting that these variables are incorporated in clinical factors used in the model 2. The full panel of determinants predicted 27.0%, 7.8%, 23.3%, 11.8% and 13.2% of variance in serum GGT, ALT, ALP, albumin and bilirubin levels, respectively. The liver markers' variance was only partially explained by the panel of determinants indicating the contribution of other regulating factors.

To further explore these observations, we constructed similar multiple linear regression analyses to predict liver markers in patients with and without malnutrition. These revealed that only in malnourished women, OC was not an independent predictor of GGT or ALT, and NTx/Cr predicted ALP activity ($\beta = 0.108$, $P = 0.042$), while all other abovementioned correlations in the entire cohort were present regardless of the nutritional status.

Predictors of bone turnover markers (multiple regression analyses)

Results from linear multiple regression with each bone turnover marker as a dependent variable and age, sex, GGT, ALT, ALP, albumin, bilirubin, 25(OH)D, PTH, leptin, adiponectin and resistin (all biochemical variables were log-transformed) as independent variables are shown in Table 4. Five factors were significantly and independently associated with OC: age, ALP, leptin (all three positively), GGT and ALT (both negatively). BAP was independently predicted by GGT and ALP. Both elevated NTx/Cr and DPD/Cr were predicted by higher

ALP activity and lower leptin concentrations; age also demonstrated a strong relationship with NTx/Cr. Finally, the independent determinants of OC/BAP ratio were GGT (inverse) and leptin (positive). Overall, the panel of determinants independently contributed to 15% of OC variance, to 20.4% of BAP, to 22.3% of NTx/Cr, to 13.5% of DPD/Cr, and to 11.6% of OC/BAP ratio variance.

Multivariate regression modeling, using abovementioned laboratory and clinical variables and eGFR confirmed also a significant inverse bidirectional link between leptin and adiponectin ($\beta = -0.331$, $P = 0.036$ for adiponectin as an independent correlate of leptin, and $\beta = -0.065$, $P = 0.036$ for leptin as a predictor of adiponectin).

Clinical implications

From the practical point of view, we tried to evaluate the diagnostic value of hepatic markers as indicators of the mineral-bone metabolism status. We focused on GGT activity which seems to be more relevant to bone metabolism and performed logistic regression analyses with GGT as a dependent variable ($\text{GGT} \leq 20 \text{ U/L}$ (cut-off level of the first tertile) vs. $> 20 \text{ U/L}$). Among patients with the $\text{GGT} > 20 \text{ U/L}$, there was a 2.3-fold increase of subjects with low circulating OC levels ($< 14 \text{ ng/mL}$, lower limit of normal range), about a 4.8-fold increase of subjects with elevated of BAP activity ($> 43 \text{ U/L}$, upper limit of normal range) and, consequently, a 2.6-fold increase of subjects with low OC/BAP ratio (< 0.6 , median level). These associations remained significant after adjustments for age, sex, serum adiponectin, leptin and resistin levels, alcohol overuse, presence of CAD or DM (Table 5). Diagnostic value of $\text{GGT} > 20 \text{ U/L}$ was as follows: for presence of low OC ($< 14 \text{ ng/mL}$) sensitivity 59.9%, specificity 60.2%, positive predictive value (PPV) 75.7%, negative predictive value (NPV) 42.1%, positive likelihood ratio (LR) 1.51; for high BAP ($> 43 \text{ U/L}$) 9.5%, 97.8%, 90.0%, 34.6%, 4.41, respectively; for low OC/BAP ratio (< 0.6) 57.9%, 65.6%, 77.5%, 43.3%, 1.68, respectively. Although the diagnostic value was relatively low, this still would have translated in identifying about 76% of patients

Table 5. Serum Gamma-Glutamyltransferase Activity (GGT > 20 U/L) as a Predictor of Abnormal Bone Formation Markers in Older Patients With Hip Fracture

Adjustments	OC < 14 ng/mL			BAP > 43 U/L			OC/BAP ratio < 0.6		
	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Unadjusted	2.26	1.32 - 3.87	0.003	4.76	1.03 - 30.33	0.044	2.62	1.52 - 4.54	0.001
+Age, sex	2.06	1.23 - 3.46	0.006	4.42	0.99 - 19.68	0.051	2.68	1.58 - 4.54	< 0.001
+Alcohol use*	2.07	1.22 - 3.50	0.007	4.88	1.10 - 21.75	0.038	2.81	1.64 - 4.82	< 0.001
+CAD	2.10	1.23 - 3.57	0.006	5.05	1.38 - 22.66	0.035	2.79	1.65 - 4.79	< 0.001
+DM	2.07	1.22 - 3.53	0.007	4.81	1.06 - 21.73	0.041	2.70	1.57 - 4.66	< 0.001
+Adipokines**	2.20	1.22 - 3.94	0.008	7.55	0.99 - 60.32	0.053	2.29	1.28 - 4.12	0.005

GGT: gamma-glutamyltransferase (> 20 U/L, above the level in the first tertile); OC: osteocalcin; BAP: bone specific alkaline phosphatase; CAD: coronary artery disease; DM: diabetes mellitus. *Three or more times a week. +Adjustments for all covariates in the above model. **Adipokines: adiponectin, leptin, resistin.

with abnormal bone formation status (PPV = 75.7% for low OC and PPV = 77.5% for low OC/BAP ratio) indicating that GGT > 20 U/L in the elderly can be used as a simple first step in detecting bone disease.

Interestingly, GGT > 20 U/L was also associated with a two-fold increase of presence of CAD (OR: 1.94, 95% CI: 1.01 - 3.65, P = 0.050) (sensitivity 25.0%; specificity 85.6%, PPV 76.7%, NPV 37.6%, LR 1.73).

Discussion

The major findings of this cross-sectional study are the significant and independent associations and bidirectional links between liver markers, indices of bone and mineral metabolism and adipokines. Two main points arise from our results: 1) the liver-bone interactions, and 2) the role of adipokines in these relationships.

Liver-bone interactions

Our results are consistent with many studies that have shown that elevated liver markers are not only sensitive indicators of hepatobiliary diseases and alcohol consumption, but even within reference range, as in the present study, are associated with a variety of common diseases, such as CAD, AF, DM, dementia, PD [28-33], and mortality [34-37] independently of alcohol consumption. Reported in the literature age and gender-based differences in liver [32, 34-36] and bone turnover markers [38-42], as well as in three studied adipokines [43-46] were confirmed within our study population, specifically, the decrease of transaminase activities and increase of bone turnover (OC and NTx/Cr) with ageing, and higher levels of urinary bone resorption markers and circulating adiponectin and leptin in women than in men.

In the present study, we focused on associations between hepatic markers (in the majority of our patients, they were within the normal range), parameters of mineral and bone metabolism, and the concentrations of three adipokines. We found significant bidirectional links between liver and bone-mineral

parameters, specifically between: 1) GGT and OC, NTx/Cr (both inverse) and BAP (positive), 2) ALT and OC (inverse), and 3) ALP and BAP (positive). In addition, ALP was an independent predictor of OC, NTx/Cr and DPD/Cr, and was negatively associated with 25(OH)D. OC was an independent negative correlate of bilirubin, and in the model which included only laboratory data, there was a significant association between PTH and bilirubin. Interestingly, the activity of ALT, a more specific hepatic marker than GGT, has shown similar to GGT inverse associations with OC but to a lesser degree.

Consistent with our findings, an inverse association of serum OC concentrations with ALT has been demonstrated in non-obese and obese subjects [13] and in patients with NAFLD [47, 48], independent of insulin resistance and the metabolic syndrome [47].

There are clinical and epidemiological reports, as well as *in vitro* and animal studies showing important physiological effects of GGT on bone metabolism. In postmenopausal women urinary GGT excretion exhibited a high correlation with DPD [49]. A large longitudinal study (16,036 Korean men aged ≥ 50 years) demonstrated that a higher serum GGT level was associated with increased development of osteoporotic fractures over a mean 3-year follow-up period [50]. Experimental data indicate that both a deficiency and an excess of GGT result in osteoporosis [49, 51-53]. GGT *in vitro* stimulates osteoclast formation (independently of its enzymatic activity) and expression of receptor activator of nuclear factor-κB ligand (RANKL) mRNA and protein from bone marrow stromal cells, and in transgenic mice promotes osteoporosis [51-53]. It has been proposed that osteopenia/osteoporosis in GGT-deficient (GGT-/-) mice is caused by suppression of bone formation, while excess of GGT results mainly in acceleration of bone resorption [53]. In our cohort of older HF patients, we found significant bidirectional inverse associations between GGT and both OC (an osteoblast-specific noncollagen protein, a bone formation marker) and NTx/Cr (a recognized bone resorption marker) and a positive association between GGT and BAP (a bone formation marker); GGT was an independent negative predictor of the OC/BAP ratio (an index of osteoblast differentiation) [27]. Our observations are in agreement with reports that OC and BAP are not uniformly associated.

For example, BAP correlates positively with OC in healthy women and negatively with OC in women with liver disease [54]. OC is in a positive and BAP is in a negative relationship with serum insulin growth factor-I (GF-I) [27] and the OC/BAP ratio is inversely associated with the number of vertebral fractures. In the current study, the OC/BAP ratio adjusted for age and sex was significantly and negatively associated with GGT, ALT, ALP and bilirubin indicating that dysregulated osteoblast formation and differentiation is linked to liver status. Moreover, GGT was an independent negative predictor of the OC/BAP ratio. The opposite associations of GGT with two osteoblast-derived bone formation markers (OC and BAP) could point to its specific effect on osteoblast differentiation, as OC is a protein that binds to hydroxyapatite deposited in the matrix, whereas BAP is essential to the process of mineralization of the matrix after formation of osteoid [55, 56]. The negative correlation between GGT and NTx/Cr (in adjusted multivariate linear regression analyses higher serum GGT was a significant independent predictor of lower NTx/Cr, and vice versa) may reflect the complex role of GGT in coordinating the tightly coupled bone formation and resorption. It appears that GGT acts as a factor that maintains bone homeostasis, and plays dual role in physiopathology: higher GGT levels may affect osteoblast differentiation and decreases bone resorption. However, in malnourished women both serum OC and urine NTx/Cr were not independent predictors of GGT activity, while BAP was significantly associated with GGT (as it was observed in the total cohort and in non-malnourished subjects), suggesting that the links between liver markers and specific metabolic indices of bone remodeling are modulated by nutritional status and gender, and these may contribute to an individual's propensity to osteoporosis and bone frailty.

The bidirectional link between BAP and ALP is not surprising as approximately half of circulating total ALP comes from bone [55, 56].

Our study also showed that serum hemoglobin is a significant and independent predictor of GGT (inverse association) and albumin, another determinant of bone health in the elderly [57]. These results are in agreement with previous epidemiologic studies that demonstrated that hemoglobin level is positively associated with risk of development of NAFLD independently of body mass index, type 2 DM, and other metabolic diseases [58]. Taken together, our results suggest reciprocal liver-bone cross-talk. Liver plays an important role in the regulation and dysregulation of bone remodeling and vice versa (bone-mineral metabolism parameters modulate various hepatic functions). In these interactions, GGT seems to be a major factor acting on and being influenced by bone turnover markers, as well as multiple other metabolic factors and conditions.

Adipokines and markers of hepatic, mineral and bone metabolism

Extensive studies of leptin, adiponectin, and resistin, the three best-studied adipokines, in both hepatic and bone metabolism have produced conflicting results, especially when comparing

data from clinical and animal reports. In HF patients, studies on adipokines are limited [44, 59-62], and the relationship of adipokines with liver and bone turnover markers have not been evaluated systematically.

The present study, to our knowledge for the first time, documents the links between adiponectin, leptin and resistin, and liver and bone-mineral indices in patients with HF. In our multiple linear regression analyses, adiponectin, but not leptin and resistin, was an independent predictor of GGT activity, resistin (in humans, unlike in rodents, it is produced mostly in macrophages) [63] was a determinant for albumin and bilirubin (the latter in a model adjusted only for laboratory variables), whereas leptin independently predicted OC, OC/BAP ratio, NTx/Cr and DPD/Cr levels (inversely for both resorption markers).

In regard to adipokines-liver relationship, our observations are consistent with current evidence that the liver is a major target organ for many of its effects [18, 64]. In our study, as in previous reports, adiponectin was negatively associated with GGT [65, 66], although others found only a weak inverse association [67], or even a positive correlation [68] between these variables; some researchers demonstrated that adiponectin was negatively associated with circulating ALT activity [65-67, 69]. In a previous study, as in ours, serum levels of resistin positively correlated with bilirubin [68]. Our data are also in line with recent experiments showing that the majority of metabolic effects of leptin essential for energy homeostasis are dependent on its action in nonhepatocyte cells and/or the central nervous system [70].

Concerning the adipokine-bone links, we found that in the entire cohort leptin (but not adiponectin or resistin) was an independent predictor of OC, OC/BAP ratio and both bone resorption markers. These findings are in contrast with the widespread (based mainly on animal studies) view that leptin acting primarily through the centrally (hypothalamus and brainstem) mediated pathways suppresses bone formation [14, 71, 72], but are in accord with some, but not all [34, 44, 73-76], clinical studies that reported a positive association between serum leptin and OC levels [77, 78] and with experiments showing that peripheral leptin increases osteoblast proliferation, differentiation and activity, inhibits osteoclast activity and bone resorption, increasing bone mass [79-81]. Furthermore, a meta-analysis (59 studies) found that leptin was positively associated with bone mineral density (BMD) and high levels of leptin were predictive of lower risk of fractures [82].

There is considerable controversy on the role of adiponectin in bone metabolism. Although *in vitro* studies demonstrated that adiponectin stimulates the differentiation and mineralization of osteoblasts and the expression of OC, directly inhibits osteoclast activity and indirectly stimulates osteoclast differentiation [83-86], it is less clear whether adiponectin plays the same role in humans. We found that in malnourished women (but not in other groups) adiponectin significantly correlated with OC, suggesting that bone-adipocyte interaction in these subjects may be regulated through adiponectin and OC. Adiponectin was positively associated with total OC [87, 88] and BAP [89] in postmenopausal women and in Chinese men [90], but in other studies no correlation with any of bone formation

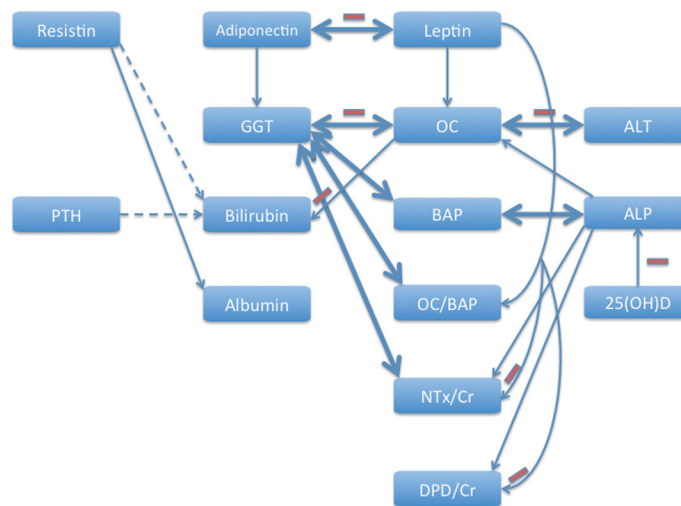


Figure 1. Links between liver markers, indices of bone metabolism and adipokines in older patients with hip fracture. GGT: gamma-glutamyltransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; OC: osteocalcin; BAP: bone specific alkaline phosphatase; NTx/Cr: cross-linked N-telopeptide of type 1 collagen corrected for urinary creatinine concentration; DPD/Cr: deoxypyridinoline corrected for urinary creatinine concentration; PTH: parathyroid hormone; Hb: hemoglobin. Bidirectional links are shown in bold arrows; – indicates inhibitory effect. The interactions between GGT and OC, BAP, and NTx/Cr, as well as between ALT and OC, and between ALP and BAP are bidirectional: the activity of hepatic enzymes contributes to synthesis and release of molecules reflecting bone formation and bone resorption, and these, in turn, influence the circulating levels of liver enzymes. ALP activity correlates with levels of serum OC and urinary resorption markers. There is a strong reciprocal association between adiponectin and leptin. Adiponectin is positively associated with GGT activity, while leptin is positively associated with OC and OC/BAP ratio and inversely with both resorption markers; therefore, adiponectin and leptin may exert opposite effects on bone metabolism; resistin is associated with serum albumin concentration. In multiple logistic regression analyses adjusted for age, sex and confounding laboratory (but not clinical) variables both resistin and PTH were independently associated with bilirubin levels (shown in dashed lines). All other data are based on fully adjusted model. Of note, in malnourished patients circulating adiponectin and OC levels are positively associated, while adiponectin does not correlate with GGT activity (not shown).

or bone resorption markers was found [45, 91]. Several clinical studies have shown an inverse relationship between circulating adiponectin and BMD independent of gender and menopausal status, and some reviewers concluded that adiponectin may be a negative regulator of bone mass [82, 92-94], while other emphasized that the data on this association are inconsistent [95].

It is also uncertain whether resistin is related to bone turnover markers. Resistin is expressed in primary human bone marrow stem cells and in mature human osteoblasts [96], but no association between resistin and BMD was found in clinical studies [92]. In our study, circulating resistin levels in line with the latter data were not associated with bone turnover markers.

In line with many previous studies, we observed a significant inverse relationship between adiponectin and leptin, both by paired comparisons adjusted for age and sex, as well as multivariate regression modeling. This indicates that metabolic functions of leptin and adiponectin, which generally affect cellular behavior in an opposing manner, should not be considered in isolation, but rather as complementary. Our data suggest that the bone remodeling, as measured by bone formation and resorption markers, depends, at least partially, on the regulatory interaction between adiponectin and leptin which may exert opposite effects on bone metabolism through different pathways. In other words, the adiponectin-leptin link appears as an important mediator of the association between

GGT and OC.

Our analysis also identified that in malnourished women, in contrast to subjects without such condition, adiponectin is not associated with GGT, but strongly correlates with OC. It appears that in different diseases and in particular conditions adiponectin may be regulated in the opposite directions and may exert opposite activities [97, 98]. At the molecular level, variations have been found at all sites of adiponectin action including different subtypes of adiponectin receptors operating in the liver and bones [94, 99, 100]. Our observations are in agreement with a recent animal study, which concluded that adiponectin has the ability to regulate the same function in two opposite manners depending on where it acts and it opposes leptin's influence [101]. Our results show that adiponectin may exert a negative net effect on bone metabolism by increasing GGT and decreasing circulating leptin levels (inverse adiponectin-leptin relationship); on the other hand, OC and adiponectin have opposite effects on GGT. However, these relationships are not consistently maintained, but expressed differently under various pathophysiological conditions indicating the level of complexity of the homeostatic system(s). Specifically, in malnourished women increases in serum adiponectin, which is produced almost exclusively by adipocytes, are associated with higher OC levels and do not correlate with GGT.

Alterations in vitamin D-PTH status are factors substantially contributing to the high prevalence of osteoporosis and fractures in the elderly [1]. Although vitamin D insufficiency and elevated PTH levels were recorded in 79.7% and 38.2% of our patients, respectively, in multivariate analyses no significant correlations were detected between GGT and ALT activities, on the one hand, and 25(OH)D and PTH concentrations, on the other, suggesting that the vitamin D-PTH axis is not an independent determinant of the transaminase activities. Also, 25(OH)D and PTH were not independent predictors of bone turnover markers, indicating that vitamin D and PTH are not involved in the relationship of these liver enzymes with bone remodeling. We found positive correlations between 25(OH)D and ALP, and between PTH and bilirubin, which indicate that vitamin D insufficiency and SHPT may affect specific liver functions, as it was previously reported for the PTH-bilirubin association [102, 103].

Potential mechanisms: combining regulatory elements

Our results illustrate complex interactions between different adipokines, liver function and mineral-bone metabolism, and extend this notion by showing these links in HF patients. The findings suggest liver function as an important component involved in bone remodeling. Significant independent correlates for OC included GGT, ALT (both negative), ALP, leptin and age (all three positive), for BAP-GGT and ALP, for NTx/Cr-GGT and leptin (both negative), ALP and age (both positive), for DPD/Cr-ALP (positive) and leptin (negative), and for OC/BAP ratio-GGT (negative) and leptin (positive). Integrating multidirectional interrelationship between adipokines, hepatic and bone metabolism, factors acting on and/or being influenced by each other, it is possible to propose a model that unifies a number of functions and observations that have been considered contradictory in the past (Fig. 1). This model based on the results of multivariate regression analyses takes into account that homeostasis necessitates reciprocal signaling between hepatic function, components of mineral-bone metabolism and adipokines. It should be viewed as a part of a complex network that integrates and orchestrates the liver-bone-adipose tissue axis in health and diseases. Of major pathophysiological interest is the feedback loop between GGT and OC, in which adiponectin and leptin appear as two important counterplayers: the former acts as a positive regulator of GGT, while the latter is a positive regulator of OC (Fig. 1, upper part). Importantly, these relationships are not invariant, but depend on multiple underlying conditions, among which the nutritional status and gender may be the key contributors.

The stronger (compared to other liver indices) and bidirectional association of GGT, an indicator of oxidative stress [28, 104], with bone turnover markers suggests that the liver-bone links might reflect systemic rather than solely hepatic processes. Several lines of evidence indicate oxidative stress, a systemic process implicated in the regulation of ageing and longevity [105], as well as numerous pathological conditions [106, 107], including liver diseases [108] and osteoporosis [109, 110], as a possible unifying factor. 1) GGT is respon-

sible for the extracellular catabolism of the main antioxidant in mammalian cells - glutathione [28, 104, 111]. 2) The liver plays a key role in the systemic glutathione (GSH) homeostasis [112, 113]. 3) Adiponectin significantly contributes to oxidative damage [114]. 4) Oxidative stress produces deleterious effects in osteoblasts [115]. In addition, our study showed involvement of other factors which have also been proposed as indices of oxidant stress status, namely serum hemoglobin levels were significantly and independently associated with GGT activity (inversely) and albumin concentration (positively), while OC correlated with bilirubin level. Hemoglobin [116, 117], albumin [118-120] and bilirubin [118, 121] exhibit potent antioxidant properties. Increases in GGT activity may reflect the responses of counteracting mechanisms to protect against oxidative damage. Our findings suggest the need to explore in depth the role of liver function in bone metabolism, in particular, the participation of GGT in these processes.

Potential clinical implications

Of practical interest is the finding that in the HF patients GGT > 20 U/L (above the cut-off level of the first tertile) corresponds with a two times higher prevalence of CAD, low OC levels (< 14 ng/mL, lower limit of normal range) and 2.7 times higher prevalence of low OC/BAP ratio. Consistent with our results, previous studies have found GGT to be associated with CAD [31, 33, 122, 123]. Collectively these results indicate that GGT within the normal range is broadly linked to health conditions, including osteoblast dysfunction, and older patients with GGT > 20 U/L need an examination of their bone status and consideration of an antiosteoporotic medication with anabolic properties. Currently, osteoporosis is predominantly treated with antiresorptive medications, despite the fact that in a significant proportion of elder patients bone loss is primarily attributed to the impaired osteoblastic activity [124]. Serum GGT > 20 U/L may be a promising indirect marker useful as the first and easy step in diagnostic evaluation of impaired bone metabolism in the elderly and pointing to the need of individualized therapy; its PPV for low OC and for low OC/BAP ratio is above 75%. The clinical significance of this marker should be confirmed and validated by longitudinal studies.

Limitations

The main limitations of this study are the cross-sectional nature of the analysis and the single point-in-time assessment of the hepatic indices, mineral-bone turnover markers and adipokines. Therefore, the associations between the variables found in the study suggest a link, but a causal relationship cannot be established. Secondly, we did not measure the undercarboxylated OC (unOC), RANKL, osteoprotegerin, high molecular weight adiponectin, and sexual hormones, each of which may be involved in the liver-bone interaction. Thirdly, we could not eliminate the possible effect of medications used on the present findings, although we attempted to control most potential confounders. Finally, because the study population

consisted predominantly of Caucasians, generalization of the results should be done with caution. The strengths of our study include the use of data from an unselected well-characterized cohort of HF patients, simultaneous measurements of multiple hepatic, mineral-bone biomarkers and three adipokines. Pearson's correlation coefficients preserved statistical significance after Bonferroni and Sidak adjustments. The variance inflation factor in our multivariate regression analyses ranged between 1.07 and 1.32 indicating that the multicollinearity phenomena were not significant.

Conclusions

In older HF patients liver functions (within normal range in the vast majority) are associated with indices of bone metabolism. The complex liver-bone-adipokine interactions include bidirectional links between GGT and markers of bone formation (OC, BAP), differentiation (OC/BAP ratio) and resorption (NTx/Cr), as well as between ALT and OC. Adiponectin is an independent predictor of GGT, whereas leptin is a determinant of OC and bone resorption markers; these relationships are modulated by nutritional status and gender. GGT > 20 U/L is associated with a two times higher prevalence of low OC levels and 2.7 times higher prevalence of low OC/BAP ratio; it may be used as a marker of impaired bone metabolism.

Acknowledgement

We thank Wichat Srikusalanukul, MD, for help with the statistical analyses.

Financial Support

None.

Potential Competing Interests

None.

Grant Support

None.

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Research Paper

Liver Function Parameters in Hip Fracture Patients: Relations to Age, Adipokines, Comorbidities and Outcomes

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Received: 2014.01.03; Accepted: 2014.04.11; Published: 2015.01.01

Abstract

Aim: To assess liver markers in older patients with hip fracture (HF) in relation to age, comorbidities, metabolic characteristics and short-term outcomes.

Methods: In 294 patients with HF (mean age 82.0±7.9 years, 72.1% women) serum alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), albumin, bilirubin, 25(OH)vitaminD, PTH, calcium, phosphate, magnesium, adiponectin, leptin, resistin, thyroid function and cardiac troponin I were measured.

Results: Elevated ALT, GGT, ALP or bilirubin levels on admission were observed in 1.7% - 9.9% of patients. With age GGT, ALT and leptin decrease, while PTH and adiponectin concentrations increase. Higher GGT (>30U/L, median level) was associated with coronary artery disease (CAD), diabetes mellitus (DM), and alcohol overuse; lower ALT (≤20U/L, median level) with dementia; total bilirubin >20μmol/L with CAD and alcohol overuse; and albumin >33g/L with CAD. Multivariate adjusted regression analyses revealed ALT, ALP, adiponectin, alcohol overuse and DM as independent and significant determinants of GGT (as continuous or categorical variable); GGT for each other liver marker; and PTH for adiponectin. The risk of prolonged hospital stay (>20 days) was about two times higher in patients with GGT>30U/L or adiponectin >17.14 ng/L (median level) and 4.7 times higher if both conditions coexisted. The risk of in-hospital death was 3 times higher if albumin was <33g/L.

Conclusions: In older HF patients liver markers even within the normal range are associated with age-related disorders and outcomes. Adiponectin (but not 25(OH)vitaminD, PTH, leptin or resistin) is an independent contributor to higher GGT. Serum GGT and albumin predict prolonged hospital stay and in-hospital death, respectively. A unifying hypothesis of the findings presented.

Key words: liver function, adipokines, hip fracture, comorbidities, outcomes

Introduction

Global population ages rapidly, and by the year 2050 the percentage of people >60 years of age is expected to reach 22%. Advanced age is the single major independent risk factor for most chronic diseases and functional deficits, accounting for 60% of all deaths

worldwide. The liver because of its multitude metabolic, homeostatic and detoxification functions plays a central role in aging and susceptibility to age-related diseases. Ageing is associated with significant loss of hepatic volume and blood flow, structural changes in

all liver cells, accumulation of ageing pigments at the cytoplasm and pseudocapillarization of the sinusoid.¹⁻⁴ Over the past several years, substantial research has shown that alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) activities decrease with old age.⁵⁻¹⁰ However, the relationship between ageing and liver function and diseases remains obscure.¹¹ For example, a number of reports concluded that in the elderly low ALT level is a strong and independent predictor of mortality,^{6, 8, 12-15} while in other studies of community-dwelling older adults^{14, 16-18} and older twins,¹⁹ elevated serum ALT,¹⁶ aspartate aminotransferase (AST), alkaline phosphatase (ALP)^{14, 17, 20} and, especially, GGT levels^{12, 14, 18, 20-29} predicted all-cause, cardiovascular, and liver mortality.

Numerous investigators found a positive association between ALT and/or GGT activities and metabolic syndrome, non-alcoholic fatty liver disease, type 2 diabetes mellitus (DM), cardiovascular disease (CVD), including coronary artery disease (CAD), hypertension, heart failure and stroke, chronic kidney disease, and cancers,^{19, 27, 30-44} conditions which are highly prevalent in older individuals. The aforementioned associations were often evident across ALT and GGT activities values within the normal range,^{13, 21, 23, 31, 45-47} independent of alcohol intake and other risk factors. Notably, although most of these studies did not focus specifically on the elderly population and the results have not been entirely consistent,⁴⁸ these associations were strong in young individuals, but weakened with age.^{21, 22, 24, 36, 42, 49, 50}

Liver is critically involved in metabolism of many factors contributing to bone health and hepatic osteodystrophy is a common well-documented complication in patients with chronic liver disease.⁵¹⁻⁵⁸ Surprisingly, limited research has examined the liver function in older patients with hip fracture (HF).^{59, 60} Liver markers, except low serum albumin concentration for mortality⁶¹⁻⁶⁸ and increased postoperative complications,^{61, 69} are not currently included in the prognostic criteria.

The potential pathogenic role of factors affecting both liver and bone, such as low vitamin D status, elevated PTH levels, dysregulation in secretion of adipokines (especially, adiponectin, leptin and resistin), all of which are common in the elderly, virtually have not been investigated systematically in HF patients, despite growing evidence that the liver, the bone and adipose tissue are functionally interrelated organs.⁷⁰ The aforementioned metabolic mechanisms are important for optimal function of many organs and tissues throughout the body and involved in numerous age-related comorbidities which may substantially contribute to poor outcomes in HF patients.

To our knowledge, no published study has evaluated the relationship between liver function parameters and age, comorbidities, adipokines, vitamin D and PTH, as well as short-term outcomes in HF patients.

The aim of this prospective observational study was three-fold: 1) to determine liver function parameters in older HF patients in relation to age, and whether markers of hepatic function are associated with comorbidities, 2) to evaluate the relationship between serum liver markers, on one hand, and serum concentrations of vitamin D, PTH, adipokines adiponectin, leptin, and resistin, on the other, and 3) to assess the value of liver function markers on admission as predictors of short-term outcomes.

Materials and Methods

Patients

The study population consisted of 294 patients (212 females and 82 males) aged 60 years and older with low-trauma osteoporotic HF admitted to The Canberra Hospital. Patients with high trauma, pathological HF, Paget's disease, primary hyperparathyroidism or who did not have all serum variables of interest measured were not considered for the study. A detailed medical history, full physical examination and medication use were obtained along with demographic and anthropometric variables in all patients.

Informed consent was obtained from all individuals or their carers. The study was approved by the regional ACT Health Human Research Ethical Committee and conducted according to the Helsinki Declaration (as revised in 2008).

Laboratory Analyses

After 12-hour overnight fast usually within 24 hours after arrival at the Emergency Department venous blood samples were taken and sera were isolated. One serum sample was frozen and stored at -70°C until further analysis of adiponectin, leptin and resistin. All other haematological and biochemical assessments were performed at the day of collection. All patients had the following tests performed: liver function markers (ALT, GGT, ALP, albumin and total bilirubin), complete blood count, urea, creatinine and electrolytes, fasting blood glucose (and HbA1C in diabetic patients), thyroid function tests (TSH, T4 and T3 if indicated), 25 (OH) vitamin D [25(OH)D], intact PTH, total calcium, phosphate, magnesium, C-reactive protein (CRP) and cardiac troponin I (cTnI), adiponectin, leptin and resistin.

Liver function tests were evaluated by using commercially available standard enzymatic reagents and diagnostic kits by spectrophotometry on the bio-

chemical autoanalyzer Abbott Architect CI16200 (Abbott Laboratories, IL 60064, USA). ALT, GGT and ALP were measured with enzymatic colorimetric methods, total bilirubin was analysed using diazonium salt, total protein was tested by a Biuret method and albumin was measured using bromocresol green. The mean inter-assay and intra-assay coefficients of variations (CV) for these tests were within 1.1% – 6.6%.

Serum levels of leptin were determined by enzyme-linked immunosorbent assay (ELISA) method (Diagnostic System Laboratories, Webster, TX, USA), total adiponectin and resistin by human ELISA kits (B-Bridge International, Mountain View, CA, USA). Intra- and interassay CV were less than 7% for these tests. All assays were performed according to the manufactures' instructions with kits of the same lot number.

Serum levels of 25(OH)D were determined by a radioimmunoassay kit (Dia Sorin, Stillwater, MN, USA; interassay CV 5.9–9.4%, intraassay CV<11.5%), intact PTH by solid-phase two-site chemiluminescent enzyme-linked immunometric assay on a DPC Immulite 2000 analyzer (Diagnostic Products, Los Angeles, CA, USA; interassay CV 6.2–7.0%, intraassay CV < 6%), cTnI by two-step chemiluminescent microparticle immunoassay (Chemiflex, Abbott Labs, Mississauga, Ontario, Canada; the minimum detectable limit 0.03 µg/L and the upper limit of reference range is 0.06 µg/L). Serum calcium concentration was corrected for serum albumin. Glomerular filtration rate (eGFR) was estimated using a standardized serum creatinine-based formula normalized to a body surface area of 1.73 m² (Levey A 2010; Johnson D 2012). All serum samples were processed in the same laboratory using the same methods and the same reference values.

For liver enzymes 2 times the upper normal limit (UNL) cut-off levels were used to define abnormal tests. When aminotransferase activities were analysed as categorical variables median values were used: for ALT 20U/L and for GGT 30U/L, levels comparable with data in the literature (Ruhl C 2012; Wu W 2012).

For the analyses, insufficiency of vitamin D was defined as 25(OH)D < 50 nmol/l and deficiency as 25(OH)D < 25 nmol/l based on current recommendations. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (>6.8pmol/l, the upper limit of the laboratory reference range).

Short-term outcomes included in-hospital all-cause mortality, prolonged length of stay (>20 days), post-operative myocardial injury defined by cardiac troponin I rise (>0.06 µg/L), high inflammatory response (CRP>100 mg/L) and being discharged to a permanent residential care facility.

Statistical Analyses

Statistical calculations were carried out using the Stata software version 10 (StataCorp, College Station, TX, USA). The summary statistics are presented as the mean ± standard deviation (SD) for continuous variables and as the number (percentages) for categorical variables. Comparisons between groups of patients were made by use Student's t test for normally distributed continuous variables and a χ^2 test for categorical variables. The relationships between variables were examined by Pearson's linear correlation test and multivariate logistic regression analyses after logarithmic transformation of continuous variables with a skewed distribution. When the dependent parameter was stratified by level to further assess the independent participation of each of the factors studied, odds ratios with 95% confidence intervals (CI) were measured in multivariate logistic regression models, incorporating into the models biomarkers as continuous variables and clinical characteristics (sex, presence of CVD, DM, excessive alcohol consumption, history of smoking, etc) as categorical/ binary variables (yes, no). Bonferroni and Sidak adjustments for multiplicity were performed. To assess the significance of multicollinearity phenomena in multivariate regression analyses, the variance inflation factor was calculated. Results were considered statistically significant if P values <0.05 (two-sided).

Results

Characteristics of patients

Demographic, biochemical and haematological characteristics of the study population on admission are summarized in Table 1. The mean age of the patients was 82.0 years, 72.1% were women who were slightly older than men (+2.0 years, $p=0.053$), and 89(30.2%) subjects were admitted from long care residential facilities. There were 152 patients with a cervical HF and 142 with an intertrochanteric HF. The prevalent comorbidities at the time of hospitalisation for HF included CVD (66.3%), chronic kidney disease (CKD)≥stage 3 (eGFR<60 ml/min/1.73m², 43.2%), dementia (27.8%), history of stroke or transient ischaemic attack (19.7%), type 2 DM (16.4%), chronic obstructive pulmonary disease (COPD, 11.0%), and Parkinson's disease (4.6%); 28(9.5%) patients consumed alcohol 3 or more times a week. In the study population 51(17.3%) of patients had neither CVD, CKD, or DM, nor were alcohol overusers. The prevalence of abnormal liver tests was as follows: ALT (>2ULN, 80 U/L) in 5(1.7%) patients, GGT (>2ULN, 128 U/L) in 23 (7.8%), ALP (>2ULN, 120 U/L) in 26 (8.8%), bilirubin (>1ULN, 20µmol/L) in 29 (9.9%) and albumin (<33 g/L) in 64 (21.8%) subjects. The liver

function abnormalities were small in magnitude and not associated with clinically apparent hepatic disease. There were no gender differences in indicators of liver functions, haemoglobin, and glycosylated haemoglobin (HbA1c) values. However, women, compared to men, had higher PTH, leptin, adiponectin and T4 concentrations and significantly lower mean 25(OH)D and eGFR levels.

Table 1. General characteristics of patients with hip fractures by gender

	Total Sample (n=294)	Men (n=82)	Women (n=212)	P Value
Age, years	82.0 ± 7.9	80.6 ± 8.3	82.6 ± 7.7	0.053
Cervical/Trochanteric fracture, n	152/142	38/44	114/98	0.298
GGT, U/L	54.1 ± 95.6	57.9 ± 106.0	52.6 ± 91.5	0.670
ALT, U/L	23.0 ± 42.6	18.9 ± 11.1	24.6 ± 49.6	0.302
ALP, U/L	105.4 ± 80.1	98.6 ± 64.6	108.2 ± 85.3	0.343
Total bilirubin, µmol/L	12.4 ± 7.4	13.6 ± 7.1	11.9 ± 7.5	0.073
Albumin, g/L	37.1 ± 6.3	37.4 ± 5.8	37.0 ± 6.5	0.573
Haemoglobin, g/L	124.9 ± 17.1	127.8 ± 16.8	123.7 ± 17.1	0.064
Leptin, ng/ml	18.5 ± 23.2	11.7 ± 18.6	21.1 ± 24.3	0.002
Adiponectin, ng/ml	17.5 ± 7.4	15.6 ± 7.6	18.3 ± 7.1	0.007
Resistin, ng/ml	18.7 ± 10.5	18.7 ± 10.7	18.6 ± 10.4	0.978
Leptin/adiponectin ratio	1.5 ± 2.4	1.1 ± 1.6	1.6 ± 2.7	0.121
Leptin/resistin ratio	1.4 ± 2.1	0.8 ± 1.0	1.6 ± 2.3	0.006
Adiponectin/resistin ratio	1.3 ± 1.5	1.1 ± 0.9	1.4 ± 1.7	0.148
25 (OH) D, nmol/L	37.3 ± 18.0	42.4 ± 18.2	35.3 ± 17.6	0.003
PTH, pmol/L	6.9 ± 5.6	5.5 ± 3.5	7.4 ± 6.1	0.009
TSH (mU/L)	1.55 ± 2.17	1.36 ± 1.35	1.62 ± 2.41	0.382
Free T4 (pmol/L)	15.9 ± 3.54	15.2 ± 3.53	16.2 ± 3.50	0.021
eGFR, ml/min/1.73m ²	65.1 ± 23.7	71.2 ± 26.4	62.7 ± 22.2	0.006
*HbA1c, %	6.6 ± 1.04	6.7 ± 1.29	6.6 ± 0.94	0.825

Data are mean ± SD. GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; 25(OH) D, 25-hydroxyvitamin D; PTH, parathyroid hormone; TSH, thyroid-stimulating hormone; T4, thyroxine; eGFR, estimated glomerular filtration rate; * HbA1c, glycosylated haemoglobin, measured only in patients with type 2 diabetes mellitus.

Ageing mediated changes in serum metabolic parameters

The results of Pearson's correlations between liver markers, other parameters and age are shown in Table 2. GGT, ALT, leptin and eGFR were inversely associated with age, whereas adiponectin, resistin, PTH, creatinine, and urea were positively correlated with age. As might be expected, age showed a significant positive association with American Society of Anaesthesiologists (ASA) score ($r=0.224$; $p=0.001$) and presence of dementia ($r=0.214$; $p=0.001$). There was no significant association between any other studied parameters and age.

Study subjects were further classified into three age groups: <75, 75 – 85 and >85 years (Table 3). In the oldest patients (aged > 85 years), compared to aged < 75 years, the mean GGT activity was 50.6% and the ALT activity 48.4% lower; the mean leptin, 25(OH)D

and eGFR levels were also 36.5%, 14.6% and 29.4% lower, respectively, while adiponectin (+42.2%), resistin (+25.1%) and PTH (+72.3%) demonstrated an opposite trend (all p for trend <0.05). No associations between age and ALP, albumin, bilirubin, TSH, T4, HbA1c were found.

Table 2. Association between age and parameters of liver, renal, thyroid functions, adipokines and haemoglobin in older patients with hip fractures (Pearson correlation coefficients)

Variable	r	P Value
GGT	-0.198	0.001
ALT	-0.134	0.022
ALP	-0.076	0.192
Bilirubin	0.006	0.920
Albumin	-0.060	0.308
Leptin	-0.159	0.007
Adiponectin	0.248	<0.001
Resistin	0.148	0.018
Egfr	-0.313	<0.001
Creatinine	0.202	0.005
Urea	0.296	0.001
25 (OH)D	-0.003	0.958
PTH	0.250	<0.001
TSH	0.032	0.594
T4	-0.027	0.662
Haemoglobin	-0.110	0.058
ASA score	0.224	0.001
Dementia	0.212	0.001

Data were log transferred before analysis (to achieve normal distribution).

GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; TSH, thyroid-stimulating hormone; T4, thyroxine; eGFR, estimated glomerular filtration rate; ASA, American Society of Anaesthesiologists.

Table 3. Liver and renal parameters, adipokines, vitamin D, PTH and haemoglobin levels in older patients with hip fracture by age

Variable	Age (years)			P Value
	<75 (n=52)	75 – 85 (n=145)	>85 (n=97)	
Age, mean ± SD	69.3±5.0	81.2±2.7	90.1±3.5	<0.001
GGT, U/L	85.6±65.1	50.7±77.2	42.3±61.5	0.010
ALT, U/L	37.8±29.0	21.1±24.1	19.5±18.5	0.010
ALP, U/L	114.6±89.5	104.1±76.1	102.5±66.4	0.564
Bilirubin, µmol/L	12.3±8.3	12.5±7.7	12.2±6.4	0.948
Albumin, g/L	38.1±5.4	36.8±6.1	36.9±6.1	0.438
*HbA1c, %	6.6±1.0	6.6±1.0	6.5±1.2	0.971
Leptin ng/ml	20.8±24.7	21.2±26.5	13.2±14.9	0.026
Adiponectin, ng/ml	13.5±6.5	17.8±7.9	19.2±6.2	0.001
Resistin, ng/ml	16.7±10.3	17.9±9.6	20.9±11.4	0.044
25 (OH) D, nmol/L	39.7±15.9	37.1±19.0	33.9±17.5	0.035
PTH, pmol/L	4.7±3.1	6.8±5.0	8.1±7.0	0.002
eGFR, ml/min/1.73m ²	82.9±25.2	63.1±21.5	58.5±21.5	<0.001
Hb, g/L	129.8±17.4	124.4±18.0	122.9±15.1	0.051

Data are the mean ± SD. P value for trend. GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; *HbA1c, glycosylated haemoglobin, measured only in patients with type 2 diabetes mellitus; 25(OH) D, 25-hydroxyvitamin D; PTH, parathyroid hormone; eGFR, estimated glomerular filtration rate; Hb, haemoglobin.

Links between liver markers and comorbidities

Parameters of liver function were compared between patients with and without specific co-morbid conditions. Lower ALT levels (≤ 20 IU/L) were associated with dementia (OR=2.11, 95%CI 1.10 – 4.05, $p=0.015$). Lower GGT levels (≤ 30 U/L) were prevalent in females (OR=1.88, 95%CI 1.09 – 3.25, $p=0.016$). Higher GGT levels (>30 U/L) were associated with coronary artery disease (CAD, OR=2.55, 95%CI 1.37 – 4.74, $p=0.003$), type 2 DM (OR=2.19, 95%CI 1.12 – 4.29, $p=0.013$), and excess alcohol consumption (>3 times a week, OR=6.39, 95%CI 1.6 – 29.57, $p=0.002$), while total bilirubin levels above $20 \mu\text{mol/L}$ were associated with CAD (OR=2.56, 95%CI 1.01 – 6.42, $p=0.025$), cervical (vs. trochanteric) HF type (OR=3.26, 95%CI 1.27 – 8.72, $P=0.006$) and alcohol overuse (OR=4.67, 95%CI 1.12 – 18.19, $p=0.008$). Albumin concentrations above 33g/L were associated with CAD (OR= 3.13, 95%CI 1.21 – 8.5, $p=0.009$). On the other hand, the majority of 51 patients without prevalent CVD, CKD ≥ 3 stage, DM, and non-alcohol overusers, had ALT ≤ 20 U/L (70.6%), GGT ≤ 30 U/L (66.7%), albumin $>33 \text{g/L}$ (74.5%) and bilirubin $<20 \mu\text{mol/L}$ (94.1%).

Correlations between liver markers and other parameters

Pearson's correlation coefficient between log ALT and log GGT was 0.372 ($p<0.001$). Both GGT and ALT were also significantly and positively associated with ALP ($r=0.426$; $p<0.001$ and $r=0.141$; $p=0.015$, respectively), and bilirubin ($r=0.218$; $p=0.001$, and $r=0.171$; $p=0.003$, respectively). ALT inversely correlated with albumin ($r=-0.124$; $p=0.034$). ALP was negatively associated with 25(OH)D ($r=-0.187$, $p=0.002$). Bilirubin correlated with resistin ($r=0.151$, $p=0.016$), and PTH with adiponectin ($r=0.193$, $p=0.002$). No other associations were found between GGT, ALT, bilirubin, albumin, on one hand, and 25(OH)D, PTH and adipokines, on the other hand. However, GGT correlated positively with TSH ($r=0.116$, $p=0.05$) and negatively with T4 ($r=-0.185$, $p=0.002$). There was also a significant inverse association between leptin and adiponectin ($r=-0.178$, $p=0.005$).

Multiple linear regression analysis with GGT as the dependent variable and age, sex, ALT, ALP, bilirubin, albumin, eGFR, three adipokines (adiponectin, leptin and resistin), 25(OH)D, PTH, T4 levels, alcohol consumption (>3 times/week, yes/no), presence of DM (yes/no), and CVD (yes/no) as independent variables revealed eight independent and significant determinants of GGT (as a continuous variable): ALT, ALP, bilirubin, adiponectin, alcohol overuse, DM (all six positively related), albumin and age (both inversely related). This model explained 54.3% of the

variance in serum GGT activity (Table 4). Similar analyses for four other liver markers, namely ALT, ALP, bilirubin and albumin, as dependent parameters in separate regression models showed that GGT was an independent and significant predictor for each of these liver function parameters ($\beta=0.112$, $p=0.10$; $\beta=0.541$, $p<0.001$; $\beta=0.019$, $p=0.004$; $\beta=-0.012$, $p=0.039$, respectively). When multiple linear regression analysis was performed with adiponectin as the dependent variable three independent significant associations were found: age ($\beta=0.232$, $p=0.001$), male sex ($\beta=-2.500$, $p=0.031$) and PTH ($\beta=0.185$, $p=0.040$).

Table 4. Multivariate linear regression analyses for serum gamma-glutamyltransferase (GGT) level as a dependant continuous (model 1) and categorical (>30 U/L, model 2) variable

	Model 1		Model 2		
	β	P Value	OR	95% CI	P Value
Age	-2.464	<0.001	0.98	0.94-1.03	0.563
Sex (m)	2.008	0.852	2.53	1.28-5.03	0.008
ALT	0.263	0.010	1.04	1.01-1.06	0.013
ALP	0.637	<0.001	1.01	1.01-1.02	0.015
Bilirubin	1.869	0.004	1.01	0.94-1.06	0.602
Albumin	-1.554	0.039	1.01	0.95-1.06	0.738
Adiponectin	1.403	0.034	1.06	1.02-1.11	0.009
*Alcohol	92.368	<0.001	6.13	1.44-26.02	0.015
DM	30.560	0.048	2.29	1.03-5.07	0.041
	$R^2=0.5426$		$R^2=0.1924$		

β , parameter estimate/regression coefficient; OR, odds ratio; CI, confidence interval; ALT alanine aminotransferase; ALP, alkaline phosphatase; DM, diabetes mellitus. *Alcohol consumption ≥ 3 times a week.

Adjustments: age, sex, ALT, ALP, bilirubin, albumin, adiponectin, leptin, resistin, 25(OH) D, PTH, estimated glomerular filtration rate (eGFR), alcohol overuse, presence of type 2 diabetes mellitus and cardiovascular disease. Only statistically significant associations (at least in one model) shown ($p<0.05$).

In multivariate adjusted regression model that included GGT >30 U/L as the dependent variable and the same independent variables, the significant associations showed adiponectin, male sex, ALT, ALP, alcohol overuse and DM (Table 4, model 2). In multivariate regression analyses, ALT <20 U/L was independently predicted only by GGT (OR=0.992, $p=0.021$), and albumin $<33 \text{g/L}$ was predicted only by GGT (OR=0.995, $p=0.033$) and ALP (OR=1.006, $p=0.040$). Compared to the rest of the cohort, the patients with GGT >30 U/L (105.7 ± 65.5 vs. 19.6 ± 5.1 U/L) had also significantly higher levels of ALT (31.9 ± 35.8 vs. 17.2 ± 8.2 U/L, $p=0.004$), ALP (129.4 ± 109.4 vs. 89.3 ± 45.8 U/L, $p<0.001$), bilirubin (13.7 ± 9.0 vs. $11.5 \pm 6.0 \mu\text{mol/L}$, $p=0.011$), adiponectin (18.8 ± 7.4 vs. $16.6 \pm 7.2 \text{ ng/ml}$, $p=0.015$) and resistin (20.3 ± 10.9 vs. $17.5 \pm 10.0 \text{ ng/ml}$, $p=0.038$).

On subgroup analysis of patients with vitamin D insufficiency (25(OH)D $<50 \text{ nmol/L}$, $n=222$), the subjects with elevated PTH levels ($>6.8 \text{ pmol/L}$, $n=98$) compared to those with PTH within the reference

range (n=124) demonstrated a significantly higher mean adiponectin concentration (19.8 ± 7.3 vs. 16.0 ± 7.1 ng/ml, $p=0.001$), while 25(OH)D levels were similar (29.0 ± 11.96 vs. 31.3 ± 11.83 nmol/L, $p=0.194$). It appears, therefore, that elevated PTH (not vitamin D insufficiency *per se*) modulates adiponectin production by adipose tissue.

Taken together, these data show strong bidirectional associations between GGT and ALT, ALP, bilirubin (all positive) and albumin (negative), and confirm that both DM and alcohol overuse are independent predictors of higher GGT. More importantly, the results indicate that in older patients with HF circulating adiponectin, which rises with ageing in parallel with PTH, is an independent and significant determinant of serum GGT activity. Multiple regression analysis adjusted for possible covariates, including alcohol consumption and DM, identified that for every 1ng/ml increment of serum adiponectin concentration the probability of higher GGT(>30 U/L) increases by 6.2%. With age both GGT and ALT decrease, while PTH and adiponectin levels increase, and the latter seems to have a greater effect on serum GGT activity than age. These complex relationships are depicted in Figure 1.

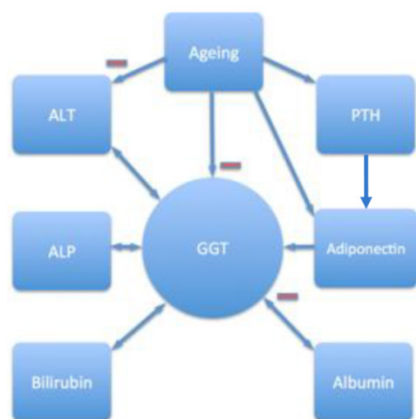


Figure 1. Relationships between age, liver function parameters, circulating levels of adiponectin and PTH (independent and significant associations shown). GGT, gamma-glutamyltransferase; ALT alanine aminotransferase; ALP, alkaline phosphatase; PTH, parathyroid hormone; — negative correlation. Ageing is associated with decreased GGT and ALT activities and higher serum PTH and adiponectin levels. PTH correlates with adiponectin and adiponectin is associated with higher GGT levels. A bidirectional relationship links GGT with ALT, ALP and bilirubin (positively) and albumin (negatively).

Liver markers and short-term outcomes

In our cohort, the prevalence of prolonged LOS (>20days) was 31.6% (n=94), and the in-hospital death rate was 4.8% (n=14). Myocardial injury (cTnI rise) was observed in 27.2% (n=80) of patients, a high post-operative inflammatory response (CRP>100 mg/L) in 60.2% (n=177) and 49% of patients admitted from home have been discharged to a permanent

residential care facility.

When serum parameters (log transformed) of liver function were analysed as continuous variables by Pearson correlation, GGT activity correlated positively with prolonged hospital stay ($r=0.140$, $p=0.020$), and ALP correlated with a higher post-operative inflammatory response ($r=0.123$, $p=0.036$); albumin correlated inversely with in-hospital death ($r= -0.137$, $p=0.005$), postoperative cTnI rise ($r=-0.292$, $p<0.001$) and need to be discharged to a permanent residential care facility ($r= -0.151$, $p=0.002$).

When each liver marker was examined as a categorical variable in multivariate adjusted analysis (with age, sex, other liver parameters and eGFR as independent variables), GGT >30U/L was associated with 1.9-fold increases in prolonged hospital stay and albumin<33g/L was associated with a 3-fold higher in-hospital mortality (Table 5). These findings remained consistent or even stronger after further adjustment for serum leptin, adiponectin, resistin levels, ratios leptin/adiponectin, leptin/resistin and adiponectin/resistin, 25(OH)D and PTH (model 2), as well as such potential confounders as alcohol consumption, smoking (current or previous), presence of DM and CVD (Table 5, model 3). Of note, in multivariate analyses, adiponectin ($p=0.031$), PTH ($p=0.001$), age ($p=0.051$) and smoking history ($p=0.007$) were also independent predictors of prolonged hospital stay, while PTH ($p=0.001$) and male sex ($p=0.03$) were independently associated with in-hospital death. In patients with LOS>20 days, compared to the rest of the cohort, the mean adiponectin levels were significantly higher (18.9 ± 7.9 vs. 16.4 ± 7.0 ng/ml, $p=0.022$). Serum adiponectin concentration above the median level (17.14 ng/ml) 2-fold increases the risk of prolonged hospital stay (OR 2.01, 95%CI 1.16-3.77, $p=0.014$) after adjustments for age, sex, liver markers, 25(OH)D, PTH, eGFR, presence of CVD and DM.

Table 5. Liver function parameters at admission as independent predictors of poorer outcomes in older patients with hip fracture

Parameter	Outcome	Model	OR (95%CI)	P Value
GGT>30U/L	LOS>20 days	1	1.86 (1.09 – 3.19)	0.023
		2	1.98 (1.12 – 3.49)	0.019
		3	1.74 (1.08 – 3.10)	0.038
Albumin<33g/l	In-hospital death	1	3.13 (1.01 – 9.09)	0.049
		2	3.45 (1.08 – 11.11)	0.038
		3	11.11 (2.33 – 50.00)	0.003

GGT, gamma-glutamyltransferase; LOS, length of hospital stay; OR, odds ratio; CI, confidence interval.

Model 1: adjusted for age, sex, liver function parameters (GGT, ALT, ALP, bilirubin, albumin, except the evaluated categorical variable), and estimated glomerular filtration rate (n=293).

Model 2: Model 1 plus serum leptin, adiponectin, resistin, ratios leptin/adiponectin, leptin/resistin, adiponectin/resistin, 25(OH)D and PTH (n=285).

Model 3: Model 2 plus alcohol consumption (≥ 3 times a week), smoking (current or previous), presence of type 2 diabetes mellitus, cardiovascular disease (hypertension, coronary artery disease, history of stroke, atrial fibrillation) (n=285).

The prognostic value of GGT>30U/L for prolonged LOS [accuracy 60.4%, 95%CI 54.7 – 66.1%, sensitivity 48.9%, 95%CI 39.9 – 57.8%; specificity 65.8%, 95%CI 61.6 – 69.9%; PPV 39.8%, 95%CI 32.5 – 47.1%; NPV 73.5%, 95%CI 68.9 – 78.1%] was comparable with that of higher adiponectin level (cut-off 17.14 ng/ml) [accuracy 57.9%, 95%CI 51.6 – 63.7%, sensitivity 61.5%, 95%CI 51.8 – 70.7%; specificity 56.1%, 95%CI 51.5 – 60.4%; PPV 40.0%, 95%CI 33.6 – 45.9%; NPV 75.4%, 95%CI 69.2 – 81.3%]. However, higher GGT and adiponectin levels show synergistic prognostic value for prolonged hospital stay: the risk increases 4.7-times in patients with both these characteristics compared to subjects with none of such features (Figure 2). These two biomarkers, although interrelated, are associated with different specific pathogenetic processes; thus, for predicting LOS a two-marker approach (two biomarkers measured in parallel) is described. Compared with presence only of one of these biomarkers, combined GGT>30U/L plus adiponectin >17.14ng/ml, as would be expected, demonstrated an improvement of prognostic accuracy (72.3%, 95%CI 66.9 – 77.3), specificity (83.2%, 95% CI 79.2 – 86.8%) and PPV (57.3%, 95%CI 47.4 – 66.6%), while the sensitivity (48.9%, 95%CI 40.4 – 56.8%), and NPV (77.8%, 95% CI 74.2 – 81.3%) unchanged.

Serum albumin <33g/L has the following prognostic value for in-hospital deaths: accuracy 77.2%, 95%CI 75.0 – 79.8, sensitivity 42.9%, 95%CI 19.1–69.7%; specificity 78.9%, 95%CI 77.7 – 80.3%; PPV 9.2%, 95%CI 4.1 – 15.0%; NPV 96.5%, 95%CI 95.1 – 98.1%.

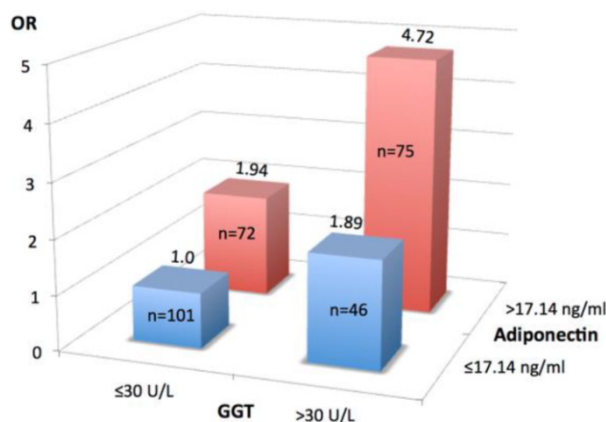


Figure 2. Association between prolonged hospital stay (>20 days) and gamma-glutamyltransferase (GGT) and adiponectin profiles. Study patients were classified into four groups according to serum levels of GGT (≤30U/L or >30U/L) and adiponectin (≤17.14 ng/ml and >17.14 ng/ml). Values are odds ratio (OR) adjusted for age, sex, alcohol consumption, presence of type 2 diabetes and cardiovascular disease. Number of subjects in each group is shown in the column. Group 1 (n=101)-reference (OR=1.0), group 2 (n=46)-high GGT and low adiponectin (OR=1.89, 95%CI 1.29 – 4.49, p=0.026), group 3 (n=72)- low GGT and high adiponectin (OR= 1.94, 95%CI 1.64–3.22, p=0.004), group 4 (n= 75)- high GGT and high adiponectin (OR=4.72, 95%CI 2.58 – 8.65, p=0.001). Of note, both higher serum GGT (>30U/L) and adiponectin (>17.14 ng/L) levels are synergistically associated with prolonged LOS.

Discussion

Main findings

The results of this observational study of older HF patients showed that: (1) the prevalence of abnormal liver function tests is relatively low, but hepatic functions (even within normal range) are associated with common age-related disorders, (2) with age GGT and ALT activities decrease, while serum PTH and adiponectin concentrations increase, (3) adiponectin is an independent contributor to higher GGT, which, in turn, demonstrates bidirectional links with ALT, ALP, bilirubin and albumin levels, and (4) GGT>30U/L and albumin<33g/L on admission, regardless of other traditional risk factors, are useful independent predictors of prolonged LOS and in-hospital mortality, respectively.

Our work corroborates previous studies showing age-related decline in hepatic function, extends the understanding of the liver status and the role of adipokines, especially adiponectin, in its regulation in the elderly patients with HF, and presents novel findings of pathophysiological and clinical importance.

Liver markers and related parameters in patients with hip fracture according to age

We found that in older HF patients the prevalence of abnormally elevated ALT, GGT, ALP or bilirubin on admission was relatively low (<10%, ranging between 1.7% and 9.9%) and comparable with or even lower than that reported in population-based studies,^{17, 71–75} as well as in a cohort of orthopaedic patients.⁵⁹

In our study, ageing was closely linked with decline in GGT, ALT and leptin concentrations and increasing adiponectin and PTH levels, both in correlation analyses and when young old (<75 years) patients were compared to the old-old (>85 years); adiponectin, leptin and PTH concentrations were significantly higher in females than in males. These findings are consistent with many, but not all^{21, 76, 77} previous reports that serum GGT^{5, 9} and ALT^{6, 8–10, 73, 78–81} activities (within reference range as well as elevated) are negatively associated with age. We observe neither a decline in serum albumin, nor an increase in bilirubin with increasing age as it was reported by some authors.⁹ No gender dependence in the mean values of liver markers was found, although lower GGT levels (≤30U/L) were about 2-times prevalent in females; this is in agreement with previous studies that men had significantly higher concentrations of GGT than women.^{5, 21, 23} Our data are in agreement with earlier studies showing age-related increase of circulating adiponectin^{82–86} and decrease of leptin

levels,^{87,88} and with the well-recognized fact that both these adipokines are higher in women than in men. In previous reports, as in ours, PTH levels were significantly associated with age and higher in women than in men.⁸⁹⁻⁹⁴ However, such influence of age and gender on PTH was not observed in a recent study of 35-65 year old healthy adults.⁹⁵ These discrepancies might be due, at least partially, to the higher mean age in our cohort.

Liver markers and comorbidities

The high burden of pre-existing comorbidities at time of HF and its important affect on outcomes are well known, but the role and impact of liver function status on comorbidities has not yet been examined in the setting of HF. In our study, higher GGT levels (>30U/L), as might be expected, were associated with CAD, DM, and excess alcohol consumption, while lower ALT levels (≤ 20 U/L) were associated with dementia; total bilirubin levels above 20 μ mol/L were associated with CAD, cervical (vs. trochanteric) HF type and alcohol overuse, and albumin concentrations above 33g/L were associated with CAD. These findings may indicate that even in the absence of overt liver disease hepatic functions are implicated in the pathogenesis of age-related disorders rather than merely be bystanders of ageing.

Our findings are consistent with numerous studies and meta-analysis showing the association between serum GGT levels (within normal limits) and CAD,^{21-23, 46, 47, 77, 96-102} DM,^{25, 47, 103, 104} alcohol consumption,^{32, 47, 105} cardiovascular and all-cause mortality in older adults,^{14, 18, 24, 105, 106} and the contribution of GGT to the pathogenesis of atherosclerotic plaques.^{46, 107, 108} Our data are also consistent with reports that ALT is poorly linked to CVD,^{35, 36} and GGT (but not ALT), which is regarded as less specific for liver injury than ALT, is significantly associated with insulin resistance in non-diabetic subjects.¹⁰⁹ Of note, results of our multivariate analysis demonstrated that DM and alcohol overuse were significant determinants of higher GGT (both as a continuous or categorical variable), but presence of CVD was not an independent predictor of GGT, suggesting that shared factors influence the pathogenetic relationship between the liver and CVD. The association of lower ALT levels with dementia is plausible given that ageing is a multidimensional process and age-related decrease in ALT, a highly specific liver enzyme restricted to the cytosolic component of hepatocytes,¹¹⁰ may signify a decrease in the mass and function of the normal liver^{6, 7} in parallel with progressive decline of other vital functions.

In our cohort, contrary to some previous studies, higher bilirubin levels were associated with CAD. Low bilirubin levels have been reported to be associ-

ated with CAD,¹¹¹ its severity,¹¹² increased carotid intima-media thickness,¹¹³ carotid atherosclerosis,¹¹⁴ peripheral arterial disease,¹¹⁵ and total mortality,¹¹⁶ while higher levels or mild to moderate elevations were associated with a protective effect against coronary microvascular dysfunction,¹¹⁷ reduced cardiovascular events¹¹⁸ and stroke,¹¹⁹⁻¹²¹ as well as CAD in subjects with Gilbert syndrome.¹²² On the other hand, total bilirubin has been shown to be a strong and independent predictor of morbidity and mortality in patients with heart failure,¹²³⁻¹²⁶ acute myocardial infarction,^{127, 128} and after heart valve surgery.¹²⁹

Reports on the relationship between serum albumin level and atherosclerotic CVD are also conflicting, demonstrating a negative,^{111, 130} positive,¹³¹⁻¹³³ or no association.^{134, 135} A recent study showed that higher albumin levels were associated with insulin resistance, but did not predict the development of diabetes.¹³⁶ In our cohort, the odds ratio for presence of CAD was 3.1 in patients with serum albumin above 33g/L. A possible explanation for the associations between higher bilirubin levels and/or normal albuminaemia with CVD is that individuals with lower bilirubin or albumin levels have already died by the time of HF. In the current as many previous studies^{61-68, 137} hypoalbuminaemia was strongly associated with mortality. Therefore, the high-normal bilirubin and/or albumin levels in our older HF patients with CAD are not contradictory to observations of negative correlations between serum albumin and/or bilirubin levels and cardiovascular risk factors, as well as CAD.

The abovementioned conflicting findings regarding the associations between liver markers and comorbidities might be attributable to different characteristics of the study populations (age and sex distribution, alcohol consumption and smoking habits, obesity and insulin resistance, presence of metabolic syndrome or DM) and emphasise the complexity of the underlying mechanisms indicating the importance of an integrated approach to interpretation.

Correlations: hepatic markers and adipokines, PTH and vitamin D

To make matters even more difficult to the interpretation of results, the literature data on the inter-correlations between liver function markers, as well as on correlations between these markers and adipokines, and between adipokines and 25(OH) D and PTH in different pathologies are controversial.

In our study, GGT activity and other hepatic markers exhibited a bidirectional relationship: a positive with ALT, ALP and bilirubin and a negative with albumin. These observations are in line with many, but not all,¹²⁰ reports showing significant positive

correlations between GGT and ALT,^{5, 8, 138, 139} ALP¹⁴⁰ and bilirubin.¹⁴¹ These bidirectional links indicate that GGT activity represents an integral component of liver function.

The adipocytokines adiponectin, leptin and resistin, three best-studied pleiotropic hormones involved in a number of physiological and pathophysiological processes from energy homeostasis to inflammation and immunity, have been implicated in hepatic dysfunction.^{70, 142-150} However, in our cohort we were not able to demonstrate an association between leptin or resistin and liver markers, except higher mean resistin levels in patients with GGT>30 U/L.

In contrast, a main novelty of this study is a significant positive correlation between serum adiponectin, the most abundant adipocytokine, and GGT activity. Yet it should be noted that the robustness of adiponectin as an independent determinant of higher GGT become obvious after adjusting for important confounders (Table 5). Our results are in contrast with those observed in patients with type 2 DM,^{151, 152} visceral obesity,¹⁵³ non-alcoholic hepatic steatosis (NASH),^{154, 155} as well as in healthy (often overweight and obese) individuals,^{96, 156-159} and Japanese male workers,¹⁶⁰ in all of which adiponectin levels were negatively correlated with GGT. Although a significant association between ALT and adiponectin was observed in young healthy men,¹⁶¹ in the majority of previous studies, as in ours, ALT and ALP levels were not associated with adiponectin.^{153, 155, 159, 162} Why adiponectin is more strongly linked to GGT than to ALT and other liver markers is not entirely clear. Although serum GGT is predominantly secreted by the liver, it is present and active on the surface of most cell types where it plays an important role in glutathione metabolism; GGT may also capture extra-hepatic processes relevant to ageing.

More importantly, while in DM, metabolic syndrome and NASH adiponectin levels are reduced and aminotransferase activities increased, in advanced liver disease, paradoxically, adiponectin levels are elevated and positively correlate with markers of liver cell injury, including GGT.¹⁶³⁻¹⁶⁵ The contrasting findings in the relationship between adiponectin and liver markers in these diseases may reflect different underlying mechanisms. For example, the strong association between adiponectin and insulin resistance in the first group of conditions^{166, 167} was not observed in cirrhotic patients;¹⁶⁴ in subjects with normal adiponectin and leptin levels liver enzyme activities did not reflect insulin resistance.¹⁶⁸ It has been suggested that the inverse association between adiponectin and insulin could be a function of suppressed adiponectin secretion by hyperinsulinemia,¹⁶⁹ although in other

studies higher adiponectin levels predicted lower incidence of diabetes independent of prevalent insulin resistance and glycemic status.^{170, 171} Possible explanations for these conflicting associations should also include other factors influencing adiponectin levels, such as adipose mass, hepatic¹⁶³ and renal¹⁷² catabolism, natriuretic peptides, which directly stimulate adiponectin production,²² and adiponectin resistance.¹⁷³

The complex pathophysiological role of adiponectin is further reflected in contradictory reports on the relationship between adiponectin levels and mortality in animal and human studies.¹⁷⁴⁻¹⁸¹

All these discrepancies should be interpreted in the context of known adiponectin "paradoxes":¹⁸²⁻¹⁸⁵ 1) inverse association of circulating adiponectin level with body weight, obesity/visceral fat percentage; 2) metabolically beneficial effects of the hormone (anti-atherogenic, anti-inflammatory, insulin sensitizing, anti-fibrinogenic and anti-apoptotic properties in the liver and other organs) well-documented in experimental and human studies, low serum adiponectin concentrations in non-alcoholic fatty liver disease, atherosclerotic CVD (CAD, stroke, peripheral artery disease), hypertension, DM, metabolic syndrome, and cancers (prostate, colon, gastric, breast, leukemia) in contrast with increased adiponectin levels in advanced liver disease, including NASH-related cirrhosis, as well as in high-risk CVD patients, subjects with chronic heart failure, kidney, pulmonary and connective tissue diseases, preeclampsia, and in critically ill; 3) its favorable (protective) associations with DM, metabolic syndrome and CVD in middle-aged cohorts and the opposite (increased risk of CVD, cardiovascular outcomes and all-cause mortality) for older populations; 4) a U-shaped relationship with CVD, particularly CAD, and mortality in older adults,¹⁸⁶ although the oldest-old individuals^{83, 187, 188} and centenarians¹⁷³ have higher adiponectin levels than younger subjects. It appears, therefore, that in different pathologies and age groups adiponectin may be regulated in opposite directions.

In the present study, multivariate analysis showed that in older HF patients adiponectin is correlated positively with GGT in contrast to the inverse association reported in other cohorts, including healthy persons and, especially, patients with obesity-associated chronic diseases. The fact that adiponectin was significantly higher in patients with GGT>30U/L, and GGT levels (after controlling for confounding factors) were bidirectionally inversely correlated with albumin (synthetic liver function), and positively with other hepatic markers (ALT, ALP, bilirubin), but did not predict serum adiponectin concentration, indicates the important regulatory role

of adiponectin on serum GGT activity.

The age-related increase in adiponectin and its positive association with GGT in our study were independent of other liver parameters (ALT, ALP, bilirubin and albumin), leptin (a sensitive marker of adiposity negatively associated with adiponectin) and resistin (a biomarker strongly associated with an inflammatory response) levels, 25(OH)D, PTH, eGFR, alcohol consumption, presence of DM or CVD, although some of these factors were also independent contributors to higher GGT levels (Table 4). In other words, the origin of higher serum GGT activity is multifactorial (eight factors accounted for 54.3% of GGT variance), and the elevated adiponectin concentration and its relation to GGT are part of and reflect the complex homeostatic dysregulation(-s) that accompany ageing. Consistent with this hypothesis, are our findings demonstrating that PTH, which also increases with age, correlated positively with adiponectin and in the multivariate analysis was an independent determinant of adiponectin (but not of leptin or resistin). Of note, despite the marked prevalence of vitamin D insufficiency in our cohort, with its associated increase in PTH, 25(OH)D levels were not independent contributors of adiponectin, indicating a specific affect of higher PTH levels on production and/or release of adiponectin by adipocytes. Our results are in line with observations that 25(OH)D is not associated with adiponectin in nondiabetic obese adults,²²⁹ while PTH is independently associated with adiponectin in patients with heart failure.¹⁸⁹ However, in patients with primary hyperparathyroidism adiponectin concentrations were found to be normal¹⁹⁰ or reduced,^{191, 192} did not changed or reversed by parathyroidectomy,^{190, 192} and were not associated with PTH levels.¹⁹³ The complex multifactorial origin of GGT activity is further suggested by our observation of its significant correlation with TSH (positive) and T4 (negative); thyroid hormones are known to play an important role in oxidative stress balance.¹⁹⁴

The positive correlation between PTH and adiponectin has not been previously documented in HF patients, and it may explain, at least partially, the adiponectin-GGT "paradox": although in general with ageing GGT activity decreases, adiponectin levels, mediated in part by elevated PTH, rise resulting in higher GGT levels. We hypothesise that PTH elevation may partly contribute to higher adiponectin concentration, which is clearly related to higher GGT.

Towards an integrated and unifying hypothesis

Our data in line with numerous previous publications showed that decline in liver functions, increase in adiponectin and PTH are interconnected universal phenomena associated with human ageing.

Age-related rise in serum adiponectin and PTH concentrations are positively correlated, but age and adiponectin are paradoxically compatible with hepatic function, especially with GGT activity. In parallel with adiponectin elevation GGT activity increases indicating that the hormonal effect of adiponectin takes precedence over age-related suppression in the enzyme activity. The serum GGT activity reflects the integrated response of these opposite effects.

These observations raised two key questions: 1) is there a special common cause that underlies the metabolic changes occurring with advancing age, although each change results from an interplay of numerous independent mechanisms, and 2) is higher serum adiponectin concentration associated with GGT elevation, as in our case, an adoptive/compensatory response or a harmful effect.

The exact answers remain largely unknown, and any attempt to adequately explain the observations should include at least two fundamental mechanisms: homeostasis and oxidative stress. Several lines of evidence suggest close but complex interactions between oxidative stress and GGT, albumin, bilirubin, adiponectin and PTH (these factors may act as causes and consequences of oxidative stress). Oxidative stress, an imbalance between the production and inactivation of reactive oxygen species in favour of oxidants accumulation, is widely accepted as an important mechanism associated with human ageing and its adverse effects.¹⁹⁵⁻¹⁹⁹ Hepatic aging is associated with greater oxidative stress and cell apoptosis.²⁰⁰⁻²⁰² GGT, a membrane-bound enzyme, plays a pivotal role in the intracellular antioxidant defence being involved in the gamma-glutamyl cycle by which extracellular glutathione is transported into cells. Depletion of intracellular glutathione, a principal intracellular antioxidant,²⁰³ in response to oxidative stress results in an increase in GGT so that the metabolic homeostasis are maintained. Serum GGT activity is inversely associated with the concentration of serum antioxidants.²⁰⁴ Serum GGT within its normal range is recognized as a sensitive marker of oxidative stress.^{30, 141, 203-207} However, in physiological conditions, GGT may also act as a pro-oxidant,^{203, 204, 208} generating reactive oxygen species,^{209, 210} which could exceed the capacity of the antioxidant system and induce cellular oxidative stress damage.

The oxidative stress responses involve also other potent antioxidants, namely albumin,²¹¹ the major protein in plasma, which accounts 80% of thiol's antioxidant effect in the body,^{212, 213} bilirubin, which protects cells from a 10 000-fold excess of oxidants through rapid regeneration of bilirubin by biliverdin reductase,^{119, 214} and adiponectin. In animal models^{215, 216} and in humans,^{217, 218} including the elderly,²¹⁹ adi-

ponectin inhibits oxidative stress, but oxidative stress suppresses adiponectin production and its powerful protective antioxidant properties.²²⁰ Importantly, adiponectin is also involved in apoptosis, an evolutionary conserved controlled-death program, which ensures proper regulation of the size and quality of cell populations in tissues.²²¹ PTH may affect oxidative stress directly or by intracellular calcium accumulation.^{222, 223} In other words, GGT, albumin, bilirubin, adiponectin and PTH, all of which play a critical role in homeostasis, may be elicited by oxidative stress and/or may have both protective and promoting effects on oxidative stress. The interplay and high degree of complexity of aforementioned and other factors involved in oxidative stress indicate the role of regulatory feedback mechanisms in different conditions. Normally, the oxidative stress responses maintain metabolic homeostasis and are beneficial for adaptation and survival, while dysregulation in the feedback processes may cause the vicious cycle(-s) of oxidants overproduction resulting in aging and aging-related diseases.²²⁴ Despite considerable gaps in our knowledge, emerging data suggests that adiponectin and oxidative stress can function in both a defence and harmful manner.

In regard to our observations, age-related decrease of GGT activity may reflect the decline not only in liver function, but in the whole antioxidant defence system (including the decrease in the antioxidant effects of albumin as one of its components), and the increase in serum GGT level may be a compensatory response to oxidative stress, a recognised hallmark of ageing and chronic diseases. This response includes increases in PTH and adiponectin. The former is at least in part responsible for the adiponectin elevation, while the later enhances the anti-oxidant potential in the cells by increasing GGT and promotes apoptosis.²²⁵ In this way, it can be hypothesized that in our patients, higher GGT activity which is positively associated with both adiponectin and bilirubin (also a potent antioxidant) levels, reflects a compensatory response to oxidative stress. However, in the setting of advanced age and co-morbidities this response is unable fully counter-regulate the oxidative damage and prevent its progression if oxidative stress increases, as in the perioperative period. Indeed, in our cohort, both GGT and adiponectin above the median levels were independently and synergistically associated with prolonged LOS, but not with in-hospital mortality, whereas even mild decrease in albumin concentration, an important extracellular antioxidant, demonstrated a strong relation to fatal outcome. These complex interactions are summarised in Figure 3.



Figure 3. Diagram illustrating complex interplay between ageing, oxidative stress, parathyroid hormone (PTH), adiponectin and gamma-glutamyltransferase (GGT) and other hepatic markers and their relation to short-term outcomes in older patients with hip fracture. LOS, length of hospital stay. In advanced age, GGT, albumin, bilirubin, adiponectin and PTH, all of which are influenced by oxidative stress, may exert both protective and promoting effects on oxidant formation, affect each other, and contribute to poor outcomes. Excessive PTH along with different other age-related factors drives adiponectin levels higher, and such elevations increase GGT activity which reflects an integrative adaptive/compensatory or pathological response (homeostatic regulation or dysregulation). In HF patients, both GGT and adiponectin concentrations above the median levels are independent markers of prolonged LOS and contribute synergistically to this outcome, hypoalbuminaemia is associated with in-hospital death, and hyperparathyroidism is an independent predictor of both fatal outcome and prolonged hospital stay.

Liver markers and short-term outcomes

Our data shows that in older HF patients liver markers (usually within the normal ranges) on admission are associated with a continuum of risk for poor outcomes. Of clinical interest, GGT, albumin and adiponectin levels analysed as categorical variables (given the practical convenience of cut-off values), independently of known risk factors, may help to identify subjects at increased risk of prolonged hospital stay (LOS>20 days) and in-hospital death. Patients with GGT>30U/L and albumin<33g/L were near two times as likely to have a LOS>20 days, and more than 3 times as likely to have died during hospitalisation, respectively, compared to those without such conditions. The calculated sensitivity and specificity of serum GGT>30U/L for discriminating those with LOS>20 days were 48.9% and 65.8%, respectively, and of albumin <33g/L for prediction in-hospital death were 42.9% and 78.9%, respectively; serum albumin<33g/L on admission had a NPV of 96.5%. The risk of LOS>20 days also doubled in subjects with adiponectin>17.14 ng/L (median level). Furthermore, joint effects of GGT>30U/L and adiponectin>17.14 ng/ml raised the odds of LOS>20 days by 4.7-fold, demonstrating synergism; the specificity of two markers in combination for predicting LOS>20 days was 83.2%. These two biomarkers, although interrelated, are associated with different pathogenetic processes, and, not surprisingly, when

measured in parallel are more informative for predicting LOS.

Our observations are in line with previous reports that low albumin is of prognostic value on mortality in HF patients.^{61, 62, 65, 66, 69, 137, 226-228} Our data are also concordant with clinical observations that demonstrate that serum GGT, a robust predictor of mortality due to CVD in younger individuals, did not predict mortality in individuals aged 70 years or more.⁶⁹ The prognostic value of GGT and/or adiponectin in HF patients has not been reported previously. Of note, in contrast to the present multivariate analyses, most of the previous prognostic studies in patients with HF examined only few liver parameters (mainly albumin) and often did not control for possible confounders, clinical and laboratory.⁶⁷

The reason(s) for prolonged LOS among patients with higher GGT and/or higher adiponectin remains unclear. The finding that only GGT and not ALT (a more specific marker of hepatic function), ALP, bilirubin or albumin was associated with LOS in adjusted models may suggest that the GGT -- LOS association reflects not only the liver status but the increased oxidative stress.

The additional (albeit moderate) prognostic information to HF outcomes provided by liver markers, accurate and not expensive laboratory tests, could be helpful in early identification and appropriate treatment of this frail population, and, consequently, improve outcomes, especially prolonged LOS. Currently, HF's represent about two-thirds of all hospital days due to fracture and account for more hospital days than any other musculoskeletal injury.²²⁸

Limitations and strength of the study

Several limitations of this study should be considered. First, it was a cross-sectional analysis and, therefore, the cause-effect relationship could not be determined. Second, we measured serum GGT activity and total adiponectin, but the GGT fractions and high-molecular weight adiponectin, shown in some, but not all,¹⁸⁵ studies to be more informative, were not assessed. Third, potential effects of confounding from variables not represented in our models (e.g. medication used) cannot be excluded. Forth, as the cohort was mainly Caucasian our findings may not be generalizable to other ethnic groups.

The strengths of this study are its prospective design, and the amount of obtained variables. Although multiple comparisons may potentiate the significance of multicollinearity phenomena in multivariate regression analysis, the variance inflation factor in all models presented in Tables 4 and 5 was less than 1.3, indicating that the amount of multicollinearity was not significant.

Conclusions

The present study of older persons with hip fracture demonstrates that biochemical parameters of liver function even within its normal range in the majority of patients are relevant to comorbidities and outcomes, documents the regulatory role of adiponectin (but not leptin or resistin) as an independent contributor to GGT activity, and shows that serum GGT (>30 IU/L) and albumin (<33g/L) levels on admission may be helpful in predicting prolonged hospital stay and in-hospital death, respectively.

Authors' contributions

Both LF and AF were involved in the design of the study, data collection and analyses, manuscript preparation and review, WS performed statistical analysis, PS was involved in drafting and review of the manuscript. All authors approved the final version of the article, including the authorship list.

Conflicts of interest

The authors report no conflicts of interest in this work.

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Relationship between Serum Gamma-Glutamyltransferase (GGT) Activity and Indices of Iron Metabolism with Bone-Mineral Parameters in Orthogeriatric Patients

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Rec Date: Jun 21, 2016; **Acc Date:** Jul 18, 2016; **Pub Date:** Jul 25, 2016

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Citation: Fisher L, Fisher A. Relationship between serum gamma-glutamyltransferase (GGT) activity and indices of iron metabolism with bone-mineral parameters in orthogeriatric patients. J Biomedical Sci. 2016, 5:4.

Abstract

Purpose: To examine serum levels of GGT activity and biomarkers of iron metabolism in relation to parameters of bone and mineral metabolism in elderly patients with and without osteoporotic bone fractures in the absence of overt liver diseases.

Methods: In a cross-sectional study of 416 older (>60 years) patients (168 subjects with hip fracture, 89 with other fractures of the peripheral skeleton and 160 without any fracture; mean age 78.9 ± 8.7 years; 282 women) we simultaneously measured serum levels of two bone formation markers (osteocalcin, OC; procollagen type 1 N-terminal propeptide, P1NP), bone resorption marker (β -isomerised carboxy-terminal cross-linking telopeptide of type I collagen, β CTX), their ratios (P1NP/OC, P1NP/ β CTX, OC/ β CTX), 25(OH) vitamin D, PTH, calcium, phosphate, magnesium, GGT activity, other liver function tests and indices of iron metabolism (serum ferritin, iron, transferrin and transferrin saturation [TSAT]).

Results: Multivariate regression analyses demonstrated significant bidirectional links between P1NP and GGT, P1NP/OC ratio and GGT, P1NP and TSAT, OC and ferritin, GGT and ferritin. GGT, ferritin and TSAT were independent indicators of β CTX, while ferritin and TSAT were also independent predictors of the P1NP/OC ratio, and TSAT was an independent predictor of the P1NP/ β CTX ratio. In multivariate regression, P1NP, β CTX, P1NP/ β CTX ratio, ferritin, magnesium and age were independent indicators of fracture.

Conclusions: The study highlighted the bidirectional links between serum GGT activity, indices of iron and bone metabolism and the role of GGT and iron homeostasis in maintaining bone health and identified indicators for osteoporotic fractures. The GGT- and iron-related factors contributing to bone integrity warrant further investigations.

Keywords: Bone turnover markers; GGT; Iron metabolism; Fracture; Elderly

Introduction

The interest in gamma-glutamyltransferase (GGT; E.C.2.3.2.2), a heterodimeric glycosylated protein embedded into outer surface of the plasma membrane of numerous cells and strongly associated with many major non-skeletal diseases[1], in osteology has recently increased considerably owing to several reports. GGT-deficient (GGT^{-/-}) mice exhibit severe osteoporosis caused by decreased osteoblast activity and increased osteoclast number and activity [2]. GGT induces osteoclast formation in vitro [3], accelerates bone resorption causing osteopenia/osteoporosis in transgenic mice [4,5], and increases in urinary excretion of GGT correlate with urinary bone resorption markers in osteoprotegerin (OPG)-deficient osteoporotic mice as well as in postmenopausal osteoporotic women [6]. In older Korean men (>50 years) higher serum GGT levels were associated with incident osteoporotic fractures over a mean 3-year follow-up period [7]. Iron, an essential trace element in all three kingdoms of living organisms, is recognized as an important determinant of bone metabolism [8-10]. However, there is disagreement as to whether iron overload or iron deficiency lead to osteopenia/osteoporosis and whether the iron-related bone loss is due to reduced bone formation or to increased resorption of bone.

Studies in humans without haematological disorders on relationship between markers of iron metabolism and bone status are scarce and controversial. A significant association between higher serum ferritin level and lower bone mineral density (BMD) was reported in healthy Koreans aged >40 years [11], especially in women ≥ 45 years of age [12], in postmenopausal Chinese women with fragility fractures [13], and in astronauts during space flights [14], whereas in other studies, increased serum ferritin levels were inversely correlated with BMD (of lumbar spine) only in premenopausal women, but not in postmenopausal women (either at the femur or lumbar spine) [15]. No significant association between ferritin levels and

osteoporosis has been observed in Turkish postmenopausal women [16]. Italian postmenopausal women with osteoporotic fractures were found to have higher transferrin levels and lower ferritin levels than controls [17] and a positive association between serum ferritin and BMD (in both femoral neck and lumbar spine). A positive ferritin-BMD correlation was also observed in adolescent girls [18] and in elderly Korean men (>65 years) [19]. Furthermore, numerous nutritional studies suggested that iron deficiency, rather than excess, may lead to osteoporosis [10].

Both GGT [20] and iron [21] are implicated in generating oxidative stress, which plays a significant role in bone pathophysiology [22,23], but these relationships remain unclear. Despite accumulating data suggesting importance of both GGT and iron in bone health, and the central role of liver in iron storage and regulation, no analysis of the potential links between serum GGT activity, markers of iron and bone-mineral metabolism in humans has been reported to date. The aim of this study was to examine serum levels of GGT and biomarkers of iron metabolism in relation to parameters of bone and mineral metabolism in elderly patients with and without osteoporotic bone fractures in the absence of overt liver diseases. We simultaneously measured serum levels of two bone formation markers (osteocalcin, OC, and N-terminal propeptide type 1 collagen, P1NP), bone resorption marker (β -isomerised C-terminal cross-linking telopeptide of type I collagen, β CTX), and their ratios (PINP/OC, PINP/ β CTX, OC/ β CTX), 25-hydroxyvitamin D (25(OH)D), intact parathyroid hormone (PTH), calcium, phosphate, magnesium in relation to serum GGT activity (and other liver function parameters) and indices of iron metabolism (serum ferritin, iron, transferrin and transferrin saturation).

Materials and Methods

Patients

We conducted a cross-sectional study of 493 older (>60 years) patients admitted to the orthopaedic unit of our hospital between 1 January 2010 and August 2011. After excluding patients with high-trauma fracture, acute or known chronic hepatobiliary diseases, iron metabolism-related diseases (hemochromatosis, thalassemia), acute infection, primary hyperparathyroidism, and Paget's disease, or who lacked adequate laboratory data, 416 patients (282 women, 134 men) were evaluated for the study.

The study was conducted according to the standards of the Declaration of Helsinki and was approved by the local Health Human Research Ethical Committee. Informed consent from each patient or carer was obtained.

Laboratory measurements

In each patient venous blood samples were collected in the morning following an overnight fast, usually within 24 h after arrival.

Markers of bone and mineral metabolism

The following serum indicators of bone and mineral metabolism were measured: bone formation markers (P1NP and OC), bone resorption marker (β CTX), PTH, 25(OH)D, calcium, phosphate and magnesium concentrations. The serum concentrations of P1NP, OC and β CTX were measured using an electrochemiluminescent immunoassay (Elecsys 2010, Roche Diagnostics, Ltd Corp., IN, USA). Intra- and inter-assay coefficients of variation (CV) for P1NP were 2.6% and 4.1%, respectively; for OC 3.6% and 6.6%, respectively, and for β CTX 3.2% and 6.5%, respectively. Serum 25(OH)D level was measured by a radioimmunoassay (Dia Sorin, Stillwater, MN, USA) and intact PTH was determined by a two-site chemiluminescent enzyme-linked immunoassay on DPC Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA, USA); the intra- and inter-assay CV ranged from 2.1% to 12.7%. Serum calcium concentrations were corrected for serum albumin. For bone turnover markers, we used reference ranges and data provided by the manufacturers. Vitamin D status was defined as deficient for circulating 25(OH)D concentration <25 nmol/L, and as insufficient for 25–50 nmol/L. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (>6.8 pmol/L, the upper limit of the laboratory reference range).

Liver function tests

The serum levels of GGT, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured enzymatically using the Abbott Architect CI16200 automatic analyser, total bilirubin was measured using diazonium salt, albumin was analysed using bromocresol green, and total protein by a Biuret method. The mean inter-assay and intra-assay CV for these tests were within 1.1% – 6.6%. For liver enzymes two times upper normal limit (UNL) cut-offs were used to define abnormal tests.

Indicators of iron status

Serum indices of iron metabolism were analysed on the Abbott Architect CI16200 (Abbott Laboratories, IL 60064, USA). Serum iron was assessed by direct colorimetric determination, serum ferritin concentration was measured by a two-step chemiluminiscent microparticle immunoassay and transferrin was measured using an immunoturbidimetric procedure. The mean inter-assay and intra-assay CV for these tests were between 1.6% and 3.7%. Transferrin saturation (TSAT) was calculated using the IFCC protein standards (1 mg of transferrin carries 1.49 μ g of iron) as follows: TSAT (%) = iron (μ mol/L) $\times$ 3.8 / transferrin (g/L); reference range 18-46%.

In addition, in all patients routine hematological and biochemical assessments (complete blood count, urea, creatinine, fasting blood glucose, haemoglobin A1c (HbA1c, only in diabetic patients), thyroid function tests (thyroid-stimulating hormone, TSH, and thyroxine, T4), C-reactive protein (CRP) were also performed by standardized methods on autoanalyzers. Estimated glomerular filtration rate (eGFR) was determined using the serum creatinine equation. Chronic kidney disease (CKD) was defined as eGFR <60 ml/1.73 m² (CKD $\geq$ 3 stage).

Statistics

Statistical analyses were performed using Stata software version10 (StataCorp., College Station, TX, USA). For summary statistics, data were untransformed and presented as mean $\pm$ standard deviation (SD) for continuous variables and as numbers (or percentages) for categorical variables. For Pearson correlations and regressions, values of all laboratory parameters were logarithmically transformed to account for the skewed nature of most of these variables. Sidak adjustments for multiplicity were performed. Linear regressions involving the predictive value of GGT, and indices of iron metabolism on bone turnover markers were performed accounting for age, sex, other liver function tests, 25(OH)D, PTH, eGFR, T4, TSH, CRP, white cell count (WCC), alcohol consumption, smoking status, presence of diabetes mellitus (DM), cardiovascular disease (CVD), hip fracture (HF) or any fracture. Similar models were used to assess independent determinants of GGT, ferritin, TSAT, HF or any fracture. When the dependent parameter was stratified by level, odds ratios with 95% confidence intervals (CI) were measured in multivariate logistic regression models. The significance of multicollinearity phenomena in multivariate regression analyses was assessed by the variance inflation factor. Two tailed tests were used and results were considered statistically significant if $P < 0.05$.

Results

Patient demographic, clinical and laboratory characteristics

Among 416 studied patients there were 168 subjects with a HF (52.2% cervical, 44.1% trochanteric and 3.7% subtrochanteric), 89 patients with a non-HF fracture of the peripheral skeleton and 160 subjects without any fracture; 67.8% of the entire cohort were women. Hypertension was diagnosed in 62.7%, coronary artery disease (CAD) in 18.3%, atrial fibrillation (AF) in 17.1%, history of stroke or transient ischemic attack in 12.3%, peripheral vascular disease in 5.0%, chronic cardiac failure (CCF) in 10.8%, DM in 19.5%, CKD (eGFR < 60 ml/min/1.73 m²) in 19.2%, dementia in 17.6%, Parkinson's disease in 3.6%, chronic obstructive airway disease (COPD) in 11.1%, and osteoarthritis in 46.8%. At least one CVD and/or DM was found in 76.0% of the cohort; 18.9% of the patients consumed alcohol > 3 times a week, 19.3% were ex-smokers and 9.1% were current smokers, 19.9% of the patients were admitted from long-term residential care facilities.

The demographic and biochemical characteristics of the study cohort are shown in **Table 1**. Patients with fractures, as could be expected, were older, had significantly lower mean levels of P1NP, transferrin, hemoglobin, serum calcium (corrected for albumin) and magnesium, and higher concentrations of PTH, β CTX, and ferritin than patients without fractures; accordingly, P1NP/ β CTX and OC/ β CTX ratios were also significantly lower in the fracture group. In addition, patients with HF compared to

the non-fracture subjects had lower serum phosphate and iron concentrations. Patients with fractures had a higher prevalence of vitamin D deficiency (11.7% vs. 5.7%, $p = 0.043$) and SHPT (43.1% vs. 34.2%, $p = 0.044$).

Low hemoglobin levels (< 120 g/L) were present in 331 (79.6%) patients. Both serum ferritin level and TSAT were in the normal range in 14.6% of our patients. High serum ferritin (> 370 μ g/L, upper limit of reference range) was found in 32.5% of patients, low TSAT ($< 18\%$, lower limit of reference range) in 78.9%, including 52.9% with ferritin in the normal range and 26.0% with a "paradoxical" combination (i.e. relatively high serum ferritin but low TSAT); only 4 (0.96%) patients demonstrated TSAT $> 46\%$ (upper limit of reference range). Compared to the rest of the cohort, patients with low TSAT ($< 18\%$) had significantly lower mean P1NP/OC (8.5 ± 8.9 vs. 12.2 ± 18.5 , $p = 0.009$) and P1NP/ β CTX (123.4 ± 96.9 vs. 156.5 ± 118.7 , $p = 0.007$) ratios, while subjects with elevated serum ferritin levels (> 370 μ g/L) had lower OC concentrations (6.5 ± 3.8 vs. 7.7 ± 4.5 μ g/L, $p = 0.009$) and higher P1NP/OC ratios (11.2 ± 16.2 vs. 8.3 ± 8.4 , $p = 0.018$).

Relationship between serum GGT activity and indices of bone-mineral and iron metabolism

When the subjects were grouped according to the GGT tertiles patients with lower GGT activity tended to be older (80.4 ± 8.4 years in the first tertile vs. 77.6 ± 9.3 years in the third tertile, p for trend 0.027). Increasing tertile of GGT was significantly associated with higher P1NP (46.1 ± 39.5 vs. 64.0 ± 70.7 μ g/L, $p = 0.028$), ferritin (262.6 ± 218.0 vs. 522.5 ± 542.1 μ g/L, $p = 0.000$), and calcium levels (2.31 ± 0.13 vs. 2.37 ± 0.13 mmol/L, $p = 0.001$), while 25(OH)D concentrations showed a downward trend (64.4 ± 27.3 vs. 55.9 ± 24.4 nmol/L, $p = 0.029$).

Pearson's correlation coefficients of logarithmically transformed variables adjusted for age and sex revealed that logGGT correlated positively with logP1NP ($r = 0.136$, $p = 0.006$), P1NP/OC ratio ($r = 0.181$, $p = 0.000$), P1NP/ β CTX ratio ($r = 0.096$, $p = 0.052$), logPTH ($r = 0.119$, $p = 0.015$), logcalcium ($r = 0.194$, $p = 0.000$), logphosphate ($r = 0.108$, $p = 0.028$), as well as with logferritin ($r = 0.182$, $p = 0.000$), logiron ($r = 0.103$, $p = 0.037$) and logTSAT ($r = 0.107$, $p = 0.030$), and negatively with log25(OH)D ($r = -0.126$, $p = 0.010$). Although logP1NP, as expected, correlated with both logOC ($r = 0.424$, $p = 0.000$) and log β CTX ($r = 0.484$, $p = 0.000$), and logOC correlated with log β CTX ($r = 0.353$, $p = 0.000$), no significant correlation between logGGT and logOC or log β CTX was observed.

Serum GGT activity and indices of iron metabolism as predictors of bone turnover markers

Stepwise multivariate linear regression analyses (**Table 2**) showed that both logGGT and logTSAT were significant independent determinants of logP1NP, log β CTX (negatively by logTSAT), P1NP/OC and P1NP/ β CTX (borderline significant by GGT, $p = 0.068$) ratios. Logferritin was inversely associated with

both logOC and log β CTX, and positively with P1NP/OC ratio independently of multiple potential confounders.

Table 1 Demographic and biochemical characteristics of the study patients overall and according to the presence of fracture.

Characteristic	Total, n=416	Without fracture, n=160 (1)	With hip fracture, n=168 (2)	With any fracture, n=256(3)	P Value	P Value
					(2 -1)	(3-1)
Age, years	78.9 $\pm$ 8.7	75.6 $\pm$ 8.1	82.8 $\pm$ 7.9	81.0 $\pm$ 8.5	0.000	0.000
Women, n (%)	282 (67.8)	101(63.1)	116(69.1)	181(70.0)	0.257	0.108
GGT, U/L	50.3 $\pm$ 57.7	53.4 $\pm$ 64.3	43.1 $\pm$ 42.6	48.4 $\pm$ 53.2	0.088	0.391
*Calcium, mmol/L	2.35 $\pm$ 0.13	2.38 $\pm$ 0.12	2.33 $\pm$ 0.13	2.34 $\pm$ 0.14	0.001	0.001
Phosphate, mmol/L	0.92 $\pm$ 0.24	0.96 $\pm$ 0.21	0.90 $\pm$ 0.28	0.91 $\pm$ 0.26	0.039	0.067
Magnesium, mmol/L	0.77 $\pm$ 0.11	0.79 $\pm$ 0.10	0.75 $\pm$ 0.13	0.76 $\pm$ 0.12	0.004	0.023
25(OH)D, nmol/l	60.2 $\pm$ 26.4	60.9 $\pm$ 24.1	60.5 $\pm$ 28.9	59.7 $\pm$ 27.8	0.878	0.645
PTH, pmol/L	7.0 $\pm$ 4.7	6.4 $\pm$ 3.5	8.0 $\pm$ 6.0	7.4 $\pm$ 5.3	0.004	0.036
OC, μ g/L	7.3 $\pm$ 4.3	7.5 $\pm$ 4.4	7.1 $\pm$ 4.5	7.1 $\pm$ 4.3	0.37	0.372
P1NP, μ g/L	57.6 $\pm$ 61.1	65.4 $\pm$ 70.1	55.1 $\pm$ 57.5	52.7 $\pm$ 54.3	0.144	0.039
β CTX, μ g/L	0.50 $\pm$ 0.33	0.44 $\pm$ 0.26	0.58 $\pm$ 0.39	0.53 $\pm$ 0.36	0.000	0.006
OC/ β CTX ratio	18.9 $\pm$ 16.6	21.2 $\pm$ 19.9	15.3 $\pm$ 9.5	17.4 $\pm$ 14.1	0.000	0.023
P1NP/ β CTX ratio	130.0 $\pm$ 102.5	156.3 $\pm$ 117.3	112.4 $\pm$ 94.5	113.8 $\pm$ 88.6	0.000	0.000
P1NP/OC ratio	9.3 $\pm$ 11.7	10.3 $\pm$ 12.3	9.5 $\pm$ 13.4	8.7 $\pm$ 11.3	0.589	0.178
haemoglobin, g/L	106.5 $\pm$ 16.9	108.6 $\pm$ 15.3	103.7 $\pm$ 17.3	105.1 $\pm$ 17.8	0.007	0.040
Iron, μ mol/L	5.8 $\pm$ 4.7	6.1 $\pm$ 4.1	4.9 $\pm$ 5.0	5.6 $\pm$ 5.0	0.018	0.333
Ferritin, μ g/L	436.1 $\pm$ 572.9	334.8 $\pm$ 404.3	592.0 $\pm$ 405.8	499.3 $\pm$ 526.6	0.001	0.019
Transferrin, g/L	1.7 $\pm$ 0.43	1.8 $\pm$ 0.43	1.6 $\pm$ 0.41	1.7 $\pm$ 0.42	0.000	0.000
Transferrin sat, %	12.5 $\pm$ 9.5	12.5 $\pm$ 8.2	11.6 $\pm$ 9.6	12.6 $\pm$ 10.2	0.377	0.919
Urea, mmol/L	6.3 $\pm$ 3.6	6.2 $\pm$ 2.8	6.8 $\pm$ 4.5	6.4 $\pm$ 4.0	0.110	0.489
Creatinine, μ mol/L	79.1 $\pm$ 49.5	78.5 $\pm$ 31.3	84.7 $\pm$ 69.5	79.5 $\pm$ 58.1	0.306	0.848
eGFR, ml/min/1.73 m ²	80.3 $\pm$ 26.4	79.0 $\pm$ 25.5	79.8 $\pm$ 28.7	81.1 $\pm$ 26.9	0.801	0.437
T4, pmol/L	16.2 $\pm$ 3.1	16.3 $\pm$ 3.0	16.0 $\pm$ 3.2	16.1 $\pm$ 3.2	0.340	0.379
TSH, mU/l	1.7 $\pm$ 3.1	1.7 $\pm$ 3.7	1.7 $\pm$ 2.9	1.7 $\pm$ 2.6	0.990	0.891

Values are means $\pm$ SD. GGT: Gamma-Glutamyl Transferase; 25(OH)D: 25 Hydroxyvitamin D; PTH: Parathyroid Hormone; OC: Osteocalcin; P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; β CTX: Crosslinked Carboxy-Terminal Telopeptide of Type I Collagen; TSAT: Transferrin Saturation; eGFR: Estimated Glomerular Filtration Rate; T4: Free Thyroxine; TSH: Thyroid Stimulating Hormone.*Albumin-corrected.

Besides, log β CTX was also predicted by logiron (positively) and logtransferrin (negatively). All three bone turnover markers were also independently predicted by logPO4 (positively) and logeGFR (negatively). LogPTH was inversely associated with logP1NP and P1NP/ β CTX ratio, while logMg was inversely associated with logP1NP and P1NP/OC ratio.

Presence of any fracture demonstrated significant negative correlations with logP1NP, P1NP/OC and P1NP/ β CTX ratios, while presence of HF was an independent indicator for log β CTX, P1NP/ β CTX and OC/ β CTX ratios (negative associations with both ratios).

Taken together, these findings show that both serum GGT activity and indices of iron metabolism are specific and important factors influencing bone turnover, which is highly relevant to the mechanical properties of bone and fractures.

Parameters of bone-mineral and iron metabolism as indicators of serum GGT activity

Multivariate logistic regression analysis (**Table 3**) revealed the following factors as independently and significantly associated with GGT activity: logP1NP, P1NP/OC ratio, logferritin (all positively), log25(OH)D and age (both inversely); logPTH levels demonstrated borderline significance ($p=0.059$).

In other words, increasing age and higher 25(OH)D are independently associated with lower GGT, whereas higher P1NP, P1NP/ OC ratio, ferritin and possible PTH are indicators of higher GGT.

Table 2 Serum GGT activity and indices of iron metabolism as independent determinants of bone turnover markers (stepwise multivariate linear regression analyses).

	Log P1NP	Log OC	Log β CTX	P1NP/OC	P1NP/ β CTX	OC/ β CTX
Log GGT	0.095 (0.019)		0.073 (0.043)	1.829 (0.008)	10.809 (0.068)	
*Log Cac		0.588 (0.003)				
Log PO4	0.421 (0.000)	0.154 (0.047)	0.358 (0.000)	2.695 (0.099)		-4.537 (0.057)
Log Mg	-0.489 (0.048)			-17.63 (0.000)		
Log PTH	-0.198 (0.004)				-22.532 (0.014)	
Log Ferritin		-0.116 (0.000)	-0.086 (0.036)	1.533 (0.016)		
Log Fe			0.347 (0.033)			
Log Transferrin			-0.740 (0.001)			8.501 (0.0.10)
Log TSAT	0.136 (0.012)	-0.356 (0.027)	3.237 (0.000)	27.535 (0.000)		
LogeGFR	-0.344 (0.000)	-0.303 (0.000)	-0.349 (0.000)			
HF			0.185 (0.004)		-20.749 (0.044)	-5.476 (0.002)
Any fracture	-0.145 (0.038)			-2.312 (0.045)	-41.184 (0.000)	
R ²	0.371	0.185	0.270	0.269	0.186	0.070

Data represented as the regression coefficient and associated significance level (in brackets) if $p < 0.100$. The R-squared value for the overall regressions are also shown. Abbreviations: GGT: Gamma-Glutamyl Transferase; P1NP: Amino-Terminal Propeptide of Type I Procollagen; OC: Osteocalcin; Bctx: Crosslinked Carboxy-Terminal Telopeptide of Type 1 Collagen; PO4: Phosphate; Mg: Magnesium; 25(OH) D: 25-Hydroxyvitamin D; PTH: Parathyroid Hormone; Fe: Iron; eGFR: Estimated Glomerular Filtration Rate; HF: Hip Fracture. *Calcium: Corrected for Albumin. In each model the bone turnover parameter was the dependent variable. Independent variables included age, gender, logGGT, logALT (alanine aminotransferase), logALP (alkaline phosphatase), logbilirubin, logalbumin, log25(OH)D, PTH, logcalcium(corrected for albumin), logphosphate, logmagnesium, logeGFR, alcohol overuse (>3 drinks/week), smoking status (current smoker, ex-smoker), presence of hip fracture or any fracture (in separate models, i.e. the model included presence of hip fracture or presence of any fracture), diabetes mellitus and cardiovascular disease.

Serum bone-mineral parameters and GGT activity as indicators of iron metabolism

Both ferritin and TSAT, the two most informative and commonly used indices of iron metabolism, were independently positively predicted by logP1NP and logALT, and negatively by logOC and eGFR (**Table 4**). In addition, higher logGGT, CRP, male gender and presence of HF or any fracture correlated positively and DM correlated negatively with logferritin levels.

CRP correlated negatively with TSAT, and there was an inverse correlation between blood white cell count (WCC) and TSAT of marginal statistical significance ($p=0.076$).

The divergent results regarding inflammatory markers (CRP and WCC) are in line with the well known fact that serum ferritin is as a positive acute phase reactant, whereas transferrin is considered a negative one.

Variables independently associated with hip fracture or any fracture

The following 6 variables were independently associated with presence of any fracture (**Table 5**): log β CTX, logferritin and age (all three positively), logP1NP, P1NP/ β CTX ratio and logMg (the last three variables inversely).

When HF patients were considered alone, the statistically significant independent associates were also logGGT and logPO4 (both inverse). In other words, the risk of HF increases with every increment of one unit of log β CTX by 127%, of one unit of logferritin by 67%, and with one year of age by 13%, while increases in logP1NP, P1NP/ β CTX ratio, logGGT, logPO4 and logMg have significant protective effects.

Similarly, the risk of any fracture increases with an increase in one unit of log β CTX by 91%, in one unit of logferritin by 30%, with one year of age by 8%, whereas increases in logP1NP, P1NP/ β CTX ratio and logMg demonstrate protective effects.

Discussion

Main findings

Our data shows a complex interplay between serum GGT activity, iron status and bone metabolism (**Figure 1**). Bone remodelling is regulated by GGT and iron status, and specific bone-derived molecules (P1NP and OC) affect both the GGT activity and iron homeostasis, while GGT and ferritin are also interrelated.

The bidirectional links between P1NP and GGT (1), P1NP/OC ratio and GGT (2), P1NP and TSAT (3), OC and ferritin (4), GGT and ferritin (5) suggest that these molecules and factors act as

feedback loop signals participating in the complex homeostatic, adaptive and compensatory mechanisms.

Furthermore, serum GGT activity, ferritin and TSAT are independent indicators of β CTX, while ferritin and TSAT are also independent predictors of the P1NP/OC ratio, and TSAT is an independent predictor of the P1NP/ β CTX ratio.

Table 3 Parameters of bone - mineral and iron metabolism and related variables as independent determinants of serum logGGT activity (stepwise multivariate linear regression analysis).

	β	95%CI	P value
LogP1NP	0.131	0.026; 0.235	0.015
P1NP/OC ¹	0.01	0.003; 0.016	0.003
Logferritin	0.331	0.226; 0.435	0.000
Logtransferrin	0.359	-0.039; 0.757	0.077
Logiron	0.112	-0.009; 0.233	0.069
Log25(OH)D	-0.207	-0.367; -0.046	0.012
LogPTH	0.139	-0.005; 0.283	0.059
Age	-0.019	-0.029; -0.009	0.000
HF	-0.147	-0.320; 0.026	0.096
R ²	0.195		

Results with $p < 0.100$ are shown. Abbreviations: P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; 25(OH) D: 25-Hydroxyvitamin D; PTH: Parathyroid Hormone; HF: Hip Fracture. logGGT was the Dependent Variable and the Independent Variables Were: Age, Gender, LogP1NP, LogOC, Log β CTX, P1NP/OC, P1NP/ β CTX, OC/ β CTX, Log25(OH)D LogPTH, Logcalcium(corrected for albumin), Logphosphate, Logmagnesium, LogALT, LogALP, Logbilirubin, Logalbumin, Logegfr, Logcrp, Logwcc, Alcohol Overuse (>3 Drinks/Week), Smoking Status (Current Smoker: Ex-Smoker), Presence of Hip Fracture or Any Fracture, Diabetes Mellitus and Cardiovascular Diseases. ¹P1NP and OC (as independent variables) were not included in the model examining P1NP/OC ratio; similarly, P1NP and β CTX were not included in the model examining P1NP/ β CTX ratio and OC and β CTX were not included in the model examining OC/ β CTX ratio.

The study provides evidence that GGT and iron status indices (even within normal or mildly-moderately abnormal range) act as independent determinants of different aspects of bone metabolism, and are, in turn, regulated by bone-derived peptides. Remarkably, both osteoblast-specific secreted

molecules - OC (well documented previously [24,25]) and P1NP (as described, for the first time, in this study) – exert multiple extra-skeletal metabolic functions. The biological and clinical relevance of our results, which confirm and extend a number of previous studies are further discussed.

Serum GGT activity

In our multivariate adjusted models serum GGT activity was an independent determinant of P1NP and β CTX levels, as well as the P1NP/OC ratio. On the other hand, higher serum GGT activity was independently predicted by higher P1NP, P1NP/OC ratio, ferritin levels, lower 25(OH)D concentrations and younger age.

These results are consistent with:

- Hxperimental data indicating important (patho)physiological role of GGT in bone turnover state involving both bone formation and bone resorption [2-6].
- Human studies which reported as (i) Increased urinary excretion of bone resorption markers in parallel with urinary GGT levels in postmenopausal women [6], (ii) Correlation between serum GGT and ferritin levels in the adult population [26], as well as in patients with chronic hepatitis C [27] and DM [28], and (iii) An inverse association between 25(OH)D and GGT activity [29].

The results from the present study show that age-related decreases in GGT activity, a well known phenomenon [30,31], is associated with suppression of bone formation (indicated by lower P1NP and P1NP/OC ratio), as well as bone resorption (lower β CTX), but more affecting the former process.

On the other hand, both lower serum GGT and P1NP levels are associated with lower ferritin concentrations which, in turn, correlate inversely with markers of bone differentiation (higher OC) and bone resorption (higher β CTX).

Lower GGT activity is also a significant independent indicator of HF. These findings suggest a complex/dual effect of lower GGT on bone turnover, and indicate that GGT and iron operate as integral components of a complex homeostatic network that regulates bone metabolism.

Table 4 Serum bone-mineral parameters, GGT activity and related variables as independent determinants of indices of iron metabolism (stepwise multivariate linear regression analyses).

	Log ferritin			Log TSAT		
	β	95%CI	P value	β	95%CI	P value
LogOC	-0.328	-0.487; -0.169	0.000	-0.143	-0.256; -0.032	0.012
LogP1NP	0.173	0.053; 0.294	0.005	0.126	0.045; 0.207	0.002
LogGGT	0.171	0.066; 0.277	0.002			
LogALT	0.217	0.093; 0.340	0.001	0.137	0.064; 0.210	0.000
CRP	0.003	0.002; 0.004	0.000	-0.003	-0.004; -0.002	0.000
WCC				-0.014	-0.029; 0.001	0.076

LogeGFR	-0.26	-0.424; -0.330	0.022	-0.2	-0.336; -0.64	0.004
DM	-0.229	-0.451; -0.046	0.016			
HF	0.328	0.166; 0.489	0.000			
Any fracture	0.202	0.032; 0.372	0.020			
Sex(m)	0.306	0.143; 0.469	0.000			
R²		0.293			0.398	

Results with $p < 0.100$ are presented. The dependent variables were logferritin and TSAT and the independent variables were age, gender, logP1NP, logOC, log β CTX, log25(OH)D, PTH, logcalcium (corrected for albumin), logphosphate, logmagnesium, logGGT, logALT, logALP, logbilirubin, logalbumin, logeGFR, CRP, WCC, alcohol overuse (>3 drinks/week), smoking status (current smoker, ex-smoker), presence of hip fracture or any fracture, DM, cardiovascular diseases. Abbreviations: TSAT: Transferrin Saturation; P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; OC: Osteocalcin; Bctx: Crosslinked Carboxy-Terminal Telopeptide of Type 1 Collagen; PTH: Parathyroid Hormone; Mg: Magnesium; ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; eGFR: Estimated Glomerular Filtration Rate; HF: Hip Fracture; DM: Diabetes Mellitus; CRP: C-Reactive Protein; WCC: White Cell Count.

Iron metabolism

Our logistic regression analyses after adjusting for demographics, liver, renal, mineral and inflammatory markers, smoking and alcohol consumption status, presence of DM, CVD and fracture indicated independent positive associations of TSAT with P1NP, P1NP/OC and P1NP/ β CTX ratios, and negative with β CTX, while ferritin was a negative regulator of both OC and β CTX and a positive determinant of the P1NP/OC ratio. On the other hand, both P1NP and OC independently predicted TSAT, as well as ferritin.

These findings suggest that:

- Higher iron status (as indicated by higher TSAT and/or ferritin levels) is associated with suppression of osteoclast function and bone resorption (lower β CTX).
- Bone formation (P1NP) increases when TSAT is higher, but higher ferritin suppresses the process of bone differentiation (lower OC and higher P1NP/OC ratio).
- Iron-related markers have different effects on specific aspects of osteoblast function; feedback signals between molecules involved in iron and bone metabolism generate a positive loop with osteoblast formation (TSAT – P1NP) and a negative loop with osteoblast differentiation (ferritin – OC).

Thus, the bidirectional links TSAT-P1NP and ferritin-OC, as well as the effects of both ferritin and TSAT on β CTX, of TSAT on P1NP/OC and P1NP/ β CTX ratios should be considered as components of homeostatic systems that maintain structural and functional integrity in the bone (as well as in different other tissues).

Experimental studies linked osteoporosis/osteopenia with both iron overload [32-34] and iron deficiency [35-37]. Few studies in humans that examined the relation between parameters of iron status and bone turnover markers have produced inconclusive results.

An inverse association between serum ferritin and OC was reported in subjects aged 55-80 years with CVD [38]. In postmenopausal women with HF, serum ferritin correlated

positively and transferrin correlated negatively with both P1NP and β CTX [13].

Table 5 Associations between serum markers of bone-mineral and iron metabolism and GGT activity with hip fracture or any fracture. Clearly, contradicting results on associations between indices of iron and bone metabolism at least in part may be explained by the heterogeneity of the study populations, effects of age, gender, ethnicity, hormonal and environmental factors, different definitions of iron status, analytical variability of assays and adjustments only for some confounding factors.

	Hip fracture			Any fracture		
	OR	95%CI	P value	OR	95%CI	P value
Log P1NP	0.59	0.39; 0.92	0.019	0.51	0.36; 0.73	0.000
*P1NP/βCTX	0.43	0.29; 0.82	0.008	0.44	0.33; 0.71	0.000
Log βCTX	2.27	1.36; 3.79	0.002	1.91	1.25; 2.91	0.003
Log PO4	0.29	0.94; 0.89	0.030			
Log Mg	0.08	0.01; 0.48	0.006	0.17	0.03; 0.81	0.027
Logferritin	1.67	1.20; 2.31	0.002	1.3	1.01; 1.68	0.040
LogGGT	0.6	0.40; 0.88	0.010			
Age	1.13	1.09; 1.17	0.000	1.08	1.05; 1.11	0.000
R²	0.311			0.176		

Multivariate linear regression analyses of the dependent variables (hip fracture or any fracture – top row) and the significant ($p < 0.05$) independent variables (left column) shown. Adjustments: age, sex, logP1NP, logOC, log β CTX, P1NP/ β CTX (in a model without LogP1NP and log β CTX), log25(OH)D, logPTH, logcalcium (corrected for albumin), logphosphate, logmagnesium, logGGT, logALT, logALP, logbilirubin, logalbumin, logeGFR, alcohol overuse (>3 drinks/week), smoking status (current smoker, ex-smoker), presence of diabetes mellitus and/or cardiovascular diseases.

In young women negative correlations were found between log-ferritin and a urinary bone resorption marker (log-NTx), and

between transferrin and P1NP [10], while serum ferritin concentration was not correlated with serum bone resorption marker (tartrate-resistant acid phosphatase type 5b) in patients with sickle cell disease [39]. In astronauts, both ferritin and TSAT positively correlated with biochemical markers of bone resorption [14].

Our observation of the inverse relationship between ferritin and OC as well as between ferritin and β CTX concur with some previous clinical observations [10,38] and is in line with animal studies which showed lower serum OC concentration in iron-deficient rats [36]. The positive associations of TSAT with P1NP and P1NP/OC ratio in this study is consistent with lower P1NP

levels in premenopausal women with iron deficient anemia [10] and decreased bone matrix formation in anemic rats [35]. Notably, in our study, three iron indices - TSAT, ferritin and transferrin - were independently and inversely associated with β CTX, suggesting that iron deficiency results not only in decreased bone formation but also in increased bone resorption, a finding consistent with significantly elevated levels of urinary resorption marker in iron deficient women [10], and data of increased bone resorption in anaemic rats [35]. Other researchers found both reduced bone formation and resorption in iron-deficient anaemic rats but bone resorption remained greater than bone formation, resulting in overall bone loss [36].

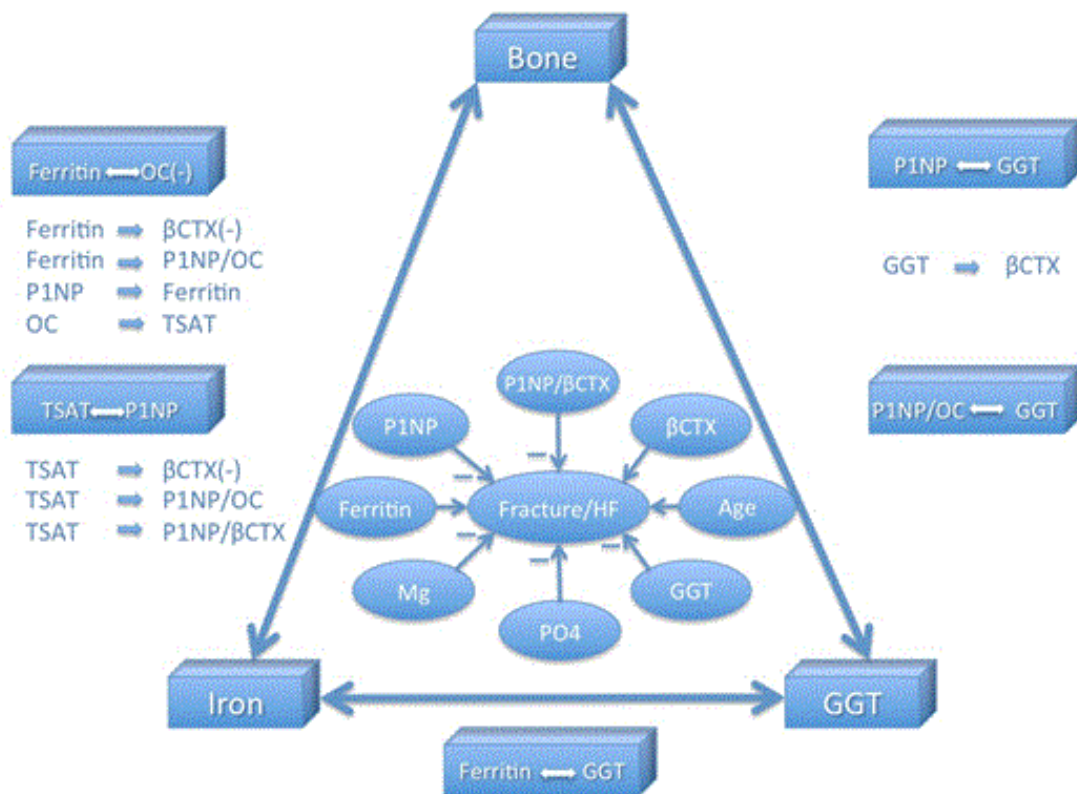


Figure 1 Schematic representation of the complex interactions between serum gamma-glutamyl transferase activity, markers of fracture and bone-mineral metabolism in the elderly; factors independently associated with bone fractures are shown in ovals. Abbreviations: GGT: Gamma-Glutamyl Transferase; TSAT: Transferrin Saturation; P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; OC: Osteocalcin; β CTX: Crosslinked Carboxy-Terminal Telopeptide of Type 1 Collagen; PO4: Phosphate. Bidirectional links are underlined; (-) indicates an inverse association; arrows indicate the direction of affect. Serum GGT activity is an independent determinant of P1NP, β CTX, P1NP/OC ratio and ferritin, which, in turn, is an independent determinant of OC, β CTX, and P1NP/OC ratio, while TSAT is an independent predictor of P1NP, β CTX, P1NP/ β CTX and P1NP/OC ratios. The bidirectional links between ferritin and GGT, ferritin and OC, GGT and P1NP, GGT and P1NP/OC ratio, TSAT and P1NP indicate feedback signals involved in the complex homeostatic mechanisms. Higher serum concentrations of β CTX and ferritin, lower P1NP, P1NP/ β CTX ratio, PO4 levels and advanced age are independent indicators of any fracture; lower GGT activity is an additional independent indicator of HF.

An important point to note is that ferritin demonstrated a bidirectional inverse link with OC, whereas TSAT showed a bidirectional positive association with P1NP, despite the fact that P1NP is correlated with OC. It appears, therefore, that the contribution of ferritin to bone metabolism may reflect an effect mediated by mechanism (-s) other than increased iron stores. Furthermore, despite the strong inverse bidirectional link between ferritin and OC, neither serum iron, nor TSAT (additional indicators of body iron stores, transport and availability) independently predicted OC in our and other studies [28].

These observations are in keeping with the existing evidence that elevated serum ferritin is a multifactorial metabolic marker which reflects in addition to body iron stores other conditions such as inflammation, oxidative stress, chronic kidney, liver and autoimmune disorders; elevated ferritin levels do not exclude underlying iron deficiency. Indeed, 78.9% of our patients had low TSAT, and 26.0% demonstrated a “paradoxical” combination of elevated ferritin levels with low TSAT, indicating functional iron deficiency [40], a condition typical for anemia of chronic disease (anemia of inflammation) and chronic kidney disease. In the present study ferritin was independently predicted by CRP (positively) and eGFR (negatively).

Although hydroxylation of 25(OH)D to 1,25(OH)D requires iron [36] and low vitamin D levels were linked with iron deficient anemia in females [10], in our study, 25(OH)D was not an independent determinant of TSAT or ferritin.

Homeostatic mechanisms

Our data indicate a negative-feedback loop ferritin – OC and a positive-feedback loop TSAT – P1NP, whereas both P1NP and P1NP/OC ratio demonstrate positive-feedback loops with GGT. These observations reflect the complexity of homeostatic mechanisms and are in line with the growing body of evidence suggesting oxidative stress as an integrative (patho)physiologic mechanism that links both GGT activity and iron status with bone integrity. Higher level of GGT, the main determinant of extracellular hydrolysis of glutathione, and elevated serum ferritin concentrations leading to release of more free iron, could facilitate glutathione catabolism, increase production of reactive oxygen species and subsequent oxidative stress, which in turn, affects bone metabolism and causes bone loss [22,23,41]. The hypothesis that oxidative stress is involved in the pathogenesis of bone loss is supported by animal studies showing that treatment with the antioxidant N-acetyl-L-cysteine, precursor of glutathione, reversed osteopenia in GGT-deficient mice [2] and prevented the development of bone abnormalities in iron-overloaded mice [33]. In ours and other studies, GGT and ferritin correlated with each other. On the other hand, these variables demonstrated associations with β CTX and presence of HF, which is in line with the dual role of reactive oxygen species (positive at moderate levels and damaging at elevated levels) in bone metabolism, as in a number of other pathological conditions. Interrelated serum GGT and ferritin levels appear as an important homeostatic bridge between metabolic, oxidative and bone status.

Taken together, the existing literature (although results are mixed) and our observations provide evidence that regulation of both osteoblastic and osteoclastic differentiation and function requires an optimal iron status: iron levels should be sufficiently high to facilitate bone formation and suppress bone resorption, but not so high that they affect bone differentiation and increase GGT activity. Importantly, these factors are in a state of mutual influence: feedback mechanisms involving both osteoblast-specific proteins (P1NP and OC) participate in maintaining iron homeostasis and GGT activity.

Biomarkers of osteoporotic fractures

In multivariate regression, P1NP, β CTX, P1NP/ β CTX ratio, ferritin, magnesium and age were independent indicators of HF or any fracture; GGT and phosphate levels were additional independent negative indicators of HF. Multivariate regression analyses revealed also that presence of HF was an independent positive predictor for logferritin, log β CTX, and a negative predictor for both P1NP/ β CTX and OC/ β CTX ratios. Interestingly, neither serum 25(OH)D, nor PTH were independently associated with fractures, and no association between serum 25(OH)D and any of serum bone markers was found, but logPTH was an independent negative predictor of logP1NP and P1NP/ β CTX ratio.

The literature on the association between fracture risk and bone turnover markers, as well as serum 25(OH)D and PTH concentrations is controversial. Our data are in agreement with previous studies showing that: (1) higher serum and urinary bone resorption markers predict risk for HF, vertebral and non-vertebral fracture [42], (2) in women low serum levels of P1NP (independent of BMD) predict increased risk of HF [43], (3) serum concentrations of PTH correlated negatively with P1NP, while there was no correlation between 25(OH)D and any bone markers [43,44].

Although P1NP and CTX were recommended as osteoporotic markers by the Bone Marker Standards Working Group [45], the P1NP/ β CTX ratio has not been evaluated previously. In our study, the P1NP/ β CTX ratio that combines two different markers reflecting both bone formation and bone resorption appears as an independent and a better indicator of fracture than absolute P1NP and β CTX values.

Practical considerations

First, the fact that abnormal iron status, a preventable and curable factor, contributes to the occurrence of osteoporotic fractures, signifies the importance of screening for and treating all elderly persons with altered iron metabolism and/or anemia for osteoporosis and vice versa.

Second, in the elderly iron deficiency, absolute (low iron stores) or functional (impaired iron mobilization from storage and transportation to target tissues despite normal or abundant body iron stores) is prevalent and multifactorial (renal insufficiency, sex hormone deficiency, limited iron intake, blood loss). Since ferritin levels are not specific for iron overload (serum ferritin is almost equally both a measure of iron stores and an inflammatory marker), this parameter has to be

interpreted with caution. Moderately elevated ferritin levels, as in our study, especially if combined with a low TSAT (an index of the amount of iron molecules available in the serum that are bound to transferrin), may reflect multiple systemic processes. Therefore, the recommendation of lowering “iron overload” (based on serum ferritin levels only) by chelation therapy [9] or by a biologically active form of hepcidin [8] for the treatment of osteoporosis seems premature if not inappropriate, specifically in the elderly, in whom osteoporosis, iron-deficiency, chronic inflammation and renal impairment are common. Therapeutic manipulation of iron balance in this population is challenging; to be effective and safe it should be based on detailed assessment of pathogenic mechanisms and individualised.

Third, because low BMD, currently the main diagnostic criterion of fracture risk, has a poor sensitivity (about half of low-trauma fractures occur in subjects with non-osteoporotic BMD), our observations together with literature reports indicate that bone turnover markers, especially the P1NP/ β CTX ratio, may be useful in detection of increased risk of osteoporotic fracture and in selection of the most suitable type of antiosteoporotic treatment in a given patient (i.e., anabolic versus antiresorptive medications in individuals with low P1NP). A combined approach with both indices of bone turnover and BMD could possibly improve fracture prediction and management.

Limitations

A number of limitations of this study need to be considered. First, because it is a cross-sectional study, it cannot prove causality. Second, a single-point measurement of the biomarkers may be subject to within-individual variation, although the majority of biochemical tests, including serum GGT, ferritin, iron, OC, P1NP, indices of mineral metabolism are relatively stable and have no circadian and between-day variations. Third, although in multivariate models we attempted to adjust for a series of potential confounders (laboratory, clinical, behavioural), the possibility of residual or unknown confounding cannot be excluded. Fourth, as we did not measure hepcidin, soluble transferrin receptor, and undercarboxylated OC, our results should be interpreted cautiously. Finally, this study was conducted at a single centre and as the cohort was almost entirely Caucasians, our findings may not necessarily translate to other centres and be applicable to other ethnic and racial populations. The strength of this study includes comprehensive clinical and laboratory evaluations and simultaneously measuring of multiple parameters of liver, iron and bone metabolism in a moderately large sample size, whereas most of previous clinical studies assessed a limited number of variables and often did not control for important factors. In all our models the variance inflation factor ranged between 1.03 and 1.17 indicating that the amount of multicollinearity was not significant.

Conclusions

The study highlighted and specified the bidirectional interconnections between serum GGT activity, indices of iron

metabolism and bone-derived factors, the role of GGT and iron homeostasis in maintaining bone health in the elderly, reinforced the need to individualize prophylactic and therapeutic strategies based on alterations in individual biochemical profiles, and identified the serum bone turnover markers, especially P1NP/ β CTX ratio, as indicators for osteoporotic fractures. The clinical significance and ways of modulation of GGT and iron-related factors warrant further investigations.

Conflict of interests

The authors declare that they have no conflict of interests.

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Research Paper

The Neutrophil to Lymphocyte Ratio on Admission and Short-Term Outcomes in Orthogeriatric Patients

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Received: 2016.03.03; Accepted: 2016.06.15; Published: 2016.07.05

Abstract

Aim: To investigate the association of the neutrophil to lymphocyte ratio (NLR) at admission with presence of fracture, comorbid conditions, and its prognostic value for short-term outcomes in orthogeriatric patients.

Methods: On 415 consecutive patients (mean age 78.8 ± 8.7 [SD] years, 281 women, 255 with a non-vertebral bone fracture, including 167 with a hip fracture, HF) admitted to the Department of Orthopaedic Surgery at the Canberra hospital (2010 - 2011) data on clinical and laboratory characteristics were collected prospectively. The validation dataset included 294 consecutive patients (mean age 82.1 ± 8.0 years, 72.1% women) with HF.

Results: Multivariate regression revealed four variables, presence of HF, hypoalbuminaemia ($<33\text{g/L}$), anaemia ($<120\text{g/L}$) and hyperparathyroidism ($\text{PTH} > 6.8 \text{ pmol/L}$), as independent determinants of admission $\text{NLR} \geq 5.1$. There was a dose-graded relationship between presence of fracture, especially HF, postoperative complications and levels of NLR categorized as tertiles. Compared to patients with $\text{NLR} < 5.1$ (first tertile), patients with $\text{NLR} 5.1-8.5$ (second tertile) had a 1.8-, 3.1-, 2.6-, and 2.5-fold higher risk for presence of any fracture, HF, developing postoperative myocardial injury (troponin I rise) and a high inflammatory response/infection ($\text{CRP} > 100\text{mg/L}$ after the 3rd postoperative day), respectively, while in subjects with $\text{NLR} > 8.5$ (third tertile) these risks were 2.6-, 4.9-, 5.9- and 4.5-times higher, respectively; subjects with $\text{NLR} > 8.5$ had a 9.7 times higher chance of dying in the hospital compared to patients with $\text{NLR} 5.1-8.5$; the NLR retained its significance on multivariate analyses. The $\text{NLR} \geq 5.1$ predicted postoperative myocardial injury with an area under the curve (AUC) of 0.626, $\text{CRP} > 100\text{mg/L}$ with AUC of 0.631 and the $\text{NLR} > 8.5$ predicted in-hospital mortality with an AUC of 0.793, showing moderately high sensitivity (86.7%, 80% and 90%, respectively) and negative predictive value (92.9%, 71.2%, 99.6%, respectively), but low specificity. Admission NLR was superior to other, except hypoalbuminaemia, prognostic markers; combined use of both $\text{NLR} \geq 5.1$ and $\text{albumin} < 33\text{g/L}$ only moderately increased the accuracy of prediction. The validation study confirmed the prognostic value of the admission NLR.

Conclusions: In orthogeriatric patients, high NLR on admission is an independent indicator of fracture presence, a significant risk factor and moderate predictor of postoperative myocardial injury, high inflammatory response/infection and in-hospital death.

Key words: neutrophil to lymphocyte ratio (NLR), orthogeriatric patients, hip fracture, outcomes

Introduction

With population ageing associated with high prevalence of osteoporosis, musculoskeletal, nervous system and cardiovascular diseases, high incidence of falls and fractures, the proportion of orthopaedic

patients is rising. Preoperative multimorbidity of older adults ($>50\%$ have three or more chronic diseases [1]) causes a significant increase in the burden of morbidity and mortality and requires

specific management. Postoperative adverse outcomes dominated by cardiovascular events [2-6] and inflammatory complications [7-10] are associated with increased hospital stay, institutionalization, poorer quality of life, higher inpatient and long-term mortality, greater use of healthcare resources and substantially increased costs [11-13]. Although post-admission and postoperative conditions and complications contribute significantly to outcomes, it is important to identify preoperative outcome-affecting risk factors related to both medical comorbidities and orthopaedic conditions and treatment. These, if modifiable, have the potential to improve the perioperative management and decrease postoperative morbidity and mortality. Unfortunately, there are no widely accepted, effective, clinically applicable predictors of outcomes to guide preventive and treatment choice in orthogeriatric patients.

Several studies which investigated the impact of different preoperative clinical factors, various scoring systems and admission blood tests [6, 14-25] on prediction of mortality risk following hip fracture (HF) surgery produced controversial results. Little is known about the usefulness of these markers and tools for prediction of other outcomes after HF repair, and it remains uncertain whether routinely available preoperative clinical and laboratory markers identify non-HF orthogeriatric patients at higher risk of major perioperative complications.

Over the past decade data have emerged that a high preoperative neutrophil to lymphocyte ratio (NLR), a systemic inflammatory-immunological marker, is an independent predictor of mortality in critically ill intensive care patients [26], after emergency abdominal surgery in the elderly [27], after major cardiac and vascular surgery [28] and after surgery for a variety of cancers [29-31]. NLR was also found to be a significant independent predictor of adverse outcomes in patients with coronary artery disease (CAD) [32-39], hypertension, ischaemic stroke [40], chronic kidney disease (CKD), diabetes mellitus (DM), chronic heart failure (CHF), peripheral arterial disease [33, 35, 37, 38, 41], and for survival in various cancer populations [31, 42, 43], conditions common in the elderly. However, some studies failed to demonstrate its prognostic value, for example, in postoperative atrial fibrillation AF [44] and in different cancer types [45-47]. Patients with complications after major abdominal surgery did not present a higher preoperative NLR than those without [48], but an increased NLR on the first postoperative day indicated a greater risk of complications after colorectal surgery [49]. Because different cut-offs of NLR (ranging between 2.10 and 22.85) were used, the

magnitude of the prognostic impact of NLR is still unclear, and controversy exist even in regard to different cancer types.

In orthopaedic patients, in contrast to that in other patient groups, this marker has been studied very little. In one study of HF patients, the preoperative NLR was not predictive of postoperative mortality, after surgery NLR decreased but $\text{NLR} > 5$ at the 5th postoperative day was associated with higher risk of postoperative mortality, cardiovascular complications and infections [50].

In the present study we aimed to investigate in orthogeriatric patients (1) the association of NLR on admission with presence of fracture and comorbid conditions known to affect outcomes, (2) to evaluate the prognostic value of NLR against established risk factors, and (3) to determine whether combined use of the NLR and other biomarkers on admission improves the prediction of short-term outcomes.

Patients and Methods

In total, 415 consecutive patients aged 60 years and over, who were admitted between 1 January 2010 and 31 August 2011 to the Department of Orthopaedic Surgery at the Canberra hospital (a 500-bed university-affiliated tertiary care centre), underwent surgery and for whom full clinical and laboratory data was available, were included in the study. The mean age of the cohort was 78.8 ± 8.7 years, 281 (67.7%) were female, and 394 (95%) were Caucasian. Of 415 patients 255 (61.4%) had a non-vertebral bone fracture, including 167 (40.2%) a HF. Among 160 non-fracture patients there were 143 subjects admitted for elective surgery, 6 patients with suspected surgical site infections (not confirmed by further investigation) and 11 patients with a prosthetic joint infection following total hip (8) or knee (3) arthroplasty. Data was collected prospectively on demographics, medical and orthopaedic diagnoses, laboratory characteristics, procedures performed, medication used, and short-term outcomes.

Validation Dataset

A retrospective analysis of a second cohort included data (obtained from electronic medical and administrative records) from 294 consecutive older (≥ 60 years of age) patients (mean age 82.1 ± 8.0 years, 72.1% women) with osteoporotic HF who were treated at the Canberra Hospital between 2005 and 2007. Among all orthogeriatric patients this group is known to contribute the greatest to postoperative morbidity and mortality.

The study was conducted according to the standards of the Declaration of Helsinki and was approved by the local Health Human Research Ethical

Committee. As only routinely collected patient data (anonymized before analysis) were used and none of the patients had a blood test for the purposes of the study itself, the need for informed consent was waived.

Laboratory measurements

In each patient venous blood samples were collected on admission and the following tests performed: complete blood count, electrolytes, renal (creatinine, urea), liver (ALT, GGT, ALP, albumin and total bilirubin) and thyroid function tests (thyroid stimulating hormone, TSH; thyroxine, T₄), C-reactive protein (CRP), cardiac troponin I (cTnI), fasting blood glucose (and HbA1C in diabetic patients), 25 (OH) vitamin D [25(OH)D], intact PTH, total calcium, phosphate and magnesium. All biochemical parameters were measured by standard automated laboratory methods and using commercially available kits according to the manufacturers' protocols. Serum cTnI was determined by a 2-step chemiluminescent microparticle immunoassay (Chemiflex, Abbott Labs, Mississauga, Ontario, Canada), 25(OH)D by a radioimmunoassay kit (Dia Sorin, Stillwater, MN, USA), intact PTH by 2-site chemiluminescent enzymelinked immunoassay on DPC Immulite 2000 (Diagnostic Products, Los Angeles, CA). According to the manufacturer, the low detection limit for cTnI assay is 0.03 µg/L and the upper limit of reference range is 0.06 µg/L. In this study all values of cTnI above this level were considered elevated, indicating myocardial injury. Glomerular filtration rate (GFR) was estimated by a standardized serum creatinine-based formula normalized to a body surface area of 1.73 m² [51, 52]. Chronic kidney disease (CKD) was defined as a glomerular filtration rate (GFR) <60 mL/min/1.73 m², which represents a loss of half or more of the normal adult renal function level [53].

For the analyses, deficiency of vitamin D was defined as 25(OH)D < 25 nmol/L and insufficiency as 25(OH)D < 50 nmol/L based on current recommendations. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (>6.8 pmol/L, the upper limit of the laboratory reference range). Cut-off values for neutrophil count (>8.0x10⁹/L), lymphocyte count (<1.2x10⁹) and serum albumin level (<33g/L) were defined as greater than the upper limit or lower than the low limit of normal range, respectively.

Short-term outcomes

These included: (1) in-hospital all-cause mortality, (2) postoperative myocardial injury defined by cardiac cTnI I rise (cTnI >0.06 µg/L), a marker unique to myocardium, (3) high inflammatory

response (CRP>100 mg/L or >150mg/L after the 3rd postoperative day), (4) prolonged length of stay (>10 days) and (5) being discharged to a permanent residential care facility (RCF). According to our standard postoperative care protocol, in all patients aged ≥60 years, CRP and cTnI measurements were performed on the first 3 days after surgery and thereafter if elevated. The postoperative cTnI rise was chosen as an important indicator of short-term outcome because myocardial injury/necrosis (diagnosed with an elevated cTnI measurement) is the most common cardiovascular complication after noncardiac surgery, asymptomatic in up to 80% of patients but known to be associated with significant in-hospital and long-term morbidity and mortality [5, 54-57].

Elevated CRP, a widely recognised parameter for early detection of postoperative infections, reflects also the extent of surgical trauma. The hepatic synthesis of CRP due to bacterial infection is known to start 6-8 hours after infection, reached its peak on the second-third postoperative day (36 - 50 hours) [58-61], a level of ≥96 mg/L after the fourth day of surgery is highly indicative for deep wound infection [62]. Importantly, the CRP response after orthopaedic surgery is more informative than white blood cell count (WBC) [59, 63], absolute neutrophil count and erythrocyte sedimentation rate [58, 64], and is not associated with age, gender, type of anaesthesia, operation time, amount of bleeding, transfusion or drugs administered [58]. On these bases persistent elevation and/or second rise in CRP concentrations (CRP>100 mg/L and CRP >150mg/L) after the 3rd postoperative day were chosen as indicators of possible postoperative infective complications.

Statistical analyses

Continuous variables are reported as means ± standard deviation (SD) and compared using the Student's t test. Categorical variables are presented as proportions/percentages and compared by Chi-square and Fisher exact tests. The admission NLR was analysed as both a continuous and a categorical variable; in the latter, NLR was categorized into 3 groups (stratified by tertiles). The associations between NLR and presence of any fracture, HF and outcomes were estimated with univariate and multivariate linear logistic regression models and reported as odds ratios (OR) with 95% confidence interval (CI); all potential confounding variables (demographic, clinical and laboratory) with statistical significance ≤ 0.15 on univariate analyses were included in multivariate models to identify independent factors associated with fractures and/or poorer short-term outcomes. In the univariate

analysis, age was explored both by category and as a continuous variable. In the multivariate analysis, age was treated as a continuous variable. We compared the predictive values of NLR in the 2nd and 3rd tertiles with that in the 1st. To quantify the potential predictive utility for NLR and other parameters of interest receiver operating characteristic (ROC) analysis was used and the predictive accuracy was expressed as area under curve (AUC). To quantify the significance of multicollinearity phenomena in regression analyses the variance inflation factor was calculated. Two-tailed p-values < 0.05 were considered statistically significant. The Stata software version 10 (StataCorp, College Station, TX, USA) was used for all statistical analyses.

Results

Patient characteristics

The main clinical and laboratory characteristics of the study population are displayed in Table 1. At least one cardiovascular disease (CVD) was present in 315 (75.9%) patients. Anaemia (haemoglobin < 120 g/L) was diagnosed in 330 (79.5%) patients, CKD in 79 (19.0%), type 2 diabetes mellitus (DM) in 80 (19.3%), dementia in 73 (17.6%), vitamin D insufficiency in 148 (35.7%) and hyperparathyroidism in 164 (39.5%) subjects.

Relationships between the NLR and clinical characteristics

On admission the mean white cells, neutrophil and lymphocyte counts were $8.91 \pm 3.67 \times 10^9/L$, $7.61 \pm 2.47 \times 10^9/L$ and $1.27 \pm 1.18 \times 10^9/L$, respectively; the mean NLR was 8.24 ± 6.58 . The mean NLRs were significantly higher in patients > 75 years of age, with any fracture, HF, dementia, AF, CHF, CKD, anaemia, hypoalbuminaemia, vitamin D deficiency and elevated PTH level; in patients with CAD the difference was of borderline significance ($p=0.052$), while patients with OA demonstrated lower mean NLR compared to the rest of the cohort (Table 1). In contrast, the mean NLRs did not differ by gender, presence of hypertension, cerebrovascular disease, peripheral vascular disease, DM, Parkinson's disease, COPD, history of cancer, smoking (current or past), alcohol consumption and use of walking aids. Preoperative NLR evaluated as a continuous variable revealed that each 1-unit increase in NLR was associated with increased probability of being >75 years of age by 9.3%, of having any fracture by 7.8%, a HF by 12.3%, dementia by 6.4%, AF by 3.4%, CHF by 4.9%, CKD by 6.1%, vitamin D deficiency by 4.2%, hyperparathyroidism by 4.9%, of being anaemic by 10.3%, and hypoalbuminaemic by 15.2% (Table 1).

Table 1. Clinical characteristics and admission neutrophil to lymphocyte ratio (NLR) in orthogeriatric patients (n=415)

Characteristics	Sign present	Sign absent	P value	OR	95%CI	P value
Age > 75 yr (n=277, 66.7%)	9.09 ± 7.23	6.54 ± 4.62	0.0002	1.093	1.042 - 1.146	0.000
Gender (females, n=281, 67.7%)	8.06 ± 6.18	8.62 ± 7.34	0.4195	1.012	0.982 - 1.044	0.421
Any fracture (n=255, 61.4%)	9.17 ± 7.45	6.79 ± 4.55	0.0003	1.078	1.033 - 1.125	0.001
Hip fracture (n=167, 40.2%)	10.57 ± 8.42	6.69 ± 4.34	0.0000	1.123	1.076 - 1.172	0.000
Hypertension (n=260, 62.7%)	8.53 ± 7.05	7.75 ± 5.71	0.2434	1.019	0.987 - 1.053	0.246
CAD (n=74, 17.8%)	9.59 ± 8.77	7.95 ± 5.98	0.0517	1.032	0.999 - 1.067	0.060
MI (n=33, 8.0%)	9.52 ± 6.91	8.13 ± 6.55	0.2453	1.023	0.982 - 1.072	0.251
CVA (n=28, 6.7%)	10.18 ± 9.37	8.10 ± 6.33	0.1074	1.035	0.991 - 1.081	0.117
TIA (n=22, 5.3%)	7.66 ± 4.48	8.37 ± 6.68	0.6688	0.984	0.912 - 1.060	0.668
AF (n=71, 17.1%)	9.71 ± 7.23	7.94 ± 6.41	0.0393	1.034	1.000 - 1.070	0.047
CHF (n=44, 10.6%)	10.85 ± 8.95	7.93 ± 6.19	0.0053	1.049	1.011 - 1.088	0.010
PVD (n=20, 4.9%)	8.59 ± 5.37	8.22 ± 6.64	0.8105	1.008	0.946 - 1.074	0.810
DM (n=80, 19.3%)	8.01 ± 6.78	8.30 ± 6.54	0.7253	0.993	0.955 - 1.032	0.725
Dementia (n=73, 17.6%)	11.0 ± 9.66	7.65 ± 5.56	0.0001	1.064	1.028 - 1.101	0.000
Parkinson's disease (n=15, 3.6%)	10.20 ± 7.40	8.17 ± 6.55	0.2403	1.034	0.977 - 1.093	0.248
Cancer (n=84, 20.2%)	9.29 ± 6.37	7.97 ± 6.62	0.1009	1.027	0.994 - 1.061	0.108
OA (n=194, 46.7%)	7.32 ± 4.78	9.05 ± 7.75	0.0074	0.955	0.923 - 0.99	0.010
Any CVD (n=315, 75.9%)	8.32 ± 6.76	7.98 ± 6.00	0.6503	1.001	0.973 - 1.045	0.650
COPD (n=46, 11.1%)	8.06 ± 6.55	8.26 ± 6.59	0.8452	0.995	0.948 - 1.045	0.845
Smoker (n=36, 8.7%)	7.65 ± 5.72	8.30 ± 6.66	0.5714	0.983	0.926 - 1.043	0.571
Ex-smoker (n=83, 20.0%)	8.03 ± 5.13	8.30 ± 6.90	0.7394	0.994	0.956 - 1.032	0.739
Alcohol over-user (n=79, 19.0%)	7.46 ± 4.62	8.43 ± 6.96	0.2422	0.974	0.932 - 1.018	0.244
Walking aids user (n=168, 40.5%)	8.28 ± 5.21	8.22 ± 7.38	0.9289	1.001	0.972 - 1.031	0.929
CKD (n=79, 19.0%)	10.74 ± 10.03	7.65 ± 5.32	0.0002	1.061	1.025 - 1.097	0.001
Albumin < 33 g/L (n=167, 40.4%)	10.86 ± 8.46	6.48 ± 4.10	0.0000	1.152	1.100 - 1.207	0.000
Haemoglobin < 120 g/L (N=330, 79.5%)	8.75 ± 6.87	6.25 ± 4.89	0.0017	1.103	1.038 - 1.173	0.002
25(OH)D < 25 nmol/L (n=39, 9.4%)	10.47 ± 7.77	8.00 ± 6.43	0.0258	1.042	1.003 - 1.082	0.034
25(OH)D < 50 nmol/L (n=148, 35.7%)	8.92 ± 8.23	7.86 ± 5.45	0.1165	1.024	0.993 - 1.055	0.123
PTH > 6.8 pmol/L (n=164, 39.5%)	9.46 ± 7.43	7.44 ± 5.85	0.0022	1.049	1.016 - 1.084	0.004

Data are mean values (±SD) and univariate logistic regression.

Abbreviations: OR, odds ratio; CI, confidence interval; CAD, coronary artery disease; MI, myocardial infarction; CVA, cerebrovascular accident; TIA, transient ischaemic attack; AF, atrial fibrillation; CHF, chronic heart failure; PVD, peripheral vascular disease; CVD, cardiovascular disease; DM, diabetes mellitus; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease (estimated glomerular filtration rate, $eGFR < 60 \text{ ml/min/1.73 m}^2$); OA, osteoarthritis; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.

We then investigated in multivariate models the associations between NLR as a continuous variable and presence of fracture including all parameters shown to be linked to NLR in univariate analyses ($p \leq 0.150$) and controlling for age and sex. These demonstrated that preoperative NLR remained an independent indicator of a HF (OR 1.060, 95%CI 1.010-1.118, $p=0.030$) but not of any fracture (OR 1.036, 95%CI 0.988-1.086, $p=0.141$). On the other hand, in a similar multivariate regression model with NLR as a continuous dependent variable dementia ($\beta = 2.038$, 95%CI 0.084 - 3.99, $p=0.041$) was the positive and albumin ($\beta = -0.357$, 95%CI -0.534 - -0.180, $p=0.000$) and eGFR levels ($\beta = -0.032$, 95%CI -0.062 - -0.001, $p=0.042$) were the negative independent determinants of higher NLR, while presence of HF was only of borderline significance ($\beta = 1.625$, 95%CI -0.069 - 3.319, $p=0.060$). In other words, higher NLR is an independent consistent indicator of presence of HF, but the opposite is not always true.

When dividing the patients according to tertiles of NLR, subjects in the highest tertile (>8.5 , mean $\pm$ SD: 14.69 ± 7.91) compared to subjects in the first tertile (<5.1 , mean $\pm$ SD: 3.42 ± 1.06) were significantly older (80.8 ± 8.5 vs. 76.8 ± 8.6 years, $p=0.0005$), more likely to have any fracture (72.1% vs. 49.3%, $p=0.000$)

or a HF (55.1% vs. 20.0%, $p=0.000$), AF (23.5% vs. 13.6%, $p=0.033$), dementia (22.1% vs. 11.4%, $p=0.018$), history of cancer (27.9% vs. 15.0%, $p=0.009$), anaemia (88.2% vs. 68.6%, $p=0.000$), and hyperparathyroidism (48.9% vs. 28.1%, $p=0.001$), as well as lower serum albumin (32.1 ± 4.7 vs. 35.7 ± 4.2 g/L, $p=0.0001$) levels, erythrocyte (3.31 ± 0.57 vs. $3.62 \pm 0.53 \times 10^{12}$ /L, $p=0.0001$) and lymphocyte counts (0.72 ± 0.25 vs. $1.94 \pm 1.80 \times 10^9$ /L, $p=0.0001$), and higher total leukocyte count (10.30 ± 3.99 vs. $7.75 \pm 4.05 \times 10^9$ /L, $p=0.0001$) and creatinine concentrations (89.4 ± 72.4 vs. 74.6 ± 27.7 μ mol/L, $p=0.0249$).

In multivariate logistic regression models which included all clinical and laboratory factors associated with higher NLR with a value of $p \leq 0.15$ in univariate analyses after adjusting for age and sex, the independent determinants of preoperative $\text{NLR} \geq 5.1$ were presence of HF (OR 2.66, 95%CI 1.38 - 5.12, $p=0.003$), lower levels of albumin (OR 0.92, 95%CI 0.86 - 0.99, $p=0.019$) and haemoglobin (OR 0.98, 95%CI 0.96 - 0.99, $p=0.028$) and higher serum PTH concentration (OR 1.09, 95%CI 1.01 - 1.19, $p=0.036$); presence of any fracture was of borderline significance (OR 1.61, 95%CI 0.95 - 2.71, $p=0.077$).

In comparison to subjects with preoperative NLR levels <5.1 (first tertile, referent category), patients with $\text{NLR} \geq 5.1$ were about 2 times more likely to present with a fracture (OR 2.12, 95% CI 1.40 - 3.22, $p=0.000$) and 3.9 times more likely to have a HF (OR 3.90, 95%CI 2.34 - 6.52, $p=0.000$). Patients with NLR in the range of 5.1 - 8.5 (intermediate tertile) were 1.75-fold more likely to have any fracture (OR 1.75, 95% CI 1.08-2.91, $p=0.022$) and 3.14-fold more likely to have a HF (OR 3.14, 95%CI 1.70- 5.80, $p=0.000$), whereas patients with NLR in the category of >8.5 (high tertile) were 2.62-fold more likely to have any fracture (OR 2.62, 95% CI 1.54-4.46, $p=0.000$) and 4.93-fold more likely to have a HF (OR 4.93, 95%CI 2.64 - 9.28, $p=0.000$) (Figure 1).

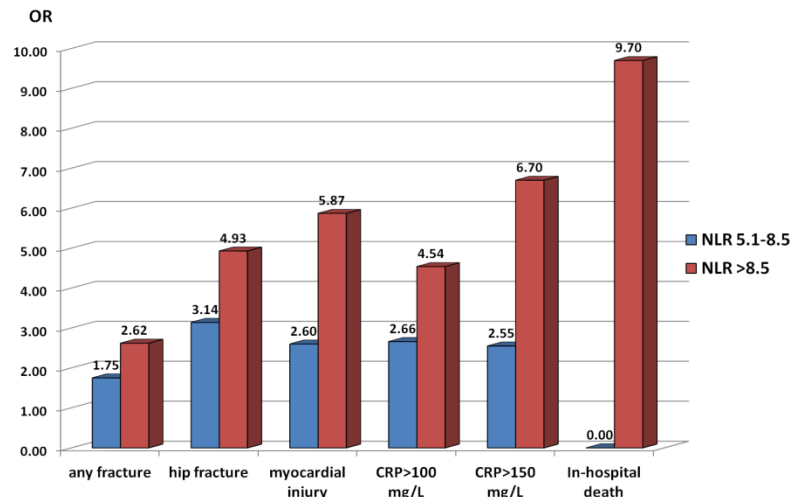


Figure 1. Odds ratios (OR) for presence of fracture and specific short-term outcomes in orthogeriatric patients according to neutrophil to lymphocyte ratio (NLR) tertiles on admission. Patients with admission NLR in the second (5.1 - 8.5) and third (>8.5) tertiles are compared to those with $\text{NLR} < 5.1$ (first tertile) for all shown outcomes except in-hospital death. The OR for in-hospital death reflects the comparison of patients with admission $\text{NLR} > 8.5$ and those with $\text{NLR} 5.1 - 8.5$ (no fatal outcomes were observed among patients with $\text{NLR} < 5.1$ on admission). *Abbreviations:* CRP, C-reactive protein; myocardial injury, postoperative cardiac troponin I rise.

Admission NLR and short-term outcomes

Postoperative complications and outcomes are shown in Table 2. Postoperative myocardial injury with cTnI rise was caused by acute pulmonary oedema due to fluid overload, myocardial ischaemia associated with anaemia and sepsis, acute coronary syndrome and pulmonary embolism; it was observed in 75 (18.1%) patients. A significant

inflammatory response which persisted 3 days after surgery with CRP>100 mg/L was seen in 200 (48.2%) patients and with CRP>150 mg/L in 129 (31.1%) patients; it was related mainly to urinary tract, pulmonary, skin or wound infections. Prolonged hospital stay (LOS>10 days) occurred in 211 (50.8%) patients. Overall postoperative in-hospital mortality rate was 2.4%; 9 of 10 patients who died presented with a HF (the mortality rate for HF was 6.0%). Of 322 patients admitted from home 22 were discharged to permanent RCF.

Patients with all above mentioned postoperative outcomes, except being discharged to RCF, had significantly higher mean NLRs on admission (Table 2) and in univariate analysis NLR was significantly associated with these short-term outcomes. With each unit increase in preoperative NLR there was a 13.6%, 12.5%, 4.3% and 9.7% increase in postoperative myocardial injury, inflammatory complications, prolonged hospital stay and in-hospital death, respectively. Admission NLR did not influence the incidence of discharges to RCF among subjects admitted from home (OR 1.26, 95% 0.93- 1.71, $p=0.141$).

We then investigated in multivariate models the associations between NLR as a continuous variable and outcomes (as dependent variables) including presence of CAD, AF, CHF, cerebrovascular disease, dementia, cancer, osteoarthritis, any fracture or HF, eGFR, haemoglobin, 25(OH)D and PTH levels, age and gender as independent variables. These demonstrated that preoperative NLR remained an independent predictor of postoperative death, myocardial injury and inflammatory complications. For every unit increase in the NLR, there was a 10.6% increased risk of in-hospital death, a 8.6% increased risk of cTnI rise, a 8.1% higher risk of developing an inflammatory response with CRP>100 mg/L and a 9.4% higher risk of having CRP>150 mg/L. NLR, however, was not an independent predictor of

prolonged LOS in multivariate analysis.

Among other laboratory variables hypoalbuminaemia preoperatively appeared as the most informative predictor of outcomes. Every 1-unit decrease in serum albumin was associated with a 15.5% higher risk of postoperative cTnI rise (OR 0.845, 95%CI 0.775-0.922, $p=0.000$), a 8.5% higher risk of having CRP>100 mg/L (OR 0.915, 95%CI 0.861-0.972, $p=0.004$), a 7.0% higher risk of CRP>150 mg/L (OR 0.930, 95%CI 0.873-0.990, $p=0.023$) and a 6.4% higher risk of hospital stay >10 days (OR 0.936, 95%CI 0.886-0.989, $p=0.018$). Higher serum PTH levels were independently associated with mortality (OR1.171, 95%CI 1.035-1.323, $p=0.012$). Lower admission haemoglobin levels were independently predictive for postoperative inflammation with CRP>100 mg/L (OR 0.964, 95%CI 0.948-0.980, $p=0.000$) and CRP>150 mg/L (OR 0.979, 95%CI 0.963-0.996, $p=0.014$).

Patients with NLR at admission in the range of 5.1 - 8.5 (intermediate tertile) compared to patients with NLR levels <5.1, postoperatively had a 2.6 times higher risk of myocardial injury (OR 2.60, 95%CI 1.12 - 6.14, $p=0.014$) and/or inflammatory complications (for CRP>100mg/L: OR 2.66, 95%CI 1.57 - 4.51, $p=0.000$; for CRP>150 mg/L: OR 2.55, 95%CI 1.34 - 4.91, $p=0.002$), whereas patients with preoperative NLR>8.5 (high tertile) had a 5.87- (OR 5.87, 95%CI 2.67 - 13.20, $p=0.000$), 4.54 - 6.70-fold (for CRP>100mg/L: OR 4.54, 95%CI 2.65 - 7.81, $p=0.000$; for CRP>150 mg/L: OR 6.70, 95%CI 3.58 - 12.64, $p=0.000$) higher risk of myocardial injury and inflammatory complications, respectively, indicating a dose-response relationship (Figure 1). None of the 10 patients who died had a preoperative NLR<5.1, and in 9 subjects it was above 8.5, suggesting that the risk of a fatal outcome in subjects with NLR>8.5 on admission was near 10 times higher in comparison with patients whose NLR was in the range of 5.1 - 8.5 (OR 9.71, 95%CI 1.24 - 207.53, $P=0.009$).

Table 2. Admission neutrophil to lymphocyte ratio (NLR) and postoperative outcomes in orthogeriatric patients

Outcome	Outcome present	Outcome absent	P value	OR	95%CI	P value
In-hospital death (n=10, 2.4%)	18.35 ± 7.88	8.00 ± 6.37	0.0000	1.097 1.106	1.044 - 1.152 1.002 - 1.221	0.000 0.045
CRP>100 mg/L (n=200, 48.2%)	10.11 ± 7.88	6.52 ± 4.45	0.0000	1.126 1.081	1.076 - 1.177 1.030 - 1.134	0.000 0.002
CRP>150 mg/L (n=129, 31.1%)	11.29 ± 8.41	6.88 ± 5.01	0.0000	1.124 1.094	1.078 - 1.171 1.045 - 1.146	0.000 0.000
Troponin rise (n=75, 18.1%)	13.18 ± 10.68	7.12 ± 4.60	0.0000	1.136 1.086	1.088 - 1.185 1.032 - 1.142	0.000 0.001
LOS>10 days (n=211, 50.8%)	9.05 ± 7.41	7.40 ± 5.49	0.0106	1.043 1.019	1.009 - 1.079 0.982 - 1.058	0.014 0.310

Data are mean values (±SD), univariate (first line) and multivariate (second line) logistic regression analyses.

Adjustments: age, sex, presence of any fractures or HF, history of coronary artery disease, hypertension, cerebrovascular disease, atrial fibrillation, chronic heart failure, peripheral vascular disease, diabetes mellitus, cancer, dementia, chronic obstructive airway disease, chronic kidney disease (eGFR<60 ml/min/1.73m²), haemoglobin<120g/L, albumin<33g/L, 25(OH)D <25 nmol/L, PTH>6.8 pmol/L, smoking status and alcohol overuse (≥3 drinks/week).

Abbreviations: OR, odds ratio; CI, confidence interval; CRP, C-reactive protein; LOS, length of hospital stay.

Table 3. Characteristics on admission independently associated with presence of any fracture, hip fracture and postoperative myocardial injury and extended inflammatory response in orthogeriatric patients (multivariate logistic regression analyses)

Characteristic	Any fracture		Hip fracture		Troponin rise		CRP>100mg/L		CRP>150 mg/L	
	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value
NLR>5.1	1.74 (1.08; 2.79)	0.023	3.11 (1.69; 5.74)	0.000	2.40 (1.11; 5.22)	0.026	2.42 (1.48; 3.95)	0.000	3.17 (1.75; 5.74)	0.000
Age	1.07 (1.03; 1.10)	0.000	1.10 (1.06; 1.15)	0.000	1.05 (1.01; 1.10)	0.011				
Sex(M)					2.32(1.21; 4.42)	0.011	1.93(1.17; 3.18)	0.010		
Dementia	2.50 (1.20; 5.25)	0.015	3.58 (1.58; 8.08)	0.002						
AF					2.29(1.08; 4.85)	0.030				
Albumin<33g/L	2.09 (1.27; 3.46)	0.004	3.27 (1.82; 5.87)	0.000	4.21(2.16; 8.2)	0.000	3.42(2.10; 5.56)	0.000	2.72(1.63; 4.56)	0.000
Hb<120g/L							2.94(1.56; 5.58)	0.001	2.53(1.14; 5.63)	0.023
eGFR <60 ml/min/1.73m ²									2.28(1.25; 4.13)	0.007
PTH>6.8 pmol/L									1.92(1.16; 3.16)	0.011

Abbreviations: OR, odds ratio; CI, confidence interval; NLR, neutrophil to lymphocyte ratio; AF, atrial fibrillation; eGFR, estimated glomerular filtration rate; Hb, haemoglobin; PTH, parathyroid hormone.

Next, we assessed in multivariate models the independent characteristics associated with presence of any fracture, HF, as well as adverse postoperative outcomes using the NLR as a categorical variable and adjusting for age, gender, history of CAD, hypertension, cerebrovascular disease, AF, CHF, PVD, DM, cancer, dementia, chronic COPD, CKD, haemoglobin<120g/L, albumin<33g/L, 25(OH)D <25 nmol/L, PTH>6.8 pmol/L, smoking status (current and former) and alcohol overuse (≥ 3 drinks/week); the postoperative outcomes were adjusted also for presence of any fracture or HF (Table 3). Patients with admission NLR ≥ 5.1 compared with those in the lowest tertile of NLR (<5.1) had significantly greater odds of presenting with any fracture (OR 1.74) or HF (OR 3.11), and of experiencing postoperative myocardial injury (OR 2.40), inflammatory complications with CRP>100 mg/L (OR 2.42) or CRP>150 mg/L (OR 3.17). Hypoalbuminaemia (<33g/L) on admission was the only other independent indicator of fracture, HF and all these postoperative complications with ORs comparable to those of NLR. Dementia was strongly associated with fractures, especially HF. Presence of AF was an independent predictor of cTnI rise. Preoperative anaemia (haemoglobin <120 g/L), CKD and elevated PTH were identified as independent risk factors for a high inflammatory response. The NLR>8.5 on admission (OR 16.63, 95%CI 1.70 - 163.09, $p=0.016$), history of CHF (OR 7.52, 95%CI 1.00 - 57.78, $p=0.050$) and cancer (OR 6.35, 95%CI 1.13 - 32.09, $p=0.025$) were the only independent predictors of in-hospital death. Thus, NLR ≥ 5.1 on admission after adjustment for variables known to predict poorer postoperative outcomes remained independently associated with presence of any fracture, HF and predictive of postoperative myocardial injury, inflammatory complications and in-hospital death.

The potential prognostic value of elevated NLR was evaluated by c-statistics on the basis of tertiles with cut-offs ≥ 5.1 and >8.5. The NLR ≥ 5.1 predicted

postoperative myocardial injury with an area under the curve (AUC) of 0.738 ($p=0.000$), CRP>100mg/L with AUC of 0.659 ($p=0.000$), CRP>150 mg/L with AUC of 0.664 ($p=0.000$) and in-hospital mortality with an AUC of 0.763 ($p=0.002$); the NLR >8.5 predicted in-hospital mortality with an AUC of 0.847 ($p=0.000$). The NLR ≥ 5.1 showed moderately high sensitivity for predicting postoperative myocardial injury (86.7%), CRP>100mg/L (80%) and CRP>150mg/L (85.3%), low specificity (38.5%, 46.3%, 42.1%, respectively) and low positive predictive value (PPV, 23.8%, 58.2% and 40.0% respectively), but a reasonable negative predictive value (NPV, 92.9%, 71.2% and 86.3% respectively). NLR>8.5 for in-hospital mortality demonstrated high sensitivity (90%) and NPV (99.6%), but was considerably less specific (68.6%). These data indicate that the prognostic value of elevated NLR, except NLR>8.5 for in-hospital mortality, is only modest (accuracy ranged between 69.1% and 47.2%).

Further we compared the predictions of NLR with that of different other factors on admission, including: (1) neutrophils>8.0x10⁹/L, (2) lymphocytes <1.2x10⁹/L, (3) haemoglobin<120g/L, (4) albumin<33g/L, (5) 25(OH)D<50nmol/L, (6) 25(OH)D<25nmol/L, (7) PTH>6.8pmol/L, (8) eGFR<60 ml/min/1.73m², (9) age>75 years, (10) presence of CVD (any), (11) presence of AF and (12) dementia. Each of these factors, except albumin<33g/L, performed worse than NLR ≥ 5.1 and yielded an AUC of 0.586 - 0.459 (for different outcomes). Comparing with NLR ≥ 5.1 , the haemoglobin<120g/L had higher sensitivity but very low specificity for predicting myocardial injury (89.5% and 22.8%, respectively), as well as for CRP>100mg/L (91.0% and 30.8%, respectively) and CRP>150 mg/L (93.1% and 26.3%). The predictive performance was comparable only for albumin <33g/L and NLR ≥ 5.1 ; although moderate by both variables, the former characteristic demonstrated higher specificity but lower sensitivity for predicting

myocardial injury and high inflammatory responses, was indicative for prolonged LOS but not for in-hospital death (Table 4).

Next we determined whether the combined use of the NLR and albumin level measured on admission can improve the prediction of postoperative outcomes. On admission, hypoalbuminaemia ($<33\text{g/L}$) was observed in 168 (40.5%) patients, $\text{NLR} \geq 5.1$ in 275 (66.3%) and both features, elevated NLR and low albumin, in 133 (32.0%) subjects. Compared to either a high NLR or low albumin level, presence of both these characteristics was a more specific and slightly more accurate predictor of postoperative myocardial injury and high inflammatory responses. However, $\text{NLR} > 8.5$ was a strong predictor of in-hospital death and prolonged LOS was predicted better by low albumin alone (Table 4). Multivariate analyses (adjusted for all the same above mentioned conditions) showed that patients with combination of these two factors compared to those with both admission $\text{NLR} < 5.1$ and $\text{albumin} > 33\text{g/L}$ had a very high risk of postoperative myocardial injury ($\text{OR} 11.54$, $95\%\text{CI}$ 3.27 - 40.77, $p=0.000$) and inflammatory complications with $\text{CRP} > 100\text{ mg/L}$ ($\text{OR} 10.94$, $95\%\text{CI}$ 4.58 - 22.89, $p=0.000$) or $\text{CRP} > 150\text{mg/L}$ ($\text{OR} 9.71$, $95\%\text{CI}$ 3.86 - 24.42, $p=0.000$). ROC characteristics curves for predicting in-hospital mortality, postoperative myocardial injury and high inflammatory response using elevated NLR, low albumin and combination of both parameters are depicted in Figure 3.

Validation of admission NLR as a risk prediction factor

Patients in the validation dataset comparing to those in the test dataset were older (+3.3 year), had a higher prevalence of CKD (43.2% vs.19.0%) and dementia (27.8% vs.17.6%), but there were no differences in other comorbidities, including CVD (66.3%), history of stroke or transient ischaemic attack (19.7%), type 2 DM (16.4%), COPD, (11.0%) and Parkinson's disease (4.6%); the proportions of current (5.4%) and former (10.0%) smokers and alcohol over-users (9.5%) were lower. Postoperatively myocardial injury ($\text{cTnI} > 0.06\mu\text{g/L}$) was observed in 27.2% ($n=80$) of patients, a high inflammatory response with $\text{CRP} > 100\text{ mg/L}$ in 60.2% ($n=177$) and with $\text{CRP} > 150\text{ mg/L}$ in 38.1% ($n=112$), a prolonged LOS ($>10\text{days}$) in 31.6% ($n=93$) patients; 49% ($n=97$) of patients admitted from home ($n=198$) have been discharged to a permanent RCF, and the in-hospital death rate was 4.8% ($n=14$).

When the admission NLR cut-off of ≥ 5.1 derived from the test dataset was applied to the validation dataset it showed significant and similar predictive value for postoperative cTnI rise (AUC 0.684, sensitivity 77.9%, NPV 82.7%), for $\text{CRP} > 100\text{ mg/L}$ (AUC 0.632, sensitivity 79.1%, NPV 72.7%), for $\text{CRP} > 150\text{mg/L}$ (AUC 0.639, sensitivity 89.1%, NPV 88.7%) and in-hospital death (AUC 0.700, sensitivity 92.9%, NPV 99.0%). $\text{NLR} \geq 5.1$ was also moderately predictive for $\text{LOS} > 10\text{ days}$ (AUC 0.572, sensitivity 51.0%, NPV 69.2%) and for being discharge to a RCF (AUC 0.594, sensitivity 72.2%, NPV 63.5%). Admission $\text{NLR} > 8.5$ was a strong predictor of fatal outcome (AUC 0.801, sensitivity 89.6%, specificity 70.6%, NPV 98.7%).

Table 4. Predictive value of selected preoperative parameters in detection poorer outcomes in orthogeriatric patients

Variable	ROC	Sensitivity,%	Specificity,%	PPV,%	NPV,%	Accuracy rate,%	P value
Post operative myocardial injury							
$\text{NLR} > 5.1$	0.738	86.7	38.5	23.8	92.9	47.2	0.000
$\text{Albumin} < 33\text{g/L}$	0.774	72.4	67.2	33.1	91.5	68.1	0.000
$\text{NLR} > 5.1 + \text{Albumin} < 33\text{g/L}$	0.774	64.0	75.4	36.6	90.4	73.4	0.000
Post operative CRP > 100 mg/L							
$\text{NLR} > 5.1$	0.659	80.0	46.3	58.2	71.2	62.6	0.000
$\text{Albumin} < 33\text{g/L}$	0.708	58.7	76.6	70.2	66.4	68.0	0.000
$\text{NLR} > 5.1 + \text{Albumin} < 33\text{g/L}$	0.711	50.5	85.0	75.9	64.8	68.4	0.000
Post operative CRP > 150 mg/L							
$\text{NLR} > 5.1$	0.664	85.3	42.1	40.0	86.3	55.6	0.000
$\text{Albumin} < 33\text{g/L}$	0.678	62.3	69.5	48.2	80.2	67.2	0.000
$\text{NLR} > 5.1 + \text{Albumin} < 33\text{g/L}$	0.680	54.3	77.9	52.6	79.0	70.5	0.000
LOS > 10 days							
$\text{NLR} > 5.1$	0.554	66.8	34.3	51.3	50.0	50.8	0.806
$\text{Albumin} < 33\text{g/L}$	0.611	49.5	69.3	62.5	57.0	59.2	0.000
$\text{NLR} > 5.1 + \text{Albumin} < 33\text{g/L}$	0.592	38.4	74.5	60.9	53.9	56.1	0.005
In-hospital death							
$\text{NLR} > 8.5$	0.847	90.0	68.6	6.6	99.6	69.1	0.000
$\text{Albumin} < 33\text{g/L}$	0.765	70.0	60.3	4.2	98.8	60.6	0.053
$\text{NLR} > 8.5 + \text{Albumin} < 33\text{g/L}$	0.792	38.8	89.8	63.3	76.4	73.9	0.000

Adjustments: age and sex.

Abbreviations: NLR, neutrophil to lymphocyte ratio; CRP, C-reactive protein; LOS, length of hospital stay; PPV, positive predictive value; NPV, negative predictive value.

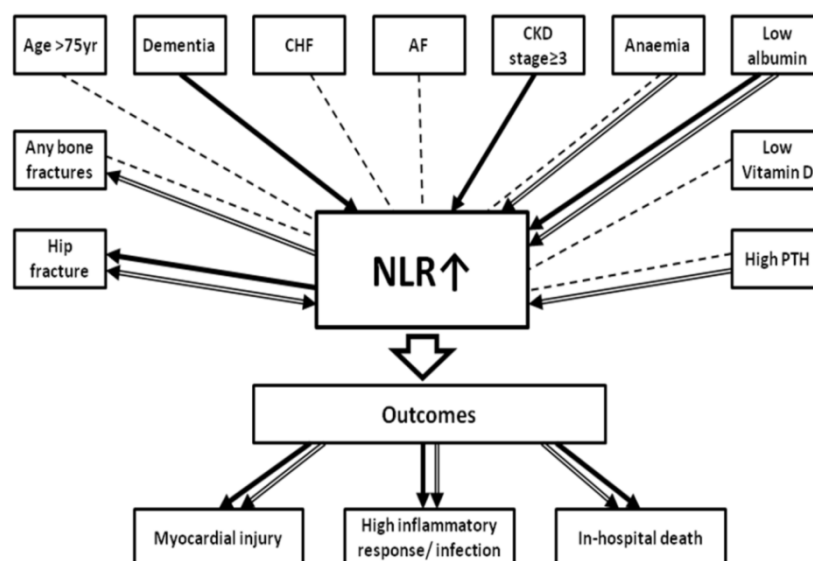


Figure 2. Associations between admission neutrophil to lymphocyte ratio (NLR) , medical conditions, presence of fracture and short-term outcomes in orthogeriatric patients. Significant associations found in univariate analyses are shown in dot lines. Independent associations revealed by multivariate analyses are shown in bold (with NLR as a continuous variable) and in double-line (with NLR $\geq$ 5). Arrow indicates the dependent variable. Higher admission NLR (both as a continuous and a categorical variable) is an independent indicator of hip fracture presence and risk factor of poorer outcomes; NLR $\geq$ 5 is an independent indicator of any fracture. Dementia, hypoalbuminaemia and CKD $\geq$ 3 stage are the independent correlates of higher preoperative NLR as a continuous variable; independent correlates of admission NLR $\geq$ 5.1 are presence of hip fracture, lower levels of albumin and haemoglobin and elevated PTH concentration. Abbreviations: CHF, chronic heart failure; AF, atrial fibrillation; CKD $\geq$ 3, chronic kidney disease (estimated glomerular filtration rate $<$ 60ml/min/1.73m ^{2}); Anaemia, haemoglobin $<$ 120g/L; Low albumin, $<$ 33g/L; Low vitamin D, serum 25-hydroxyvitamin D $<$ 25nmol/L; High PTH, parathyroid hormone $>$ 6.8pmol/L.

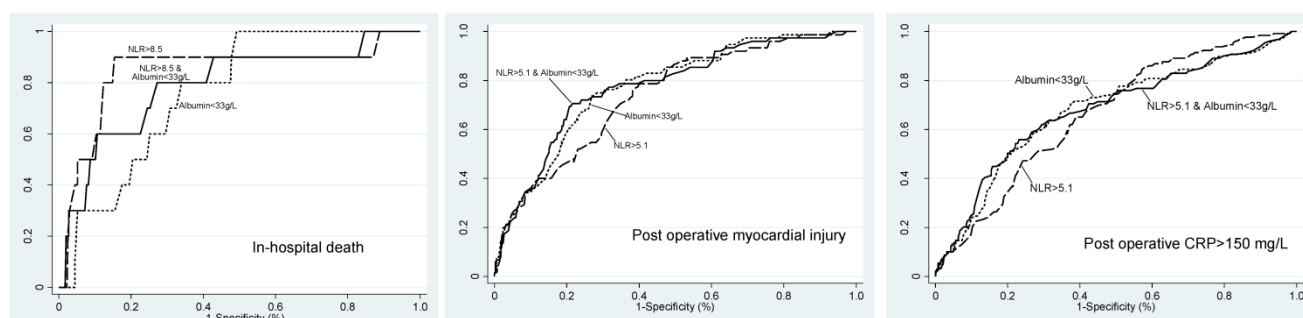


Figure 3. Receiver operating characteristics curves for predicting in-hospital mortality, postoperative myocardial injury with troponin I rise and high inflammatory response with CRP $>$ 150 mg/L. NLR, neutrophil to lymphocyte ratio; CRP, C-reactive protein.

Discussion

Main findings

In orthogeriatric patients at the time of hospital admission higher NLR (analysed both as a continuous and categorical variable) is: (1) an independent indicator of HF presence (although a variety of medical conditions affect NLR), and (2) an independent risk factor and modest predictor of poorer short-term postoperative outcomes such as myocardial injury (identified by cTnI rise), inflammatory complications (with high CRP levels), and in-hospital mortality (Figure 2). To our best knowledge, these results are the first to show that higher preoperative NLR, a widely available and inexpensive marker, may be helpful in improving the

prognosis of elderly patients undergoing orthopaedic treatment.

NLR, comorbidities and fractures

In agreement with many studies [33, 65-67] in our univariate analyses, admission NLR was significantly associated with 10 variables: age $>$ 75 years, presence of any fracture, dementia, AF, CHF, CKD $\geq$ 3 stage, anaemia, vitamin D deficiency, hyperparathyroidism and hypoalbuminaemia. Multivariate regression revealed only three variables, dementia, hypoalbuminaemia and CKD $\geq$ 3 stage, as independent determinants of higher preoperative NLR as a continuous variable; presence of HF showed borderline significance ($p=0.060$). Independent determinants of admission NLR $\geq$ 5.1 were presence of HF, lower levels of albumin and haemoglobin and

elevated serum PTH concentration. On the other hand, the analyses demonstrated a robust and independent association of higher NLR on admission and presence of fracture. There was a dose-graded relationship between presence of fracture, especially HF, and levels of NLR categorized as tertiles. The crude odds ratios (OR) for presence of HF were 3.14 for NLR 5.1-8.5 (second tertile) and 4.93 for NLR >8.5 (third tertile), the ORs for any fracture were 1.75 and 2.62, respectively. Adjusted ORs (multivariate regression analyses), demonstrated a 3.11- and 1.74-fold increases in presence of HF or any fracture, respectively, when patients with NLR ≥ 5.1 were compared with those in the first tertile.

In patients with a fracture, the cause (-s) of elevated NLR at admission may be multifactorial and related to a variety of pre-fracture co-morbid conditions, a concurrent clinical or subclinical infection, as well as to the responsive process to fracture *per se*. The association of fractures with higher NLR, a marker of dysregulated immune system and chronic inflammation, is in line with an increasing body of evidence linking immune status/low-grade inflammation (affecting both process - osteogenesis and bone resorption) with bone homeostasis and, consequently, with pathogenesis of osteoporosis [68-76] and higher fracture rates [77, 78]. Several recent studies demonstrated that NLR levels are significantly elevated in the elderly with osteoporosis and inversely correlated with BMD [79-81]. In other words, elevated NLR, an indicator of a systemic inflammatory-immunological process, not merely reflects a response to fracture and/or infection (in some patients at admission) but appears to be a significant factor linked to osteoporotic fractures.

NLR and short-term outcomes

The high incidence of concurrent medical comorbidities amongst orthogeriatric patients emphasises the importance of identification of vulnerable persons, recognition of potentially reversible risk factors and preoperative stabilisation. However, little is known about preoperative markers that can identify orthogeriatric patients at high risk of adverse outcomes. Most studies focussed on preoperative predictors of mortality in HF patients [15, 19, 25, 82-86]. The prognostic role of preoperative NLR has not been systematically investigated in orthopaedic surgery, in contrast to that in patients with cancer, CVD, AF, DM, inflammatory diseases, chronic renal and hepatic failure.

In this study, it was demonstrated that higher NLR at admission not only correlates with presence of fracture and comorbidities but also closely relates to key adverse outcomes - postoperative myocardial

injury, high inflammatory response and in-hospital death. The incidence of these three outcomes in our cohort was consistent with data reported in the literature. In our cohort which included both emergency and elective surgery patients, postoperative myocardial injury (diagnosed with an elevated cTnI measurement) occurred in 18.1%. Perioperative cTnI elevation, the most common cardiovascular complication associated with significant morbidity and mortality [57, 87-89], was reported in 8% of adults undergoing major noncardiac surgery [57], in 19% of aged >60 years after noncardiac surgery [87], in 17% of subjects undergoing major orthopaedic surgery [5], in 22% - 52.9% after emergency orthopaedic operations [2], and in 26.7% - 39.0% of elderly HF patients [55, 56, 90].

Increased inflammatory response as measured by CRP, an acute-phase protein, is well known as a useful indicator of infection after operative fracture treatment and a major predictor of mortality in the elderly [91, 92]. In this study, after the 3rd postoperative day CRP >100mg/L was found in 48.2% of patients and CRP >150mg/L in 31.1%. Previous research has shown that infective complications occur primarily in patients with persistent elevation and/or second rise in CRP concentrations (CRP >96 mg/L) after the first 3 postoperative days [62]. Postoperative infections complicating HF surgery have been reported in 8.9% - 61% [10, 93-97] and were associated (particularly deep wound and chest infections) with delirium, increased length of hospital stay, 30-day and 1-year mortality [2, 10, 98]. The increased susceptibility of orthogeriatric patients to postoperative infective complications, one of the main factors affecting outcomes, is, at least partially, a result of age-related decline and dysregulation in immune functions [99-103]. Of note, although both NLR and CRP are well recognized inflammatory biomarkers and both increase following the elevation of circulating IL-6, which is produced by several types of cells (monocytes, macrophages and endothelial cells), other mechanisms underlying the development of a high NLR and elevated CRP differ. Whereas the production of CRP in human hepatocytes is mainly induced by circulating IL-6, lymphocytes play a central role in the immune reaction and NLR is a marker of systemic inflammation representative of innate and adaptive immunity. Not surprisingly, therefore, patients with a high NLR do not always have an elevated CRP as we observed in our cohort on admission, but elevated preoperative NLR indicates predisposition to postoperative infective complications with a high CRP.

In-patient mortality in our study was 2.4% (6.0% for HF patients) which is compatible with that in other

centres. Reported short-term mortality for HF ranged between 1.14 - 4.6% [104, 105] - 10.9% [106] and 13.3% [16], but reaches 43% in patients with postoperative chest infection and 65% in patients with acute heart failure [2].

We observed a dose-graded relationship between increasing admission levels of NLR and the proportion of patients with postoperative myocardial injury, high inflammatory markers and fatal outcomes. Compared to patients with $\text{NLR} < 5.1$, patients with $\text{NLR} 5.1\text{--}8.5$ had a 2.6-fold higher risk for developing postoperative myocardial injury and about 2.5-fold higher risk for a high inflammatory response, while in subjects with $\text{NLR} > 8.5$ these risks were 5.9 and 4.5-6.7-times higher, respectively. Patients with admission $\text{NLR} > 8.5$ had a 9.7 times higher chance of dying in the hospital compared to patients with $\text{NLR} 5.1\text{--}8.5$ (no deaths occurred in patients with $\text{NLR} < 5.1$). Although in our study 10 patients who died in hospital do not provide good statistical power, all of them had admission $\text{NLR} \geq 5$ and 9 subjects had $\text{NLR} > 8.5$; 14 patients with HF and fatal outcome in the validation cohort demonstrated similar NLR patterns.

In patients with $\text{NLR} \geq 5.1$ at admission the increased risks of postoperative myocardial injury and high inflammatory response persisted after accounting for multiple confounding factors known to be associated with postoperative complications and death; similarly $\text{NLR} > 8.5$ was an independent and significant predictor of a fatal outcome. In other words, the risks of poor outcomes were dependent on the degree of the NLR elevation but independent of preoperative cardiovascular, metabolic, renal, nutritional and behavioural factors associated with adverse outcomes (as showed the multiple logistic regression analyses).

Forget et al. [50] reported that the preoperative NLR level did not predict short-term outcomes in HF patients. However, our data which identified higher NLR at admission as a significant risk factor for unfavourable outcomes in orthogeriatric patients is consistent with numerous studies demonstrating a strong association between elevated NLR levels and increased mortality in different cardiovascular, noncardiac and malignant diseases [28, 31-34, 67]. High NLR was found to be a significant predictor of mortality and worse outcomes in patients with acute coronary syndromes [32, 107], advanced heart failure [38], different cancers [29, 30, 108-110], in elderly patients who have underwent major vascular [111], abdominal [27] and lung cancer surgery [112].

Interestingly, although different factors were independently associated with presence of fracture and specific outcomes, elevated $\text{NLR} (\geq 5.1)$ and low

albumin ($< 33\text{g/L}$) on admission were the only two independent variables associated with presence of any fracture, HF, as well as with poorer outcomes. The other independent factors for any fracture and HF were older age and dementia, for postoperative myocardial injury - AF and male sex, for high inflammatory responses - CKD, anaemia and elevated PTH (Table 3).

Prognostic value of NLR on admission

Although there is no consensus of what the normal NLR levels are, we identified < 5.1 (upper limit in the first tertile) as the cut-off value to predict adverse outcomes. Of note, the threshold $\text{NLR} > 5$ ("classical") has been used in many other studies [30, 109, 112]. We found that elevated $\text{NLR} \geq 5.1$ significantly differentiated subjects with poorer outcomes from the rest of the cohort, but only at a modest level (accuracy: 47.2% for myocardial injury, 62.6% for postoperative $\text{CRP} > 100\text{mg/L}$ and 55.6% for postoperative $\text{CRP} > 150\text{mg/L}$, $p = 0.000$ in all cases). $\text{NLR} > 8.5$ (third tertile) demonstrated a moderately better performance (accuracy: 69.5%, 61.4% and 69.8%, respectively). $\text{NLR} \geq 5.1$ showed a considerably high sensitivity (86.7%, 80.0% and 85.3%, respectively) and negative predictive value (NPV, 92.9%, 71.2% and 86.3%, respectively) indicating that these complications are unlikely in patients with a lower ratio. However, positive predictive value (PPV) was only 23.8%, 58.2% and 40.0%, respectively, indicating that a higher NLR does not necessarily predict postoperative complications. $\text{NLR} > 8.5$ had a moderately high sensitivity (90%) and accuracy (69.1%) for in-hospital death; the NPV was 99.6% showing that patients with a ratio lower than 8.5 were likely to survive, but $\text{NLR} > 8.5$ does not predict a fatal outcome (PPV was only 6.6%).

In this study, we have also evaluated twelve most widely recommended and used predictor markers for determining outcomes among orthogeriatric patients: age > 75 years [113-115], dementia, presence of CVDs, AF, high white blood cell (WBC) count [19], low lymphocyte count [19, 23, 25, 86, 116, 117, 118], low serum albumin [15, 25, 84-86, 117], anaemia/reduced haemoglobin levels ($\text{Hb} < 120\text{g/L}$) [15, 18, 19, 22, 82, 83, 94, 119], vitamin D deficiency ($25(\text{OH})\text{D} < 25\text{nmol/L}$) and insufficiency ($25(\text{OH})\text{D} < 50\text{nmol/L}$), elevated PTH (6.8 pmol/L) [115, 120], and $\text{CKD} \geq 3$ stage. The performance of these markers, which have been suggested as independent prognostic factors for unfavourable outcomes in HF patients, was variable. For postoperative myocardial injury, for example, accuracy ranged from 35.0% ($\text{Hb} < 120\text{g/L}$) to 75.5% ($25(\text{OH})\text{D} < 25\text{nmol/L}$) with sensitivity 89.5% and 9.2%, respectively, and

specificity 22.8% and 90.5%, respectively. Similarly, other markers comparing to NLR, were either more specific (AF, dementia, CKD) but lacked sensitivity (26.3% -32.9%) or, as age>75 years, had low specificity (37.0%) and higher sensitivity (85.5%). As NLR integrates two important and opposite immune pathways (neutrophils represent non-specific systemic inflammation and lymphocytes are a marker of the immune system physiological stress response) it is not surprisingly, that it performs better than absolute neutrophil and lymphocyte count separately. The predictive value examined using ROC curve analysis revealed that the highest discriminative ability for predicting in-hospital death has NLR>8.5 (AUC 0.847), albumin<33g/L (AUC 0.765) and neutrophils>8.0x10⁹/L (AUC 0.720). Low admission albumin and elevated NLR were the two variables which demonstrated the highest prognostic values for postoperative myocardial injury and high inflammatory responses. On the whole, among the orthogeriatric patients, the NLR was superior to age and other routinely used admission characteristics, except hypoalbuminaemia, for determining these outcomes; only hypoalbuminaemia, in contrast to NLR, indicated LOS>10 days (ROC 0.594). However, patients with both elevated NLR and low albumin demonstrate only a moderately increased risk of adverse outcomes compared to subjects with either biomarker abnormal (Table 4; Figure 3).

Our validation study that included older patients with HF confirmed that admission NLR ≥5.1 was a moderate predictor for patients' postoperative myocardial injury and high inflammatory responses but also for in-hospital death, LOS>10 days, and for being discharge to a RCF; NLR>8.5 was a strong predictor of fatal outcome.

Taking together, the admission NLR, except NLR>8.5 for in-hospital mortality, as well as all other above mentioned factors showed a modest/ relatively low performance to predict postoperative adverse outcomes (AUC < 0.700), and, therefore, should be considered mainly as risk factors than predictive ones. Clearly, NLR and other biomarkers to give reasonable prognostic information should be evaluated and interpreted in the context of a complete clinical assessment.

Clinical implications

Our results showing that higher NLR on admission is associated with fracture and worse outcomes suggest that NLR may be a useful additional biomarker for therapeutic selection and preventive intervention. Firstly, it is important to recognise that in the complex interplay of modifiable and non-modifiable factors which determine

outcomes cTnI elevation, regardless of etiology, is associated with short- and long-term morbidity/mortality [2, 5, 54, 56, 57, 87-89]. The clinical relevance of preoperative identification of subjects with a high risk of this serious and silent (asymptomatic in>80% of patients) complication is further emphasized by the reports that beta-blockers, alfa-2 agonists, calcium channel blockers, statins, and aspirin can prevent postoperative myocardial injury and reduce the risk of mortality [121-124]. Secondly, although this study was not designed to assess clinically apparent infection, elevated NLR (≥5.1) on admission, indicates a high probability of perioperative infectious complications and might be useful when considering empirical antibiotic therapy. Although a higher NLR on admission could be an initiative for preventive treatment, whether patients with elevated NLR may benefit from preoperative cardiovascular medications and/or antibiotics must be elucidated. Thirdly, NLR>8.5 which was most discriminatory for survival may be (in conjunction with a comprehensive clinical assessment) helpful in preoperative predicting a fatal outcome and aid decision making with regard to operative plan. Fourthly, our findings showed that in orthogeriatric patients the systemic inflammatory-immunological process as indicated by elevated NLR may be an important contributor to the pathogenesis of osteoporotic fractures and other age-related diseases, rather than only a secondary reflection of fracture and/or concurrent infection. In other words, higher NLR which represents age-related changes in the immune system (immunosenescence, [125, 126]) and chronic inflammation, a phenomenon known as "inflamm-ageing" [127, 128], reflects and is influenced by a variety of age-related co-morbid conditions and fractures. Therefore, it appears that NLR≥5.1, an easily obtained clinical test, as a global index of inflammatory-immunological status may be a useful marker for screening and preventing multimorbidity including risk of osteoporotic fracture in older adults. However, it should be emphasised that although the elevated NLR at admission demonstrates high statistical significance as an independent risk factor for poorer outcomes, its prognostic value in relation to individual patients is modest (but superior to the majority of other recommended predictors) and it does not indicate the type of possible adverse outcome. Obviously, any decision on prophylactic treatment (use of antibiotics and/or cardioprotective drugs) requires full clinical assessment. Further investigations of the predictive value of NLR in orthogeriatric patients are needed to provide more insight into the pathophysiology of its elevation as well as in specific and individualised perioperative

therapeutic interventions to improve outcomes for these complex patients.

Limitations and Strength

Several limitations of this study should be considered. First, because our study is observational, the inference of a causal relationship between admission NLR and outcomes is limited. Second, as we have included all orthogeriatric patients, without any exclusion criterion, the contribution of age-related impairment of the immune system, stress response to the fracture, active preoperative infection, and/or combination of these factors to elevated NLR was difficult to differentiate and determine. However, the substantial heterogeneity of our cohort is typical and reflects the real-world clinical practice. Third, the rate of in-hospital mortality was low, so caution is necessary when interpreting this result. Finally, it was a single-center study mainly of Caucasians and, therefore, may not necessarily translate to other centers with differing management practices and be representative of other racial and ethnic populations. The strengths of this prospective study are that it (1) focused on all orthogeriatric patients whereas previous studies have targeted selected patient groups, mainly with HF, (2) included multiple NLR-affecting factors, (3) analysed demographic, co-morbid and laboratory variables previously implicated as admission markers of poorer outcomes, but in most studies not assessed in the same patients in relation to postoperative outcome prognosis, and (5) validated the results in a cohort of HF patients. Of note, in multivariate regression analyses the variance inflation factor in all models presented in Tables 2 and 3 was less than 1.3, indicating that the amount of multicollinearity was no significant.

Conclusions

In orthogeriatric patients, the high NLR (≥ 5.1) on admission is an independent indicator of fracture presence, a significant risk factor and moderate predictor of poorer postoperative outcomes including myocardial injury, high inflammatory response/infection and in-hospital death. This simple and inexpensive biomarker could be used for risk stratification and individualized perioperative management. Multi-centre prospective studies are required to explore whether interventions to decrease NLR levels reduces fractures and improves outcomes.

Competing Interests

The authors have declared that no competing interest exists.

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Lower serum PINP/ β CTX ratio and hypoalbuminemia are independently associated with osteoporotic nonvertebral fractures in older adults

This article was published in the following Dove Press journal:
Clinical Interventions in Aging
19 July 2017
Number of times this article has been viewed

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Purpose: To estimate the discriminative value of serum PINP/ β CTX ratio and albumin levels in hospitalized orthogeriatric patients with and without nonvertebral fractures.

Methods: In 1,239 orthogeriatric patients (mean age 78.1 $\pm$ 9.52 years, 69.1% women) including 854 (68.9%) with osteoporotic nonvertebral fractures (455 [36.7%] with hip fracture [HF]) and 385 (31.1%) without fractures, markers of bone formation (procollagen type 1 N-terminal propeptide [PINP], osteocalcin [OC]), and bone resorption (beta-C-terminal cross-linking telopeptide of type 1 collagen [β CTX]), indices of mineral metabolism, and parameters of liver and renal functions were assessed; data on clinical and laboratory characteristics were collected prospectively.

Results: Both lower serum PINP/ β CTX ratio and albumin concentration (as continuous or categorical variables) were independently associated with fracture presence in multivariate logistic regressions. Compared with the highest PINP/ β CTX tertile, the prevalence of HF, after adjustment for multiple covariates, was 3-fold higher in the lowest tertile and 1.5 times higher in the middle tertile; presence of any fracture was 2.3- and 1.6-fold higher, respectively; patients with albumin levels in the lowest tertile had multivariate odds ratio (OR) of 4.6 for HF and 2.8 for any fracture, in the middle tertile the ORs were 2.2 and 1.3, respectively. The PINP/ β CTX <100.0 (median) and hypoalbuminemia (<33 g/L) demonstrated area under the curve values for HF of 0.802 and 0.806, respectively, and for any fractures of 0.711 and 0.706, respectively. When both characteristics were combined, the ORs for HF or any fracture, compared with the nonfractured group, were 7.8 and 3.2, respectively, with an accuracy of 79.6% and 71.6%, respectively.

Conclusions: In orthogeriatric patients, both serum PINP/ β CTX ratio and albumin levels demonstrated an inverse dose-effect relationship with the prevalence of nonvertebral fractures and independently indicated fracture presence with acceptable discriminatory power. Lower PINP/ β CTX (<100) and hypoalbuminemia could be useful simple additive prognostic tools for fracture risk stratification in the elderly.

Keywords: nonvertebral fractures, PINP/ β CTX ratio, albumin, elderly, orthopedic patients

Introduction

The ability to predict and prevent fragility fractures is limited.¹⁻³ Currently, the predicting is largely based on bone mineral density (BMD) testing and several clinical risk factors (the World Health Organization's fracture risk assessment tool FRAX,^{4,5} Garvan and QFracture).¹ However, BMD indicated osteoporosis only in 30%–50% of patients with major fragility fracture⁶ and in 4% of women with a distal radial

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fracture.⁷ The prognostic value of clinical risk factors alone in FRAX is comparable to that of BMD alone.⁸ There is an obvious need of identifying additional fracture risk factors not included in currently available strategies.

Numerous studies on the prognostic value of bone turnover markers (BTMs) yielded conflicting results.^{9–17} BTMs display significant analytical and biological variability^{18,19} and currently are recommended only for monitoring the efficacy of osteoporosis treatment and compliance.^{11,20,21} Bone formation and resorption are coupled but not always remain in balance: in the elderly bone is lost because remodeling becomes unbalanced.²² However, most of the reports focused on separate BTMs. One way to overcome the existing discrepancies between studies would be to characterize the balance between total bone formation and resorption. In the only prognostic study, which assessed such index, the ratio of a urinary resorption (N-telopeptide of type 1 collagen [u-NTx]) to serum formation marker (osteocalcin, OC) (u-NTx/OC) predicted fractures independently of FRAX, but did not significantly improve the accuracy of fracture risk prediction in addition to FRAX.²³ Recently the joint international working group proposed serum procollagen type 1 N-terminal propeptide (P1NP) and beta-C-terminal cross-linking telopeptide of type 1 collagen (β CTX) as the reference BTMs to evaluate bone formation and bone resorption, respectively.¹¹ To our knowledge, no studies of P1NP/ β CTX ratio measurements have been undertaken.

Albumin, one of the major proteins synthesized in the liver and the most abundant protein in the circulatory system, has pleiotropic physiologic effects.^{24,25} Hypoalbuminemia is strongly associated with inflammation–malnutrition complex²⁵ and various systemic disorders (liver, kidneys, cardiovascular, diabetes, and malignancy), many of which are particularly common in the elderly, increases the risk of falls and fractures,^{26,27} and is linked to poor prognosis and mortality in the general population^{28,29} as well as in the orthopedic patients.^{30,31} Only limited information with conflicting results is available regarding the role of hypoalbuminemia in osteoporosis,^{32,33} and its value in predicting fractures has not been sufficiently investigated.^{34,35}

We hypothesized that in orthogeriatric patients lower serum P1NP/ β CTX ratio and/or hypoalbuminemia, two indices that current algorithms do not take into consideration, may be associated with the presence of fracture, indicating a greater osteoporosis-related fracture risk. In this study, we aimed to investigate the relationship between serum P1NP/ β CTX ratio, an index of bone turnover balance, and hypoalbuminemia with the presence of osteoporotic hip or other nonvertebral fractures in a cohort of hospitalized

orthogeriatric patients, which reflects the real world in regard to the prevalence of major fractures.

Methods

Patients

This was an observational study using prospectively collected data on 1,899 consecutive patients >60 years of age who were admitted to the Department of Orthopedic Surgery at the Canberra Hospital (a 500-bed university-affiliated tertiary care center, Australian Capital Territory, Australia) between 1 January 2012 and 31 December 2014. After excluding patients with high-trauma fractures, vertebral and periprosthetic fractures, primary hyperparathyroidism, Paget's disease, metastatic cancer to bone, or who lacked adequate laboratory data, 1,239 patients were evaluated for the study. Of these 1,239 hospitalized patients (mean age 78.1 $\pm$ 9.52 years, 69.1% women), 854 (68.9%) had a low-energy trauma (falls from standing height) which resulted in a nonvertebral bone fracture: 455 (36.7%) had a hip fracture (HF) (52.0% cervical and 48.0% trochanteric) and 399 (32.2%) had other nonvertebral fractures (humerus –79, femur –75, ankle –68, tibia or/and fibula –27, knee –16, wrist –16, forearm –15, others –103). There were 385 (31.1%) patients without fractures (elective hip or knee replacement –343, suspected surgical site infections not confirmed by further investigation –15, and 27 patients with a prosthetic joint infection following total hip [n=20] or knee [n=7] arthroplasty). Data were collected on demographics, orthopedic and medical diagnoses, procedures performed, laboratory characteristics, medication used, and outcomes.

The study was approved by the Australian Capital Territory Ethical Review Board and performed in accordance with the principles of the Helsinki declaration. All study patients or the legally authorized carers gave their informed consent.

Validation dataset

A retrospective analysis of a second cohort included data obtained from electronic medical and administrative records from 417 consecutive orthogeriatric patients (mean age 78.9 $\pm$ 8.7 years, 68.2% women) admitted to the same department between October 2011 and August 2012. Among these patients, there were 152 (36.5%) subjects with an HF, 103 (24.7%) with other nonvertebral fractures, and 162 (38.8%) without fractures.

Laboratory evaluation

In each patient, fasting venous blood samples were collected within 24 hours of admission and the following tests were performed: serum concentrations of P1NP, OC, and β CTX

using an automated electrochemiluminescent immunoassay (Elecsys 2010, Roche Diagnostics, Ltd Corp., Indianapolis, IN, USA), 25 (OH) vitamin D [25(OH)D] by a radioimmunoassay kit (DiaSorin, Stillwater, MN, USA), intact PTH by 2-site chemiluminescent enzyme-linked immunoassay on DPC Immulite 2000 (Diagnostic Products, Los Angeles, CA, USA), total calcium, phosphate and magnesium, as well as routine laboratory investigations, including complete blood count, electrolytes, renal (creatinine, urea), liver (alanine aminotransferase [ALT], gamma-glutamyltransferase [GGT], alkaline phosphatase [ALP], albumin, and total bilirubin) and thyroid function tests (thyroid-stimulating hormone [TSH]; free thyroxine [fT4]), by standard automated laboratory methods. Intra- and inter-assay coefficients of variation (CV) for PINP were 2.6% and 4.1%, respectively, for OC 3.6% and 6.6%, respectively, and for β CTX 3.2% and 6.5%, respectively; for 25(OH)D and PTH, the intra- and inter-assay CV ranged from 2.1% to 12.7%. Calcium concentrations were corrected for serum albumin. Vitamin D status was defined as deficient for circulating 25(OH)D concentration <25 nmol/L and as insufficient for 25–50 nmol/L. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (>6.8 pmol/L, the upper limit of the laboratory reference range). The glomerular filtration rate (GFR) was estimated³⁶ and chronic kidney disease (CKD $\geq$ stage 3) was defined as GFR <60 mL/min/1.73 m². Anemia was defined as hemoglobin <120 g/L. Similar laboratory tests, equipment, methods, and definitions were used in the validation cohort.

Statistical analyses

The statistical analysis was performed with Stata software version 10 (StataCorp, College Station, TX, USA). Continuous variables are expressed as mean $\pm$ SD and compared using analysis of variance. Categorical variables are presented as numeral/percentages and compared by chi-square and Fisher exact tests. The correlations between the variables were determined by Pearson's coefficients. For Pearson correlations and regressions, values of all continuous laboratory parameters were logarithmically transformed to account for the skewed nature of most of these variables. The admission PINP/ β CTX ratio was analyzed as both a continuous and a categorical variable; in the latter, PINP/ β CTX ratio was categorized into three groups (tertiles) or as the median value. Univariate and multivariate logistic analyses were performed to identify factors associated with the presence of HF or of any nonvertebral fracture. Multivariate forward stepwise procedures (covariates with $P \leq 0.100$ in univariate analysis were selected for entry) were performed. To quantify the significance of multicollinearity phenomena in regression analyses, the variance

inflation factor was calculated. To quantify the discriminative utility for serum PINP/ β CTX ratio, albumin concentration, other parameters of interest, and their combination receiver operating characteristic (ROC), analysis was used and the predictive accuracy was expressed as area under curve (AUC). All statistical tests were two tailed and P -values <0.05 was considered statistically significant.

Results

Patient characteristics

In the entire cohort, patients averaged 2.7 chronic diseases per person, and the most common were hypertension (60.0%), osteoarthritis (42.5%), abnormal gait with the use of an assistive device (42.0%), diabetes mellitus type 2 (DM, 22.0%), CKD (21.3%), coronary artery disease (CAD, 17.1%), chronic obstructive airway disease (COPD, 15.4%), atrial fibrillation (AF, 14.8%), dementia (14.4%), cerebrovascular disease (12.2%), malignancy (10.4%), and chronic heart failure (7.8%). Four and more chronic conditions were identified in total in 28.5% of patients with the greatest burden, as expected, among the HF patients (36.0% vs 29.4% in the nonfracture group, $P=0.040$). Prior to admission, osteoporosis has been diagnosed in 239 (19.3%) patients and 182 (14.7%) subjects were receiving antiresorptive treatment.

Regarding laboratory parameters, both groups with fracture (HF and other nonvertebral fractures), compared to the nonfracture group, had significantly higher mean levels of β CTX, lower PINP/ β CTX ratios, and concentrations of calcium and higher prevalence of hyperparathyroidism (Table 1). Subjects with HF in addition exhibited higher levels of PTH, lower OC/ β CTX ratios, phosphate, magnesium, hemoglobin, albumin concentrations and GFR, as well as a higher prevalence of hypoalbuminemia, vitamin D deficiency, and anemia. On admission, hypoalbuminemia (<33 g/L) was observed in 688 (55.6%) patients including 342 (75.2%) with HF, 185 (46.4%) with other fractures, and 161 (42.2%) without fractures. Of note, mean serum levels of PINP, OC, PINP/OC ratio, 25(OH)D, as well as creatinine, ALP, TSH, and fT4 did not differ between the three groups.

Patients receiving antiosteoporotic therapy (a bisphosphonate plus vitamin D and calcium supplements) at least for 3 months prior to hospital admission ($n=182$) compared to those who were not treated ($n=1,057$) had significantly higher mean levels of serum PINP/ β CTX ratio (+16.7%: 141.8 ± 120.7 vs 121.5 ± 90.8) and 25(OH)D (+19.6%: 73.1 ± 24.4 vs 61.1 ± 26.3 nmol/L) and lower β CTX (−7.3%: 0.38 ± 0.27 vs 0.52 ± 0.36 μ g/mL) (all $P < 0.01$), whereas the PINP, PTH, and albumin levels were not different.

Table 1 Demographic, clinical, and laboratory characteristics of orthogeriatric patients by fracture status

Variables	Total (n=1,239)	HF (n=455)	Non-HF (n=399)	No fracture (n=385)
Age, years	78.1±9.52	83.0±8.48 ^{c,f}	76.6±9.49 ^c	73.9±8.06
Females, n (%)	855 (69.1)	331 (73.2) ^c	292 (72.8) ^c	232 (60.6)
RCF resident, n (%)	190 (15.4)	126 (27.7) ^{c,f}	42 (10.5) ^a	22 (5.7)
Osteoporosis, n (%)	239 (19.3)	120 (26.4) ^{c,e}	73 (18.3) ^a	46 (12.0)
Dementia, n (%)	178 (14.4)	125 (27.5) ^{c,f}	33 (8.3)	20 (5.2)
PINP, µg/L	58.7±89.3	56.5±101.6	58.0±71.2	62.0±90.8
OC, ng/mL	6.8±4.7	6.5±4.8	7.1±4.6	6.9±4.7
βCTX, µg/L	0.50±0.35	0.56±0.36 ^{c,e}	0.49±0.36 ^a	0.43±0.32
PINP/βCTX	123.3±101.9	103.4±93.9 ^{c,f}	127.1±83.8 ^b	147.2±104.9
OC/βCTX	16.2±11.1	13.6±9.4 ^{c,f}	18.1±12.5	19.4±12.2
25(OH)D, nmol/L	62.9±26.3	61.6±27.6	64.8±26.5	62.4±24
<25 nmol/L, n (%)	96 (7.8)	49 (10.8) ^{a,e}	22 (5.6)	25 (6.7)
<50 nmol/L, n (%)	394 (31.9)	155 (34.1)	122 (30.8)	117 (31.2)
PTH, pmol/L	7.4±5.36	8.1±5.99 ^{c,e}	7.0±4.96	6.8±4.84
>6.8 pmol/L, n (%)	472 (38.2)	206 (45.6) ^{c,d}	147 (37.3) ^a	119 (31.5)
Calcium,* mmol/L	2.41±0.13	2.39±0.14 ^{c,d}	2.41±0.13 ^b	2.43±0.13
Phosphate, mmol/L	0.91±0.25	0.87±0.24 ^{c,f}	0.93±0.25	0.94±0.24
Magnesium, mmol/L	0.76±0.10	0.74±0.10 ^{c,f}	0.78±0.09	0.77±0.10
Albumin, g/L	32.1±4.41	30.2±3.90 ^{c,f}	32.8±4.29	33.5±4.35
<33 g/L, n (%)	688 (55.6)	342 (75.2) ^{c,f}	185 (46.4)	161 (42.2)
Hemoglobin, g/L	109.5±17.9	104.8±17.2 ^{c,f}	112.5±17.6	118.1±17.9
<120 g/L, n (%)	876 (70.8)	366 (80.4) ^{c,f}	254 (63.7)	256 (66.8)
GFR, mL/min/1.73 m ²	72.6±18.9	70.7±19.8 ^e	74.7±17.4	72.5±19.0

Notes: Data expressed as mean ± SD or number (percentage). Comparison with no fracture group: ^a*P*<0.05, ^b*P*<0.01, and ^c*P*<0.001. Comparison patients with hip fracture and other nonvertebral fractures: ^d*P*<0.05, ^e*P*<0.01, and ^f*P*<0.001. *Calcium corrected for albumin. Of note, the proportion of patients with hypertension, chronic heart failure (CHF), diabetes mellitus (DM), chronic obstructive airway disease (COPD), chronic kidney disease (CKD, GFR <60 mL/min/1.73 m²), history of malignancy, as well as current smokers and warfarin users were similar in the three groups; the mean serum levels of alkaline phosphatase (ALP), thyroid-stimulating hormone (TSH), and free thyroxine (FT4) did not differ between the three groups (data not shown).

Abbreviations: βCTX, β C-terminal βcross-linked telopeptide of type I collagen; HF, hip fracture; OC, osteocalcin; PINP, N-terminal propeptide of type I procollagen; PTH, parathyroid hormone; RCF, residential care facility.

Osteoporotic fractures and laboratory parameters (correlation analyses)

These relationships have been evaluated for laboratory parameters expressed as both continuous and categorical variables. Among 13 studied laboratory parameters (PINP, OC, βCTX, PINP/βCTX ratio, OC/βCTX ratio, PTH, 25(OH)D, calcium, phosphate, magnesium, ALP, albumin, and hemoglobin), analyzed as continuous log-transformed variables adjusted for age and gender, the highest Pearson correlation coefficients in relation to the presence of HF or any fracture demonstrated the PINP/βCTX ratio and albumin: −0.3015 and −0.3667, respectively, for HF, and −0.1971 and −0.2133, respectively, for any fracture (all *P*=0.000). Only age showed higher correlation coefficients (*r*=0.4736, *P*=0.000 for HF; *r*=0.2902, *P*=0.000 for any fracture). Other clinical (female, dementia, AF, CAD, anemia, arthritis, osteoporosis, history of stroke or transient ischemic attack (TIA), use of walking aid) and laboratory (PINP, OC, βCTX, OC/βCTX ratio, PTH, calcium, phosphate, magnesium) parameters were also significantly but weaker associated with the presence

of fracture (*r* ranged between 0.2930 for dementia and HF, and 0.075 for stroke and any fracture).

Multivariate logistic regressions performed with HF or any fracture as a dependent variable and all clinical and laboratory characteristics with *P*≤0.100 in univariate analysis as independent variables after adjusting for age and gender revealed that both lower serum PINP/βCTX ratio and lower albumin concentration (as continuous variables) are independent and significant factors associated with these conditions (β coefficients 0.897 and 0.869, respectively, *P*=0.000 for both variables). These models explained 28.5% and 22.3% of variance among patients with an HF or any fracture, respectively, correctly classifying 77.4% and 70.5% of cases, respectively. For HF, the model's sensitivity was 79.4%, specificity 74.9%, positive predictive value (PPV) 79.2% and negative predictive value (NPV) 75.1%, and for any fracture, 89.9%, 26.7%, 73.5%, and 53.8%, respectively. For the presence of HF, the AUC was 0.799 for PINP/βCTX and 0.816 for albumin, for the presence of any fracture, the AUC was 0.701 and 0.713, respectively.

Because of practical considerations, we further examined the impact and clinical usefulness of the PINP/ β CTX ratio and albumin level as categorical variables. First, we examined the association of fracture presence and the serum PINP/ β CTX ratio divided into tertiles. Table 2 shows the tertile groupings and the percentage of individuals in each tertile, as well as the ORs for fracture presence. Proportion of patients with fractures decreased sharply from tertile 1 (lowest) to tertile 3 (highest). With tertile 3 (PINP/ β CTX >129.2, mean 219.3 $\pm$ 112.6) used as the reference, the odds of fracture were significantly higher in tertiles 2 (PINP/ β CTX 78.6–129.2, mean 100.7 $\pm$ 14.3) and 1 (PINP/ β CTX <78.6, mean 53.9 $\pm$ 15.8). After adjusting for age and gender, patients in tertile 2 had a 1.6-fold higher risk of HF or any fracture, while for those in tertile 1, the risk of HF was 3.4-fold higher and the risk of any fracture was 2.5-fold higher. In other words, the ORs for the presence of fracture linearly increased across decreasing PINP/ β CTX ratio tertiles, indicating a dose–response effect.

We further performed multivariate forward stepwise logistic regression analyses for the presence of HF or of any fracture according to serum PINP/ β CTX ratio tertiles including in the models the following variables: age, gender, 25(OH)D, PTH, calcium (corrected for albumin), phosphate, magnesium, OC, albumin, alkaline phosphatase, presence of dementia, cardiovascular diseases (CAD, AF, CVA, and CHF), diabetes mellitus, history of smoking, and alcohol consumption status. The adjustment for all these confounding factors did not significantly alter the results. Lower PINP/ β CTX ratio remained an independent and powerful indicator for fracture presence. The ORs for HF and any fracture demonstrated a similar linearly increased pattern across decreasing PINP/ β CTX ratio tertiles (Table 2, model 2). Compared with the highest tertile, the presence of

HF among patients in the lowest tertile was more than 3-fold higher and among patients in the middle tertile 1.5 times higher, whereas the presence of any fracture was 2.3-fold and 1.6-fold higher, respectively.

Albumin levels on admission analyzed in tertiles and adjusted for age and gender also demonstrated a dose–response relationship with fracture presence. Compared to tertile 3 (the highest: >34 g/L, mean 37.3 $\pm$ 2.2 g/L), the HF patients in tertile 2 (16–34 g/L, mean 32.4 $\pm$ 1.04 g/L) had an OR of 2.2 (95% CI: 1.4–3.3, $P=0.000$) and in tertile 1 (the lowest: <31 g/L, mean 27.6 $\pm$ 2.5 g/L), an OR of 4.6 (95% CI: 3.0–7.1, $P=0.000$); similarly, patients with any fracture had ORs of 1.3 (95% CI: 0.98–1.8, $P=0.065$) and 2.8 (95% CI: 2.0–3.8, $P=0.000$), respectively.

Next, we dichotomized subjects using the median value of the PINP/ β CTX ratio (100.0) in our cohort. Of 612 orthogeriatric patients with the PINP/ β CTX <100.0 (under median level) on admission, 484 (79.1%) presented with a fracture. In stepwise multiple linear regression analyses which included all laboratory indices along with sociodemographic and clinical characteristics, PINP/ β CTX <100.0 and hypoalbuminemia (<33 g/L) were independent indicators of an HF (OR 2.8, 95% CI: 2.0–3.8, $P=0.000$ and OR 3.1, 95% CI: 2.2–4.3, $P=0.000$, respectively) or any fracture (OR 2.1, 95% CI: 1.6–2.8, $P=0.000$ and OR 1.7, 95% CI: 1.3–2.3, $P=0.000$, respectively).

Determinants of lower PINP/ β CTX ratio and hypoalbuminemia

In multivariate forward stepwise regression analyses, which included all univariate clinical (dementia, CAD, AF, history of stroke, TIA, CKD, osteoporosis, antiosteoporotic medications use, smoking status), sociodemographic (age, gender, residence type, and use of walking aids), laboratory

Table 2 Presence of fracture in hospitalized orthogeriatric patients according to serum PINP/ β CTX ratio tertiles

PINP/ β CTX ratio	Model 1 ^a			Model 2 ^b		
	OR	95% CI	P-value	OR	95% CI	P-value
Hip fracture						
Tertile 1 (<78.6), n=221 (48.1%)	3.44	2.32–5.12	0.000	3.05	2.02–4.63	0.000
Tertile 2 (78.6–129.2), n=140 (30.8%)	1.62	1.10–2.38	0.016	1.54	1.03–2.31	0.036
Any fracture						
Tertile 1 (<78.6), n=334 (39.1%)	2.51	1.80–3.48	0.000	2.30	1.63–3.23	0.000
Tertile 2 (78.6–129.2), n=287 (33.9%)	1.61	1.19–2.18	0.002	1.56	1.14–2.13	0.005

Notes: Logistic regression models for fracture presence by tertiles of serum PINP/ β CTX ratio; as the reference used tertile 3 (PINP/ β CTX >129.2, the highest), n=94 (20.4%) in the hip fracture group and n=230 (27.0%) among all patients with fractures. ^aAdjustment only for age and gender. ^bAdjustment for age, gender, 25(OH)D, PTH, calcium (corrected for albumin), phosphate, magnesium, osteocalcin, albumin, alkaline phosphatase, GFR, presence of dementia, cardiovascular diseases (coronary artery disease, atrial fibrillation, and chronic heart failure), cerebrovascular diseases, diabetes mellitus, history of smoking, and alcohol consumption status.

Abbreviations: PINP, N-terminal propeptide of type I procollagen; β CTX, cross-linked carboxy-terminal telopeptide of type I collagen; OR, odds ratio; CI, confidence interval.

parameters with $P \leq 0.100$ and the presence of an HF or any fracture (separate analyses), the PINP/ β CTX ratio as a continuous variable was independently predicted by the presence of HF ($\beta = -28.602$, $P = 0.000$) or of any fracture ($\beta = -23.4845$, $P = 0.000$), age ($\beta = -1.3032$, $P = 0.000$), OC ($\beta = 2.574$, $P = 0.000$), GFR ($\beta = 0.482$, $P = 0.002$), and the use of antiosteoporotic medications ($\beta = 29.761$, $P = 0.000$). These data indicate that lower PINP/ β CTX ratio is largely determined by increasing age, lower OC, and decline of renal function, and is strongly associated with any nonvertebral fracture and the nonuse of antiosteoporotic therapy.

Independent determinants of PINP/ β CTX < 100.0 (under median level) were also assessed in a similar stepwise multiple linear regression analyses which included the laboratory, sociodemographic, and clinical characteristics. The probability of PINP/ β CTX < 100.0 increased with increment in each year of age by 4% (OR 1.04, 95% CI: 1.02–1.06, $P = 0.000$), the presence of HF by 2.8-fold (OR 2.8, 95% CI: 2.0–3.8, $P = 0.000$), and the presence of any fracture by 2.1-fold (OR 2.1, 95% CI: 1.6–2.8, $P = 0.000$), and decreased with the use of antiosteoporotic medications by 34.6% for HF (OR 0.65, 95% CI: 0.43–1.00, $P = 0.050$) and 32.4% for any fracture (OR 0.68, 95% CI: 0.48–0.96, $P = 0.027$).

With regard to hypoalbuminemia (< 33 g/L), a similar multiple regression revealed that the presence of HF (OR 2.8, 95% CI: 2.0–3.9, $P = 0.000$) or of any fracture (OR 1.5, 95% CI: 1.2–2.0, $P = 0.002$) is an independent determinant of this condition and its probability with each year of age increases by 3% in HF patients (OR 1.03, 95% CI: 1.01–1.05, $P = 0.005$) and by 4% in the group with any fracture (OR 1.04, 95% CI: 1.02–1.05, $P = 0.000$).

Taken together, these data suggest that among the studied laboratory parameters both lower serum PINP/ β CTX ratio and hypoalbuminemia are strongly associated with and are the best to indicate a nonvertebral osteoporotic fracture.

Informative/predictive values of lower PINP/ β CTX ratio and hypoalbuminemia

In the attempt to give to practicing physicians a simple tool, we focused on the median PINP/ β CTX ratio (< 100.0) and low albumin (< 33 g/L) as cutoff values. We additionally evaluated the discriminative values of recently recommended treatment targets for antiosteoporotic therapies: PINP > 62 μ g/L for bone-forming agents³⁷ and β CTX < 0.250 μ g/L for antiresorptive therapy.^{20,38} In our cohort, there were only nine (0.73%) patients (including five without fractures, one with an HF, and three with other fractures) in whom both these markers were within the targeted zone; none of them had PINP/ β CTX < 100.0 and eight subjects (including all four with fractures) have been receiving antiosteoporotic treatment. In other words, both markers were in the desired zone only in 1.3% of patients without fractures and in 0.47% of patients with any fracture (0.22% among HF). However, PINP < 62 μ g/L was found in 942 (76.6%) patients (in 368 without fracture, 304 with HF and 270 with other fractures), and β CTX > 0.250 μ g/L in 955 (77.7%) patients (in 376, 302 and 277, respectively).

The results of the analyses performed with five explanatory variables are displayed in Table 3 and Figure 1A and B. AUC values after adjustment for age and gender for all markers were between 0.691 and 0.855, indicating a mild-to-moderate discriminatory ability. The PINP/ β CTX ratio < 100.0 and

Table 3 Bone turnover markers and hypoalbuminemia as indicators of nonvertebral osteoporotic fractures in orthogeriatric patients

Variables	OR (95% CI)	AUC	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %
Hip fracture							
PINP/ β CTX < 100	2.8 (2.0; 3.8) ^a	0.802	78.5	68.9	75.2	72.8	74.1
PINP < 62.0 μ g/L	1.7 (1.2; 2.4) ^b	0.785	76.3	65.2	72.3	69.8	71.2
β CTX > 0.250 μ g/L	1.3 (0.9; 1.9) ^c	0.781	77.0	65.5	72.9	70.4	71.8
Albumin < 33 g/L	3.1 (2.2; 4.3) ^a	0.806	78.7	71.5	76.7	73.8	75.4
PINP/ β CTX < 100 + albumin < 33 g/L	7.8 (4.9–12.4) ^a	0.855	83.0	75.6	80.4	78.7	79.6
Any fracture							
PINP/ β CTX < 100	2.1 (1.6; 2.8) ^a	0.711	90.1	27.1	73.5	54.8	70.7
PINP < 62.0 μ g/L	1.5 (1.1; 2.0) ^b	0.695	90.3	18.7	71.3	46.4	68.2
β CTX > 0.250 μ g/L	1.2 (0.9; 1.6) ^c	0.691	91.3	17.2	71.3	46.8	68.5
Albumin < 33 g/L	2.2 (1.3; 2.7) ^a	0.706	89.3	19.9	71.4	45.5	67.9
PINP/ β CTX < 100 + albumin < 33 g/L	3.2 (2.2–4.6) ^a	0.754	85.7	41.8	75.7	58.0	71.6

Notes: Data adjusted for age and gender; ^a $P < 0.001$; ^b $P < 0.01$; ^c $P > 0.200$.

Abbreviations: PINP, N-terminal propeptide of type I procollagen; β CTX, cross-linked carboxy-terminal telopeptide of type I collagen; OR, odds ratio; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

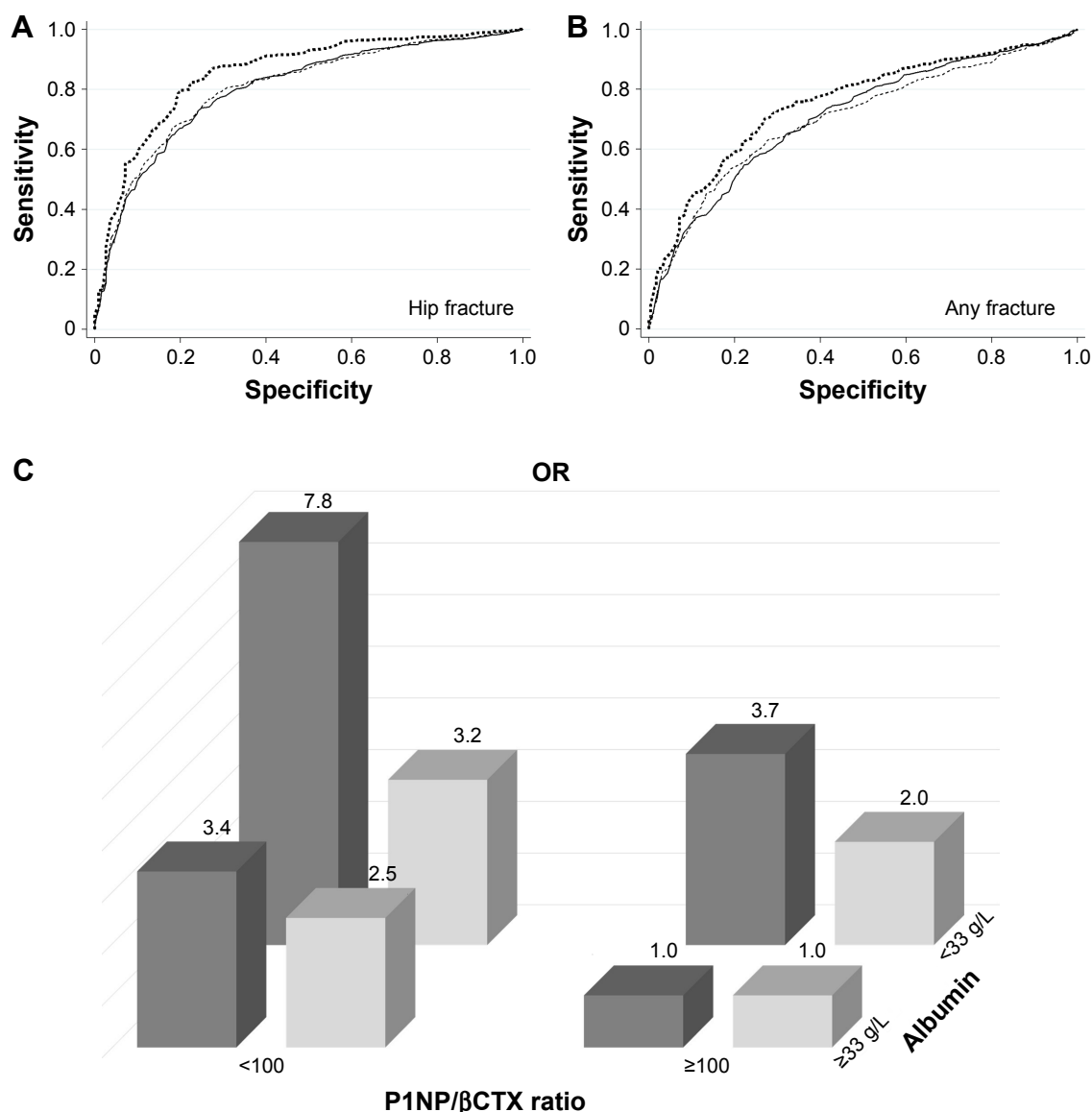


Figure 1 Discriminative information on nonvertebral fracture presence according to serum PINP/ β CTX ratio and albumin concentrations in orthogeriatric patients.

Notes: (A, B) Receiver operating characteristic curves (ROC) adjusted for age and gender for PINP/ β CTX < 100 (solid line), albumin < 33 g/L (thin dashed line), and their combination (thick dashed line) as prognostic tests for HF (A) or for any fracture (B). (C) odds ratios (ORs) adjusted for age and gender for the presence of an HF or of any nonvertebral fracture. The group with PINP/ β CTX > 100.0 and albumin > 33 g/L on admission used as the reference one. Among patients with only PINP/ β CTX < 100.0, the ORs are 3.4- and 2.5-fold higher in subjects with an HF or any nonvertebral fracture, respectively, and among patients with only albumin < 33 g/L, the ORs are 3.7- and 2.0-fold higher, respectively; if both conditions are present (combined), the ORs are 7.8- and 3.2-fold higher, respectively.

Abbreviations: β CTX, cross-linked carboxy-terminal telopeptide of type I collagen; HF, heart failure; PINP, amino-terminal propeptide of type I procollagen.

albumin < 33 g/L yielded the best AUC measures for HF (0.802 and 0.806, respectively) or for any fractures (0.711 and 0.706, respectively).

Interestingly, among patients with only PINP/ β CTX < 100.0, the ORs were 3.4-fold (95% CI: 2.0–5.7, $P=0.000$) and 2.5-fold (95% CI: 1.7–3.7, $P=0.000$) higher in subjects with HF or any nonvertebral fracture, respectively, and among patients with only albumin < 33 g/L, the ORs were 3.7-fold (95% CI: 2.7–6.0, $P=0.000$) and 2.0-fold (95% CI: 1.4–2.8, $P=0.000$) higher, respectively (Figure 1C). The data

further suggest the independent and strong association of each of these two factors with nonvertebral fractures.

When PINP/ β CTX ratio < 100.0 and albumin < 33 g/L were combined, the ORs for HF or for any fracture, compared with the nonfractured group, were 7.8 and 3.2, respectively, and the AUC improved (0.855 and 0.754, respectively). The presence of both these characteristics was a more sensitive (83.0% for HF and 85.7% for any fracture) and accurate indicator of fracture presence (79.6% and 71.6%, respectively) compared to other variables.

Validation of serum P1NP/ β CTX ratio and albumin levels as indicators of nonvertebral fracture

Patients in the validation dataset comparing to those in the test dataset did not show significant differences in sociodemographics, comorbidities, and antiresorptive medication use. When the P1NP/ β CTX ratio cutoff of <100.0 and albumin <33 g/L were applied to the validation dataset, they showed significant and similar discriminative values. P1NP/ β CTX <100 : for HF AUC 0.810 (sensitivity 78.7%, PPV 75.9%), for any fracture AUC 0.710 (sensitivity 89.3%, PPV 73.8%); albumin <33 g/L: for HF AUC 0.811 (sensitivity 78.3%, PPV 71.7%), for any fracture AUC 0.705 (sensitivity 88.3%, PPV 71.7%); both factors combined: for HF AUC 0.861 (sensitivity 86.2%, PPV 81.1%), for any fracture AUC 0.765 (sensitivity 88.0%, PPV 76.7%).

Discussion

In this study, in a large cohort of consecutive hospitalized orthogeriatric patients, lower levels of serum P1NP/ β CTX ratio and albumin concentration were 1) strong independent indicators of HF or of any nonvertebral fracture, 2) showed a dose-dependent relationship with the prevalence of fractures, and 3) demonstrated a discrimination ability of acceptable precision that exceeded the discrimination ability of other studied laboratory parameters. To the best of our knowledge, this is the first study to demonstrate the clinical utility of lower serum P1NP/ β CTX ratio and hypoalbuminemia as promising biomarkers for predicting osteoporotic fractures in older adults.

The prevalence of both lower P1NP/ β CTX ratio (reflects an imbalance between total bone formation and resorption in favour of the latter) and hypoalbuminaemia increase with age, and both factors are independently associated with osteoporotic fractures; lower P1NP/ β CTX ratio is also largely determined by decline of renal function and the nonuse of antiosteoporotic therapy. It appears that these two characteristics – serum P1NP/ β CTX ratio and albumin – accumulate key determinants of fracture risk incorporating the effects of multiple clinical and metabolic abnormalities reported in the literature and observed in our univariate analysis. Although there are no prior studies using P1NP/ β CTX ratio, our results are consistent with the only previous report showing that the ratio of a urinary resorption to serum formation marker (u-NTx/OC) was predictive of fractures independent of FRAX.²³ A number of reports, but not all,^{11,20,39,40} indicated a link between abnormalities in BTMs, bone loss, and increased risk of fracture independent of BMD.^{10,12,13,41–44}

The pathophysiological mechanism(s) underlying the relationship between altered albumin homeostasis and osteoporotic fractures is not fully understood. Hypoalbuminemia could be caused and/or aggravated by numerous chronic diseases associated with increased risk of falls and fractures.^{26,27} Hypoalbuminemia may directly and indirectly influence bone status, shifting the balance toward bone resorption via its effects on the nuclear factor- κ B, disturbed inflammatory and antioxidant responses, reduced flux of minerals to and from the bone, decreased formation of calcium phosphate apatite crystals, as well as affecting the metabolism of PTH, vitamin D binding protein, and Gla-protein.^{32,45}

Our data showed that among hospitalized orthogeriatric patients, the serum P1NP/ β CTX <100 or/and albumin <33 g/L at admission outweighed other laboratory parameters in its discriminatory ability of fracture presence, especially for HF, and the combination of both signs doubles the ORs. The fact that near equal proportions of patients admitted with a fracture had only one of these characteristics (Figure 1C) reflects the complexity, multifactorial nature, and heterogeneity of metabolic mechanisms underlying osteoporotic fractures and indicates the usefulness to include in the screening strategy measuring of both parameters, each of which demonstrated a strong independent association with fractures.

Taken together, in older patients, serum BTMs and albumin may perhaps help distinguish subgroups with different prognoses for osteoporotic fracture: 1) high risk if P1NP/ β CTX <100.0 and albumin <33 g/L (OR 7.8 for HF and 3.2 for any fracture), 2) intermediate risk if P1NP/ β CTX <100.0 (OR 3.4 and 2.5, respectively) or albumin <33 g/L (OR 3.7 and 2.0), and 3) low risk ($<0.5\%$) in older adults with β CTX <0.250 μ g/L and P1NP >62.0 μ g/L. Although the discriminative ability of these markers is only moderate (but higher when compared with other currently available indices), they may be particularly useful in persons who have negative BMD test. It should be, however, emphasized that the fracture risk remains substantial in subjects with P1NP/ β CTX >100.0 and albumin >33 g/L; such characteristics demonstrated 58.1% of orthogeriatric patients without fracture but also 18.7% of all fracture patients including 9.0% with HF, indicating that in near 1/5 of subjects with fragility fractures other factors rather than the total balance between bone formation and resorption and/or albumin homeostasis are important in the development of fractures.

Limitations of the study include: 1) cross-sectional design (results describe associations rather than causation),

2) comparison with nonfractured elderly orthopedic patients (not a healthy control group), a significant proportion of which may have undiagnosed/undocumented osteoporosis, 3) reliance on single measurement, and 4) data from one medical center, mostly on white older adults, limiting the generalizability of the results. Of note, within 26 hours after fracture, BTMs are not altered from the preinjury levels,⁴⁶ but both bone formation and bone resorption markers significantly increase within 6 weeks to 6 months after fracture, reflecting the fracture healing process, and these changes may persist for up to a year.^{46–49} As in all our patient, fasting venous blood samples were collected within 24 hours of admission to the hospital it is unlikely that the fracture per se contributed to the observed changes in BTMs.

This study also has several strengths: the relatively large number of patients, adjustment for a wide range of confounding factors, and the use of validation cohort. In multivariate regression analyses, the variance inflation factor was between 1.07 and 1.18, indicating that the amount of multicollinearity was not significant.

Conclusion

In an unselected cohort of hospitalized consecutive orthogeriatric patients, both serum PINP/ β CTX ratio and albumin levels demonstrated an inverse dose–effect relationship with the prevalence of nonvertebral fractures and independently indicated fracture presence with acceptable discriminatory power. Lower PINP/ β CTX (<100) and hypoalbuminemia (<33 g/L) could be useful simple and inexpensive tools to obtain additive prognostic information on fracture risk in the elderly. However, confirmation in other cohorts is needed to further support the applicability of these characteristics to the total population.

Summary

In a cohort of unselected orthogeriatric patients ($n=1,239$), serum PINP/ β CTX ratio and albumin levels demonstrated an inverse dose–effect relationship with the prevalence of nonvertebral fractures. PINP/ β CTX <100 and hypoalbuminemia (<33 g/L) could be useful additive prognostic tools for fracture risk stratification in the elderly.

Author contributions

AF was the coordinator of the study and together with LF participated in the study design, data collection, analysis, interpretation, and article writing. PS operated on the patients and contributed to data interpretation. WS performed statistical analysis and took part in interpretation of data. The final

version of the manuscript was approved by all authors. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Research Paper

Bone Turnover Status: Classification Model and Clinical Implications

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Received: 2017.09.08; Accepted: 2017.11.23; Published: 2018.02.01

Abstract

Aim: To develop a practical model for classification bone turnover status and evaluate its clinical usefulness.

Methods: Our classification of bone turnover status is based on internationally recommended biomarkers of both bone formation (N-terminal propeptide of type I procollagen, PINP) and bone resorption (beta C-terminal cross-linked telopeptide of type I collagen, bCTX), using the cutoffs proposed as therapeutic targets. The relationships between turnover subtypes and clinical characteristic were assessed in 1223 hospitalised orthogeriatric patients (846 women, 377 men; mean age 78.1 ± 9.50 years): 451 (36.9%) subjects with hip fracture (HF), 396 (32.4%) with other non-vertebral (non-HF) fractures (HF) and 376 (30.7%) patients without fractures.

Results: Six subtypes of bone turnover status were identified: 1 - normal turnover (PINP $>32 \mu\text{g/L}$, bCTX $\leq 0.250 \mu\text{g/L}$ and PINP/bCTX >100.0 [(median value)]; 2- low bone formation (PINP $\leq 32 \mu\text{g/L}$), normal bone resorption (bCTX $\leq 0.250 \mu\text{g/L}$) and PINP/bCTX >100.0 (subtype 2A) or PINP/bCTX <100.0 (subtype 2B); 3- low bone formation, high bone resorption (bCTX $>0.250 \mu\text{g/L}$) and PINP/bCTX <100.0 ; 4- high bone turnover (both markers elevated) and PINP/bCTX >100.0 (subtype 4A) or PINP/bCTX <100.0 (subtype 4B). Compared to subtypes 1 and 2A, subtype 2B was strongly associated with nonvertebral fractures (odds ratio [OR] 2.0), especially HF (OR 3.2), age >75 years and hyperparathyroidism. Hypoalbuminaemia and not using osteoporotic therapy were two independent indicators common for subtypes 3, 4A and 4B; these three subtypes were associated with in-hospital mortality. Subtype 3 was associated with fractures (OR 1.7, for HF OR 2.4), age >75 years, chronic heart failure (CHF), anaemia, and history of malignancy, and predicted post-operative myocardial injury, high inflammatory response and length of hospital stay (LOS) above 10 days. Subtype 4A was associated with chronic kidney disease (CKD), anaemia, history of malignancy and walking aids use and predicted LOS >20 days, but was not discriminative for fractures. Subtype 4B was associated with fractures (OR 2.1, for HF OR 2.5), age >75 years, CKD and indicated risks of myocardial injury, high inflammatory response and LOS >10 days.

Conclusions: We proposed a classification model of bone turnover status and demonstrated that in orthogeriatric patients altered subtypes are closely related to presence of nonvertebral fractures, comorbidities and poorer in-hospital outcomes. However, further research is needed to establish optimal cut points of various biomarkers and improve the classification model.

Key words: bone turnover markers; classification; nonvertebral fracture; prediction

Introduction

As the world's population ages, the prevalence of osteoporotic fractures is increasing, but the existing

prevention strategies are only partially effective. Although altered bone and mineral metabolism is

considered as one of the most important and modifiable risk factors for osteoporotic fractures, the diagnostic and prognostic value of bone turnover markers (BTMs) is still disputed. Currently BTMs, which reflect the status of total bone metabolism, are recommended only for the monitoring the efficacy of osteoporosis treatment and compliance [1-7]. The reasons for scepticism about the practical value of BTMs include their significant analytical and biological variability [8-11], parallel dynamics (due to coupling bone formation and resorption), and, more importantly, large overlap in BTMs values between those with and without fractures [2, 4, 7, 12]. Moreover, both increased and low bone turnover have been shown to be associated with bone gain or loss as well as with increased risk of fracture [13-20]. Despite accumulating evidence suggesting heterogeneity of the osteoporotic processes as a reflection of sophisticated and multifactorial regulation of bone metabolism, osteoporosis is still often considered as a single entity. One possible way to deal with this complex disorder is to identify clinical subtypes based on selected variables. However, there is currently no international consensus regarding characteristics (absolute values) of normal, high or low bone turnover, and the balance between bone formation and resorption is mostly neglected, although after midlife bone is lost because remodelling, despite of coupling, becomes unbalanced [13, 20, 21].

In light of paucity of studies investigating the phenomenon of variants of BTMs we attempted to develop and introduce a practical classification model based on both bone formation and resorption biomarkers and their ratio. We aimed to identify distinct subtypes of bone metabolism and analysed in a cohort of hospitalised orthogeriatric patients the relationships between these subtypes and (1) presence and type of a non-vertebral fracture, (2) clinical and laboratory characteristics (2) and (3) in-hospital outcomes.

Patients and Methods

Patients

This was an observational study using prospectively collected data on 1899 consecutive older (>60 years) patients admitted to the Department of Orthopaedic Surgery at the Canberra hospital (a university-affiliated tertiary care centre, Australian Capital Territory, Australia) between 1 January 2012 and 31 December 2014. After excluding patients with high-trauma fracture, primary hyperparathyroidism, Paget's disease, metastatic cancer to bone, or who lacked adequate laboratory data, 1223 patients (846

women, 377 men) were evaluated for the study. Of these 1223 hospitalized orthogeriatric patients 847 (69.3%) had a non-vertebral bone fracture. Patients with hip fracture (HF, n=451) constituted 53.2% among all fracture patients, and 36.9% of the total cohort. There were 396 (32.4%) patients with other non-vertebral (non-HF) fractures (humerus -79, femur -74, ankle -68, tibia or/and fibula -27, knee -16, wrist -16, forearm -15, other -101) and 376 (30.7%) patients without fractures (elective hip or knee replacement -340, suspected surgical site infections not confirmed by further investigation -12, and 24 patients with a prosthetic joint infection following total hip [n=17] or knee [n=7] arthroplasty).

Data on demographics, orthopaedic and medical diagnoses, chronic comorbid conditions, residential and smoking status, alcohol consumption, laboratory characteristics, procedures performed, medication used, and short-term (in-hospital) outcomes were analysed.

The study was conducted according to the ethical guidelines of the current Declaration of Helsinki and was approved by the local Health Human Research Ethical Committee. Informed consent from each patient or carer was obtained.

Laboratory measurements

In each patient fasting venous blood samples were collected in the morning, usually within 24h after arrival. The following serum indicators of bone and mineral metabolism were measured: two bone formation markers (N-terminal propeptide of type 1 procollagen, P1NP, and osteocalcin, OC), bone resorption marker (beta C-terminal cross-linked telopeptide of type I collagen, bCTX), parathyroid hormone (PTH), 25 hydroxyvitamin D [25(OH)D], calcium, phosphate and magnesium concentrations. The serum concentrations of P1NP, OC and bCTX were measured using an electrochemiluminescent immunoassay (Elecsys 2010 analyser, Roche Diagnostics, Ltd Corp., IN, USA). Intra- and inter-assay coefficients of variation (CV) for P1NP were 2.6% and 4.1 %, respectively; for OC 3.6% and 6.6%, respectively, and for bCTX 3.2% and 6.5%, respectively. Serum 25(OH)D level was measured by a radioimmunoassay (Dia Sorin, Stillwater, MN, USA) and intact PTH was determined by a two-site chemiluminescent enzyme-linked immunoassay on DPC Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA, USA); the intra- and inter-assay CV ranged from 2.1% to 12.7%. Calcium concentrations were corrected for serum albumin. The ratio of P1NP to bCTX was calculated by dividing the P1NP by bCTX. Vitamin D status was defined as deficient for circulating 25(OH)D concentration <25nmol/L, and

as insufficient for 25–50 nmol/L. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (>6.8 pmol/L, the upper limit of the laboratory reference range). Chronic kidney disease (CKD) was defined as glomerular filtration rate (GFR) <60 mL/min/1.73 m² (CKD stage ≥3), anaemia as haemoglobin <120 g/L and hypoalbuminaemia as albumin <33 g/L.

Classification criteria for bone turnover status

In line with the recommendations of the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine on BTMs [7], in our classification we used P1NP as a formation marker and bCTX as a resorption marker. There is to date no consensus on normal reference intervals for BTMs. Because the reports on thresholds of optimal bone metabolism, particularly in the older age, are controversial, to classify bone turnover status we used the cutoffs proposed as therapeutic (fracture-protective) targets, though some researchers concluded “that absolute values for BTMs are not suited as treatment targets” [12]. Two approaches were recommended to choose treatment targets for osteoporotic therapy: (1) provisional threshold values derived from community-dwelling observations [22–24] and (2) the mean/median of premenopausal reference intervals [7, 25, 26]. As a provisional treatment target /threshold for optimal anti-resorptive response values of bCTX ≤0.230 µg/L (Chubb S 2016; 2017) and ≤0.250 µg/L (the equivalent of urinary NTX <21 nmol BCE/mmol [22]) were recommended. In 17 studies, the mean/median reference intervals for bCTX in premenopausal women ranged between 0.217 µg/L and 0.484 µg/L [27–41] being ≤0.260 µg/L in seven reports. In 8 studies, the mean/median reference intervals for bCTX in adult men ranged between 0.260 µg/L and 0.490 µg/L [34–36, 42–45] being ≤0.270 in two studies. Even more controversy exists in relation to the target/desired level of P1NP during osteoporosis treatment because of the direction of changes associated with different classes of drugs: P1NP increases greatly with teriparatide administration (Sugimoto T 2014) and decreases (but less than bCTX) with antiresorptive therapy [3, 46–48]. In 15 studies, the mean/median reference intervals for P1NP in premenopausal women ranged between 33.0 µg/L and 47.7 µg/L [27, 29–31, 33–41, 49, 50]; similarly, in 8 studies, the mean/median reference intervals for P1NP in adult men ranged between 32.7 µg/L and 64.9 µg/L [34–36, 38, 42–45]. Based on data from a cohort of community-dwelling older men receiving antiresorptive therapy, serum P1NP concentrations of <32 µg/L (equivalent to the

provisional βCTX threshold of <0.230 µg/L) has recently been recommended as an indicator of optimal therapeutic response to bisphosphonate treatment [23].

In the present study, to classify the bone turnover status we have chosen as the cut points for serum P1NP 32 µg/L and for bCTX 0.250 µg/L; these arbitrary levels are relatively close to those recommended by the majority of experts and based on data reported by both abovementioned approaches.

Our classification of bone turnover status combines analysis of P1NP, bCTX and their ratio, assuming that the circulating concentrations of these markers are related to and reflect the integrated formation and resorption processes of the skeleton, while the ratio P1NP/bCTX <100 (median value) indicates a shift towards accelerated bone resorption.

Outcomes

The following short-term outcomes have been analysed: in-hospital death, myocardial injury (as reflected by cardiac troponin I rise), high postoperative (>3 days) inflammatory responses (CRP >100 mg/L and CRP >150 mg/L), length of hospital stay (LOS >10 days and >20 days), and new discharges to a permanent residential care facility (RCF).

Statistical analyses

Data analyses were performed using Stata software version 10 (StataCorp., College Station, TX, USA). The patient characteristics were summarised using descriptive statistics; data presented as mean ± standard deviation (SD) for continuous variables and as numbers (and percentages) for categorical variables. Associations between bone turnover subtypes and fracture prevalence as well as comorbid conditions and outcomes were assessed using multiple linear regression models with a backward stepwise approach adjusting for age and gender. For multivariate logistic regression models all variables with $p \leq 0.100$ at univariate analysis were selected. The discriminative accuracy of each bone turnover subtype was expressed with two descriptors: (1) the area under the receiver operating characteristic curve (ROC), and (2) the percentage of correctly classified patients. Two tailed tests were used and results were considered statistically significant if $p < 0.05$.

Results

Patient characteristics

In the total cohort of orthogeriatric patients the mean age was 78.1 ± 9.50 years, 846 (69.2%) were women, and 190 (15.4%) were living in a RCF. Patients

averaged 2.7 chronic diseases per person. Four or more chronic conditions were identified in 28.5% of patients with the greatest burden among individuals with HF (36.0% vs. 29.4% in the non-fracture group, $p=0.040$). The most common comorbidities were hypertension requiring medications (60.0%), osteoarthritis (42.5%), abnormal gait with use of an assistive device (42.0%), diabetes mellitus type 2 (DM, 22.0%), CKD (21.3%), coronary artery disease (CAD, 17.1%), chronic obstructive airway disease (COPD, 15.4%), atrial fibrillation (AF, 14.8%), dementia (14.4%), cerebrovascular disease (12.2%), malignancy (10.4%) and chronic/congestive heart failure (CHF, 7.8%).

On admission, vitamin D insufficiency exhibited 295(24.1%) patients, vitamin D deficiency 95(7.8%), hyperparathyroidism 468(38.3%), anaemia 872(71.3%) and hypoalbuminaemia 680(55.6%) subjects. There were 17.3% ex-smokers and 8.0% current smokers, and 31.8% of patients consumed alcohol on average ≥ 3 times per week. At the time of admission antiresorptive treatment (bisphosphonates or denosumab) received 182(14.9%) patients (26.4% with HF, 18.2% with a non-HF and 11.2% without fractures). Compared with patients without a fracture, subjects with a nonvertebral fracture were significantly older (for HF 83.0 ± 8.48 years, for non-HF 76.6 ± 9.49 years vs. 73.9 ± 8.06), much more frequent female (73.2%, 72.8 vs. 60.6%, respectively), more often living in a RCF (27.7%, 10.5% vs. 5.7%, respectively). The proportion of patients with hypertension, CHF, DM, COPD, CKD, history of malignancy, as well as current smokers and anticoagulation medication (mainly warfarin) users were similar in the three groups. Patients with fracture had significantly higher mean values of serum bCTX (+20.9%, $p=0.000$) and PTH (+11.8%, $p=0.021$), lower P1NP/bCTX ratio (-22.1%, $p=0.000$), haemoglobin ($p=0.001$) and albumin ($p=0.000$) levels. The mean serum levels of P1NP, OC, P1NP/OC ratio, 25(OH)D, creatinine, alkaline phosphatase (ALP), thyroid-stimulating hormone (TSH), free thyroxine (fT4) on admission did not differ between the three groups.

Classification of bone turnover status and fracture prevalence by subtypes

To classify bone turnover status we integrated the evidence available in the literature and used the cutoffs proposed as fracture-protective targets for osteoporotic therapy (see Methods). We used three criteria: 1) serum P1NP concentrations of $32 \mu\text{g/L}$, 2) serum bCTX of $0.250 \mu\text{g/L}$, and (3) P1NP/bCTX ratio of 100.0 (the median value in our cohort). In this study, serum bCTX $< 0.250 \mu\text{g/L}$ is referred as “normal”, and the serum P1NP $< 32 \mu\text{g/L}$ is referred as

low. Subjects were initially divided into 4 groups according to bone turnover marker levels: 1) normal bone turnover- both markers (P1NP and bCTX) are normal; 2) low bone formation (P1NP $\leq 32 \mu\text{g/L}$) and normal bone resorption (bCTX $\leq 0.250 \mu\text{g/L}$); 3) low bone formation (P1NP $\leq 32 \mu\text{g/L}$) and high bone resorption (bCTX $> 0.250 \mu\text{g/L}$); 4) high bone turnover- both markers are high (P1NP $> 32 \mu\text{g/L}$ and bCTX $> 0.250 \mu\text{g/L}$). All subjects in group 1, as would be expected, had P1NP/bCTX > 100.0 , indicating that bone formation was equal or exceeded bone resorption; the absolute majority of patients in group 3 had P1NP/bCTX < 100.0 (97.6% among patients with fractures). Groups 2 and 4 were further divided into two subtypes (A and B) on the basis of the ratio P1NP/bCTX (≥ 100.0 or < 100.0). In this paper, for simplicity, we are referring to six subtypes (avoiding terms “variant” or “group”). Figure 1 illustrates the principles of classification and the prevalence of each subtype among patients admitted with and without fracture. In our cohort in total, the prevalence of elevated bCTX was 78.1%, and the prevalence of low P1NP was 38.7%; ratio P1NP/bCTX < 100.0 (bone resorption predominates bone formation) was observed in 300 (66.5%) patients with HF, but only in 116(30.9%) individuals without a fracture.

Subtype1 (normal bone turnover) was found in 67 (5.5% of the total cohort) subjects: in 39 patients with fractures (4.6% among all fractures), including 11 with HF (2.4% of all HFs), and in 28 patients without fractures (7.4% among the non-fractured). In subjects with subtype 1, compared to the rest of the cohort, risk of HF (but not other nonvertebral fractures) was 2.3 times lower (inverse association: OR 0.43, Table 1), and receiver operating characteristic (ROC) curve analysis showed the area under the curve (AUC) value of 0.7837 (75% sensitivity, 67.6% specificity and 71.6% accuracy). Interestingly, P1NP $> 62 \mu\text{g/L}$ (treatment target for anabolic therapy/ teriparatide) and normal serum bCTX ($< 0.250 \mu\text{g/L}$) was observed in total only in 9 (0.74%) patients, including 5(1.3%) without fracture, 3(0.76%) with non-HF and 1(0.22%) subject with a HF; 8 of these 9 patients (including all 4 with fractures) have been receiving antiresorptive medications.

Subtypes2A and 2B (low P1NP and normal bCTX) were observed in 201(16.4%) patients: in 64 with HF (14.2% of all HFs), 66 with non-HF (16.6% among the non-HFs) and in 71 without fracture (18.9% of all non-fractured). Among 57 subjects with subtype 2B (an imbalance between bone formation and resorption) 44(77.2%) patients presented with fractures, including 26 with HF. There was no significant difference between subjects with subtype 2A and subtype1 in prevalence of HFs ($p=0.155$) or

non-HFs ($p=0.300$), whereas in patients with subtype 2B the risk of any fracture was 3.3 times higher (OR 3.3, 95% CI 1.45-7.61, $p=0.004$) and risk of HF was 4.3 times higher (OR 4.3, 95% CI 1.73-10.68, $p=0.001$) than in subjects with subtype 1. After adjustment for age and gender, compared to the rest of the cohort, patients with subtype 1 and subtype 2A did not show significant association with presence of nonvertebral fractures, while subjects with subtype 2B, had 2.1-fold increased risk of HF (OR 2.12, 95% CI 1.00-4.53, $p=0.050$; AUC value 0.7821, 76.7% sensitivity, 66.0% specificity and 71.8% accuracy). In other words, despite low/normal levels of both BTMs contrasting association with fracture prevalence were related to the inadequate formation /resorption balance.

Subtype3 (low P1NP and elevated bCTX) accounted for 272(22.2%) patients in the total cohort, including 137 with HF (30.4% among all HFs), 74 with non-HF (18.7% among the non-HFs) and in 61 patients without fractures (16.2% among the non-fractured). Compared to the rest of the cohort (adjusted for age and gender), patients with subtype3 had a 1.5-fold

increased risk for any fracture (OR 1.45, 95%CI 1.04-2.01, $p=0.027$) and 1.8-fold increased risk for HF (OR 1.77, 95%CI 1.21- 2.58, $p=0.003$) with AUC values of 0.6920 and 0.7852, respectively.

Subtypes 4A and 4B (high bone turnover) were found in 683 (55.8%) patients, and in 272 (39.8%) of them the P1NP level was $>62\mu\text{g/L}$. The subtype 4B (bone resorption predominating the formation) demonstrated 295 subjects (24.1% of the total cohort), including 137 with HF (30.4% among the HFs), 97 with non-HF (24.5% among the non-HFs) and 61 without fractures (16.2% among the non-fractured). When compared to the rest of the cohort and adjusted for age and gender, subtype 4B was a significant indicator of presence of both HF (OR 1.78, 95%CI 1.121-2.62, $p=0.003$; AUC value 0.7853) or non-HF (OR 1.64, 95%CI 1.14-2.36, $p=0.008$; AUC value 0.6172). Comparison of subtypes 4A and 4B showed that in the latter odds ratio (OR) for presence of HF was 2.4-fold higher (OR 2.41, 95%CI 1.74-3.40, $p=0.000$) and for any fracture 2.6-fold higher (OR 2.55, 95%CI 1.78-3.67, $p=0.000$).

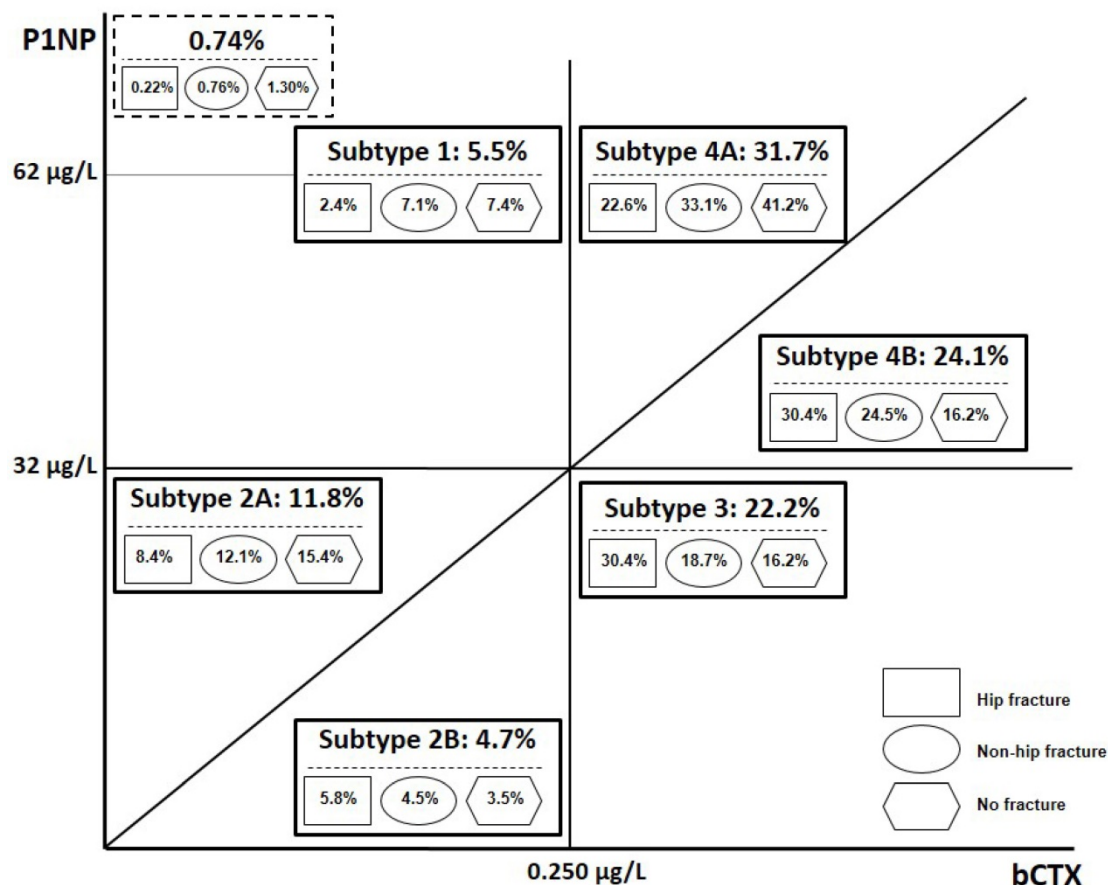


Figure 1. Schematic presentation of principles of classification of bone turnover marker status and the prevalence (%) of each subtype among hospitalised orthogeriatric patients. In subtypes 2A and 4A the ratio P1NP/bCTX >100.0 , while in subtypes 2B and 4B the ratio P1NP/bCTX <100.0 . The proportion (%) of patients with each subtype among all subjects admitted with a hip or non-hip fracture and without a fracture is shown in geometrical figures. Abbreviations: P1NP, N-terminal propeptide of type I procollagen; bCTX, C-terminal β cross-linked telopeptide of type I collagen; HF, hip fracture; non-FH, other non-vertebral fracture.

Table 1. Discriminative value of bone turnover status for non-vertebral fracture presence/prediction

Bone turnover status	Fracture site	OR	95%CI	AUC	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %
1*. P1NP>32 µg/L, bCTX<0.250 µg/L, P1NP/ bCTX >100.0 (n=67)	Hip	0.43	0.19-0.97 (p=0.043)	0.7837	75.0	67.6	73.3	69.4	71.6
	Any fracture	0.77	0.46-1.30 (p=0.324)	0.6916	91.2	18.02	71.3	47.9	68.6
2A*. P1NP<32 µg/L, bCTX <0.250 µg/L, P1NP/ bCTX >100.0 (n=144)	Hip	0.70	0.43-1.14 (p=0.151)	0.7815	76.7	66.0	73.0	70.3	71.8
	Any fracture	0.73	0.50-1.06 (p=0.095)	0.6918	91.3	17.8	71.4	47.5	68.7
2B. P1NP<32 µg/L, bCTX <0.250 µg/L, P1NP/ bCTX <100.0 (n=57)	Hip	3.23	1.37-7.65 (p=0.008)	0.8061	66.7	84.9	76.9	77.1	71.0
	Any fracture	2.04	1.00-4.17 (p=0.051)	0.7220	82.3	39.4	69.9	56.5	66.4
3. P1NP<32 µg/L, bCTX >0.250 µg/L, P1NP/ bCTX >100.0 (n=272)	Hip	2.40	1.42-4.06 (p=0.001)	0.8124	79.0	70.1	77.0	72.5	75.1
	Any fracture	1.74	1.14-2.65 (p=0.010)	0.7194	89.9	27.9	74.0	54.7	71.0
4A. P1NP>32 µg/L, bCTX>0.250 µg/L, P1NP/ bCTX >100.0 (n=388)	Hip	0.94	0.58-1.53 (p=0.815)	0.7597	57.6	84.7	70.2	76.1	74.2
	Any fracture	0.94	0.66-1.34 (p=0.714)	0.6647	77.7	38.2	65.1	53.5	61.8
4B. P1NP>32 µg/L, bCTX >0.250 µg/L, P1NP/ bCTX <100.0 (n=295)	Hip	2.53	1.48-4.33 (p=0.001)	0.8247	81.2	72.8	79.1	75.4	77.5
	Any fracture	2.08	1.37-3.16 (p=0.001)	0.7412	91.4	26.5	75.2	55.7	72.5

The asterisk (*) on the subtypes 1 and 2A indicates comparison with the rest of the cohort. For all other subtypes comparison was made with combined data for subtypes 1 and 2A.

Abbreviations: P1NP, N-terminal propeptide of type I procollagen; β-CTX, C-terminal βcross-linked telopeptide of type I collagen; HF, hip fracture; OR, odds ratio; AUC, area under the receiver operating characteristic curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

As can be seen, the most common subtypes were 4A, 4B and 3, representing 31.7%, 24.1% and 22.2%, respectively, of patients in the total cohort and 27.5%, 27.6% and 24.9%, respectively, among patients with fractures. Among individuals with P1NP/bCTX<100, patients with fractures comprised 79.2%. In patients with nonvertebral fractures, subtypes 2B, 3 and 4B were found in 5.2%, 24.9% and 27.6%, respectively, compared to 3.5%, 16.2% and 16.2% among subjects without a fracture. Conversely, among patients with subtypes 2B, 3 and 4B nonvertebral fractures had 77.2%, 77.6% and 79.3% (including a HF - 45.6%, 50.4% and 46.4%, respectively).

Because both subtypes 1 and 2A, compared to the rest of the cohort, were not significantly associated with presence of nonvertebral fractures (except an inverse association of subtype 1 with HF presence) and there were no major differences between subtype 1 and 2A in regard to fracture prevalence, we further evaluated the relationship between subtypes 2B, 3, 4A and 4B and fracture presence in comparison with combined data for subtypes 1 and 2A (Table 1). These analyses revealed that subtype 2B increases the risk of HF by 3.2-fold and the risk of any non-vertebral fracture by 2.0-fold, subtype 3 by 2.4- and 1.7-fold, respectively, and subtype 4B by 2.5- and 2.1-fold, respectively, whereas subtype 4A does not show such discriminative value (Table 1). Receiver operating characteristic (ROC) curve analyses for distinguishing HF and non-fracture patients showed the highest area under the curve (AUC) values for subtype 1 when compared to the rest of the cohort (0.7837), and for subtypes 2B (0.8061), 3 (0.8124,) and 4B (0.8247) when

compared to subtypes 1 and 2 combined. For distinguishing any non-vertebral fracture the AUC values were lower (0.7220, 0.7194, and 0.7412 for subtypes 2B, 3 and 4B, respectively). For HF, subtypes 2B, 3 and 4B had, respectively, an accuracy of 71.0%, 75.1% and 77.5%, sensitivity of 66.7%, 79.0% and 81.2%, specificity of 84.9%, 70.1% and 72.8%; for any non-vertebral fracture the corresponding values for sensitivity were 82.3%, 89.9% and 91.4%, and for specificity 39.4%, 27.9% and 26.5%, respectively.

On the other hand, subtypes 2A and 4A, both with P1NP/bCTX>100.0, were not discriminative for fracture presence, although in 37.7% of patients with fractures these subtypes of bone turnover were observed. These findings suggest that in subjects with subtypes 2A and 4A metabolic factors other than reflected by serum P1NP and bCTX may be more relevant for assessing bone quality and fracture development.

Bone turnover status and other parameters related to bone and mineral metabolism

The profiles of bone-mineral metabolism in subjects with different subtypes of bone turnover demonstrated, as would be expected, significant differences in a number of parameters in addition to the variables used for classification (Table 2). Subtype 1, compared to subtype 2A, showed higher mean levels of bone formation markers (P1NP, OC, alkaline phosphatase [ALP]), bone resorption (bCTX), as well as P1NP/bCTX and P1NP/OC ratios. Comparison with combined data from subtypes 1 and 2A revealed the following statistically significant

differences in the mean values. For subtype 2B: lower P1NP, OC, ALP, phosphate, albumin and haemoglobin concentrations, P1NP/bCTX and P1NP/OC ratios, and higher bCTX and PTH levels. For subtype3: higher bCTX (2.7-fold) and lower P1NP (2-fold), ALP, magnesium, albumin, haemoglobin, transferrin saturation and GFR levels, P1NP/bCTX and P1NP/OC ratios. For subtype 4A: higher concentrations of P1NP (3.7-fold), bCTX (3.3-fold) OC, ALP (about 2-fold each), phosphate, calcium (corrected for albumin) and GGT, significantly elevated P1NP/OC ratio, but lower magnesium, albumin and haemoglobin levels. For subtype 4B: a 4.3-fold higher bCTX concentration, higher P1NP, OC (both about 2-fold), ALP, PTH, phosphate, and lower magnesium, albumin, haemoglobin and GFR levels, as well as P1NP/bCTX ratio. Subtypes 4A and 4B, despite similarities in the direction of changes in P1NP, OC, ALP, bCTX, phosphate, magnesium, albumin, haemoglobin and GFR, demonstrated significant differences. Patients with subtype 4A comparing to those with subtype 4B exhibited higher mean values for P1NP, calcium (in absence of overt

hypercalcaemia), P1NP/OC ratio and lower values for bCTX and PTH ($p<0.001$ for all variables), indicating a higher bone formation, lower bone resorption as well as a strong coupling of bone formation and resorption.

Bone turnover status and clinical characteristics

We analysed the associations of bone turnover subtypes with the following chronic comorbidities: dementia, hypertension, coronary artery disease (CAD), atrial fibrillation(AF), chronic heart failure (CHF), history of myocardial infarction, stroke, transitional ischaemic attack, malignancy, peripheral vascular disease (PVD), diabetes (DM), chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD), anaemia, Parkinson's disease, osteoarthritis, and use of osteoporotic medications prior to admission. The analysis also included relation to smoking (current or ex-smoker), alcohol consumption (more than 3 times a week), use of a walking device and residential status (living in a long-term RCF).

Table 2. Parameters of mineral-bone metabolism and related variables in orthogeriatric patients by bone turnover status

Variable	Bone turnover status						
	Subtype 1	Subtype 2A	Subtypes 1+2A	Subtype 2B	Subtype 3	Subtype 4A	Subtype 4B
P1NP, $\mu\text{g/L}$	46.20 $\pm$ 18.71	22.85 $\pm$ 5.85***	30.26 $\pm$ 15.88	14.83 $\pm$ 3.98***	23.02 $\pm$ 5.84***	112.02 $\pm$ 142.45***	50.45 $\pm$ 19.57***
bCTX, $\mu\text{g/L}$	0.195 $\pm$ 0.038	0.160 $\pm$ 0.049***	0.171 $\pm$ 0.048	0.190 $\pm$ 0.036**	0.458 $\pm$ 0.210***	0.563 $\pm$ 0.405***	0.740 $\pm$ 0.317***
P1NP/bCTX	247.62 $\pm$ 112.04	151.88 $\pm$ 52.31***	182.28 $\pm$ 88.34***	78.30 $\pm$ 15.52***	57.17 $\pm$ 22.82***	187.12 $\pm$ 113.49	71.96 $\pm$ 17.98***
OC, pg/ml	5.91 $\pm$ 2.35	4.02 $\pm$ 1.86***	4.62 $\pm$ 2.21	3.86 $\pm$ 2.65*	4.60 $\pm$ 2.30	8.48 $\pm$ 5.32***	8.60 $\pm$ 5.13***
P1NP/OC	9.36 $\pm$ 6.58	6.82 $\pm$ 3.51***	7.63 $\pm$ 4.83	4.75 $\pm$ 2.04***	6.15 $\pm$ 3.30***	15.48 $\pm$ 17.21***	7.28 $\pm$ 4.16
PTH, pmol/L	6.88 $\pm$ 6.33	6.80 $\pm$ 4.99	6.82 $\pm$ 5.44	8.49 $\pm$ 5.69*	7.67 $\pm$ 5.21	6.30 $\pm$ 4.52	8.65 $\pm$ 6.09***
25(OH)D, mmol/L	62.55 $\pm$ 21.54	63.22 $\pm$ 23.67	63.00 $\pm$ 22.97	62.23 $\pm$ 23.67	62.78 $\pm$ 26.67	64.53 $\pm$ 25.85	61.04 $\pm$ 29.37
Ca (corrected), mmol/L	2.41 $\pm$ 0.14	2.38 $\pm$ 0.11	2.39 $\pm$ 0.12	2.36 $\pm$ 0.14	2.38 $\pm$ 0.14	2.44 $\pm$ 0.13***	2.41 $\pm$ 0.14
PO ₄ , mmol/L	0.87 $\pm$ 0.22	0.83 $\pm$ 0.24	0.84 $\pm$ 0.23	0.77 $\pm$ 0.24*	0.84 $\pm$ 0.23	0.98 $\pm$ 0.23***	0.97 $\pm$ 0.25***
Mg, mmol/L	0.79 $\pm$ 0.08	0.77 $\pm$ 0.09	0.78 $\pm$ 0.09	0.77 $\pm$ 0.10	0.76 $\pm$ 0.11*	0.76 $\pm$ 0.10*	0.76 $\pm$ 0.09*
ALP, IU	78.73 $\pm$ 27.66	66.85 $\pm$ 26.47*	72.00 $\pm$ 27.18	59.32 $\pm$ 17.62***	73.48 $\pm$ 38.20	112.28 $\pm$ 104.33***	91.43 $\pm$ 72.98***
GGT, IU	44.30 $\pm$ 33.46	47.02 $\pm$ 72.78	46.15 $\pm$ 62.88	40.32 $\pm$ 51.53	49.41 $\pm$ 64.69	62.88 $\pm$ 82.82*	51.16 $\pm$ 84.28
Albumin, g/L	34.49 $\pm$ 3.87	33.90 $\pm$ 4.06	34.09 $\pm$ 4.00	32.82 $\pm$ 3.6*	31.03 $\pm$ 3.85***	32.34 $\pm$ 4.72***	31.07 $\pm$ 4.40***
TSAT, %	12.52 $\pm$ 7.68	11.51 $\pm$ 7.63	11.82 $\pm$ 7.64	9.68 $\pm$ 6.38	9.65 $\pm$ 7.00***	12.63 $\pm$ 8.38	12.04 $\pm$ 9.54
Hb, g/L	117.10 $\pm$ 17.90	115.01 $\pm$ 17.56	115.7 $\pm$ 17.65	107.37 $\pm$ 16.66**	107.71 $\pm$ 18.03***	109.00 $\pm$ 16.93***	106.89 $\pm$ 18.02***
GFR, ml/min/1.73m^2	79.43 $\pm$ 10.50	78.47 $\pm$ 14.11	78.77 $\pm$ 13.05	74.96 $\pm$ 14.75	74.24 $\pm$ 16.77***	71.58 $\pm$ 19.51***	67.39 $\pm$ 22.45***
Age, years	74.4 $\pm$ 8.63	75.5 $\pm$ 8.66	75.1 $\pm$ 8.65	77.6 $\pm$ 8.60	80.4 $\pm$ 9.17***	76.6 $\pm$ 9.25	80.3.5 $\pm$ 9.92***

Subtype 2A is compared with subtype1, while subtypes 2B, 3, 4A and 4B are compared with combined data for subtypes1 and 2A; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

Abbreviations: P1NP, N-terminal propeptide of type I procollagen; bCTX, C-terminal β cross-linked telopeptide of type I collagen; OC, osteocalcin; PO₄, phosphate; Ca, calcium corrected for albumin; Mg, magnesium; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; 25(OH)D, 25hydroxyvitamin D; PTH, parathyroid hormone; TSAT, transferrin saturation; Hb, haemoglobin; GFR, glomerular filtration rate.

There was no significant difference between patients with subtypes 1 and 2A in regard to sociodemographic parameters, prevalence of fractures (including HF) and comorbid conditions, as well as in mean values of most laboratory variables (except P1NP, OC, ALP, bCTX, P1NP/bCTX and P1NP/OC ratios) and short-term outcomes. Therefore, data for types 1 and 2A were combined, and other subtypes were compared with the combined data. Patients with 2B, 3, 4A and 4B subtypes showed remarkable differences in regard to clinical characteristics. Compared to subtypes 1 and 2A, individuals with 3, 4A and 4B subtypes were more likely to have CKD (18.0%, 23.5% and 30.2% vs. 10.4%, respectively), anaemia (76.5%, 72.7% and 75.3% vs. 56.9%), history of malignancy (12.1%, 12.1% and 11.9% vs. 6.2%), to use a walking device (42.9%, 45.4% and 48.1% vs. 26.1%), and least likely to receive anti-osteoporotic treatment (13.6%, 11.9% and 10.5% vs. 23.2%). Patients with subtypes 3 and 4B were significantly older (+ 5 years on average) and demonstrated a significantly higher prevalence of dementia (20.2% and 17.3% vs. 9.0%, respectively), CHF (9.2% and 11.5% vs. 2.8%) and hyperparathyroidism (43.8% and 49.7% vs. 32.2%). Subtype 2B was also associated with hyperparathyroidism (49.1% vs. 32.2%). Subjects with subtype 3 were more likely to be residents of RCF (21.7% vs. 12.8%). Subtype 4B demonstrated a lower prevalence of diabetes mellitus (DM, 18.6% vs. 26.5%) and alcohol over-users (26.8% vs. 31.8%).

Independent clinical indicators/predictors of bone turnover status

We further performed multivariate logistic regression analyses with a backward stepwise approach for presence of bone turnover subtypes 2B, 3, 4A and 4B, including in the models the following variables: dementia, CHF, anaemia, CKD, history of malignancy, DM, vitamin D status, hyperparathyroidism, hypoalbuminaemia, use of walking aids, RCF residence, alcohol overuse, smoking (current and previous), use of anti-resorptive medications (>3 months), gender and age; age was evaluated as a continuous and as a categorical (>75 years) variable in separate models. As can be seen in Table 3, following these analyses, subtype 2B was independently predicted by 2 variables, subtype 3 by 6 variables, subtype 4A by 6, and subtype 4B by 4 variables. For every year increase in age there was a 6% increase in probability of subtype 3 and a 5% increase in probability of subtype 4B. Compared to subjects with subtypes 1 and 2A, among aged >75 years the presence of subtype 2B was 1.9-fold higher and presence of subtypes 3 and 4B was 2.5-fold higher. Hyperparathyroidism was the only other

independent predictor for subtype 2B. For subtypes 3, 4A and 4B hypoalbuminaemia on admission was a significant independent positive indicator while use of osteoporotic treatment was an independent negative predictor. Anaemia and history of malignancy were independent predictors of subtypes 3 and 4A, presence of CHF strongly indicated subtype 3, and CKD correlated independently with subtypes 4A and 4B.

Taken together, these results suggest that different bone turnover subtypes are linked to specific clinical characteristics (constellation of specific clinical variables) which can be used as indicators/predictors of altered bone turnover status. In other words, the clinical profile may serve as an early warning sign indicative of a possibly abnormal bone turnover status, and, *vice versa*, the bone turnover subtype may suggest the need of further evaluation for extraskeletal diseases. For example, subtype 3 is associated with and can be predicted by a clinical profile encompassing advanced age, CHF, anaemia, hypoalbuminaemia and history of malignancy. Presence of any of these conditions should raise the alarm regarding bone status and associated high risk for nonvertebral fracture, especially HF; conversely, in a patient with subtype 3 presence of previously non-diagnosed chronic conditions (e.g., CHF, anaemia, hypoalbuminaemia) as well as lack of osteoporotic treatment should be considered.

Bone turnover status and short-term outcomes

The association between bone turnover subtypes and comorbidities led us to investigate whether bone status can predict adverse in-hospital outcomes. In total, there were 32 deaths corresponding to in-hospital mortality of 2.6%: 25 (5.5%) deaths occurred among patients admitted with HF, and 7 (1.8%) among subjects with non-HF. Among patients with subtype 3 there were 11 (4.0%) non-survivors, among subjects with subtype 4A - 11 (2.8%), among patients with subtype 4B - 9 (3.0%) and among patients with subtype 2B - 1 (1.8%). None of the patients with subtypes 1 or 2A died.

Post-operative myocardial injury with cardiac troponin I rise was observed in 444 (36.4%) patients including 16 (24.2%) with subtype 1, 36 (25.0%) with subtype 2A, 16 (28.1%) with subtype 2B, 125 (46.0%) with subtype 3, 112 (28.9%) with subtype 4A and 139 (47.3%) patients with subtype 4B. Comparing to subjects with subtypes 1 and 2A, the OR for this complication obtained in patients with subtype 3 was 2.6 (95%CI 1.7-3.9, $p=0.000$) and in subjects with subtype 4B 2.7 (95%CI 1.8-4.1, $p=0.000$); after adjustment for age and gender the ORs were 2.1 and

2.2, respectively (Table 4); however, these associations become non-significant in fully adjusted models.

A high and persistent (≥ 3 days) post-operative inflammatory response was mostly related to urinary tract, respiratory or skin infections; elevated CRP of $>100\text{mg/L}$ and $>150\text{mg/L}$ demonstrated 553(45.3%) and 348(28.5%) patients, respectively. In models adjusted for age and gender, subtype 3 was a significant predictor of both CRP $>100\text{mg/L}$ (OR 2.4, $p<0.001$) and CRP $>150\text{mg/L}$ (OR 1.7, $p=0.006$), subtype 4B predicted CRP $>150\text{mg/L}$ (OR1.5, $p=0.038$), while subtypes 2B and 4A were not predictive for inflammatory marker raise. In fully adjusted models, only subtype 3 showed a significant link with CRP $>100\text{mg/L}$ (OR1.8, $p=0.013$).

The length of hospital stay (LOS) was ≥ 10 days in 530(43.3%) patients and ≥ 20 days in 256(20.9%). Compared to patients with subtypes1 and 2, in

subjects with subtypes 3, 4A and 4B the corresponding ORs for LOS ≥ 10 days were 1.8 (95%CI 1.2-2.7, $p=0.004$), 2.3 (95%CI 1.6-3.1, $p=0.000$) and 2.3 (95%CI 1.6-3.4, $p=0.000$), and for LOS ≥ 20 days 1.8 (95%CI 1.1-3.1, $p=0.026$), 2.6 (95%CI 1.6-4.2, $p=0.000$), and 1.7 (95%CI 1.1-2.9, $p=0.044$), respectively. After adjusting for age and gender the ORs did not change significantly, although subtypes 3 and 4B showed borderline significance for LOS ≥ 20 days. In fully adjusted models, a strong association remained only for subtype 4A.

New discharges to a RCF required 45(5.7%) patients: 1.9% of subjects with subtype1, 5.1% with subtype 2A and 6.8%, 6.8%, 5.2% and 6.8% of patients with subtypes 2B, 3, 4A and 4B, respectively; the differences between subtypes in the percentage of patients being discharged to RCFs did not reach statistical significance (Table 4).

Table 3. Independent and significant clinical and biochemical correlates/predictors of bone turnover status in orthogeriatric patients

Variables	Bone turnover status											
	Subtype 2B			Subtype 3			Subtype 4A			Subtype 4B		
	OR	95%CI	P Value	OR	95%CI	P Value	OR	95%CI	P Value	OR	95%CI	P Value
Age				1.06	1.03-1.08	<0.001				1.05	1.03-1.07	<0.001
Age $>75\text{yrs}^*$	1.87	1.01-3.47	0.048	2.49	1.64-3.79	<0.001				2.51	1.65-3.82	<0.001
Anaemia				1.82	1.15-2.87	0.010	1.57	1.00-2.47	0.048			
CKD							2.04	1.16-3.58	0.013	3.08	1.78-5.32	<0.001
CHF				3.01	1.13-7.97	0.027						
History of malignancy				2.16	1.04-4.48	0.039	2.17	1.02-4.60	0.045			
Hyperparathyroidism	1.87	1.01-3.46	0.046									
Hypoalbuminaemia				2.48	1.62-3.80	<0.001	1.6	1.03-2.49	0.036	2.14	1.44-3.19	<0.001
OPT				0.32	0.19-0.56	<0.001	0.29	0.17-0.49	<0.001	0.25	0.15-0.44	<0.001
Walking aids use							2.3	1.32-3.12	0.001			

Only statistically significant associations (compared to subjects with subtypes 1 and 2A) are shown. The backward stepwise regression models included dementia, CHF, anaemia ($<120\text{g/L}$), CKD ($\text{GFR}<60\text{ ml/min/1.73m}^2$), history of malignancy, diabetes mellitus, vitamin D insufficiency ($25(\text{OH})\text{D}<50\text{ nmol/L}$) or deficiency ($25(\text{OH})\text{D}<25\text{ nmol/L}$), hyperparathyroidism ($\text{PTH}>6.8\text{pmol/L}$), hypoalbuminaemia ($<33\text{g/L}$), use of walking aids, nursing home residence, alcohol use ($>3\text{ times/week}$), smoking (current and previous), use of anti-osteoporotic medications ($>3\text{ months}$) and adjusted for age and gender. * evaluated in separate models.

Abbreviations: OR, odds ratio; CI, confidence interval; CKD, chronic kidney disease; CHF, chronic heart failure; OPT, osteoporotic therapy; GFR, estimated glomerular filtration rate.

Table 4. Bone turnover status and in-hospital outcomes

Outcomes	Bone turnover status											
	Subtype 2B			Subtype 3			Subtype 4A			Subtype 4B		
	OR	95%CI	P Value	OR	95%CI	P Value	OR	95%CI	P Value	OR	95%CI	P Value
cTnI rise	0.97	0.49-1.92	0.934	2.05	1.36-3.09	0.001	1.13	0.77-1.68	0.535	2.17	1.44-3.26	<0.001
	0.80	0.38-1.68	0.561	1.40	0.90-2.19	0.139	0.84	0.55-1.30	0.440	1.38	0.88-2.17	0.162
LOS $>10\text{days}$	1.08	0.57-2.04	0.891	1.54	1.03-2.28	0.034	2.20	1.54-3.15	<0.001	2.08	1.41-3.06	<0.001
	0.95	0.49-1.84	0.876	1.16	0.76-1.76	0.496	2.18	1.48-3.20	<0.001	1.74	1.17-2.61	0.007
LOS $>20\text{days}$	0.88	0.36-2.17	0.783	1.65	0.99-2.75	0.058	2.49	1.57-3.96	<0.001	1.54	0.92-2.56	0.098
	0.80	0.32-2.01	0.640	1.30	0.76-2.23	0.333	2.61	1.60-4.26	<0.001	1.40	0.82-2.36	0.209
CRP $>100\text{mg/L}$	1.74	0.92-3.29	0.088	2.40	1.59-3.63	<0.001	1.23	0.87-1.74	0.238	1.30	0.88-1.91	0.184
	1.67	0.81-3.43	0.165	1.75	1.12-2.72	0.013	0.97	0.66-1.42	0.883	0.94	0.61-1.44	0.778
CRP $>150\text{mg/L}$	1.24	0.68-2.26	0.483	1.70	1.16-2.49	0.006	1.28	0.91-1.81	0.156	1.49	1.02-2.17	0.038
	1.18	0.62-2.24	0.622	1.44	0.96-2.15	0.078	1.04	0.72-1.52	0.821	1.20	0.80-1.78	0.374
New RCF d/c	1.31	0.31-5.50	0.709	1.26	0.47-3.42	0.649	1.09	0.42-2.83	0.858	1.36	0.50-3.68	0.858
	0.85	0.17-4.30	0.847	1.18	0.38-3.67	0.781	1.09	0.41-2.89	0.857	1.48	0.47-4.66	0.507

Multivariate regression comparisons with subtypes1and 2A combined.

Model 1 (1st line): adjustment for age and gender. Model 2 (2nd line): included chronic heart failure, dementia, chronic kidney disease ($\text{GFR}<60\text{ml/min/1.73m}^2$), history of malignancy, $\text{PTH}>6.8\text{pmol/L}$, albumin $<33\text{ g/L}$, anaemia (haemoglobin $<120\text{g/L}$), hip or any non-vertebral fracture, use of osteoporotic treatment, age and gender.

Abbreviations: OR, odds ratio; CI, confidence interval; cTnI, cardiac troponin I; LOS, length of hospital stay; CRP, C-reactive protein; RCF d/c, new discharges to a permanent residential care facility.

Discussion

Main findings

In the current study, we proposed a model for classification bone turnover status and evaluated the clinical usefulness (advantages and limitations) of such approach. The classification scheme is based on optimal treatment targets and captures three significant and widely accepted factors of bone metabolism - bone formation, bone resorption and their ratio, indices that reflect bone remodelling in the entire skeleton. We defined six subtypes of bone turnover and showed that among hospitalized orthogeriatric patients these subtypes differed substantially in terms of clinical characteristics, including prevalence of nonvertebral fractures, especially HF, chronic comorbid conditions and in-hospital outcomes. Subtypes suggestive an imbalance in bone turnover favouring an increase in bone resorption demonstrated a good/moderate discriminative ability in regard to non-vertebral fracture presence. The study highlights the similarities and differences between subtypes and indicates that the future classification should also include other indices of bone metabolism which may better reflect bone health and fracture risk.

In osteoporosis, a multifactorial heterogeneous disease, bone formation and bone resorption, though mutually dependent through crosstalk between osteoblasts and osteoclasts, may be affected differently, and, not surprisingly, various patterns of bone metabolism (determined by specific genetic, metabolic and clinical factors) occur. The present study, to our knowledge, is the first of its kind, evaluating the clinical significance of different subtypes of bone turnover markers in the elderly. Identifying the bone turnover status in the elderly is an important key to better understand underlying pathophysiological mechanisms and may have an advantage in at least three areas: individualized management, prediction of nonvertebral fractures, and prognosis of in-hospital outcomes. Table 5 presents an overview of our findings.

Classification of bone turnover status

Our classification is three-fold: it takes into account bone formation, bone resorption and the balance between these processes. Due to existing controversy concerning reference intervals of BTMs, for classification we used as cutoffs values recently proposed optimal treatment targets for anti-resorptive therapy. It worthy of mention in this connection that many, but not all [1, 12], studies suggested that P1NP and bCTX may provide information about both response to treatment and reduction of fracture risk

following osteoporotic therapy with antiresorptive [7, 23, 24, 26, 48, 51, 52] or anabolic [6, 53-59] agents. Almost all published studies demonstrated reduction in serum bCTX during antiresorptive therapy and rise in serum P1NP during therapy with teriparatide; these changes have been associated with an improvement in BMD and reduced fracture risk. The importance to examine the balance between formation and resorption when evaluating bone turnover has also been recognized [60-64].

Table 5. Overview of the relationships between altered bone turnover status and presence of non-vertebral fracture, clinical characteristics and in-hospital outcomes

	Bone turnover status			
	Subtype 2B	Subtype 3	Subtype 4A	Subtype 4B
Fracture risk (compared to subtypes 1 and 2):				
HF, (OR)	↑,3.2	↑,2.4		↑,2.5
Any fracture, (OR)	↑,2.0	↑,1.7		↑,2.1
Independent clinical indicators/predictors:				
Age>75years, (OR)	↑,1.9	↑,2.5		↑,2.5
Hypoalbuminaemia,(OR)		↑,2.5	↑,1.6	↑,2.1
Anaemia, (OR)		↑,1.8	↑,1.6	
CKD, (OR)			↑,2.0	↑,3.1
CHF, (OR)		↑,3.0		
Hyperparathyroidism, (OR)	↑,1.9			
History of malignancy, (OR)		↑,2.1	↑,2.2	
Walking aids use, (OR)			↑,2.3	
OPT, (OR)		↓,0.32	↓,0.29	↓,0.25
In-hospital outcomes:				
Myocardial injury with cTnI rise, (OR)		↑, 2.1*		↑,2.2*
LOS>10 days, (OR)		↑,1.5*	↑,2.2	↑,1.7
LOS>20 days, (OR)			↑,2.6	
CRP>100mg/L, (OR)		↑,1.8		
CRP>150mg/L, (OR)		↑,1.7*		↑,1.5*
In- hospital death, (%)	1.8	4	2.8	3

Data reflect only statistically significant results compared to subtypes 1 and 2A (combined) in multivariate adjusted regression models; the asterisk (*) indicates statistical significance in models adjusted only for age and gender.

Abbreviations: OR, odds ratio; CHF, chronic heart failure; CKD, chronic kidney disease; cTnI, cardiac troponin I; LOS, length of hospital stay; CRP, C-reactive protein (marker of systemic inflammatory response).

Bone turnover status and fractures

Despite the wide heterogeneity of bone turnover markers, from low to significantly elevated, even within the same fracture type, specific subtypes of turnover status demonstrate different impact on fracture development, and may, therefore, provide a rough estimate of individual risk. Elevated BTMs, a sign of an increased turnover rate, is commonly reported as a factor which adversely influences BMD and increases fracture risk. Consistent with this data, in our study, high levels of both biomarkers were found in 55.1% of all patients with fractures. In this context, the low prevalence of fractures among subjects with subtype1 (normal bCTX and P1NP>32

$\mu\text{g/L}$ and, especially, with $\text{P1NP} > 62 \mu\text{g/L}$), appears to contradict previous studies, who found a protective effect of low P1NP. In the present study, among patients with $\text{bCTX} < 0.250 \mu\text{g/L}$, higher levels of P1NP were associated with lower prevalence of fractures: with $\text{P1NP} > 32 \mu\text{g/L}$ there were in total 67(5.5%) patients [11(2.4%) among HFs], including with P1NP levels between $32 \mu\text{g/L}$ and $62 \mu\text{g/L}$ – 35(4.1%) patients [10(2.2%) among HFs] and with $\text{P1NP} > 62 \mu\text{g/L}$ only 4(0.47%) patients [including 1(0.22%) among HFs], whereas with $\text{P1NP} < 32 \mu\text{g/L}$ there were 130(15.3%) patients [64(14.2%) among HFs]. On the other hand, bCTX was elevated in 683(91.1%) of 750 subjects with $\text{P1NP} > 32 \mu\text{g/L}$ and in 272(96.8%) of 281 patients with $\text{P1NP} > 62 \mu\text{g/L}$. These data strongly suggest that higher P1NP becomes a risk factor for fracture only in the presence of elevated bCTX and lower P1NP/bCTX ratio (subtype 4B), showing that the ratio is of major importance in assessing bone status rather than each of the markers taken alone; it appears that well-balanced formation/resorption processes are protective. These observations indicate that an increase or a decrease in serum P1NP can be related to positive or negative effects, depending on the bCTX level and P1NP/bCTX ratio, and should not be interpreted in isolation. These data may explain the conflicting results in the literature on the associations between P1NP, BMD and fractures [42, 65-67].

Obviously, bone turnover status as defined by subtypes is more closely related to presence of osteoporotic fractures compared with separate indicators of bone metabolism. Patients with subtypes 2B, 3 and 4B, compared to those with subtypes 1 and 2A, have a 3.2-, 2.4- and 2.5-fold increase in risk for HF, respectively, and a 2.0-, 1.7- and 2.1-fold increase in risk for any nonvertebral fracture, respectively. Subtypes 2B, 3 and 4B showed good AUC values (0.8061- 0.8247) for HF, and fair AUC values (0.7194 - 0.7412) for any nonvertebral fracture, and had an acceptable sensitivity for estimating risk of any fracture of 82.3%, 89.9% and 91.4%, respectively, but a low specificity (39.4%, 27.9% and 26.5%, respectively). These data shows that dysregulation in skeletal metabolism causing bone frailty is a necessary but not a sufficient condition for fracture development. A nonvertebral bone fracture usually occurs as a result of a fall in a person with frail bones. The deterioration of main components of bone strength - bone mass, microarchitecture and remodelling/metabolism - not always remains in parallel; bone quality is only partially reflected by BTMs. Most patients with osteoporosis (as defined by BMD) do not have a fracture and at least half of fractures occur in patients without osteoporosis, while both high and low BTMs

are associated with increased fracture risk. Subtypes 2B, 3 and 4B because of their relatively high sensitivity can be useful as screening tools to identify patients at fracture risk. However, subtype 4A (both markers elevated and the ratio $\text{P1NP/bCTX} > 100.0$) which constituted 27.5% of all patients with fractures (22.6% among all HF patients), does not discriminate presence of nonvertebral fractures against subtypes 1 and 2A (seen in 14.8% of all patients with fractures and in 10.9% of HF patients). The fact that fractures in subjects with subtype 4A are not entirely explained by elevated P1NP and bCTX may suggest that high bone turnover with preserved P1NP/bCTX ratio occur rather as a compensatory/adaptive response to bone loss but it is not sufficient enough to provide adequate bone quality and prevent fracture. Apparently, the described classification model does not completely represent the (patho)physiology of bone metabolism; P1NP, bCTX and their ratio reflect important but not all factors contributing to bone health and fragility fractures, and additional biomarkers are required to characterise more accurately bone quality and predict fractures, especially in subjects with subtype 4A.

Nevertheless, the results of the study raise the possibility that identifying subtypes of bone turnover may provide the basis for pharmaceutical treatment decisions: (a) focus on those at greatest risk and avoid complications of therapy (although current osteoporotic drugs are relatively benign) in individuals with low risk, and (b) choose between therapeutics which mainly suppress bone resorption or stimulate bone formation.

Bone turnover status and other indices of bone and mineral metabolism

Compared to subjects with subtypes 1 and 2A, patients with subtypes 2B-4B demonstrated significant differences in calcium (higher in subtype 4A), phosphate (lower in subtype 2B and higher in 4A and 4B), magnesium (lower in subtypes 3, 4A and 4B) and PTH concentrations (elevated in subtypes 2B and 4B), as well as in P1NP/OC ratio (decreased in subtypes 2B and 3 but elevated in subtype 4A). These heterogeneous disturbances of parameters of mineral-bone metabolism reflect the complexities underneath the bone turnover subtypes indicating, among other mechanisms, the important role of fibroblast growth factor 23 (FGF23), a hormone secreted mainly by osteocytes that modulates serum phosphate balance, PTH and 1,25(OH) vitamin D synthesis [68-71] and sclerostin, a glycoprotein also produced by mature osteocytes that influences differentiation and survival of osteoblasts [72-74]. It could be speculated that these and other hormones, not evaluated in this study, may be the modifying

factors contributing to bone fragility and fractures, especially in subjects with subtype 4A.

Our findings add to previous studies linking separate BTMs with fracture risk evidence of higher discriminative value of specific bone turnover subtypes, and also suggest that other biomarkers of bone metabolism need to be included in the classification to improve fracture prediction and identify novel therapeutic targets in the geriatric population.

Bone turnover status and chronic clinical conditions

Although age-related chronic diseases and frailty [75-81] are among known factors predisposing to bone loss, falls and fractures, no previous studies evaluated the relationship between bone turnover status and clinical characteristics. Our data clearly showed that in the elderly, subtypes of bone metabolism are associated with and can be predicted by specific clinical conditions. On univariate analyses 16 different variables were associated with distinct bone turnover subtypes. Backward stepwise linear regression analyses after adjusting for main confounders revealed several chronic conditions as independent and significant predictors/indicators of specific bone turnover subtypes (Tables 3 and 5). Among patients with subtypes 2B, 3, and 4B the proportion of aged >75 years was 1.9-2.5 times higher than in those with subtypes 1 and 2A. Other independent correlates included the following: for subtype 2B - hyperthyroidism; for subtype 3 - hypoalbuminaemia, anaemia, CHF, and history of malignancy; for subtype 4A - hypoalbuminaemia, anaemia, CKD, history of malignancy and use of walking aids; for subtype 4B - hypoalbuminaemia and CKD. Despite an overlap between the variables, specific conditions characterised the respective subtype. Each of these clinical variables indicated the presence of a specific bone turnover subtype with an OR of 1.6-3.1 (Tables 3 and 5). As would be expected, OPT was inversely associated with subtypes 3, 4A and 4B (risk decreased by 68-75%). The observation that OPT may be highly effective is important because in recent years worldwide the percentage of OPT users has declined [82-84]. In our cohort, approximately 85% of patients did not receive OPT.

Our results are in line with numerous evidences of multidirectional (patho)physiological links between the skeleton as a dynamic, metabolically active organ and the cardiovascular, renal, liver, endocrine, nervous and immune systems. Our observations complement the results from many previous studies showing positive bi- and multi-directional relationships between osteoporosis,

falls and fractures, on one hand, and chronic conditions such as CKD [71, 85-90], CVDs [91-104], anaemia [105-111], hypoalbuminaemia [112-114] and hyperthyroidism [115-119] on the other, supporting the concept that osteoporosis is a systemic disease.

Despite the growing knowledge of bone-extraskeletal interactions, the reasons and implications for this connection(s) remain not fully understood. These associations may be driven by shared risk factors (advanced age, reduced physical activity, malnutrition, disturbed inflammatory and antioxidant responses, etc.), common genetic basis and multiple common biochemical pathways involved in the regulation of both bone and extraskeletal metabolism (PTH, 25(OH) vitamin D, osteoprotegerin/RANK ligand/RANK axis, FGF23, sclerostin-Klotho axis, bone morphogenetic proteins, adipocytokines, autonomic nervous system). Given the wide range of genetic, metabolic and environmental (medications, smoking, alcohol, etc) factors influencing production, release and removal of P1NP and bCTX, it is not surprising that different subtypes of bone turnover status reflecting altered mechanisms of either bone formation or bone resorption or both were found to be associated with different chronic conditions. It can be assumed that common factors contribute to constellation of specific patterns of abnormal metabolism (including bone metabolism) and diseases, predisposing to falls and osteoporotic fractures. The presence of relatively specific clinical profiles for each turnover subtype implies that altered turnover status may serve as a warning sign for both frail bones and extraskeletal diseases. The provided data suggests that in an elderly person presenting with the abovementioned clinical conditions it would be worth considering impaired bone turnover (with increased fracture risk) and the need for special bone examination, and *vice versa*, presence of an unfavourable turnover status may help to identify earlier individuals with serious medical problems requiring intervention (e.g. CKD, CHF, anaemia), despite the absence of clinical signs and symptoms.

Bone turnover status and outcomes

In older orthopaedic patients, hospital complication rates are high (up to 50 % after surgery for hip fracture) indicating a vital need of early identification of individuals prone to postoperative morbidity and mortality. This study demonstrated for the first time that in the orthogeriatric population, altered bone turnover status is associated with poorer in-hospital outcomes, and that specific subtypes independently of established clinical risk factors (age, gender and presence of multi-morbidities) predict

myocardial injury, high inflammatory response, prolonged LOS, and all-cause mortality. Subtypes 3, 4A and 4B accounted for 31 (96.9%) of 32 in-hospital deaths, while no fatal outcomes occurred among 211 subjects with subtypes 1 and 2A. This observation is in line with studies on older adults living in the community or RCFs showing a positive association between all-cause mortality and bCTX levels [120-123] as well as osteoporosis defined by BMD [124-128].

Models adjusted for age and gender demonstrated that, compared to subjects with subtypes 1 and 2A, patients with subtype 3 had a significantly higher risk of developing postoperative myocardial injury (OR 2.1), inflammatory/infective complications with CRP>100 mg/L (OR 2.4) and LOS>10 days (OR 1.5). Similarly, subtype 4A was predictive for prolonged hospitalisation (for LOS>10 days OR 2.2 and for LOS>20 days OR 2.5), and subtype 4B indicated an increased risk of myocardial injury (OR 2.2), high inflammatory response with CRP>150 mg/L (OR 1.5) and LOS>10 days (OR 2.1). After controlling for multiple comorbidities, presence of HF or any non-vertebral fracture, OPT, age and gender (fully adjusted models, Tables 4 and 5), subtype 3 remained a significant independent predictor for CRP>150 mg/L (OR 1.7), subtype 4A for prolonged LOS (OR 2.2 and 2.6 for LOS>10 days and >20 days, respectively), and subtype 4B for LOS>10 days (OR 1.7). Compared to subtypes 1 and 2A, the subtype 2B did not demonstrate significant associations with the outcomes. These analyses show that turnover subtypes independently of a variety of clinical characteristics (on admission) known to adversely affect outcomes can help in individualized risk assessment identifying patients in whom poorer outcomes to be expected and additional interventions planned.

No studies on predictive value of bone turnover status for hospital outcomes in orthogeriatric patients have been reported. Previous studies found in critically ill patients on admission significantly elevated bone resorption markers, including bCTX levels ([129-133]), and low-normal P1NP levels [132, 133]. Lower BMD was shown to predict myocardial infarction in men and women during 5.7 years of follow-up [97]. Other studies identified higher P1NP levels as a risk factor for incident myocardial infarction in older men followed for 7 years [134] and as a biomarker of frailty [77].

Taking together, the study illuminates the close and complex relationship between bone turnover status, nonvertebral fractures, functioning of other systems and in-hospital outcomes.

The proposed classification model of bone turnover status may be of relevance for clinical

management, as well as for research. While requiring further replication, our data highlights the heterogeneity of bone turnover status, identifies distinct subtypes, their association with chronic conditions and usefulness for better patient stratification for more individualised approaches. This pilot classification is a first step towards integrative understanding bone metabolism, further exploration of the underlying pathophysiologic differences between various turnover subtypes is needed. Subtyping could be improved by adding new biomarkers. The combination of bone turnover subtypes with other diagnostic and prognostic tools may improve the preventive and treatment strategies for the elderly.

Limitations and strengths

Several limitations of our study should be considered. Firstly, we recognize the limitations of the classification model: it is based on BTM values proposed as treatment targets, the cutoffs are arbitrarily chosen, the two markers analysed reflect mainly the function of osteoblasts and osteoclasts, but biochemical indicators of the osteocyte activity, important factors in the maintenance of skeletal integrity [135], are not included (currently these markers are used for research purposes). Therefore, the pathogenesis of bone metabolism is only partially represented by various subtypes, and there is no evidence at present that all patients with a particular subtype share the same metabolic defect(s). Although the proposed classification brings a conceptual shift in our understanding of the pathophysiology and clinical applications of bone turnover status, additional biochemical indicators of the complex multilayered regulatory mechanisms need to be found and considered in future classification. The model, despite its limitations, illustrates the clinical opportunities of an integrative approach based on simultaneous use of a formation and resorption marker and their ratio compared to those which relied on analysis of these biomarkers separately. Secondly, the subjects in our study do not represent the general population, they were selected from hospitalised orthogeriatric patients, and a significant proportion of individuals admitted without fractures may have had undiagnosed/undocumented osteoporosis. Thirdly, the study has been done in a single centre, and the patients were mainly Caucasian; that could affect the generalizability of the results. Fourthly, the cross-sectional design of the study does not allow causal conclusions, and, despite multivariate analyses with extensive adjustment for potential confounders, the possibility of residual unmeasured confounders could not be excluded.

Our study has also several strengths. For the first time, a practical method for subtyping bone turnover status using three criteria (both serum bone formation and bone resorption markers and their ratio as a reflection of the bone turnover balance) was provided. Six bone turnover subtypes were identified and in a relatively large cohort of orthogeriatric patients clear relationships between bone turnover status and presence of nonvertebral fracture, chronic comorbid conditions and in-hospital outcomes have been shown.

Conclusions

We proposed a classification model of bone turnover status based on a combination of serum bone formation and resorption markers and demonstrated that in orthogeriatric patients altered subtypes are closely related to presence of nonvertebral fractures, comorbidities and poorer in-hospital outcomes. However, the pathogenesis of bone metabolism is only partially represented by this classification, and the future subtyping model could be improved by adding other biomarkers. Further research is needed to establish optimal cut points of various BTMs, improve the classification and achieve more-individualized prognosis and treatments.

Competing Interests

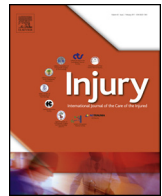
The authors have declared that no competing interest exists.

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Usefulness of simple biomarkers at admission as independent indicators and predictors of in-hospital mortality in older hip fracture patients

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ARTICLE INFO

Article history:

Accepted 7 March 2018

Keywords:

Hip fracture
In-hospital mortality
Biomarkers
Prediction

ABSTRACT

Introduction: The data on predictive value of the routinely obtained preoperative biochemical parameters in hip fracture (HF) patients are limited. The aims of this study were to examine in older HF patients (1) the relationships between a broad set of routine laboratory parameters at admission and in-hospital mortality, and (2) evaluate the prognostic value the biomarkers and clinical characteristics (alone or in combination) provide to predict a fatal outcome.

Patients and methods: In 1820 consecutive patients with low-trauma osteoporotic HF aged >60 years (mean age 82.8 ± 8.1 years; 76.4% women; 65% community-dwelling) 35 laboratory variables along with 20 clinical and socio-demographic characteristics at admission were analysed. The validation cohort included data on 455 older (≥ 60 years of age) HF patients (mean age 82.1 ± 8.0 years, 72.1% women). **Results:** The mortality rate was 6% ($n = 109$). On univariate analysis 14 laboratory and 8 clinical parameters have been associated with in-hospital mortality. Multiple regression analyses determined 7 variables at admission as independent indicators of a fatal outcome: 4 biomarkers (albumin < 33 g/L; alanine aminotransferase/gamma-glutamyl transferase ratio [GGT/ALT] > 2.5 ; parathyroid hormone [PTH] > 6.8 pmol/L; 25(OH)vitamin D < 25 nmol/L) and 3 pre-fracture clinical conditions (history of myocardial infarction, chronic kidney disease [GFR < 60 ml/min/1.73 m²] and chronic obstructive pulmonary disease); the area under the receiver operating characteristic curve (AUC) was 0.75 (95%CI 0.70–0.80). The risk of in-hospital death was 1.6–2.6 times higher in subjects with any of these risk factors (RFs), and increased by 2.6–6.0-fold in patients with any two RFs (versus no RFs). The mortality rate increased stepwise as the number of RFs increased (from 0.43% – none RF to 16.8% – ≥ 4 RF). The prognostic value of a single RF was low (AUC ≤ 0.635) but combination of 2 or more RFs improved the prediction significantly; AUC reached 0.84 (95%CI 0.77–0.90) when ≥ 4 RFs (versus 0–1RF) were present. In the validated and main cohorts the number of predicted by 1, 2, 3 or ≥ 4 RFs and observed deaths were practically similar.

Conclusions: In HF patients, seven easily identifiable at admission characteristics, including 4 biomarkers, are strong and independent indicators of in-hospital mortality and can be used for risk stratification and individualised management.

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Introduction

The number of hip fractures (HF), one of the most common and challenging clinical conditions in the elderly, is expected to rise worldwide from 1.66 million in 1990 to 6.26 million in 2050 [1],

despite declines (mainly among western populations) in the incidence rates [2,3]. The reported one-year mortality rates range between 12%–37% [4–9], and 3.3%–19.5% of HF patients die during hospitalization or in the first month following injury [4,10]–19.5%, [6,11–17]. Early prediction of outcome and the ability to identify at admission patients at a higher risk of morbidity and mortality can help optimise their management and reduce the burden of this disease.

Numerous studies on mortality following a HF focussed predominantly on clinical characteristics [4,6,15,18–24] and rarely

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took into consideration laboratory, especially biochemical, data [25–28].

Among hepatic-related markers only low serum albumin concentration has been found to be a prognostic factor for mortality [6,26,29–36], and one study documented a positive association between alanine aminotransferase (ALT) levels and mortality within 3 months [37]. The pathophysiologic and prognostic input of the available on admission spectrum of biochemical markers has not been studied, and systematic characterization of metabolic, specifically liver-related factors, in regard to survival in a large cohort of patients with HF has not been performed. However, there are numerous reports linking all-cause mortality in community-dwelling older adults and various groups of hospitalised patients with serum activities (even in the normal range) of gamma-glutamyl transferase (GGT), ALT and alkaline phosphatase (ALP) [38–46]. Recently, combined indices (ratios and scores) – serum GGT/ALT [47], albumin/bilirubin [48–51], albumin/GGT [52], and ALT/ALP [53,54], were proposed as simple and objective tools for evaluation of hepatic reserve function and prediction survival and therapeutic outcomes in different settings including non-liver disease-related mortality, especially in various malignancies. None of these ratios which represent simultaneous changes of two liver function indices have been intended for HF patients.

Among routine biochemical factors as potential predictors of early mortality in HF patients should also be noted vitamin D, parathyroid hormone (PTH), indices of iron metabolism and thyroid function, vitamin B12 and folate. These candidate biomarkers have pluripotent metabolic effects, play a vital role in a myriad of biologic functions, are often abnormal in the elderly, particularly in subjects with a HF, and, most importantly, have been shown to interfere with survival in different diseases. However, these biomarkers remain outside the models proposed for predicting mortality in HF patients. It is unknown whether any of abovementioned metabolic parameters (alone or in combination) may present an objective and reliable prognostic guide for prediction in HF patients the short-term outcome.

Given the potential effects of metabolic characteristics on outcomes, the aims of the present study were: (1) to examine in a large, well-characterised cohort of older patients with HF the relationships between a broad set of routine biochemical, mainly liver-related, parameters at admission and in-hospital mortality, and (2) to evaluate the prognostic value such biomarkers provide alone or in combination to predict a fatal outcome. We analysed in total 35 laboratory variables along with 20 clinical and socio-demographic characteristics at admission to ascertain their feasibility to predict in-hospital death.

Patients and methods

Patients

Data for this study were obtained from a prospective electronic database on all adult patients with fracture of the upper femur admitted to the Department of Orthopaedic Surgery of The Canberra Hospital (university-affiliated tertiary care centre) from January 2000 to January 2013. Patients who had subtrochanteric and shaft fracture, high trauma and pathological HF due to primary or metastatic bone cancer, multiple myeloma, Paget disease or primary hyperparathyroidism, or who had incomplete data on admission were excluded. In total, 1820 consecutive older (≥ 60 years of age) patients (mean age 82.8 ± 8.1 years; 76.4% women; 94.6% Caucasian) with low-trauma osteoporotic HF were finally included into the study. Socio-demographic, anthropometric, clinical (HF type, comorbidities, complications, medication use) and laboratory data as well as outcomes were recorded.

The study was conducted according to the standards of the Declaration of Helsinki and was approved by the Australian Capital Territory Health Human Research Ethical Committee. As only routinely collected patient data (anonymized before analysis) were used and none of the patients had a blood test for the purposes of the study itself, the need for informed consent was waived.

Validation dataset

A retrospective analysis of a second cohort included data (obtained from electronic medical and administrative records) from 455 consecutive older (≥ 60 years of age) patients (mean age 82.1 ± 8.0 years, 72.1% women) with osteoporotic HF who were treated at the Canberra Hospital between 2013 and 2015.

Laboratory tests

In each patient fasting venous blood samples were collected on admission and the following assays performed: complete blood count, GGT, ALT, ALP (all 3 enzymes measured enzymatically using the Abbott Architect C16200 automatic analyser), total bilirubin (measured using diazonium salt), albumin (analysed using brom-cresol green), total protein (Biuret method), iron (direct colorimetric determination), ferritin (two-step chemiluminescence microparticle immunoassay), transferrin (immunoturbidimetric procedure), vitamin B12 (two step chemiluminescence microparticle intrinsic factor assay), folate (chemiluminescence microparticle folate binding protein assay), 25 (OH) vitamin D (25(OH)D, radioimmunoassay kit, Dia Sorin, Stillwater, MN, USA), intact PTH (2-site chemiluminescence enzyme-linked immunoassay on DPC Immulite 2000, Diagnostic Products, Los Angeles, CA), electrolytes (sodium, potassium, total calcium, phosphate and magnesium), renal (creatinine, urea) and thyroid function tests (thyroid stimulating hormone, [TSH], thyroxine, T4). All biochemical parameters were measured using commercially available kits according to the manufacturers' protocols. The serum calcium level was corrected for albumin concentration. The mean inter-assay and intra-assay CV for these tests were within 1.1%–12.7%. In all patients the GGT/ALT, ALT/ALP, GGT/ALP, albumin/GGT, albumin/ALT, albumin/ALP, albumin/bilirubin ratios were calculated, and serum transferrin saturation (using the IFCC protein standards) and glomerular filtration rate (GFR, by standardized serum creatinine-based formula normalized to a body surface area of 1.73 m^2) estimated. All reference ranges used are the ranges used by the Pathology Department of our hospital. Continuous variables were converted to categorical groups based on generally accepted cutoffs. For the analyses, deficiency of vitamin D was defined as $25(\text{OH})\text{D} < 25 \text{ nmol/L}$ and insufficiency as $25(\text{OH})\text{D} < 50 \text{ nmol/L}$ based on current recommendations. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH ($> 6.8 \text{ pmol/L}$, the upper limit of the laboratory reference range). Chronic kidney disease (CKD $\geq$ stage 3) was defined as a $\text{GFR} < 60 \text{ ml/min/1.73 m}^2$, which represents a loss of half or more of the normal adult renal function level [55].

In total, 27 metabolic and haematological variables (albumin, ALT, GGT, ALP, bilirubin, $25(\text{OH})\text{D}$, PTH, calcium, phosphate, magnesium, TSH, T4, urea, creatinine, GFR, iron, ferritin, transferrin, transferrin saturation, vitamin B12, folate, haemoglobin, erythrocytes, white blood cell count, neutrophils, mean corpuscular volume, and haematocrit) along with 8 ratios (GGT/ALT, GGT/ALP, GGT/bilirubin, ALT/ALP, albumin/GGT, albumin/ALT, albumin/ALP and albumin/bilirubin) were analysed.

Statistical analyses

Statistical analyses were performed using the Stata software version 10 (StataCorp, College Station, TX, USA). Descriptive statistics

and frequency distributions were generated on the socio-demographic, clinical and laboratory characteristics. Continuous variables are presented as mean $\pm$ standard deviation (SD) and compared using analysis of variance. Categorical variables are reported as proportions/percentages and compared by Chi-square and Fisher's exact tests. The associations between enzymatic activities of serum ALT, GGT, ALT and their ratios (GGT/ALT, ALT/ALP, GGT/ALP) as well as between albumin, 25(OH)D, PTH levels, and albumin/ALT, albumin/GGT, albumin/ALP, albumin/bilirubin ratios and other studied laboratory variables at admission and in-hospital mortality were assessed as continuous and as categorical variables using univariate and multivariate logistic regression models. Pearson's correlation was used to evaluate the relationship among statistically significant biomarkers. All potential confounding variables (demographic, clinical and laboratory) with statistical significance ≤ 0.100 on univariate analyses were included in multivariate models to identify independent factors associated with in-hospital mortality. The final model was developed by stepwise logistic regression. To quantify the significance of multicollinearity phenomena in regression analyses the variance inflation factor was calculated.

The predictive abilities of the laboratory and clinical parameters of interest were evaluated by the receiver operating characteristic (ROC) analyses and the accuracy was expressed as area under curve (AUC); the Hosmer-Lemeshow goodness of fit test was used to assess model performance. Data are presented with 95% confidence intervals (CI). The cut-off values for GGT/ALT ratio and other abovementioned ratios were calculated based on ROC analyses (Youden Index). Two-tailed *p*-values < 0.05 were considered statistically significant.

Results

Survival differences by patient characteristics at admission

The in-hospital mortality rate was 6.0% (109/1820); there were no on-table deaths during surgery. Among 109 HF non-survivors there were 57(52.3%) patients with a cervical fracture and 52 (47.7%) with a trochanteric fracture, 75 (68.8%) females and 34 (31.2%) males (Table 1). The mortality rate in men was slightly higher than in women (7.9% vs. 5.4%, $p = 0.053$). In both genders, non-survivors were significantly older but the mean age of deceased men and women was similar, while in the group of

survivors women were significantly older (+3.2 years, $p < 0.001$). The risk of in-hospital mortality increased with advancing age on average by 5% for each year; 100 non-survivors were aged > 75 years (91.7% vs. 82.6% among survivors, $p = 0.012$).

Non-survivors were more likely to have been admitted to hospital from a permanent residential care facility (RCF), diagnosed with CKD, coronary artery disease (CAD), had a history of myocardial infarction (MI), chronic obstructive pulmonary disease (COPD), and to use walking aids. The incidence of other common chronic comorbidities including hypertension, anaemia, dementia, diabetes mellitus (DM), history of cerebrovascular accident (CVA), transient cerebral ischaemic attack (TIA), Parkinson's disease, as well as current or ex-smoking, and alcohol over-use (> 3 times per week) as factors possible predisposing to poor outcome were not different between survivors and non-survivors (Table 1).

Analysis of 35 blood parameters at admission showed that in the mortality group, compared to survivors, the mean values for GGT, ALT, ALP, bilirubin, PTH and ferritin as well as for the GGT/ALT, ALT/ALP, and GGT/bilirubin ratios were significantly higher, whereas mean levels of albumin, albumin/ALP and albumin/bilirubin ratios were significantly lower (Table 2). Only in the non-survivors the admission mean serum GGT, ALT and ALP levels were above the reference ranges. Of note, while the mean values of both GGT and ALT were 1.6 and 1.7 times, respectively, higher in non-survivors than survivors, the GGT/ALT ratio was also 1.5 times higher in non-survivors indicating that more deceased patients possessed higher GGT and contrarily lower ALT.

Given that 14 continuous laboratory variables, age and gender demonstrated significant difference between survivors and non-survivors, we performed a multivariate analysis which included all variables with $p < 0.10$ on univariate analysis. This analysis, as would be expected, showed that the majority of the observed risk factors did not operate independently of one another. The analysis revealed as independent and significant determinants of in-hospital death the following five continuous variables: albumin (OR 0.94, 95%CI 0.90–0.98, $p = 0.008$), GGT/ALT ratio (OR 1.05, 95%CI 1.01–1.08, $p = 0.005$), PTH (OR 1.03, 95%CI 1.00–1.05, $p = 0.021$), GFR (OR 0.98, 95%CI 0.97–0.99, $p = 0.001$), and age (OR 1.05, 95%CI 1.02–1.09, $p = 0.005$). When admission laboratory parameters were modelled as categorical variables fatal outcome was significantly associated with (1) hypoalbuminaemia (< 33 g/L), (2) GGT/ALT

Table 1

On-admission demographic and clinical characteristics significantly associated with in-hospital death in older hip fracture patients.

Notes: There was no significant differences between survivors and non-survivors in prevalence of hip fracture type (cervical fracture: 53.5% vs. 52.3%, $p = 0.892$), hypertension (51.6% vs. 52.3%, $p = 0.890$), anaemia (haemoglobin < 120 g/L: 38.0% vs. 45.9%, $p = 0.099$; haemoglobin < 100 g/L: 7.7% vs. 9.2%, $p = 0.583$), dementia (32.2% vs. 37.6%, $p = 0.240$), diabetes mellitus (9.4% vs. 13.8%, $p = 0.130$), history of stroke (12.2% vs. 11.0%, $p = 0.712$), transient cerebral ischaemic attack (7.7% vs. 9.2%, $p = 0.588$), Parkinson's disease (5.5% vs. 4.8%, $p = 0.720$), current (5.6% vs. 4.6%, $p = 0.663$) or ex- (12.8 vs. 14.7%, $p = 0.568$) smokers, as well as alcohol over-users (≥ 3 drinks/week: 5.2% vs. 2.8%, $p = 0.255$).

Variable	All patients (n = 1820)	Non-survivors (n = 109)	Survivors (n = 1711)	OR	95%CI	AUC	P Value
Age, years (mean $\pm$ SD):							
Females	83.4 $\pm$ 7.7	85.4 $\pm$ 7.8	83.3 $\pm$ 7.7	1.04	1.01–1.07	0.587	0.022
Males	80.6 $\pm$ 9.0	85.7 $\pm$ 7.6	80.1 $\pm$ 9.0	1.09	1.04–1.15	0.677	< 0.001
Both genders	82.8 $\pm$ 8.1	85.5 $\pm$ 7.7	82.6 $\pm$ 8.1	1.05	1.02–1.08	0.608	< 0.001
Age > 75 years, n (%)	1512(83.2)	100(91.7)	1412(82.6)	2.34	1.17–4.68	0.546	0.012
Males/Females, n	429/1391	34/75	395/1316	1.51	0.97–2.30	0.541	0.053
From RCF, n (%)	637(35.0)	50(45.9)	587(34.3)	1.62	1.10–2.40	0.558	0.014
CKD, n (%)	789(43.4)	75(68.8)	714(41.8)	3.07	2.02–4.66	0.635	< 0.001
CAD, n (%)	491(27.1)	45(41.3)	446(26.1)	1.99	1.34–2.95	0.576	< 0.001
MI, n (%)	134(7.4)	18(16.5)	116(6.8)	2.71	1.58–4.65	0.549	< 0.001
COPD, n (%)	210(11.6)	22(20.2)	188(11.0)	2.04	1.25–3.34	0.546	0.004
Walking aids use, n (%)	689(38.0)	55(50.5)	634(37.2)	1.72	1.14–2.58	0.566	0.031

Abbreviations: OR, odds ratio; CI, confidence interval; AUC, area under the curve; RCF, residential care facility (permanent); CKD, chronic kidney disease (GFR < 60 ml/min/1.73m²); CAD, coronary artery disease; MI, history of myocardial infarction; COPD, chronic obstructive pulmonary disease.

Notes: There was no significant differences between survivors and non-survivors in prevalence of hip fracture type (cervical fracture: 53.5% vs. 52.3%, $p = 0.892$), hypertension (51.6% vs. 52.3%, $p = 0.890$), anaemia (haemoglobin < 120 g/L: 38.0% vs. 45.9%, $p = 0.099$; haemoglobin < 100 g/L: 7.7% vs. 9.2%, $p = 0.583$), dementia (32.2% vs. 37.6%, $p = 0.240$), diabetes mellitus (9.4% vs. 13.8%, $p = 0.130$), history of stroke (12.2% vs. 11.0%, $p = 0.712$), transient cerebral ischaemic attack (7.7% vs. 9.2%, $p = 0.588$), Parkinson's disease (5.5% vs. 4.8%, $p = 0.720$), current (5.6% vs. 4.6%, $p = 0.663$) or ex- (12.8 vs. 14.7%, $p = 0.568$) smokers, as well as alcohol over-users (≥ 3 drinks/week: 5.2% vs. 2.8%, $p = 0.255$).

Table 2

Biomarkers at admission in older hip fracture patients significantly associated with in-hospital mortality.

Notes: Data are mean $\pm$ SD. Only statistically significant associations shown ($p < 0.05$). The mean values of Albumin/ALT, ALP/SGT, ALT/Bilirubin, and ALP/Bilirubin ratios, as well as 25(OH) vitamin D, thyroid-stimulating hormone, thyroxine, calcium, phosphate, magnesium, haemoglobin, iron, transferrin, transferrin saturation, erythrocytes, white blood cell count, neutrophils, mean corpuscular volume, haematocrit, vitamin B12, and folate did not differ significantly between the groups of survivors and non-survivors.

Variable	All patients (n = 1820)	Non-survivors (n = 109)	Survivors (n = 1711)	OR	95%CI	AUC	P Value
ALT, IU/L	24.8 $\pm$ 65.0	40.5 $\pm$ 125.1	23.8 $\pm$ 59.1	1.002	1.000–1.003	0.509	0.010
GGT, IU/L	56.9 $\pm$ 102.8	89.4 $\pm$ 165.2	54.9 $\pm$ 91.3	1.002	1.001–1.003	0.563	<0.001
ALP, IU/L	100.8 $\pm$ 70.7	118.7 $\pm$ 115.7	99.6 $\pm$ 66.8	1.002	1.000–1.004	0.562	0.007
Bilirubin, μ mol/L	12.3 $\pm$ 8.1	13.8 $\pm$ 9.0	12.3 $\pm$ 8.0	1.02	1.000–1.040	0.548	0.049
Albumin, g/L	36.8 $\pm$ 5.9	35.0 $\pm$ 5.4	37.0 $\pm$ 6.0	0.95	0.920–0.980	0.601	0.001
GGT/ALT	2.9 $\pm$ 4.4	4.2 $\pm$ 8.8	2.8 $\pm$ 3.9	1.04	1.010–1.070	0.537	0.003
ALT/ALP	0.26 $\pm$ 0.38	0.34 $\pm$ 0.88	0.26 $\pm$ 0.33	1.34	0.990–1.810	0.471	0.005
Albumin/ALP	0.45 $\pm$ 0.24	0.39 $\pm$ 0.17	0.45 $\pm$ 0.24	0.41	0.040–0.480	0.592	0.007
Albumin/Bilirubin	4.0 $\pm$ 2.6	3.5 $\pm$ 2.0	4.1 $\pm$ 2.7	0.89	0.800–0.980	0.567	0.035
GGT/Bilirubin	5.4 $\pm$ 8.9	8.6 $\pm$ 16.7	5.2 $\pm$ 8.2	1.02	1.010–1.040	0.511	<0.001
Albumin/SGT	1.45 $\pm$ 1.41	1.20 $\pm$ 0.88	1.47 $\pm$ 1.43	0.73	0.570–0.930	0.583	0.010
GFR, ml/min/1.73m ²	62.5 $\pm$ 22.1	48.4 $\pm$ 21.6	63.4 $\pm$ 21.9	0.97	0.960–0.980	0.688	<0.001
Ferritin, μ g/L	337.0 $\pm$ 348.8	422.6 $\pm$ 619.0	331.9 $\pm$ 325.9	1.001	1.000–1.001	0.502	0.024
PTH, pmol/L	8.5 $\pm$ 12.0	13.4 $\pm$ 12.0	8.2 $\pm$ 6.9	1.05	1.030–1.070	0.675	<0.001

Abbreviations: OR, odds ratio; CI, confidence interval; AUC, area under the curve; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; PTH, parathyroid hormone; GFR, glomerular filtration rate.

Notes: Data are mean $\pm$ SD. Only statistically significant associations shown ($p < 0.05$). The mean values of Albumin/ALT, ALP/SGT, ALT/Bilirubin, and ALP/Bilirubin ratios, as well as 25(OH) vitamin D, thyroid-stimulating hormone, thyroxine, calcium, phosphate, magnesium, haemoglobin, iron, transferrin, transferrin saturation, erythrocytes, white blood cell count, neutrophils, mean corpuscular volume, haematocrit, vitamin B12, and folate did not differ significantly between the groups of survivors and non-survivors.

Table 3

On-admission laboratory parameters (as categorical variables) significantly associated with in-hospital mortality in older hip fracture patients.

Notes: Only statistically significant associations shown ($p < 0.05$).

There was no significant differences between the groups of survivors and non-survivors in regard to the proportion of patients with 25(OH)D <50 nmol/L (60.1% vs.64.0%, $p = 0.443$), ALT >33 U/L (11.3% vs. 14.2%, $p = 0.378$), ALT <9 U/L (9.2% vs.10.4%, $p = 0.682$), GGT >56 U/L (21.5% vs. 28.3%, $p = 0.099$), ALP >110 U/L (25.6 vs.34.0, $p = 0.057$), ferritin >370 μ g/L (28.6% vs.32.5, $p = 0.459$), and transferrin saturation <18% (86.0% vs.90.0%, $p = 0.307$).

Variable	All patients (n = 1820)	Non-survivors (n = 109)	Survivors (n = 1711)	OR	95%CI	AUC	P Value
PTH >6.8 pmol/L, %	48.4	70.1	47.0	2.64	1.69–4.12	0.615	0.000
GGT/ALT >2.5, %	32.3	43.4	31.6	1.66	1.11–2.47	0.559	0.012
Albumin <33 g/L, %	21.1	31.5	20.4	1.79	1.18–2.74	0.556	0.006
25(OH)D <25 nmol/L, %	25.6	49.0	24.7	1.94	1.28–2.95	0.571	0.002
Bilirubin >20 μ mol/L, %	10.3	16.0	9.9	1.74	1.01–2.99	0.531	0.046

Abbreviations: OR, odds ratio; CI, confidence interval; AUC, area under the curve; GFR, glomerular filtration rate; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase;

Notes: Only statistically significant associations shown ($p < 0.05$).

There was no significant differences between the groups of survivors and non-survivors in regard to the proportion of patients with 25(OH)D <50 nmol/L (60.1% vs.64.0%, $p = 0.443$), ALT >33 U/L (11.3% vs. 14.2%, $p = 0.378$), ALT <9 U/L (9.2% vs.10.4%, $p = 0.682$), GGT >56 U/L (21.5% vs. 28.3%, $p = 0.099$), ALP >110 U/L (25.6 vs.34.0, $p = 0.057$), ferritin >370 μ g/L (28.6% vs.32.5, $p = 0.459$), and transferrin saturation <18% (86.0% vs.90.0%, $p = 0.307$).

ratio >2.5, (3) elevated PTH (>6.8 pmol/L), (4) vitamin D deficiency (<25 nmol/L) and (5) bilirubin >20 μ mol/L (Table 3).

The final multiple logistic regressions model which included all clinical and laboratory characteristics (as categorical variables) with $p < 0.100$ on univariate analysis identified seven independent factors associated with in-hospital mortality (Table 4). Consistent with the univariate analyses, these indicators were four of five aforementioned laboratory parameters (except bilirubin), and three (of eight) clinical pre-fracture characteristics (history of MI, CKD and COPD). The model correctly classified 81.6% of the cases. These 7 variables achieved a test AUC of 0.7469 (95%CI 0.6989–0.7950) suggesting that the model was able to identify HF persons with a high risk for in-hospital death. Calibration for the model was adequate (goodness-of-fit test: $\chi^2 = 1213.63$, $p = 0.1424$).

In order to make sure that CKD, hyperparathyroidism and vitamin D deficiency are independently correlated with in-hospital mortality additional analysis was done. There was no significant correlation between 25(OH)D and GFR (Pearson's correlation coefficient $r = -0.016$, $p = 0.519$), the strength of relationship between 25(OH)D and PTH was significant but very weak ($r = -0.086$, $p = 0.001$), and there was a moderate correlation between PTH and GFR ($r = -0.329$, $p < 0.001$); similar results were obtained when these three parameters were analysed as

Table 4

Independent predictors at admission of in-hospital death in older hip fracture patients.

Notes: Only statistically significant associations shown ($p < 0.05$). The multiple logistic regressions models included all clinical and laboratory characteristic (as categorical variables) with $p < 0.10$ on univariate analysis and adjusted for hip fracture type, age and gender; in separate models age >75 years, age >80 years, haemoglobin <120 g/L and haemoglobin <100 g/L have been evaluated.

Variable	OR	95%CI	P value
History of MI	2.56	1.38–4.74	0.003
CKD	2.34	1.45–3.78	0.001
COPD	2.16	1.25–3.74	0.006
Albumin <33 g/L	2.07	1.28–3.33	0.003
25(OH)D <25 nmol/L	1.94	1.23–3.04	0.004
PTH >6.8 pmol/L	1.94	1.20–3.13	0.007
GGT/ALT >2.5	1.63	1.05–2.54	0.030

Abbreviations: OR, odds ratio; CI, confidence interval; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease (glomerular filtration rate <60 ml/min/1.73 m²); PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase.

Notes: Only statistically significant associations shown ($p < 0.05$). The multiple logistic regressions models included all clinical and laboratory characteristic (as categorical variables) with $p < 0.10$ on univariate analysis and adjusted for hip fracture type, age and gender; in separate models age >75 years, age >80 years, haemoglobin <120 g/L and haemoglobin <100 g/L have been evaluated.

categorical variables [5(OH)D < 25 nmol/L, PTH > 6.8 pmol/L, GFR < 60 ml/min/1.73m²]: $r = -0.002$, $p = 0.949$; $r = -0.074$, $p = 0.003$; and $r = -0.223$, $p < 0.001$, respectively. Therefore, the risk factors (RFs) for in-hospital death were further assessed after excluding the patients with GFR < 60 ml/min/1.73m² ($n = 789$). Of 1031 individuals with GFR > 60 ml/min/1.73m², 34 (3.3%) had died. The multivariate logistic regression confirmed that elevated PTH (OR 1.94), vitamin D deficiency (OR 2.06), as well as hypoalbuminaemia (OR 2.34), GGT/ALT > 2.5 (OR 1.78) and history of MI (OR 4.74) remained independent predictors of in-hospital mortality (all $p < 0.010$) even in patients with GFR > 60 ml/min/1.73 m²; in this cohort/model only COPD lost its significance as an independent predictor (OR 1.71, $p = 0.271$).

The in-hospital mortality rate was 13.4% among individuals with a previous history of MI, 10.5% among patients with COPD, 9.5% – with CKD, 8.9% – with hypoalbuminaemia, 8.8%–with vitamin D deficiency, 8.3% –with hyperparathyroidism and 8.0% – with the ratio GGT/ALT > 2.5, while among those without the indicated characteristic the mortality rate was 5.4%, 5.4%, 3.3%, 5.2%, 5.1%, 4.1% and 5.1%, respectively. The in-hospital mortality rate was 0.48% (1/210) among patients without any of the seven RFs on admission, 2.0% among subjects with 1 RF, 5.2% – with 2 RFs, 9.3% – with 3 RFs, 13.0% – with 4 RFs, and 33.3% – with 5 RFs; in total, in the group with three or more RFs 11.8% died, and in the group with four or more RFs 16.8% died. The difference in mortality rates between subjects with 0 and only 1 RF was not statistically significant ($p = 0.257$). Comparison to those without any of the RFs, showed an OR for a fatal outcome of 11.5 in patients with 2 RFs, 21.4 in patients with 3 RFs and 42.1 in patients with ≥ 4 RFs. The proportion of patients with ≤ 1 RF was near 4 times higher among survivors than among those who died in the hospital (42.7% vs. 10.9%). Comparing to patients with ≤ 1 RF, the OR for in-hospital death was 3.6 in subjects with 2 RFs, 6.7 in subjects with 3 RFs and 13.2 in subjects with ≥ 4 RFs.

Taken together, these results imply that in older HF patients presence of ≥ 2 RFs contribute significantly and independently to the poor short-term outcome and may predict in-hospital death.

Prognostic impact of combined variables

The discriminatory ability of laboratory and clinical characteristics to identify at admission HF patients with a high risk to die during hospitalization was further assessed using the area under the receiver operating characteristic curve (AUC). The above-mentioned seven individual factors showed relatively low prognostic efficacy for predicting mortality. The greatest numerical AUC among the laboratory markers was only 0.615 (for PTH > 6.8 pmol/L) and among clinical conditions only 0.635 (for CKD); the AUC was lower than 0.600 for all other (except male gender) variables (Tables 1–3). Therefore, we evaluated the prognostic value of combined parameters focussing on the impact of metabolic variables.

Utilizing two admission parameters and adjusting for age and gender, the ORs for in-hospital death were 6.0–2.6 times higher compared to patients without the two tested abnormal characteristics (Table 5, Fig. 1). Combination of elevated PTH (> 6.8 pmol/L) with history of MI or combination of vitamin D deficiency (25(OH) D < 25 nmol/L) with CKD provided the best mortality risk estimates (OR 5.95, and OR 5.37, respectively). In individuals with hypoalbuminaemia (< 33 g/L) or GGT/ALT > 2.5 and elevated PTH, or CKD, or history of MI, or COPD the ORs for in-hospital death were 5.3–3.8-fold higher than in subjects without indicated combinations, while in patients with only one of these variables odds of death were only 3.3–1.3-times higher compared to subjects with both characteristics normal (Fig. 1). For example, compared to patients who had both albumin and PTH levels in the normal range, OR of death was 5.3-fold greater in individuals with both hypoalbuminaemia and elevated PTH (OR 5.23, 95% CI 2.84–9.82; $p < 0.001$), 2.1-fold higher in patients with only PTH elevated (OR 2.06, 95% CI 1.20–3.52; $p = 0.008$) and 1.3-fold higher in patients who had only hypoalbuminaemia (OR 1.35, 95% CI 0.59–3.10; $p = 0.481$). In general, the ORs in patients with any two of the seven independent RFs were about 2–3-fold greater than in subjects with only one of the two presented (Fig. 1).

ROC curve analysis of 2-factor combination for the discrimination of in-hospital HF non-survivors revealed the highest AUC for

Table 5

Prognostic value of combined biochemical and clinical parameters on admission for prediction in-hospital death in older hip fracture patients.

Notes: Only statistically significant associations shown ($p < 0.05$). Comparisons made to patients without indicated characteristics and data adjusted for age and gender.

*Comparison to patients with ≤ 1 risk factor and adjusted for age and gender.

Variables	OR	95%CI	P Value	AUC	Sensitivity, %	Specificity, %	PPV, %	NPV, %
PTH > 6.8 pmol/L + MI	5.95	2.83–12.51	< 0.001	0.674	32.4	93.0	16.9	96.9
25(OH)D < 25 nmol/L + CKD	5.37	2.77–10.44	< 0.001	0.698	58.5	81.0	12.6	97.7
Albumin < 33 g/L + PTH > 6.8 pmol/L	5.28	2.84–9.82	< 0.001	0.677	53.3	85.6	18.3	96.8
PTH > 6.8 pmol/L + CKD	5.20	2.74–9.89	< 0.001	0.695	79.7	59.1	11.4	97.8
GGT/ALT > 2.5 + MI	5.19	2.44–11.05	< 0.001	0.659	16.1	96.4	20	95.4
Albumin < 33 g/L + CKD	4.87	2.65–8.95	< 0.001	0.698	51.1	84.1	13.8	97.2
PTH > 6.8 pmol/L + COPD	4.80	2.36–9.74	< 0.001	0.671	37.8	89.7	14.1	97.0
GGT/ALT > 2.5 + PTH > 6.8 pmol/L	4.62	2.33–9.12	< 0.001	0.673	67.5	70.3	9.9	97.8
25(OH)D < 25 nmol/L + MI	4.54	1.63–12.66	0.004	0.657	8.9	98.1	18.5	95.7
GGT/ALT > 2.5 + CKD	4.33	2.39–7.84	< 0.001	0.694	62.7	74.1	11.7	97.8
GGT/ALT > 2.5 + COPD	3.72	1.91–7.41	< 0.001	0.662	19.0	94.1	15.8	95.3
25(OH)D < 25 nmol/L + PTH > 6.8 pmol/L	4.21	2.21–8.00	< 0.001	0.672	59.5	75.9	10.8	97.4
GGT/ALT > 2.5 + 25(OH)D < 25 nmol/L	3.16	1.74–5.76	< 0.001	0.658	32.7	86.8	12.5	95.7
Albumin < 33 g/L + 25(OH)D < 25 nmol/L	2.86	1.45–5.67	0.003	0.666	23.1	91.1	11.0	96.1
GGT/ALT > 2.5 + Albumin < 33 g/L	2.64	1.37–5.06	0.004	0.665	24.1	89.2	10.4	95.8
Any two factors*	3.12	1.46–6.63	0.003	0.699	71.4	59.1	5.2	98.5
Any three factors*	6.18	2.94–13.00	< 0.001	0.756	75.0	69.1	9.3	98.5
Any four factors*	7.88	3.46–17.98	< 0.001	0.816	63.0	85.2	13.0	98.5
Four or more factors*	11.12	5.19–23.85	< 0.001	0.839	73.0	83.0	16.8	98.5

Abbreviations: OR, odds ratio; CI, confidence interval; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; PTH, parathyroid hormone; MI, myocardial infarction; 25(OH)D, 25-hydroxyvitamin D; CKD, chronic kidney disease (glomerular filtration rate < 60 ml/min/1.73m²); GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; COPD, chronic obstructive pulmonary disease.

Notes: Only statistically significant associations shown ($p < 0.05$). Comparisons made to patients without indicated characteristics and data adjusted for age and gender.

*Comparison to patients with ≤ 1 risk factor and adjusted for age and gender.

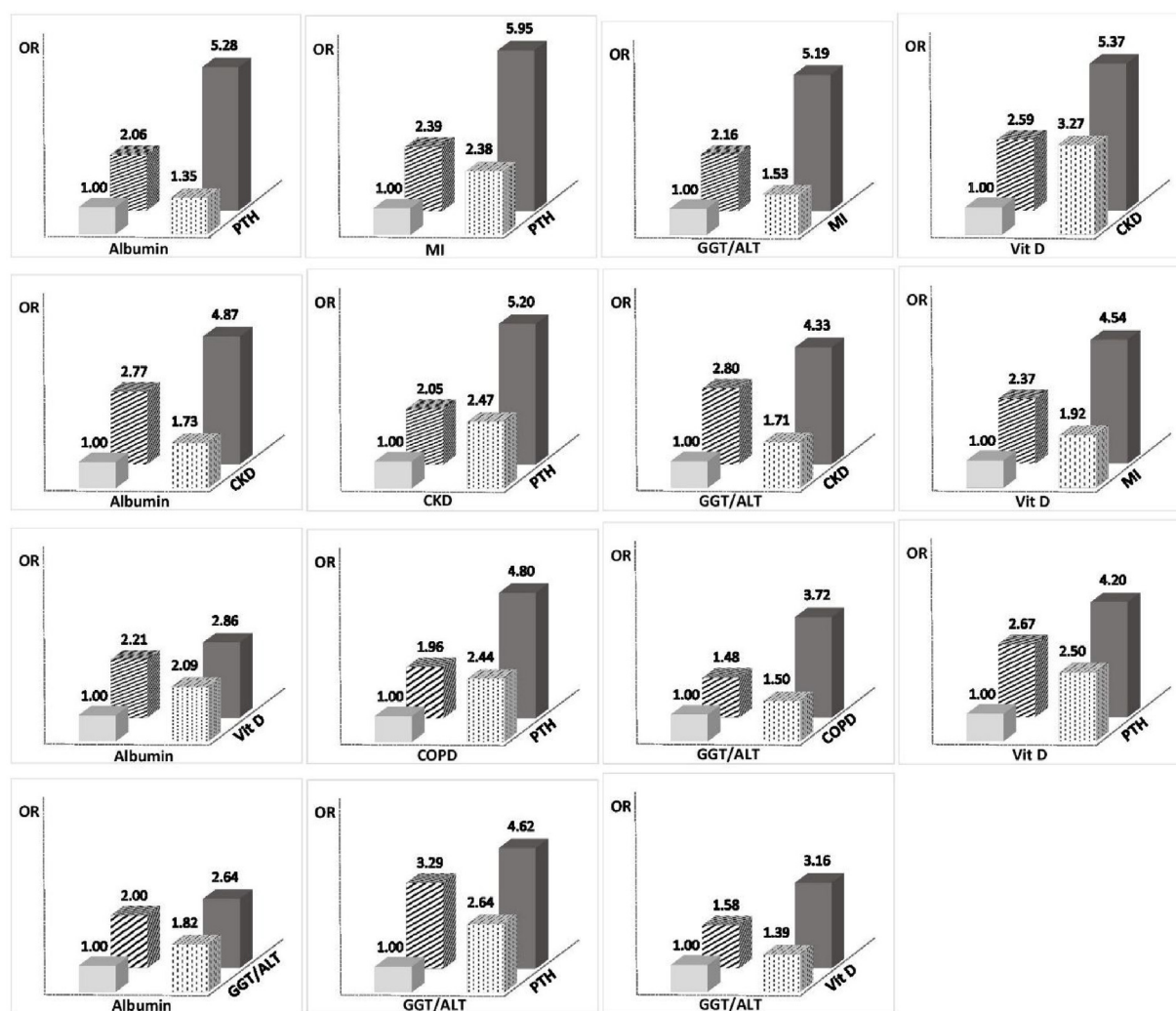


Fig. 1. Prognostic performance of the 2-marker combinations (comparing patients with and without indicated factors).

The laboratory parameters were dichotomised as follows: albumin <33 g/l vs. ≥33 g/l; PTH >6.8 pmol/L vs. ≤6.8 pmol/L; GGT/ALT >2.5 vs. ≤2.5; 25-hydroxyvitamin D <25 nmol/L vs. ≥25 nmol/L. All clinical characteristics were dichotomised as yes vs. no. The abnormal conditions are depicted on the right hand side.

Abbreviations: PTH, parathyroid hormone; CKD, chronic kidney disease; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; Vit D, 25-hydroxyvitamin D.

the combination of CKD with vitamin D deficiency (AUC 0.698, sensitivity 58.5%, specificity 81.0%) or with hypoalbuminaemia (AUC 0.698, sensitivity 51.1%, specificity 84.1%). The highest sensitivity demonstrated the combination of hyperparathyroidism with CKD (79.7%) or with GGT/ALT >2.5 (67.5%) and the highest specificity showed the combination of history of MI with hypoalbuminaemia (99.0%) or vitamin D deficiency (98.1%) or GGT/ALT >2.5 (96.4%), as well as the combination of COPD with hypoalbuminaemia (96.4%) or GGT/ALT >2.5 (94.1%). The high specificity and high negative predictive value (NPV) most of the combined characteristics indicate that the number of false positive tests is low. Presence of any two abovementioned characteristics would miss fatal outcome in 1.5–4.7 subjects for every 100 with a negative result (NPV = 95.3–98.5%).

Comparing to patients with ≤1 RF (after adjusting for age and gender), the risk of in-hospital death in HF subjects with any two RFs was 3.1 times higher (OR 3.12, AUC 0.699, sensitivity 71.4%, specificity 59.1%, PPV 5.2% and NPV 98.5% with positive and negative likelihood ratios of 1.746 and 0.483, respectively) and in subjects with three RFs was 6.2 times higher (OR 6.18, AUC 0.756, sensitivity 75.0%, specificity 69.1%, PPV 9.3% and NPV 98.5% with positive and negative likelihood ratios of 2.429 and

0.362, respectively), in patients with four or more RFs was 11.1 times higher (OR 11.12, AUC 0.839, sensitivity 73.0%, specificity 83.0%, PPV 16.8% and NPV 98.5% with positive and negative likelihood ratios of 4.302 and 0.325, respectively) (Table 5, Fig. 2). Each additional RF increased the OR for death 2.7-fold (OR 2.70, 95%CI 2.00–3.65, $p < 0.001$). These results further indicate the prognostic significance of combination of admission RFs. In subjects with ≤1 RF the probability of in-hospital death is low, but 1 in 19 patients with two RFs, 1 in 11 with three RFs and 1 in 4 with ≥4 RFs will die during hospitalization. The ROC analysis showed a fair predictive ability of ≥3 risk factors (vs. ≤1RF), with an AUC of 0.756 (95%CI 0.695–0.818); presence of any 4 RFs (vs. ≤1RF) reached an AUC of 0.816 (Table 5; Fig. 2). The small increase in AUC (0.023) when the cut-point was raised from 4 RF to ≥4 RF [0.816 vs. 0.839] suggests that presence of more than 4RF only slightly increases risk of death compared with those who already had 4 RF. Of note, the AUC of three or more RFs (vs. ≤1RF) for predicting in-hospital death showed better prognostic performance to that of the full model containing seven RFs (vs. 0 RF): AUC 0.756–0.839 vs. 0.747. The combination of any 2, 3 or 4 RFs (vs. ≤1 RF) achieved a NPV of 98.5% (95% CI 97.6–99.2%).

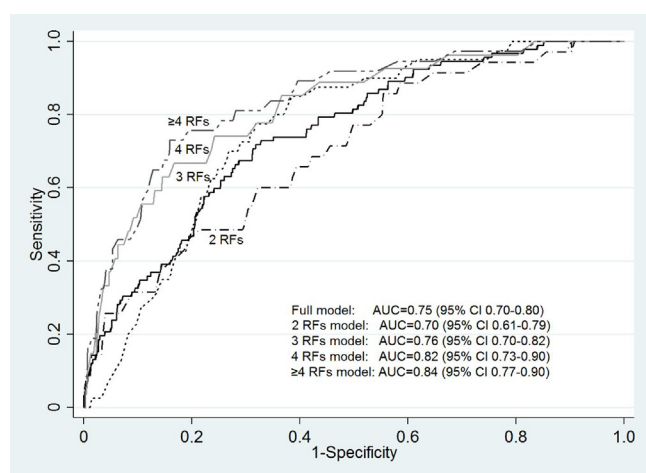


Fig. 2. Receiver operating characteristic (ROC) curves depicting the ability of the identified risk factors (laboratory and/or clinical) to predict in-hospital mortality in hip fracture patients. Solid line: ROC curve based on data from the full model (7 risk factors vs. 0 risk factors); 2 RFs – ≥4RFs: ROC curves based on combination of any 2, 3, 4 or ≥4 risk factors (vs. ≤1RF), respectively.

Abbreviations: AUC area under the curve; CI confidence interval.

Validation

In the validation cohort 23 (5.1%) of 455 HF patients died in the hospital. When the identified in the main cohort seven RFs were applied the AUC was 0.775 (goodness-of-fit test $p > 0.05$). In 29.3% of patients there was found 0 to 1 RF [mortality rate: observed 2.3%, predicted 2.0%]; 56% of patients had 2–3 RFs [mortality rate: observed 5.2%, predicted 6.9%], and 14.7% of patients had ≥4 RF [mortality rate: observed 14.6%, predicted 16.8%]. Comparing to low mortality risk (≤1 RF), presence of 2 RFs produced an AUC of 0.696, presence of 3 RFs produced an AUC of 0.761, presence of 4 RF produced an AUC of 0.779, and presence of ≥4 RFs produced an AUC of 0.813. These data are similar to that in the main cohort.

Discussion

The main findings of this single-centre study of 1820 consecutive older HF patients are as follows: (1) among 35 evaluated laboratory variables and 20 clinical characteristics at admission seven factors (four metabolic – hypoalbuminaemia, vitamin D deficiency, hyperparathyroidism and GGT/ALT ratio >2.5 and three clinical – history of MI, CKD and COPD) were found to be strong and independent indicators of in-hospital mortality; (2) the mortality rate increased stepwise as the number of these RFs increased (from 0.43% – none RF to 16.8% – ≥4RF); (3) the prognostic value of any single RF was low but combination of 2 or more RFs improved the prediction significantly, and AUC reached 0.839 when ≥4 RFs (vs. 0–1RF) were present. These easily identifiable RFs appeared as a useful objective tool for clinical risk stratification of HF patients at admission and, when combined, provided prognostic information more accurate than most of currently proposed models.

Indicators of in-hospital mortality

The reported in-hospital mortality rate in older HF persons ranges between 3.1%–3.3% [10,56] and 19.5% [11], and numerous studies found that short- and long-term mortality in both sexes is 2–8-fold higher compared to non-fractured community-dwelling subjects [7,57–59]. The excess in-hospital deaths among older patients with osteoporotic HF, a very heterogeneous population with multiple comorbidities, is a result of a cascade of different

pre- and post-fracture pathophysiological changes, involving multitude mechanisms and complex interplay in metabolic factors. Obviously, in-hospital mortality, in addition to on-admission characteristics, is significantly influenced by a variety of other biological and non-biological factors, including surgical delay [60], anaesthetic type [61,62], inappropriate planning, technique or management [63], post-operative complications (especially cardiovascular and infectious/inflammatory), hospital type and staffing volume [16]. Because of its integrative complexity the prognosis at admission remains a challenge.

Despite the paramount importance of understanding metabolic and cellular abnormalities underlying the multi-morbidity and poor outcomes of older HF patients, most of the previous studies on predictors of survival in the HF population centred on clinical characteristics. Limited research has examined the predictive value of the routinely obtained preoperative biochemical parameters in HF patients. In this study, a total of 55 laboratory and clinical on-admission variables were analysed for possible association with a fatal outcome by using univariate and multiple regression models.

According to our univariate analysis 14 of 35 laboratory parameters as continuous variables, age and male gender have been associated with in-hospital mortality (Tables 1 and 2). The multivariate analysis showed that only 5 variables (albumin, GGT/ALT ratio, PTH, GFR, and age) were independently associated with fatal outcome.

Because liver function markers (GGT, ALT, ALP and albumin, bilirubin levels) might change unequally it was recently proposed to evaluate the metabolic status/liver function reserve by combination of different biomarkers and to use such indices for prognosis and prediction. Following this logic approach we assessed 10 ratios (combined indices) regarding the outcome. Univariate analysis showed that 6 of 10 ratios were associated with in-hospital death. In non-survivors the GGT/ALT, ALT/ALP and GGT/bilirubin ratios were significantly higher, while albumin/ALP and albumin/bilirubin ratios were significantly lower than in survivors. However, in multivariate analysis only the GGT/ALT ratio remained as a significant and independent determinant of in-hospital mortality.

In our cohort, as in another [64], serum vitamin B12 levels were not associated with increased mortality, although in some [65–67], but not all [68] studies higher vitamin B12 concentration was reported as an independent predictor of mortality, especially in critically ill patients. Similarly, serum folate levels, indices of iron metabolism, thyroid function tests, and calcium, phosphate and magnesium concentrations did not differ in survivors and non-survivors.

The further multivariate analyses of laboratory parameters as categorical variables revealed four on-admission biomarkers, namely hypoalbuminaemia, elevated PTH, vitamin D deficiency and higher GGT/ALT ratio (>2.5), as independent and significant indicators of in-hospital death. Our findings are in line with other studies which reported in patients with HF the prognostic utility of hypoalbuminaemia [6,26,29–36,69–72], hyperparathyroidism [73–75], and vitamin D deficiency [75,76].

In addition to these previously established markers of poor/fatal outcome, we found the GGT/ALT >2.5 as a novel indicator of in-hospital mortality. The GGT/ALT ratio was more closely related to survival than the serum activities each of the two enzymes alone [of note, in the majority of the patients the activities of both enzymes were within the normal range]. The multivariate analyses of GGT/ALT ratio both as a continuous and as a categorical variable demonstrated its significant association with in-hospital mortality. The GGT/ALT ratio which represents the simultaneous alteration of GGT, a key enzyme of glutathione metabolism and a major antioxidant, and ALT, a specific marker of hepatocytes synthetic function, demonstrated the ability to capture outcome-associated

changes more adequate with a higher predictive value. The exact mechanism(s) underlying the association of this parameter with mortality remains unclear. Three dose-response meta-analyses found that the incidence of all-cause and cardiovascular mortality increases with the elevation of serum GGT level [46,77,78]. Low ALT was shown to be a strong and independent predictor of frailty and mortality in some studies [40,41,43,79–82], whereas other investigators reported a bimodal, U-shaped association of ALT with mortality [41,83]. Our observations are consistent with the data suggesting that mortality is associated with higher GGT and ALT [37] levels, but these markers did not reached significance in the multivariate analysis. The prognostic significance of GGT/ALT ratio has gained interest in various clinical circumstances, including prediction of post-surgical survival [84–90]. To our knowledge, this is the first report on the potential prognostic role of ten different liver function marker ratios in older HF patients suggesting that the GGT/ALT ratio >2.5 at admission is an acceptable parameter discriminating between survivors and non-survivors.

Analysis of 20 clinical and socio-demographic characteristics showed, in line with many previous studies, that the group of non-survivors was older, with a higher prevalence of males, subjects admitted from RCFs, complex comorbidities, including CKD, CAD, history of MI, COPD and impaired mobility requiring walking aids [4–6,10,12,13,15,17,19,21,23,91–98,99]. Although women had a higher prevalence of HF (3.2:1 in our cohort), the in-hospital mortality rate in men was almost 1.5-fold higher (7.9% vs. 5.4%). Interestingly, the mean age of non-survivors men and women was identical, although in the total cohort women were significantly older. HF type did not indicate a risk of a fatal outcome, as it was documented previously.

In contrast to some previous reports, in our study, dementia [14,19,100], anaemia [99,101–103], history of stroke or TIAs, hypertension, DM, Parkinson's disease, smoking status, and alcohol over-use did not significantly affect the mortality rate. In the multivariate analysis only three clinical characteristics – CKD, history of MI, and COPD – remained independent RFs for in-hospital mortality; apparently detrimental changes in renal, cardiovascular and pulmonary functions significantly and independently elevate an individual risk for fatal outcome. Associations between 5 other on-admission clinical parameters and in-hospital mortality found by the univariate analysis, however, disappeared, indicating that these factors are incorporated in the underlying chronic diseases. It appears that age [5,104,105] and male gender [5,106], traditional mortality indicators in HF patients, are confounding factors affecting survival, rather than independent prognostic factors.

The final multivariate model based on 14 candidate laboratory variables and 8 clinical characteristics (each with $p \leq 0.10$ on univariate analyses) identified the following seven factors as independent statistically significant on-admission indicators of in-hospital mortality: albumin <33 g/L, 25(OH)D <25 nmol/L, PTH >6.8 pmol/L, GGT/ALT >2.5 , CKD, history of MI, and COPD. Patients with any of these conditions died 2.6–1.6 times more than those without such characteristics. Among non-survivors 99.1% (108/109) of patients had at least one of these RFs. The in-hospital mortality rate increased stepwise as the number of risk factors increased: in subjects with none RF – 0.48%, with 1 RF – 2.0%, with 2 RFs – 5.2%, with 3RFs – 9.3%, and with ≥ 4 RFs – 16.8%. This observation suggests that the RFs influence the fatal outcome in a synergistic manner and could effectively stratify patients according to clinically meaningful differences in the expected outcome. We categorised the number of RFs into three different groups for prognostic stratification purposes: 0–1 RF – low risk (mortality rate 1.5%), 2–3 RFs – moderate risk (mortality rate 6.9%), and ≥ 4 RF – high risk (mortality rate 16.8%). Presence of ≥ 4 RF on admission identifies a subset of patients with a mortality rate 11-fold greater

compared to that with 0–1 RF. Importantly, despite the known interactions between the metabolic factors (GGT, ALT, albumin, 25 (OH)D and PTH), cardiac, renal and pulmonary systems [e.g., the weak but significant correlations between GFR and PTH as well as between 25(OH)D and PTH when evaluated both as continuous or categorical variables has been once again shown in this study], multivariate regression revealed each of the abovementioned RFs as independent indicators of in-hospital mortality. This observation is suggestive that there are other and more important determinants for each of these factors, and, therefore, presence of two or more of them resulted in a synergistic effect on poor outcome. Our results are novel with respect to combined use of specific laboratory and clinical parameters at admission as independent indicators of in-hospital mortality and provide an important pathophysiological characterisation of high risk HF patients. Knowledge of the underlying mechanisms may help to individualise interventions and target modifiable factors with a negative effect on time of admission.

Prognostic value of selected biochemical and clinical indicators of in-hospital mortality

Whilst ORs obtained from logistic regressions are useful for explaining associations of individual factors with a clinical event, the OR does not describe RF's ability to predict the event. For example, elevated PTH is a strong independent indicator of in-hospital death, but the ability of this variable to correctly predict whether or not an individual will die is limited. Each of the 7 laboratory and clinical on-admission characteristics identified as independent RFs for in-hospital mortality exhibited low prognostic performance for revealing a fatal outcome (AUCs of ≤ 0.635). These disappointing results are not surprising in such complex disease as osteoporotic HF with multiple conditions contributing to the outcome. The accuracy in predicting improved when based on combination of ≥ 2 RFs. With regard to presence of 2 RFs (vs. no such RFs), the highest AUC showed the combination of CKD with albumin <33 g/L (AUC 0.698), 25(OH)D <25 nmol/L (AUC 0.698), PTH >6.8 pmol/L (AUC 0.695) or GGT/ALT >2.5 (AUC 0.694), followed by combination of elevated PTH with low albumin (AUC 0.677) or history of MI (AUC 0.674) or GGT/ALT >2.5 (AUC 0.673). As in HF patients the in-hospital death is a relatively low-incidence outcome, high specificity of prognostic parameters is an important indicator for management decisions; the specificity was 98.1%–91.1% in five combinations and 84.1%–89.2% in five other combinations (Table 5). Furthermore, comparing to subjects with ≤ 1 RF, the AUC in patients with any 2 RFs was 0.699, with any 3 RFs 0.756 and with ≥ 4 RFs 0.839 (Table 5; Fig. 2).

Taken together, our study found that each of the on-admission RFs has only minimal prognostic value for prediction in-hospital mortality in HF patients, but combination of any 2 RFs increases significantly their discriminatory power, combination of 3 RFs shows a fair predictive ability and combination of ≥ 4 RFs demonstrates a good discrimination ability to identify subjects with the highest risk of a fatal outcome.

Importantly, when the RFs identified in the present study were used on a new dataset, the number of predicted and observed deaths were comparable to that in the main cohort: among patients with ≤ 1 RF (2.3% vs. 2.0%), with 2–3 RFs (5.2% vs. 6.9%) and with ≥ 4 RF (14.6% vs. 16.8%). Moreover, comparing to low mortality risk (≤ 1 RF), the AUC of any 2 RFs (0.696 vs. 0.699), any 3 RFs (0.761 vs. 0.756) and ≥ 4 RFs (0.813 vs. 0.839) in the validated and main cohorts practically were similar.

The reported in the literature instruments for predicting in-hospital or 30-day mortality had an AUC of: <0.68 – 0.74 [107], 0.71 [108], 0.72–0.76 [109], 0.76 [12], 0.78 [24], 0.80 [110], and 0.83 [111]. These data indicate that the seven on-admission RFs

identified in the present study exhibit an acceptable degree of predictive ability performing better than most of previously proposed prediction tools based on 6–14 mainly clinical variables [6,17,18,20,112,113].

Our study, in contrast to other models and scoring systems proposed for prediction HF mortality, does not include subjective variables (as The American Society of Anesthesiologists score, Charlson Comorbidity Index [13], does not require detailed information on comorbidities, peri-operative complications [16] and intraoperative data (as orthopaedic physiologic and operative severity score for the enumeration of mortality and morbidity [111,114], factors which make these systems too complex, time-consuming and difficult to use; not surprisingly, these instruments are relatively rarely used in clinical practice.

Practical implications and therapeutic considerations

Currently, there is no consensus in the literature on RFs on-admission that provide reliable prognostic information to stratify risk and guide clinical management of HF [16] as well as other surgical patients [115,116].

The causes and pathogenic mechanisms underlying in-hospital mortality in HF patients comprise a spectrum of many different factors including pre-fracture health state, response to injury and operative stress, and post-operative complications. Although the identified in this study independent RFs on-admission are only a part of complex conditions contributing to the fatal outcome and comprise several modifiable and irreversible factors, they offer an opportunity for both risk stratification and individualised therapeutic decisions. Clearly, accurate define of low, intermediate and high risk for in-hospital death in HF patients at admission is a key factor to improve outcomes. It may assist in surgical planning (appropriateness and most optimal choice of surgery) and counselling patients and their carers (more accurate informed consent taking into account patient's wishes for end-of-life care), help to optimise anaesthesiology strategy, timely suggest valid targets for preventive perioperative treatment and more efficient clinical care resources utilization.

Our findings show that subjects with ≥ 2 RFs on-admission are at greater risk of in-hospital death and need specific attention, more aggressive and appropriately individualised peri-operative management. Constellations of specific pre-fracture comorbidities and biomarkers generate valuable information to individualise the peri-operative treatment. Particular attention should be paid to those with a history of MI (careful haemodynamic monitoring, proper fluid resuscitation, use of beta-blockers, angiotensin converting enzyme-inhibitors, angiotensin receptor blockers, aldosterone antagonists, neprilysin inhibitor), CKD (avoidance of nephrotoxic medications, contrast-media insult), and COPD (appropriate long-acting inhaled therapy, good pulmonary toilet, avoidance of operative delays); regional or spinal (instead of general) anaesthesia should be considered [117–119], especially in COPD patients [120].

When any of these comorbidities is combined with higher PTH, or GGT/ALT ratio, or hypoalbuminaemia or vitamin D deficiency the risk of fatal outcome increases significantly. Our observations delineate the role of serum metabolic markers in prognostic assessment of HF patients on admission, and also indicate the importance to address the modifiable metabolic alterations in the pre-fracture management of elderly persons. The impact of correction of nutritional status (hypoalbuminemia) on postoperative outcomes has already been recognized [72,121,122]. Moreover, hypoalbuminaemia, vitamin D deficiency, hyperparathyroidism and higher GGT/ALT ratio appear to be among the main modifiable factors and pathways contributing to both osteoporotic fractures and in-hospital mortality in the elderly patients. Therefore, the pre-fracture preventive strategies should focus on treatments that

integrate these targets. Such approach (e.g. addressing vitamin D deficiency) may exert numerous beneficial effects on falls, fractures and common chronic diseases prevention [123,124].

Limitations

Several potential limitations of this study should be noted. First, our analyses were based on single on-admission measurements of routine laboratory parameters, deaths were not stratified according to causes (e.g., cardiovascular, infection) (because the number of observations for each cause was small). Second, time and type of surgery, type of anaesthesia and other factors which may influence in-hospital survival were not analysed. Third, the observational nature of the study precludes from concluding causality. Fourth, the effects of unmeasured potential confounders (such as time/delay of surgery, socioeconomic factors) or complex interactions between covariates cannot be excluded. Finally, the study population consisted mainly of Caucasian patients at only one centre, and therefore, the outcomes cannot yet be generalized and may not be applicable to other race/ethnicity.

The strengths of the study include the relatively large number of HF patients, extensive evaluation and validation of preoperative data on multiple laboratory and clinical variables, biomarker ratios and biomarker-clinical combinations with a focus on prediction fatal outcome at admission. In all our models the variance inflation factor ranged between 1.03 and 1.08 indicating that the amount of multicollinearity was not significant. We are unaware of any study to date that has examined the topic integrating detailed laboratory and clinical datasets.

Conclusions

In elderly HF patients, seven easily identifiable at admission characteristics, including four routine inexpensive biomarkers, are strong independent indicators of in-hospital mortality. These RFs when used in combination (≥ 2 RFs) show a fair predictive ability and can be helpful for timely risk stratification and individualised management of these complex patients by surgeons and physicians. Further studies are needed to better understand the pathophysiology underlying fatal outcome in HF patients and to validate our findings.

Conflict of interest statement

The authors have no conflicts of interest to report. There was no funding for this study.

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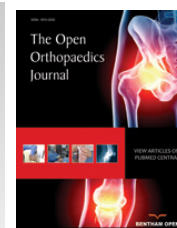
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The Open Orthopaedics Journal

Content list available at: www.benthamopen.com/TOORTHJ/

DOI: 10.2174/1874325001812010486, 2018, 12, 486-503



RESEARCH ARTICLE

Prognostic Significance Of Serum Urea Concentration at Admission in older patients with hip fracture

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Received: July 26, 2018

Revised: October 31, 2018

Accepted: November 09, 2018

Abstract:

Background:

There are unmet needs in objective prognostic indicators for Hip Fracture (HF) outcomes.

Objectives:

To evaluate the determinants and prognostic impact of elevated serum urea, a key factor of nitrogen homeostasis, in predicting hospital mortality, inflammatory complications and length of stay in HF patients.

Methods:

In 1819 patients (mean age 82.8±8.1 years; 76.4% women) with osteoporotic HF, serum urea level at admission along with 22 clinical and 35 laboratory variables were analysed and outcomes recorded. The results were validated in a cohort of 455 HF patients (age 82.1±8.0 years, 72.1% women).

Results:

Elevated serum urea levels (>7.5mmol/L) at admission were prevalent (44%), independently determined by chronic kidney disease, history of myocardial infarction, anaemia, hyperparathyroidism, advanced age and male gender, and significantly associated with higher mortality (9.4% vs. 3.3%, $p<0.001$), developing a high postoperative inflammatory response (HPIR, 22.1% vs. 12.1%, $p=0.009$) and prolonged hospital stay (>20 days: 31.2% vs. 26.2%, $p=0.021$). The predictive value of urea was superior to other risk factors, most of which lost their discriminative ability when urea levels were normal. Patients with two abnormal parameters at admission, compared to subjects with the normal ones, had 3.6-5.6 -fold higher risk for hospital mortality, 2.7-7.8-fold increase in risk for HPIR and 1.3-1.7-fold higher risk for prolonged hospital stay. Patients with increased admission urea and a high inflammatory response had 9.7 times greater mortality odds compared to patients without such characteristics.

Conclusion:

In hip fracture patients admission serum urea is an independent and valuable predictor of hospital outcomes, in particular, mortality.

Keywords: Serum urea, Hip fracture, Hospital outcomes, Prognosis, Prediction, Mortality.

1. INTRODUCTION

In the last two decades, serum urea concentration, the terminal product of protein metabolism, received much

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attention as a simple and reliable biochemical parameter for predicting adverse outcomes, especially short- and long-term mortality, in various medical and surgical settings including cardiovascular, cerebrovascular, respiratory, liver, pancreatic, septic and critically ill patients [1 - 11]. Elevated urea has been shown to be an independent and a better prognostic indicator of mortality than the Glomerular Filtration Rate (GFR) and creatinine in patients with acute and chronic cardiovascular diseases [2, 5 - 7, 12, 13].

However, the value of urea for risk stratification of hospital outcomes in Hip Fracture (HF) patients has not been evaluated systematically. Reports on the prognostic significance of serum urea level in HF patients are limited, conflicting and focussed only on predicting mortality [14 - 18]. Moreover, although liver is the main ureagenic organ and routine hepatocyte function parameters (albumin levels, enzyme activities) have been found to be valuable prognostic indicators for poor outcomes in HF patients [19 - 22], no study was performed on the relationship between serum urea, liver function characteristics at admission and outcomes in HF.

The aims of this study were to assess in a representative sample of older HF patients (1) the prevalence and determinants of elevated serum urea level at admission, (2) its association with short-term outcomes, and (3) the potential prognostic impact alone and in combination with clinical and other biochemical characteristics, focussing on liver-specific and protein metabolism-related variables.

2. PATIENTS AND METHODS

2.1. Patients

This study is based on prospectively collected socio-demographic, clinical (comorbidities, complications, medication used, hospital outcomes) and laboratory data on consecutive patients with Hip Fracture (HF) admitted to the Department of Orthopaedic Surgery of the Canberra Hospital (university-affiliated 672-bed tertiary care center) from January 2000 to January 2013. Patients who had high trauma or subtrochanteric fracture as well as pathological HF due to primary or metastatic bone cancer, multiple myeloma, Paget disease or primary hyperparathyroidism, or who had incomplete data on admission were excluded. In total, 1819 older (≥ 60 years of age) patients (mean age 82.8 ± 8.1 years; 76.4% women; 94.6% Caucasian) with low-trauma osteoporotic HF were finally included in the study.

The study was approved by the Australian Capital Territory Health Human Research Ethical Committee and waived the requirement for written consent as only routinely collected and anonymized before analysis data were used.

2.2. Validation Dataset

A retrospective analysis of a second cohort included data (obtained from electronic medical and administrative records) from 455 consecutive older (≥ 60 years of age) patients (mean age 82.1 ± 8.0 years, 72.1% women) with osteoporotic HF who were treated at the Canberra Hospital between 2013 and 2015.

2.3. Laboratory Tests

In each patient, fasting venous blood samples were collected on admission and the following assays performed: urea and electrolytes, complete blood count, C-Reactive Protein (CRP), liver (Alanine aminotransferase [ALT], Gamma-Glutamyl Transferase [GGT], alkaline phosphatase [ALP], bilirubin and albumin) and thyroid (thyroid stimulating hormone [TSH], and free thyroxine [T4]) function tests, 25 (OH) vitamin D [(25(OH)D], intact parathyroid hormone (PTH) and indices of iron metabolism (iron, ferritin, transferrin, Transferrin Saturation [TSAT]) using standard methods and commercially available kits as we described previously [20]. In all patients, glomerular filtration rate (GFR, by standardized serum creatinine-based formula normalized to a body surface area of 1.73 m^2) was estimated. Chronic kidney disease (CKD, $\geq$ stage 3) was defined as a $\text{GFR} < 60 \text{ mL/min/1.73 m}^2$. The cut-off level for elevated serum urea was set at $> 7.5 \text{ mmol/L}$ (upper limit of normal range). The cut-offs selected by other researchers varied between 5 and 15.4 mmol/L , but in most studies were between 5 and 10.0 mmol/L [7, 18, 23 - 28], therefore, we also analysed the effects of $\text{urea} > 10.0 \text{ mmol/L}$. Deficiency of vitamin D was defined as $25(\text{OH})\text{D} < 25 \text{ nmol/l}$ and insufficiency as $25(\text{OH})\text{D} < 50 \text{ nmol/l}$. Hyperparathyroidism (SHPT) was defined as elevated serum PTH ($> 6.8 \text{ pmol/l}$). In total, 22 clinical and 35 biochemical and haematological variables were analysed in relation to admission serum urea concentration and its prognostic and predictive value.

2.4. Outcomes

The main postoperative outcomes studied were: (1) in-hospital mortality, (2) complications associated with a High Postoperative Inflammatory Response (HPIR) defined as CRP>150mg/L after the 3rd postoperative day and (3) Length Of Stay (LOS).

2.5. Statistical Analyses

All continuous variables are expressed as mean $\pm$ Standard Deviation (SD) and compared using analysis of variance; categorical parameters are presented as frequency (percentage) and compared by Chi-square and Fisher's exact tests. The associations between urea at admission with each other studied variable (continuous and categorical) and with hospital outcomes were assessed using Pearson's correlation coefficients (log-transformed variables), univariate and multivariate logistic regression models. Potential confounding variables (demographic, clinical and laboratory) with statistical significance ≤ 0.100 in the univariate analysis were included in multivariate models to identify independent factors associated with elevated admission urea and with hospital outcomes. Data are presented with 95% Confidence Intervals (CI). The final predicting models were developed by stepwise logistic regression. The Hosmer-Lemeshow goodness of fit test was used to assess model performance. To quantify the significance of multicollinearity phenomena in regression analyses the variance inflation factor was calculated.

The individual predictive abilities of urea levels, other laboratory and clinical parameters of interest were evaluated by the Receiver Operating Characteristic (ROC) analyses and the accuracy was expressed as area under curve (AUC); the sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), accuracy, positive likelihood ratio (LR+), and negative likelihood ratio (LR-) were also calculated. Similarly, we assessed the predictive performances of any two eligible variables combined (pairs). Statistical significance was accepted at the $p < 0.05$ level (two-tailed). All statistical analyses were performed using the Stata software version 10 (StataCorp, College Station, TX, USA).

3. RESULTS

3.1. Clinical Characteristics of Patients With and Without Elevated Urea at Admission

The patients' socio-demographic, clinical and laboratory characteristics and short-term outcomes categorised by serum urea at admission are shown in Table 1. Urea levels were elevated in 800 (44.0%) HF patients, and in both genders, these patients were older (on average +2.6 years for females and +5.4 years for males); men were significantly younger than women, but the difference in mean age in the group with increased urea was 1.5 years vs. 5.3 years in the group with normal urea. Patients with elevated urea compared to those with normal urea levels were more likely to be admitted from a permanent Residential Care Facility (RCF), more commonly had a history of CKD, hypertension, Coronary Artery Disease (CAD), Myocardial Infarction (MI), anaemia and diabetes mellitus type 2 (DM), but were less likely to have previously a Cerebrovascular Accident (CVA), to be current smokers or alcohol over-users (>3 times per week). There was no significant difference between the two groups in terms of 7 other chronic comorbidities and lifestyle factors, including HF type and dementia. Among 35 laboratory parameters tested, 7 demonstrated statistical significance. Patients with elevated urea compared to those with normal urea, in addition to expected worse renal function (higher creatinine levels, lower GFR) had significantly higher levels of PTH, ALP and 25(OH)D and lower bilirubin concentrations (Table 1).

3.2. Determinants of Elevated Serum Urea Levels at Admission

Multivariate logistic regression analysis adjusting for all of the univariate clinical and laboratory variables associated with elevated serum urea on admission (with $p \leq 0.100$), as well as for HF type and pre-admission use of any diuretics (frusemide, thiazides, spironolactone), angiotensin-converting enzyme inhibitors and angiotensin 2-receptor blockers, revealed as significant and independent correlates of urea>7.5mmol/L the following six factors: CKD, history of MI, anaemia, hyperparathyroidism, advanced age and male gender (Table 2).

Table 1. Socio-demographic, clinical and laboratory characteristics and in-hospital outcomes in older hip fracture patients by serum urea levels on admission.

Variables	All patients (n=1819)	Urea>7.5 mmol/L (n=800)	Urea≤7.5 mmol/L (n=1019)	P Value
Age, years				
Females	83.4±7.7	84.9±7.3	82.3±7.9	<0.001
Males	80.6±9.0	83.4±7.2	78.0±9.7	<0.001
Both genders	82.8±8.1	84.5±7.3	81.4±8.5	<0.001
Age >75 years, n (%)	1511(83.1)	719(89.9)	792(77.7)	<0.001
Age >80 years, n (%)	1288(70.8)	629(78.6)	659(64.7)	<0.001
Males/Females, n	429/1390	206/594	223/796	0.054
From RCF, n (%)	636(35.0)	300(37.5)	336(33.0)	0.044
CKD, n (%)	789(43.4)	566(70.8)	223(21.9)	<0.001
CAD, n (%)	49(26.9)	261(32.6)	229(22.5)	<0.001
MI, n (%)	133(7.3)	84(10.5)	49(4.8)	<0.001
HT, n (%)	937(51.5)	451(56.4)	486(47.7)	<0.001
Anaemia:				
Hb<110g/L, n (%)	355(19.5)	194(24.3)	161(15.8)	<0.001
Hb<120g/L, n (%)	699(38.4)	370(46.3)	329(32.3)	<0.001
CVA, n (%)	219(12.0)	83(10.4)	136(13.3)	<0.053
DM, n (%)	175(9.6)	92(11.5)	83(8.1)	0.016
Smoker, n (%)	100(5.5)	33(4.1)	67(6.6)	0.024
Alcohol+, n (%)	92(5.1)	25(3.1)	68(6.6)	0.001
Urea, mmol/L	8.5±7.4	12.4±9.8	5.4±1.4	<0.001
Creatinine, µmol/L	92.9±60.0	119.0±80.3	72.3±20.5	<0.001
GFR, ml/min/1.73m ²	62.5±22.1	49.1±20.5	72.9±17.2	<0.001
PTH, pmol/L	8.5±7.4	10.4±9.1	7.1±5.3	<0.001
25(OH)D, mmol/L	48.3±28.8	50.3±29.2	46.7±28.4	0.016
ALP, IU/L	100.8±70.7	106.5±86.7	96.3±54.7	0.003
Bilirubin, µmol/L	12.4±8.1	11.7±6.8	12.9±8.9	0.003
Outcomes:				
LOS, days	19.0±22.3	20.5±25.7	17.8±19.2	0.011
LOS>20 days, %	28.4	31.2	26.2	0.021
HPIR, %	16.5	22.1	12.1	0.012
Died, %	6.0	9.4	3.3	<0.001

Abbreviations: RCF, residential care facility (permanent); CKD, chronic kidney disease (GFR<60 ml/min/1.73m²); CAD, coronary artery disease; MI, history of myocardial infarction; CVA, cerebrovascular accident; DM, diabetes mellitus type 2; Hb, haemoglobin; Alcohol+, alcohol over-user (≥3 drinks/week); GFR, glomerular filtration rate; PTH, parathyroid hormone; 25(OH)D, 25(OH) vitamin D; ALP, alkaline phosphatase; LOS, length of hospital stay; HPIR, high postoperative inflammatory response (C-reactive protein >150mg/L after the 3rd postoperative day).

Notes: Continuous data are expressed as mean ± SD and categorical data as number (%). Only statistically significant associations shown. There was no significant differences between patients with and without elevated serum urea on admission in prevalence of hip fracture type (cervical fracture: 51.0% vs. 55.2%, p=0.074), dementia (33.7% vs. 31.5%, p=0.335), chronic obstructive pulmonary disease (11.7% vs. 11.4%, p=0.900), transient cerebral ischaemic attack (7.8% vs. 7.9%, p=0.956), Parkinson's disease (4.9% vs. 4.7%, p=0.855), walking aids users (40.0% vs. 36.1%, p=0.077) or ex- smokers (12.6 vs. 13.2%, p=0.713). There were also no significant differences between the two groups with respect to mean values of albumin (36.9 vs. 36.8g/L, p=0.921), alanine aminotransferase (ALT, 23.6 vs. 25.9IU/L, p=0.471), gamma-glutamyl transferase (GGT, 55.8 vs. 57.8 IU/L, p=0.696), GGT/ALT ratio (2.9 vs. 2.9, p=0.970), transferrin saturation (10.9 vs. 10.9%, p=0.948) and CRP (99.8 vs. 89.2mg/L, p=0.338).

3.2. Correlations of Urea, Clinical and Other Laboratory Parameters with Outcomes (Pearson Correlation Coefficients)

In Pearson correlation analysis, urea as a log-transformed continuous variable showed stronger correlation with in-hospital death ($r=0.181$, $p<0.001$) than log-GFR ($r=-0.174$, $p<0.001$), log-creatinine ($r=0.169$, $p<0.001$), log-PTH ($r=0.145$, $p<0.001$), log-albumin ($r=-0.073$, $p=0.002$), log-25(OH)D ($r=-0.049$, $p=0.041$) and log-age ($r=0.081$, $p<0.001$). Log-urea did not correlate with HPIR ($r=0.090$, $p=0.092$) and LOS >20 days ($r=0.038$, $p=0.106$), while log-creatinine ($r=0.128$, $p=0.017$; $r=0.071$, $p=0.003$, respectively) and log-25(OH)D ($r=0.118$, $p=0.031$; $r=-0.077$, $p=0.001$, respectively) did. However, pairwise comparisons among all abovementioned variables showed, as would be expected,

that log-urea significantly correlated with log-creatinine ($r=0.634$, $p<0.001$), log-GFR ($r=-0.621$, $p<0.001$), log-PTH ($r=0.277$, $p<0.001$), log-haemoglobin ($r=-0.125$, $p<0.001$), log-25(OH)D ($r=0.049$, $p=0.043$) and log-age ($r=0.254$, $p<0.001$), but not with log-albumin ($r=-0.016$, $p=0.488$) or log-GGT/ALT ratio ($r=0.022$, $p=0.349$). All described associations were significant in both males and females when checked separately.

Table 2. Independent and significant correlates of elevated serum urea (>7.5 mmol/L) on admission in older patients with hip fracture.

Variables	OR	95%CI	P value
CKD	7.08	5.61-8.94	<0.001
History of MI	2.25	1.46-3.48	<0.001
PTH >6.8 pmol/L	1.69	1.35-2.12	<0.001
Anaemia (Hb <120 g/L)	1.45	1.15-1.83	0.002
Gender (male)	1.63	1.25-2.14	<0.001
Age (year)	1.02	1.00-1.03	0.040

Abbreviations: CKD, chronic kidney disease (GFR <60 ml/min/1.73m²); MI, myocardial infarction; PTH, parathyroid hormone; Hb, haemoglobin; OR, odds ratio; CI, confidence interval.

Notes: The multivariate logistic regression model included all variables with $p\leq 0.100$ on univariate analysis as well as hip fracture type (cervical, trochanteric) and pre-admission use of any diuretics (frusemide, thiazides, spironolactone), angiotensin-converting enzyme inhibitors and angiotensin 2-receptor blockers.

When we analysed the data as categorical variables, among all clinical and laboratory variables tested, urea >7.5 mmol/L demonstrated the third highest Pearson's correlation coefficient with mortality ($r=0.126$, $p<0.001$) comparable to that of creatinine $>90\mu$ mol/L ($r=0.131$, $p<0.001$) and GFR <60 ml/min/1.73m² ($r=0.129$, $p<0.001$), and higher than all other parameters, including PTH >6.8 pmol/L ($r=0.108$, $p<0.001$), 25(OH)D <25 nmol/L ($r=0.076$, $p=0.002$), albumin <33 g/L ($r=0.065$, $p=0.006$), GGT/ALT >2.5 ($r=0.060$, $p=0.012$), haemoglobin <110 g/L ($r=0.057$, $p<0.015$), history of MI ($r=0.088$, $p<0.001$), COPD ($r=0.068$, $p=0.004$), male gender ($r=0.045$, $p=0.053$), and age >75 years ($r=0.058$, $p=0.014$). Urea >7.5 mmol/L and creatinine $>90\mu$ mol/L were the only two parameters which correlated significantly with each of the three studied outcomes; in addition to in-hospital death, both elevated urea and creatinine were associated with HPIR ($r=0.133$, $p=0.012$; $r=0.162$, $p=0.002$, respectively) and LOS >20 days ($r=0.054$, $p=0.021$; $r=0.082$, $p=0.001$, respectively). Other parameters associated with HPIR were anaemia (haemoglobin <110 g/L, $r=0.140$, $p<0.009$) and 25(OH)D <25 nmol/L ($r=-0.121$, $p=0.026$), whereas LOS >20 days correlated with CKD ($r=0.065$, $p=0.006$), PTH >6.8 pmol/L ($r=0.050$, $p=0.039$) and albumin <33 g/L ($r=0.049$, $p=0.037$). The analysis showed also a significant interaction between in-hospital death and HPIR ($r=0.166$, $p=0.002$), but not between mortality and LOS or between HPIR and LOS.

3.3. Urea and Hospital Outcomes (Regression Multivariate Analyses)

Admission urea as a continuous variable (adjusted for age and gender) was significantly associated with in-hospital mortality: for each mmol/L increment in urea concentration the risk of a fatal outcome increased by 3.3% (OR 1.033; 95%CI 1.016-1.050; $p<0.001$). Other laboratory parameters (as continuous variables adjusted for age and gender) significantly associated with mortality were albumin (OR 0.948; 95%CI 0.917-0.98; $p=0.002$), GFR (OR 0.970; 95%CI 0.961-0.980; $p<0.001$), GGT/ALT ratio (OR 1.049; 95%CI 1.017-1.082; $p=0.002$), creatinine (OR 1.005; 95%CI 1.003-1.007; $p<0.001$) and ALT (OR 1.002; 95%CI 1.000-1.004; $p=0.042$). None of these biochemical variables was significantly associated with HPIR, but admission concentrations of albumin (OR 0.978; 95%CI 0.961—0.996; $p<0.015$) and creatinine (OR 1.002; 95%CI 1.000-1.004; $p<0.021$) were indicative for LOS >20 days.

Serum urea on admission >7.5 mmol/L was found in 76(69.7%) of 109 non-survivors [in 51(46.8%) of them urea was >10 mmol/L], in 177(59.0%) of 300 patients with a HPIR and in 254 (49.1%) of 517 patients with LOS >20 days. In comparison to individuals with normal urea, patients with urea above 7.5mmol/L evidenced adverse in-hospital outcomes (Tables 1 and 3): significantly higher mortality rates (9.4% vs. 3.3%, OR 2.60, $p<0.001$), higher incidence of HPIR (22.1% vs. 12.1%, OR 2.21, $p=0.009$), prolonged hospital stay (+2.7 days, $p=0.011$) and a higher proportion of long-stayers (>20 days: 31.2% vs. 26.2%, $p=0.021$, OR 1.20, $p=0.087$); all presented ORs were adjusted for age and gender. Analysis of the urea/albumin ratio >5.5 (median) showed that this parameter was no better than urea >7.5 mmol/L for predicting risks of mortality (OR 2.64, 95%CI 1.69-4.14, $p<0.001$) or HPIR (OR 1.96, 95%CI 1.09-3.54, $p=0.025$).

Table 3. Risk of in-hospital death and developing a high postoperative inflammatory response (HPIR) in older patient with hip fracture by the level of urea at admission.

Model	Urea 7.5 -10.0 mmol/L (n=410)			Urea >10.0 mmol/L (n=390)			Urea >7.5 mmol/L (n=800)		
	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
In-hospital mortality									
1	1.82	1.07-3.10	0.028	4.41	2.80-6.96	<0.001	3.00	1.98-4.54	<0.001
2	1.62	1.02-2.78	0.049	3.70	2.31-5.91	<0.001	2.60	1.70-3.98	<0.001
3	1.52	0.91-2.87	0.102	1.96	1.03-3.73	0.041	1.82	1.07-3.10	0.027
4	4.09	1.05-22.43	0.05	6.62	1.32-33.16	0.021	3.10	1.03-10.16	0.044
High postoperative inflammatory response (HPIR)									
1	1.82	1.02-3.61	0.046	2.17	1.06-4.43	0.034	2.05	1.16-3.64	0.014
2	2.17	1.06-4.43	0.034	1.91	1.02-3.90	0.04	2.21	1.21-4.01	0.009
3	2.15	0.99-4.73	0.052	2.12	1.02-3.02	0.041	2.18	1.07-4.42	0.031

Abbreviations: OR, odds ratio; CI, confidence interval.

Notes: Patients with admission urea<7mmol/L (n=1019) was the reference group (OR 1.0). Model 1, unadjusted analysis; Model 2, adjustment for age and gender; Model 3, adjustment for all admission variables with p≤0.010 on univariate analysis; Model 4, adjustment as in Model 3 and for HPIR (with CRP>150mg/L).

After further adjustments for potential confounders known to be associated with poor outcomes (all variables with p value≤0.100 on univariate analysis) including history of CKD, MI, DM, COPD, anaemia, albumin<33g/L, GGT/ALT>2.5, PTH>6.8pmol/L and 25(OH)D<25nmol/L, the significant relationships remained between admission urea >7.5mmol/L and hospital mortality (OR 1.82, p=0.027) as well as with HPIR (OR 2.18, p=0.031); in a fully adjusted model elevated urea failed to identify patients with LOS>20 days (Table 3). In the multivariate analysis, in addition to elevated urea, independent predictors of mortality included history of MI (OR 2.35, 95%CI 1.26-4.38, p=0.007), COPD (OR 2.16, 95%CI 1.24-3.74, p=0.006), albumin<33g/L (OR 2.08, 95%CI 1.28-3.35, p=0.003), 25(OH)D<25nmol/L (OR 1.99, 95%CI 1.26-3.14, p=0.003), CKD (OR 1.80, 95%CI 1.06-3.07, p=0.030), PTH>6.8pmol/L (OR 1.79, 95%CI 1.10-2.91, p=0.019) and GGT/ALT>2.5 (OR 1.64, 95%CI 1.05-2.55, p=0.029); the Hosmer–Lemeshow goodness of-fit test indicated good calibration of the model (p=0.853). Independent predictors of HPIR, in addition to elevated urea, were COPD (OR 2.62, 95%CI 1.18-5.84, p=0.018), 25(OH)D<25nmol/L (OR 2.50, 95%CI 1.09-5.56, p=0.032) and GGT/ALT>2.5 (OR 1.88, 95%CI 1.01-3.50, p=0.047); goodness of-fit test p=0.7258. Independent predictors of LOS>20 days were COPD (OR 1.46, 95%CI 1.01-2.12, p=0.043), albumin<33g/L (OR 1.32, 95%CI 1.01-1.72, p=0.041) and age (OR 1.02, 95%CI 1.00-1.04, p=0.024), but not elevated urea; goodness of-fit test p=0.3996.

We also evaluated in multivariate logistic models (all preoperative parameters with p<0.100 on univariate analysis included) predictors of poor outcomes in the subgroup of patients with admission urea >7.5mmol/L. Independent factors for a fatal outcome in this group were PTH>6.8pmol/L (OR 2.53, 95%CI 1.36-4.69, p=0.003), history of MI (OR 2.47, 95%CI 1.25-4.88, p=0.009) and albumin<33 g/L (OR 2.30, 95%CI 1.29-4.07, p=0.005); the model explained 19.7% of the total variance (R^2) in mortality. As independent predictors for HPIR were identified COPD (OR 4.96, 95%CI 1.38-17.81, p=0.014) and anaemia (haemoglobin<110g/L, OR 3.32, 95%CI 1.28-8.63, p=0.014); R^2 =17.4%. In patients with elevated urea at admission who developed a HPIR the mortality risk increased by 4.6 fold (OR 4.56, 95%CI 1.59-13.07, p=0.005). LOS>20 days was predicted by on admission low transferrin saturation (TSAT<18%, OR 1.72, 95%CI 1.23-2.39, p=0.001), vitamin D insufficiency (25(OH)D<50nmol/L, OR 1.32, 95%CI 1.03-1.70, p=0.027) and advanced age (OR 1.04, 95%CI 1.02-1.05, p<0.001); R^2 =5.3%. The persistence of main independent risk factors for hospital outcomes in the total cohort and in patients with raised urea indicates the consistency of our models.

To better characterize the association between preoperative urea levels and postoperative outcomes, patients were categorized into 3 subgroups (Table 3): with normal (<7.5mmol/L, n=1019[56.0%]), moderately increased (7.5 - 10.0mmol/L, n=410 [22.5%]) and high urea levels (>10.0mmol/L, n=390 [21.4%]). In the 3 subgroups, the postoperative mortality rates were 3.3%, 5.8% (OR 1.82, p=0.028), and 13.1% (OR 4.41, p < 0.001), respectively, HPIR developed in 12.6%, 20.7% (OR 1.82, p=0.046), and 21.5% (OR 2.17, p =0.034) of patients, respectively, and LOS>20 days had 26.1%, 30.1% (OR 1.22, p= 0.120), and 32.4% (OR 1.36, p = 0.019), respectively. After adjustments for age and gender, compared to patients with normal admission urea, the risk of a fatal outcome in patients with urea between 7.5 and 10.0mmol/L was 1.6 times higher, and in subjects with urea>10mmol/L was 3.7 times higher being 2.3 times higher than in patients with urea 7.5-10mmol/L (OR 2.34, p=0.001). After further adjustments for all abovementioned

confounders, the risk of hospital death for patients with urea 7.5-10 mmol/L was 1.5 times higher ($p=0.102$) and in subjects with urea >10 mmol/L was 2-times higher ($p<0.041$). Serum urea levels were also significantly associated with the risk of HPIR (the ORs were 2.2 in both groups with urea 7.5 -10 mmol/L and >10.0 mmol/L). Prolonged hospital stay was associated with admission urea >10 mmol/L (OR 1.36, 95%CI 1.05-1.75, $p=0.019$), but this relationship lost statistical significance after adjustment for age and gender ($p=0.087$). When the fully adjusted models included also HPIR, the risk of hospital death was 4.1 and 6.6 times higher in patients with admission urea 7.5-10 mmol/L and >10 mmol/L, respectively (Table 3).

Taken together, the presented data indicate that in older patients with HF even mildly-moderately elevated serum urea at admission is independently and strongly associated with poor outcomes, in particular with in-hospital death and developing a HPIR.

3.4. Predictive Efficacy/Accuracy of Elevated Admission Urea Levels (Single and Combined With Other Variables)

Next we performed ROC analysis (adjusting for age and gender) for elevated admission urea concentration and compared it to abnormal creatinine, GFR, PTH, and albumin levels, GGT/ALT and urea/albumin ratios. For predicting a fatal outcome, urea >10.0 mmol/L yielded the best results (the largest AUC value of 0.7252, 95%CI 0.6696-0.7807); the AUC's for urea >7.5 mmol/L (0.6792, 95%CI 0.6302-0.7283) and for urea/albumin >5.5 (0.6762) were slightly lower and comparable to those for creatinine >90 μ mol/L (0.6823) and GFR <60 ml/min/1.73m² (0.6813), but higher than those for three other on-admission indices (all AUCs <0.6570). The sensitivity, specificity, positive predictive value (PPV), negative predicting value (NPV), accuracy, positive likelihood ratio (LR+) and negative likelihood ratio (LR-) were 13.1%, 96.7%, 73.3%, 60.7%, 74.1%, 3.97 and 0.90 at urea >10 mmol/L; the corresponding values for admission urea >7.5 mmol/L were 9.3%, 96.7%, 57.4%, 69.7%, 56.6%, 2.82 and 0.94%. For predicting HPIR, the best results showed creatinine >90 μ mol/L (AUC 0.6263) followed by elevated urea (AUC 0.6062). For predicting LOS >20 days, the tested variables demonstrated low discrimination ability (all AUC <0.5648) and elevated urea levels did not reach statistical significance. The level of serum urea 7.5-10.0 mmol/L, predicted hospital mortality with 69.5% accuracy and HPIR with 67.4% accuracy, whereas the admission urea >10 mmol/L showed an accuracy of 73.3% and 68.1%, respectively.

These results indicated an acceptable AUC value to predict fatal outcome when admission urea >10 mmol/L, but the discriminatory performance of other individual parameters [despite being independent and significant prognostic indicators] was low. Therefore, we evaluated in logistic regression models (adjustment for age and gender) whether an approach combining two biomarkers can improve the accuracies of predictions for poor outcomes (Tables 4 and 5; Fig. 1). Patients with two abnormal parameters at admission, compared to subjects with the normal ones, had 2.9-5.6 -fold higher risk for hospital mortality, 2.6-7.8-fold increase in risk for HPIR and 1.3-1.7-fold higher risk for prolong LOS (Table 4). The mortality rate in patients with both urea and albumin admission levels in the normal ranges was 2.9%, in subjects with only urea >7.5 mmol/L - 8.1%, in subjects with only albumin <33 g/L - 4.6% and in individuals with both abnormalities - 14.6%.

Admission urea >7.5 mmol/L remained a significant indicator of in-hospital mortality even when all other tested clinical and laboratory parameters, except PTH, were normal; the discriminative significance of elevated urea was borderline when accompanied with GFR above 60 ml/min/1 ($p=0.062$). In patients with normal urea at admission all studied clinical and laboratory variables, except CKD, were not relevant in predicting mortality (Table 4). In patients with increased both urea and PTH the mortality risk was 4.5 times higher comparing to patients with these two markers in the normal range, but neither elevated urea, nor high PTH separately were statistically significant indicators for a fatal outcome if the other variable was normal suggesting that the effects of increased urea and PTH on mortality risk are not independent, but co-determined by some metabolic mediators.

Similarly, admission urea >7.5 mmol/L was indicative for HPIR when other studied parameters were normal [borderline significance when accompanied by anaemia with haemoglobin >110 g/L ($p=0.072$) or creatinine ≤ 90 μ mol/L ($p=0.099$)], whereas in patients with normal urea, COPD, anaemia, hyperparathyroidism and GGT/ALT >2.5 failed as prognostic indicators for HPIR. Combination of elevated urea and low albumin on admission did not achieve statistical significance as an indicator for HPIR (OR 2.2, $p=0.159$) suggesting opposite roles of these metabolic alterations in developing HPIR.

Table 4. Prognostic value of combined indices in older hip fracture patients.

Variables: Urea>7.5 mmol/L plus	Group 1 (Both variables abnormal)			Group 2 (Only urea >7.5mmol/L)			Group 3 (Only the second variable abnormal)		
	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
In-hospital mortality									
+MI	5.59	2.82-11.09	<0.001	2.52	1.59-3.98	<0.001	2.53	0.85-7.54	0.095
+Albumin<33g/L	4.97	2.71-9.12	<0.001	2.56	1.54-4.27	<0.001	1.61	0.75-3.45	0.217
+GGT/ALT>2.5	4.65	2.51-8.60	<0.001	3.01	1.68-5.39	<0.001	1.95	0.96-3.96	0.066
+PTH>6.8pmol/L	4.46	2.44-8.12	<0.001	1.74	0.82-3.66	0.148	1.53	0.74-3.18	0.255
+CKD	3.86	2.29-6.51	<0.001	1.97	0.97-4.02	0.062	2.11	1.02-4.33	0.043
+Hb<110g/L	3.74	2.10-6.64	<0.001	2.31	1.42-3.74	<0.001	1.09	0.44-2.69	0.845
+Creatinine >90µmol/L	3.55	2.17-5.82	<0.001	2.02	1.10-3.72	0.023	1.93	0.85-4.39	0.115
+Hb<120g/L	2.88	1.65-5.00	<0.001	2.52	1.46-4.37	0.001	1.10	0.53-2.26	0.795
+HPIR	9.68	3.14-29.83	<0.001	2.89	1.04-8.01	0.041	1.31	0.15-11.43	0.809
High postoperative inflammatory response (HPIR)									
+COPD	7.76	2.54-23.70	<0.001	2.07	1.06-4.04	0.032	1.74	0.58-5.23	0.323
+Hb<110g/L	4.59	1.96-10.76	<0.001	1.89	0.94-3.77	0.072	1.75	0.59-5.16	0.312
+Hb<120g/L	3.98	1.76-9.00	0.001	2.57	1.15-5.75	0.022	2.75	1.14-6.65	0.025
+GGT/ALT>2.5	3.73	1.57-8.88	0.003	2.40	1.08-5.34	0.032	1.86	0.78-4.40	0.161
+Creatinine >90µmol/L	3.48	1.66-7.34	0.001	2.01	0.88-4.60	0.099	2.78	1.03-7.50	0.044
++CKD	2.66	1.27-5.55	0.009	2.75	1.13-6.71	0.026	1.96	0.76-5.08	0.039
+PTH>6.8pmol/L	2.62	1.15-5.94	0.022	2.82	1.18-7.08	0.027	1.35	0.55-3.33	0.510
Length of hospital stay>20 days									
+25(OH)D<50nmol/L	1.72	1.25-2.36	0.001	1.25	0.88-1.77	0.215	1.45	1.07-1.97	0.016
+Albumin<33g/L	1.47	1.02-2.11	0.041	1.24	0.98-1.58	0.076	1.41	1.02-1.96	0.039
2+ Creatinine >90µmol/L	1.39	1.08- 1.79	0.010	1.13	0.84-1.51	0.408	1.56	1.05-2.32	0.027
+ CKD	1.31	1.03-1.68	0.030	1.05	0.76-1.47	0.755	1.11	0.79-1.56	0.541

Abbreviations:OR, odds ratio; CI, confidence interval; GFR, glomerular filtration rate; MI, history of myocardial infarction; COPD, chronic obstructive pulmonary disease; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; PTH, parathyroid hormone; Hb, haemoglobin; CKD, chronic kidney disease (glomerular filtration rate<60ml/min/1.73m²; 25(OH)D, 25-hydroxyvitamin D.

Notes: Comparison with patients in whom both variables were in the normal range. All data adjusted for age and gender. Group 1- both variables abnormal; Group 2-only urea>7.5mmol/L, the other variable normal; Group 3- urea normal (<7.5mmol/L) but the other variable abnormal. Only statistically significant data for combined two abnormal indices (group 1) are presented.

The impact of elevated urea combined with other parameters for prognostication LOS>20 days was minimal. The combination of increased urea and vitamin D insufficiency yielded the highest OR (1.72, $p<0.001$), which was only a little improvement comparable to that of vitamin D insufficiency and normal urea (OR 1.45, $p=0.016$); likewise, urea combined with hypoalbuminaemia demonstrated ORs of 1.47 and 1.41, respectively (Table 4).

Next, we characterized the association between mortality and HPIR. Compared to patients with normal admission urea, the risk of hospital death in patients with elevated urea who did not developed HPIR was 2.9 times higher (OR 2.89, $p<0.041$) and 9.7 times higher in patients who experienced both conditions (OR 9.68, $p<0.001$), but in subjects with normal urea levels at admission HPIR was not predictive for a fatal outcome (OR 1.3, $p<0.809$) (Table 4). Of note, some combinations have more than an additive effect on outcomes. It appears that co-occurrence of raised urea with history of MI or low albumin or GGT/ALT>2.5 and, especially, with HPIR has synergistic effect on mortality, whereas presence of COPD together with elevated urea demonstrates a synergistic effect on HPIR.

ROC-analyses of hospital death occurrence with each pair of on-admission variables showed that this outcome is best predicted by combination of urea>7.5mmol/L with albumin<33g/L (AUC 0.7731), or with GGT/ALT>2.5 (AUC 0.7698), followed by combination of elevated urea with anaemia (AUC 0.7190) or PTH>6.8pmol/L (AUC 0.7132) or history of MI (AUC 0.7093) or CKD (AUC 0.7079) or creatinine >90µmol/L (AUC 0.7045). Among admission parameters, the highest sensitivity demonstrated the combination of elevated urea with hyperparathyroidism (77.9%) or CKD (74.7%), and the highest specificity showed the combination of urea with history of MI (93.1%) or with hypoalbuminaemia (84.7%) or with anaemia (83.1%). The high NPV ($\geq 95.0\%$) of all combined characteristics indicate that the number of false positive tests is low. Presence of any two abovementioned characteristics on admission would miss a fatal outcome in 2.5-5.3% of patients with a negative result.

The highest predictive value for HPIR showed the combinations of urea>7.5mmol/L with anaemia (haemoglobin<120g/L, AUC 0.6928) or with creatinine >90µmol/L (AUC 0.6561) or with GGT/ALT>2.5 (AUC 0.6418), indicating a modest-low ability of these combinations to predict HPIR. None of the studied combinations showed a satisfactory predictive value for prolonged LOS (all AUCs under 0.5929).

Elevated urea at admission and HPIR demonstrated the highest discrimination power of predicting a fatal outcome (AUC 0.8176) and the latter result did not differ significantly from that of the full model (8 independent variables at admission and HPIR, AUC 0.8249, 95%CI 0.7442-0.9056, $p<0.001$) (Table 5, Fig 1).

Table 5. Prognostic accuracy of a combined two factor approach in predicting outcomes in older hip fracture patients.

Variables: Urea>7.5 mmol/L plus	AUC	95%CI	Se,%	Sp,%	PPV.%	NPV,%	Ac,%	P value
In-hospital mortality								
+Albumin<33g/L	0.7731	0.7072-0.8390	51.1	84.7	14.6	97.1	83.0	<0.001
+GGT/ALT>2.5	0.7698	0.7059-0.8337	64.6	74.3	11.8	97.5	73.8	<0.001
+25(OH)D<25nmol/L	0.7258	0.6325-0.8190	59.5	81.4	12.2	97.9	80.5	<0.001
+Hb<120g/L	0.7190	0.6588-0.7793	50.7	66.8	10.3	94.7	65.6	0.002
+Hb<110g/L	0.7180	0.6456-0.7904	47.2	83.1	12.9	96.7	81.3	<0.001
+PTH>6.8pmol/L	0.7132	0.6569-0.7695	77.9	58.8	11.9	97.4	60.0	<0.001
+MI	0.7093	0.6274-0.7913	31.8	93.1	16.7	96.9	90.5	<0.001
+CKD	0.7079	0.6539-0.7620	74.7	60.6	11.0	97.4	61.4	<0.001
+Creatinine >90µmol/L	0.7045	0.6492-0.7598	68.3	66.6	11.5	97.1	66.7	<0.001
+HPIR	0.8176	0.7008-0.9275	62.5	87.5	29.4	96.6	85.6	<0.001
High postoperative inflammatory response (HPIR)								
+Hb<120g/L	0.6928	0.5888-0.7968	58.1	71.8	26.1	90.9	69.8	0.001
+Creatinine >90µmol/L	0.6561	0.5695-0.7428	57.5	68.9	25.3	89.9	67.2	0.001
+GGT/ALT>2.5	0.6418	0.5268-0.7567	55.6	73.5	27.3	90.2	70.8	0.003
+COPD	0.6389	0.5161-0.7617	29.6	94.3	47.1	88.8	84.9	<0.001
+Hb<110g/L	0.6353	0.5190-0.7516	40.6	85.8	34.2	88.8	78.8	<0.001
+CKD	0.6250	0.5348-0.7152	60.0	60.2	21.4	89.3	60.2	0.018
Length of hospital stay>20 days								
+25(OH)D<50nmol/L	0.5928	0.5500-0.6355	64.6	49.6	34.3	77.5	53.9	<0.001
+Creatinine >90µmol/L	0.5686	0.5349-0.6022	42.1	67.2	33.2	75.0	60.2	0.001
+CKD	0.5683	0.5348-0.6018	47.8	61.0	32.9	74.5	57.2	0.003
+Albumin<33g/L	0.5544	0.5254-0.5834	21.8	84.8	34.0	75.1	68.1	0.017

Abbreviations: OR, odds ratio; CI, confidence interval; AUC, area under the receiver operating characteristic curve; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; Ac, accuracy; MI, history of myocardial infarction; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease (glomerular filtration rate <60ml/min/1.73m²); Hb, haemoglobin; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.

Notes: All data adjusted for age and gender. Only variables significantly associated with outcomes shown.

Taken together, these results show that the discriminatory performance of elevated urea (alone and combined with other markers) differs for different outcomes and can be used for predicting unfavorable survival outcome and developing HPIR, but not for predicting prolonged LOS.

3.5. Validation

The validation and main cohorts were comparable in mean age, gender distribution, comorbidities, fracture types and outcomes. In the validation cohort, 23(5.1%) of 455 patients died in the hospital, 99 (21.7%) developed HPIR and 93(20.4%) had LOS>20 days. Admission urea>7.5mmol/L (after adjustment for age and gender) was predictive for in-hospital death: OR 3.60, 95%CI 1.45-8.90, $p=0.006$; AUC 0.7739, 95%CI 0.7045-0.8433; mortality rate: observed 10.3%, predicted 9.4%. Similarly, urea>10.0mmol/L was indicative for a fatal outcome: OR 3.10, 95%CI 1.20-8.03, $p=0.020$; AUC 0.7179, 95%CI 0.6291-0.8067; mortality rate: observed 11.9%, predicted 13.1%. Among subjects with urea>7.5mmol/L, the observed/predicted proportions of patients who developed HPIR were 23.8%/22.1% and the proportions of individuals with LOS>20 days were 29.0%/31.1%, respectively. Analyses of the prognostic performance of two combined parameters showed also comparable results in the validation and main cohorts. Urea>7.5mmol/L combined with albumin<33g/L yielded an observed/predicted mortality rate of 12.7%/14.4%, combined with

GGT/ALT>2.5-15.8%/11.8%, combined with creatinine>90 μ mol/L-11.3%/11.5%, and combined with PTH>6.8pmol/L-11.8%/11.9%, respectively. These results indicate that the proportion of observed poor outcomes in the validation cohort matched the proportion predicted by elevated urea levels alone and/or in combination with other parameters.

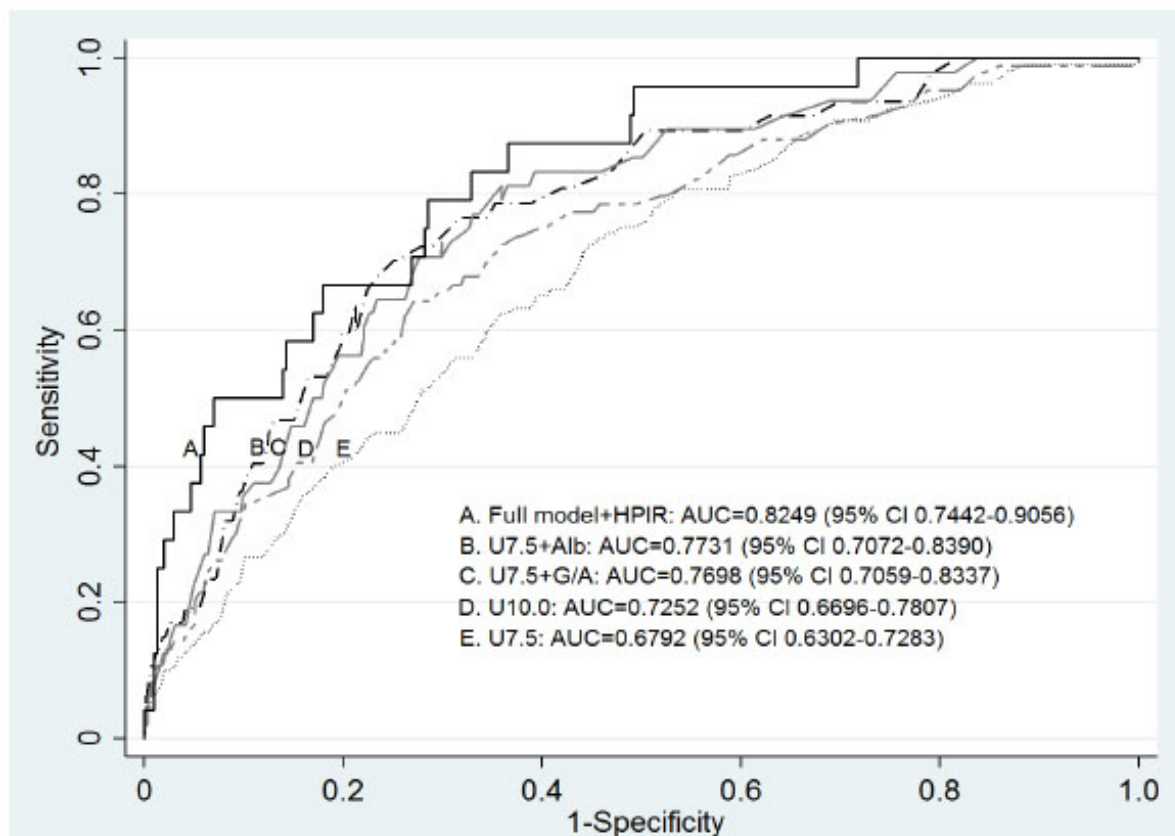


Fig. (1). Receiver-operating characteristic curves (ROC) of elevated serum urea levels at admission (alone and in combination with other factors) for predicting the all-cause in-hospital mortality in older patients with hip fracture. A (solid line), ROC curve based on data from the full model (8 on admission independent risk factors plus HPIR vs. 0 risk factors); B, ROC curve based on combination of urea>7.5mmol/L and albumin <33g/L; C, ROC curve based on combination of urea>7.5mmol/L and GGT/ALT>2.5; D, ROC curve based on urea>10.0mmol/L and E, ROC curve based on urea>7.5mmol/L.

Abbreviations: AUC, area under receiver operating characteristic curve; CI, confidence interval; HPIR, high postoperative inflammatory response; U7.5, serum urea>7.5mmol/L; Alb, serum albumin<33g/L; G/A, gamma-glutamyl transferase/alanine aminotransferase ratio (GGT/ALT); U10.0, serum urea>10.0mmol/L.

4. DISCUSSION

4.1. Main Findings

This study based on a relatively large and well defined cohort of older HF patients with parallel assessment of a broad spectrum of clinical and laboratory parameters showed that elevated serum urea levels (>7.5mmol/L) at the time of admission (1) were prevalent (44%), (2) independently determined by CKD, history of MI, anaemia, hyperparathyroidism, advanced age and male gender, and (3) had a substantial impact on prognostication hospital outcomes, in particular, mortality and developing HPIR, being associated with 2.6-fold and 2.2-fold greater odds, respectively. Patients with increased admission urea and HPIR demonstrated almost 10 times greater mortality odds compared to patients without such characteristics. Fig. (2) depicts a summary of the key findings of our study.

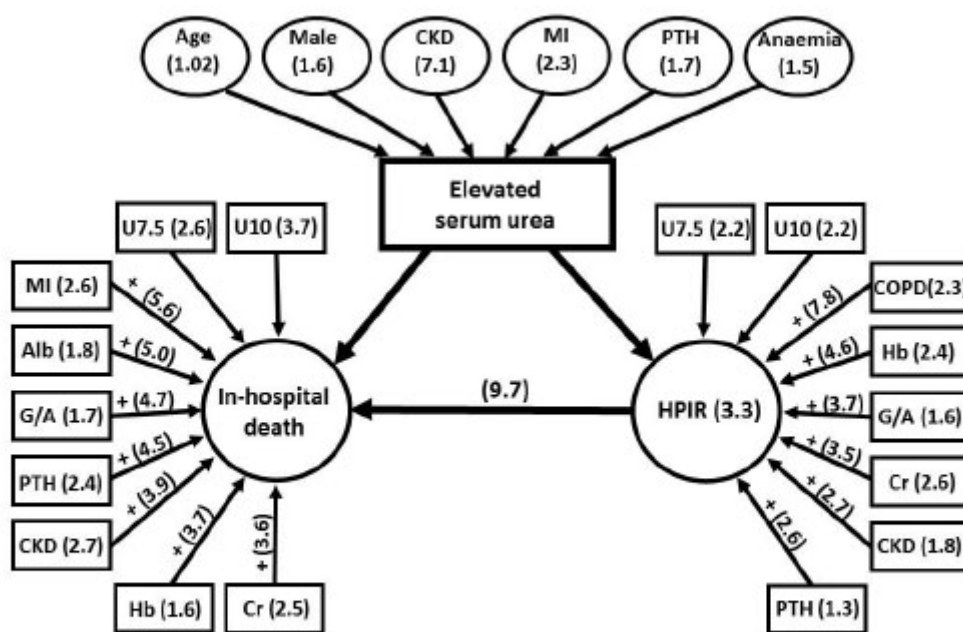


Fig. (2). Integrative schematic illustration of the prognostic impact of elevated urea at admission alone and in combination with other factors for predicting poor in-hospital outcome in older patients with HF.

Conditions independently associated with elevated urea at admission are shown in ovals. Square boxes indicate on admission factors (adjusted for age and gender) influencing the specific outcome. Odds ratios for each parameter are shown in brackets; + above the arrow indicates combined effect of two parameters on the outcome.

Abbreviations: CKD, chronic kidney disease; MI, history of myocardial infarction; COPD, chronic obstructive pulmonary disease; HPIR, high postoperative inflammatory response; PTH, hyperparathyroidism (>6.8pmol/L); Alb, serum albumin<33g/L; G/A, gamma-glutamyl transferase/alanine aminotransferase ratio (GGT/ALT); Hb, anaemia (haemoglobin<110g/L); Cr, creatinine >90μmol/L

Notes: CKD, history of MI, hyperparathyroidism, anaemia, advanced age and male gender are independent and significant factors associated with elevated urea at admission, which is a strong indicator of both in-hospital death and developing a HPIR.

Increased urea at admission combined with any other risk factor has an additive prognostic effect. Elevated urea predisposes to a HPIR, which carries a high risk of a fatal outcome by itself, and in patients exposed to both conditions a synergistic effect on mortality occurs.

4.2. Serum Urea And Prognosis

Serum urea, the terminal product of protein and amino acid metabolism and the major circulating pool of nitrogen, is produced exclusively in the hepatocytes in five reactions (two first steps in the mitochondrial matrix and the last 3 in the cytosol - Krebs-Henseleit urea cycle), and is excreted in the urine (about 80% of the eliminated waste nitrogen). Urea plays key roles in detoxification of harmful ammonia produced by amino acid catabolism and in osmoregulation [29]. Serum urea concentration is a cumulative result of multiple influences, including protein intake and catabolism, physical activity, fluid balance, liver synthetic function, urea recycling in the colon, tubular urea reabsorption, endocrine (especially, adrenal gluco- and mineralocorticoids) and renal neurohormonal activation [2, 3, 5, 9, 29 - 37]. Although the liver is the main ureagenic organ, and urea plays multiple roles in different homeostatic mechanisms, in clinical studies until now, urea is often interpreted as a biochemical marker of renal function and its relation with other liver-specific functions and protein/nitrogen metabolism remains largely neglected. It should, however, be realised that serum urea concentration is maintained through tight coordination of many metabolic pathways and reflects the complicated interrelations between nutritional and hydration status, protein metabolism, liver, renal, gut, muscle, cardiovascular and endocrine systems. Compared to creatinine and GFR, changes in urea levels are more dependent on neurohumoral mechanisms regulating the reabsorption process (renin-angiotensin-aldosterone system and vasopressin) [3, 5, 9, 11, 36]. Elevated blood urea levels by inhibiting nitric oxide production may contribute to atherogenesis [38].

Increased urea should be considered not as mere biomarker of kidney damage, but an important factor in the multi-organ pathogenetic interactions. Abnormal urea, which may result from and be pathophysiologically linked to a wide variety of conditions, is prevalent in the elderly with multiple comorbidities, can lead to poor outcomes and, therefore, constitute a useful parameter for predicting hospital outcomes, especially mortality, in different medical and surgical settings [2 - 7, 9 - 11, 28, 39 - 46]. Our findings in HF patients are in keeping with these observations.

Renal dysfunction (using GFR or creatinine levels) has been widely [14, 16, 47, 48], though not universally [49, 50], accepted as a significant risk factor for post-HF mortality. Only few studies focussed on urea, and the published data have been conflicting. Elevated urea on admission has been reported as an independent predictor of in-hospital mortality [14] and included in some scoring models [17, 18, 51], but according to other studies urea was only marginally related [16] or not correlated with fatal short- and long-term outcome [15, 52].

In our study, urea level expressed both as a continuous and categorical variable demonstrated a significant association with poor outcomes in HF patients. Admission urea was the strongest correlate for hospital mortality and the second strongest for HPIR in comparison with all other tested biochemical parameters. Urea $>7.5\text{mmol/L}$ and $>10\text{mmol/L}$ performed best for predicting in-hospital death, and were associated with 2.6- and 3.7- fold (adjusted for age and gender) increased mortality, respectively, and with 2.2-fold higher incidence of HPIR compared to patients with normal admission urea. For fatal outcome urea level is a stronger contributor than any other biochemical or clinical variable at admission; it appears to be an integrative measure reflecting the most severe changes in the metabolism and, therefore, its quantification should not be ignored in predictive models.

Our work confirms the few previous reports that have shown that urea is associated with increased mortality in HF patients [14, 18] and numerous studies demonstrating such link in older adults with serious illnesses. Our results, however, are the first to demonstrate in HF patients an association between admission urea and HPIR, indicating that blood nitrogen homeostasis is an important regulator of susceptibility and response to inflammation/infection. Importantly, elevated admission urea had a strong effect on hospital mortality and HPIR independent of various clinically relevant covariates including preoperative history of MI, COPD, CKD, anaemia, low albumin, GGT/ALT >2.5 , high creatinine, increased PTH, vitamin D deficiency, fracture type, medication used, age and gender, making our findings more special since this was not done in previous studies.

Serum urea, however, did not show an independent association with LOS; its significant univariate association with prolonged LOS appears to be secondary to pre-fracture socio-demographic and clinical conditions and postoperative complications; it would be more meaningful when admission urea used for predicting primarily hospital death and developing HPIR.

We have further extended previous findings by exploring the prognostic value of admission urea ($>7.5\text{mmol/L}$) and outcomes in HF patients with and without other well-established preoperative risk factors, which allows to separate the contribution of elevated urea and other abnormal characteristics on the risks of adverse outcome. Increased urea as a prognostic indicator interacted with other risk factors in an additive and multiplicative fashion (Fig. 2). The highest mortality risk was documented by combination of elevated urea with history of MI (OR 5.7), or hypoalbuminaemia (OR 5.0), or GGT/ALT >2.5 (OR 4.7), or hyperparathyroidism (OR 4.6), or CKD (OR 3.9). The highest risk of developing HPIR demonstrated patients in whom elevated urea was accompanied by COPD (OR 7.8), or anaemia (OR 4.6), or GGT/ALT >2.5 (OR 3.7), or creatinine $>90\mu\text{mol/L}$ (OR 3.5). Some of these factors operate synergistically and together markedly enhance the risk of fatal outcome or/and HPIR. Patients with both increased urea and HPIR had a 9.7-fold higher risk of hospital death. These observations highlight the complex nature of postoperative outcomes, demonstrating that alterations in protein/nitrogen homeostasis on admission (as assessed by elevated urea, hypoalbuminaemia, higher GGT/ALT ratio), history of CKD, MI, COPD, increased creatinine levels, anaemia, and hyperparathyroidism have a strong detrimental prognostic influence per se, whereas presence of two conditions (elevated urea plus any of the abovementioned) further significantly aggravates prognosis (by 1.5-2-fold on average).

Furthermore, closer examination revealed that most of the abovementioned laboratory and clinical variables previously recognised as risk factors for poor outcomes were not predictive in patients with normal admission urea. Although in the regression models (adjusted for age and gender) serum albumin, PTH, creatinine, GFR (both as a continuous and as a categorical variable), as well as history of MI, anaemia and COPD showed significant associations with increased risk of mortality and/or HPIR, our analyses demonstrated, for the first time, that this effects achieved statistical significance in patients with elevated admission urea but not in subjects with normal urea; this aspect has been masked to date. Indeed, in patients with normal urea at admission, only CKD was a significant risk factor for

hospital death and HPIR, and anaemia ($Hb < 120g/L$) indicated a risk of HPIR, whereas other studied factors lost their discriminative value; even developing a HPIR did not increase mortality risk, but elevated urea remained a significant predictor when most of the abovementioned parameters were normal (Table 4). These results emphasise that the prognostic performance of characteristics previously recognised as risk factors for poor hospital outcomes depends on urea status, therefore, their clinical interpretation should be in conjunction with urea level.

Why urea is associated with increased mortality and HPIR is not yet completely understood. Considering the many urea-related complex metabolic and pathophysiological pathways, including inflammation [53, 54], oxidative stress [53 - 57], and stress-induced hypermetabolic/catabolic response [58 - 60], elevated urea in HF (as in other) patients may be viewed as an integrative (but unspecific) outcome-relevant marker of severity of chronic and acute conditions and ageing; it mirrors the patient's degree of physiological reserves/frailty and altered homeostasis [61, 62] explaining the high risks for adverse effects.

4.3. Predictive Value of Elevated Urea

In older HF patients, admission urea $> 10mmol/L$ as a single parameter has a reasonable ability to predict hospital death (AUC 0.7252, accuracy 73.3%), while the discriminative accuracy of other studied clinical and biochemical variables is relatively low. An integration of two characteristics (urea $> 7.5mmol/L$ plus one traditional risk factor) materially improves the prediction of mortality and HPIR, as evidenced by increases in AUCs (Table 5). Among combined parameters at admission highest AUC for prediction mortality yielded elevated urea ($> 7.5mmol/L$) plus low albumin (AUC 0.7731) or GGT/ALT > 2.5 (AUC 0.7698) and for prediction HPIR elevated urea plus anaemia (AUC 0.6928) or increased creatinine (AUC 0.6561) or GGT/ALT > 2.5 (AUC 0.6418). Urea combined with other easily obtainable parameters at admission reached reasonable accuracy in identifying HF patients who are at risk of hospital mortality (sensitivity of 51-78%, NPV $> 95\%$) and HPIR (sensitivity 30-60%, NPV $> 90\%$). Elevated urea at admission is a strong independent predictor for hospital death, in particular among subjects who developed HPIR; these two conditions combined demonstrate the highest predictive value for unfavorable survival outcome (AUC 0.8176) with sensitivity of 62.5%, high specificity (87.5%) and high NPV (96.6%).

In the past decade a number of studies have aimed to identify clinical factors and/or biomarkers of poor outcome in HF patients. The available prediction tools [63 - 68] are complex (need of special rating scales and calculations), time consuming, some include subjective assessment or show significant lack of fit [18, 69], and are not widely used in clinical practice. The presented here approach is based on routinely collected objective data, simple, easy to use and has a fair precision rate.

4.4. Practical Implications / Therapeutic Considerations

Elevated urea at admission signifies the presence of a serious underlying condition(s) which may negatively impact survival and response to stressors (fracture, surgery, blood loss, infection, etc.), and, therefore, should be properly assessed and treated. Urea status may help to optimise risk stratification, to identify modifiable conditions and timely initiate individualized appropriate management. In patients even with mildly increased serum urea at admission, especially when it is accompanied by hypoalbuminaemia, GGT/ALT > 2.5 , hyperparathyroidism, renal impairment, history of MI, anaemia or COPD, poor outcome should be suspected and, if possible, prevented; more than 50% of hospital deaths are classified as "at least possibly preventable" [70]. The identified in this study comorbid conditions independently associated with elevated serum urea offer additional avenues for risk stratification and preventive strategies. By reducing negative effects of these comorbidities (prophylaxis of myocardial necrosis, optimising pharmacotherapy for COPD, correcting anaemia), preventing contrast-related kidney injury, inflammatory/infectious and opioid-induced complications, optimising perioperative intravenous fluid management, addressing malnutrition, use of probiotics (shown to decrease the serum urea concentrations in patients with CKD [71]) and rapid mobilization it may be possible to improve hospital outcomes.

5. LIMITATIONS AND STRENGTHS

The following limitations of this single-centre study should be noted. Firstly, its observational design does not allow a causal conclusion. Secondly, the causes of hospital death as well as the causes of HPIR were not classified. Thirdly, our results are based on a single measurement on hospital admission, and these may change and fluctuate. Fourthly, the majority of patients were Caucasian, therefore, the results might not be applicable for other ethnicities.

The strengths of our study include its relatively large sample size, measurements of urea in parallel to a variety of

clinical and laboratory parameters, use of multivariate regression models to investigate the independent association of urea and comorbid conditions and outcomes, analyses of differential performance of previously established risk factors in patients with and without elevated serum urea at admission, and validation of the prognostic value of urea for hospital outcomes in an independent cohort. The variance inflation factor in all our regression models was ≤ 1.16 , indicating that the amount of multicollinearity was not significant.

CONCLUSION

In older HF patients, serum urea, a routinely measured key factor of nitrogen homeostasis, was identified as an independent and valuable prognostic indicator for predicting poor hospital outcomes, in particular, mortality and developing HPIR. The predictive value of elevated urea was superior to other risk factors, most of which lost their discriminative ability when the admission urea levels were normal. The predictive performance of urea increased significantly when it was combined with other prognostic indicators. The risk of in-hospital death was almost 10 times higher in patients with elevated admission urea who developed HPIR.

LIST OF ABBREVIATIONS

Ac	=	Accuracy
Alb	=	Serum albumin
ALP	=	Alkaline phosphatase
ALT	=	Alanine aminotransferase
AUC	=	Area under receiver operating characteristic curve
CAD	=	Coronary artery disease
CVA	=	Cerebrovascular accident
CI	=	Confidence interval
CKD	=	Chronic kidney disease
COPD	=	Chronic obstructive pulmonary disease
CRP	=	C-reactive protein
DM	=	Diabetes mellitus type 2
G/A	=	Gamma-glutamyl transferase/alanine aminotransferase ratio
GGT	=	Gamma-glutamyl transferase
GFR	=	Glomerular filtration rate
Hb	=	Haemoglobin
HF	=	Hip fracture
HPIR	=	High postoperative inflammatory response
LOS	=	Length of hospital stay
MI	=	Myocardial infarction
NPV	=	Negative predictive value
OR	=	Odds ratio
PPV	=	Positive predictive value
PTH	=	Parathyroid hormone
RCF	=	Residential care facility (permanent)
25(OH)D	=	25(OH) vitamin D
ROC	=	Receiver-operating characteristic curves
Se	=	Sensitivity
Sp	=	Specificity

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Australian Capital Territory Health Human Research Ethical Committee and waived the requirement for written consent as only routinely collected and anonymized before analysis data were used.

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Written consent as only routinely collected and anonymized before analysis data were used.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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Cardiopulmonary complications of ERCP in older patients CME

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Background: Biochemical markers of ERCP-related myocardial injury have not previously been investigated.

Objective: To evaluate ERCP-related cardiac troponin I (cTnI) release, myocardial ischemia, hemodynamic changes, and arterial hypoxemia in a series of consecutive patients according to age and to determine their relationship to preexisting cardiovascular risk factors (RF) and the development of post-ERCP pancreatitis.

Design: Prospective cohort study.

Setting: Tertiary teaching hospital, Canberra, Australia.

Patients: Data were collected on 130 consecutive ERCPs performed on 100 unselected patients (aged 18-93 years) by one endoscopist. Patients were divided into two groups: 65 years of age and older (group 1, $n = 53$; 27 women) and less than 65 years of age (group 2, $n = 47$; 33 women).

Interventions: ERCP.

Main Outcome Measurements: Cardiovascular RFs were identified, and electrocardiogram (ECG), cTnI, creatine kinase (CK), amylase, and lipase were measured before and 24 hours after ERCP. Oxygen saturation (SpO_2), heart rate (HR), blood pressure (BP), and ECG were monitored continuously during each procedure.

Results: New ECG changes (ischemia, arrhythmias) occurred in 24% of procedures in group 1 and in 9.3% in group 2 ($p = 0.168$), and episodic arterial hypoxemia ($SpO_2 < 90\%$) in 16.2% (group 1) and 21.4% (group 2) ($p = 0.596$). A post-ERCP rise in cTnI levels was documented in 6 patients in the older group. Two of these patients died: one from acute myocardial infarction and one from undiagnosed ascending aortic aneurysm. A cTnI rise was not related to any comorbid conditions, total number of RFs, hemodynamic or ECG changes, or arterial desaturation. In patients with a new cTnI rise, the duration of ERCP was significantly longer (59.5 vs. 26.4 minutes, $p = 0.026$), being 30 minutes or longer in 5 of 6 patients. Post-ERCP pancreatitis was associated with desaturation (relative risk [RR] = 5.9; 95% confidence interval [CI] [1.2, 32.0], $p = 0.027$) and myocardial ischemia/injury (RR = 4.4; 95% CI [1.4, 7.8]; $p = 0.009$).

Conclusions: Although the majority of older patients tolerated ERCP well, in 8% of procedures, most of which were prolonged (> 30 minutes), myocardial injury, as defined by the release of cTnI, occurred. Desaturation and myocardial ischemia/injury were associated with post-ERCP pancreatitis. (Gastrointest Endosc 2006;63:948-55.)

ERCP, a well-established and highly effective diagnostic and therapeutic procedure, is frequently indicated in older patients, and it may become increasingly used, because the population is rapidly aging. In older patients with endoscopically remediable pancreaticobiliary pathology, therapeutic ERCP has been shown to lead to less mortality and morbidity than surgery. ERCP has been reported to be safe even in elderly, frail, and seriously ill patients

with significant risk factors (RF),^{1,2} e.g., recent myocardial infarction.³ Notwithstanding this, the well-documented risks of ERCP, including pancreatitis, cholangitis, cardiopulmonary complications, and even death,^{1,4,5} should not be underestimated. In addition, patients undergoing ERCP are sometimes systemically unwell because of cholangitis or pancreatitis and can be malnourished secondary to pancreatic and biliary malignancies. In a recent large study,⁶ a high frequency of ERCP-related complications was found, and the need to minimize the number of diagnostic procedures was emphasized.

Arterial hypoxemia, arrhythmias, and myocardial ischemia in association with ERCP have been reported.⁶⁻⁹

See CME section; p. 1030.

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0016-5107/\$32.00

doi:10.1016/j.gie.2005.09.020

Similar problems have been found with other endoscopic procedures, especially in older patients with heart disease.¹⁰⁻¹² Cardiopulmonary complications are thought to account for at least 50% of the mortality and 60% of the morbidity associated with upper-GI endoscopy,^{13,14} and led to as much as 50% of the deaths related to ERCP.^{6,15}

Advanced age has been considered an important RF for the development of complications of GI endoscopy by some investigators¹⁰ but not by others.^{1,16,17} Although ERCP is more hazardous than other endoscopic procedures, with markedly higher complication rates, there is only one study⁹ reporting intraoperative cardiovascular responses to ERCP in 30 older adults. The incidence, the clinical significance, and the mechanisms of ERCP-related cardiopulmonary complications, as well as the effect of preexisting RFs on the development of such complications in the elderly remain uncertain. Moreover, the relationship between periprocedural arterial hypoxemia and myocardial ischemia, the role of supplemental oxygen, and the need for pulse oximetry and hemodynamic monitoring is still debated.^{6,14}

In all previous studies, endoscopy-related myocardial ischemia was assessed only by electrocardiographic (ECG) ST segment changes. To our knowledge, no studies have specifically addressed biochemical markers of myocardial injury after ERCPs. Cardiac troponin I (cTnI), a human cardiac myocyte regulatory protein isoform, is a highly sensitive and specific marker of myocardial injury and necrosis.¹⁸ Even minor elevations of cTnI are important in early detection of acute myocardial lesions, whereas ST deviation may be short lasting and not detectable.¹⁹

The aim of this prospective study was to determine, in a series of consecutive older patients, the incidence of cardiopulmonary effects (arterial hypoxemia, myocardial ischemia, arrhythmias, heart rate and blood pressure changes, and cardiac cTnI rise) of ERCP and to evaluate their relationship to preexisting RFs, type of anesthesia, and development of post-ERCP pancreatitis.

PATIENTS AND METHODS

Subjects

In this study, 130 consecutive ERCP procedures performed at the Canberra Hospital by an experienced endoscopist (A.T.) on 100 unselected consecutive patients were analyzed. The patients were divided into two groups according to their age: 65 years of age and older (group 1, $n = 53$) and under 65 years (group 2, $n = 47$). Before ERCP, a complete medical history was obtained and a physical examination was performed. Patients' demographic and clinical data were recorded, including age, gender, comorbid medical conditions, cardiac RFs, current medications, and indications for ERCP. Particular attention was paid to RFs for developing myocardial ischemia, such as arterial hypertension, known coronary artery disease,

Capsule Summary

What is already known on this topic

- ERCP is commonly associated with significant hemodynamic changes, ECG signs of myocardial ischemia, and arterial hypoxemia.
- Cardiopulmonary complications account for up to 50% of mortality and morbidity associated with ERCP.
- Advanced age may increase the risk of these events.

What this study adds to our knowledge

- In this prospective study, the incidence and the severity of ERCP-related transient hypoxemia, tachycardia, blood pressure, and ECG changes in patients more than 65 years of age and those less than this age were similar.
- Although the majority of older patients tolerated ERCP well, 8% of procedures, especially if prolonged (> 30 minutes), resulted in silent or symptomatic myocardial injury as defined by the release of cardiac troponin I.
- Occurrence of myocardial injury could not be predicted on the basis of comorbid status, cardiac risk factors, ECG, or arterial oxygen saturation changes during ERCP; therefore, measurement of cardiac troponins before and 24 hours after the procedure may be advisable.
- Desaturation and myocardial ischemia/injury are associated with post-ERCP pancreatitis.

diabetes mellitus, hyperlipidemia, and smoking history. Before each procedure, blood was drawn for a full blood cell count, liver function tests, serum amylase and lipase, cTnI, and creatine kinase (CK). Within 2 hours before ERCP, a 12-lead ECG was recorded. Abdominal US results were also documented. All patients continued their usual medications to the day of ERCP, with the exception of oral anticoagulants. The study was in accordance with the Helsinki Declaration II, and written informed consent, covering all parts of the study, was obtained from all patients before ERCP.

ERCP procedure

The subjects fasted for at least 6 hours before ERCP. The ERCP was performed in a standard fashion with the patient in the prone position, with an Olympus side-viewing video duodenoscope (Olympus Optical Co, Ltd, Tokyo, Japan). An anesthetist was in attendance during all procedures. All subjects received supplementary oxygen (at least 4 L/min) via nasal cannulas during the procedure. In 7 subjects, general anesthesia with intubation was used; 123 patients were sedated with a combination of midazolam, fentanyl, and propofol administered intravenously. To reduce duodenal motility and secretion, intravenous hyoscine butylbromide or glucagon was given.

Monitoring

Arterial oxygen saturation (SpO_2), heart rate, blood pressure, and a 3-lead ECG strip were monitored continuously throughout the procedure (Datex As-3 monitor;

Soma Technology Inc, Cheshire, Conn). The ECG was repeated, and levels of cTnI, CK, amylase, and lipase were determined 24 hours after ERCP and thereafter if clinically indicated. Particular attention was paid to reductions in SpO₂, and changes in heart rate (HR) and blood pressure (BP), as well as ST segment deviations, arrhythmias, and conduction defects. The steady (mean arterial pressure [MAP]) and pulsatile (pulse pressure [PP]) components of BP were calculated: $MAP = SBP \text{ (systolic blood pressure)} + 2 \times DBP \text{ (diastolic blood pressure)}/3$; $PP = SBP - DBP$.

Rate pressure product (RPP) and pressure-rate quotient (PRQ), two indices that reflect myocardial oxygen consumption,^{20,21} were also calculated: $RPP = SBP \times HR/1000$; $PRQ = MAP/HR$. It has been suggested that $RPP \geq 12$ indicates myocardial ischemia,²⁰ and that $RPP \geq 20$ is a threshold for angina pectoris.²¹ $PRQ < 1.0$ is considered to be an even more accurate measure of myocardial ischemia.²⁰

Definitions

The following definitions were used:

- Hypoxemia: SpO₂ less than 90% for at least 15 seconds.
- Myocardial injury: rise in cTnI level of 0.4 µg/L or greater.
- Myocardial ischemia: new down-sloping or horizontal ST segment depression of 0.1 mV or greater or ST segment elevation of 0.2 mV or greater from electric baseline measured 60 ms from the J point and lasting for at least 1 minute.
- Tachycardia: HR exceeding 100 beats per minute (bpm). Bradycardia: HR less than 50 bpm.
- Intraprocedure hypertension: MAP greater than 100 mm Hg.
- Intraprocedure hypotension: MAP less than 55 mm Hg.
- Post-ERCP pancreatitis: significant persisting (≥ 24 hours) abdominal pain associated with the elevation of amylase and/or lipase levels at least 3 times the upper reference limit (110 U/L and 300 U/L, respectively).

Statistical analyses

Data were expressed as mean, standard deviation (SD) or relative risk (RR) with 95% confidence intervals (CI). Statistical analysis was performed by using the Student *t* test and the chi-square test with the Pearson correction and the Yates correction. A *p* value less than 0.05 was considered significant.

RESULTS

Baseline characteristics

The demographic and clinical characteristics of the two groups studied are outlined in Table 1. As expected, comorbid diseases were more common in the older patients (group 1) compared with the younger patients (group 2). The most common conditions were hypertension,

congestive heart failure, diabetes mellitus, coronary artery disease, and a history of smoking; as a consequence, the total number of cardiovascular RFs in the older group was significantly higher (2.5 vs. 1.7). There were no significant differences between the two groups with regard to gender, and the number of patients with obesity, hypercholesterolemia, or hypertriglyceridemia.

Procedure-related data are presented in Table 2. Multiple procedures were performed in 22 patients: 15 had 2 ERCPs, 6 had 3 ERCPs, and 1 patient had 4 ERCPs. The two groups were similar with respect to indications for ERCP, as well as the use of sedative drugs and anesthesia. The main indications for ERCP were suspected choledocholithiasis, pancreatitis, cholestatic liver function tests, and painless jaundice. Glucagon was mainly used in the older patients, whereas hyoscine butylbromide was more often used in the younger group. Endoscopic sphincterotomy was performed in 33 procedures (group 1) and in 32 procedures (group 2); biliary stents were placed in 32 (group 1) and 11 procedures (group 2), and pancreatic stents in 4 (group 1) and 1 procedure (group 2). Three ERCPs were performed as emergency procedures in group 1 and none in group 2. Two procedures in group 1 and 5 in group 2 were associated with the development of post-ERCP pancreatitis. One 76-year-old man who received warfarin had a postsphincterotomy bleed that required hospitalization and a blood transfusion. There were no perforations.

No significant differences were observed between the two groups in baseline HR, DBP, and PRQ; however, the older patients had a significantly higher SBP (137.4 ± 21.6 mm Hg vs. 122.1 ± 18.6 mm Hg, $p = 0.001$), MAP (95.8 ± 13.3 mm Hg vs. 90.6 ± 12.1 mm Hg, $p = 0.022$), and PP (62.4 ± 15.6 mm Hg vs. 47.3 ± 12.8 mm Hg, $p = 0.001$).

The ECG recordings before the procedure revealed, in group 1, preexisting atrial fibrillation in 9% and a bundle-branch block in 15% (8% left, 4% right, and 3% bifascicular). Before the procedure, a 74-year-old patient with diabetes mellitus and chronic renal impairment, and a 90-year-old patient with diabetes mellitus and hypertension had serum cTnI levels of 0.8 and 0.6 µg/L (normal: 0.4 µg/L), respectively, but normal CK levels. Both patients were asymptomatic. One patient in the younger group had ECG signs of an old inferior myocardial infarction, and one other had a left bundle-branch block.

Hemodynamic and respiratory responses to ERCP

During ERCP, significant hemodynamic changes occurred in both groups. There was an increase in the older group and in the younger group in the following: HR (+29.5% and +47%), SBP (+28.6% and +31.2%), DBP (+35.6% and +34.4%), MAP (+32.2% and +32.9%), PP (+20.0% and +26.2%), and RPP (+68.2% and +94.8%). During the procedure in the older patients compared with the younger patients, SBP (176.6 ± 29.7

TABLE 1. Demographic and clinical characteristics of patients, by age, who underwent ERCP

Characteristics	Group 1 (≥ 65 y)	Group 2 (< 65 y)	<i>p</i> Value
No. patients	53	47	
Female/male	27:26	33:14	0.079
Age range, y	65-93	18-64	
Age, mean, SD, y	77.6 ± 7.2	46.2 ± 12.9	0.001
No. ERCP procedures	74	56	
Hypertension, n (%)	29 (54.7%)	9 (19.1%)	0.001
Coronary artery disease, n (%)	11 (20.8%)	1 (2.1%)	0.011
History of heart failure, n (%)	18 (34.0%)	2 (4.2%)	0.001
Diabetes mellitus, n (%)	13 (24.5%)	4 (8.5%)	0.063
Current smokers, n (%)	1 (1.9%)	11 (23.4%)	0.003
History of smoking, n (%)	14 (26.4%)	4 (8.5%)	0.039
Obesity (BMI > 30 kg/m ²), n (%)	6 (11.3%)	9 (19.1%)	0.416
Hypercholesterolemia (> 5.5 mmol/L), n (%)	17 (32.1%)	16 (34.0%)	1.000
Hypertriglyceridemia (> 1.75 mmol/L), n (%)	20 (37.7%)	16 (34.0%)	0.861
Total no. RFs	2.5 ± 1.5	1.7 ± 2.0	0.028

BMI, Body mass index.

TABLE 2. Indications for ERCP, type of anaesthesia, medications administered, and duration of the procedure

	Group 1 (≥ 65 y, n = 74)	Group 2 (< 65 y, n = 56)	<i>p</i> Value
Indication for ERCP			
Gallstones	22 (29.7%)	21 (37.5%)	0.457
Pancreatitis	11 (14.9%)	10 (17.9%)	0.827
Cancer	17 (23.0%)	6 (10.7%)	0.113
Cholestatic liver function tests	8 (10.8%)	10 (17.9%)	0.371
Stricture or occluded stent	16 (21.6%)	6 (10.7%)	0.160
Postoperative leak	0	3 (6.4%)	0.200
Anesthesia			
General with intubation, n (%)	6 (8.1%)	1 (1.8%)	0.234
Midazolam + fentanyl + propofol	68 (90.5%)	55 (98.2%)	0.235
Antispasmodic			
Hyoscine butylbromide, n (%)	43 (58.1%)	53 (94.8%)	0.001
Glucagon, n (%)	23 (31.1%)	1 (1.8%)	0.001
Duration of ERCP, min	29.1 ± 19.9	22.6 ± 9.9	0.039
No. (%) patients with ≥ 2 ERCPs	15 (28%)	7 (15%)	0.170

vs. 160.2 ± 26.8 mm Hg, $p = 0.001$) and PP (74.9 ± 24.7 vs. 59.7 ± 17.9 mm Hg, $p = 0.001$) remained markedly higher. However, there were no statistically significant differences between the two groups in the maximum change for each of these parameters except HR and RPP. In the younger group, the mean maximum HR during ERCP was significantly higher than in the older group (116.5 ± 18.1 vs. 102.0 ± 17.4 bpm, $p = 0.001$), although, at baseline, the HR was similar in both groups (78.8 ± 13.0 vs. 78.1 ± 13.3 bpm, $p = 0.764$). This effect was related to more frequent use of hyoscine butylbromide in the younger patients. There was no difference in the mean maximum HR during ERCP between the older patients and the younger patients in whom this drug was not used (99.5 ± 16.1 vs. 101.7 ± 16.5 , $p = 0.603$).

The incidences of abnormal hemodynamic and respiratory responses to ERCP are shown in Table 3. The proportion of patients with hypertension, high RPP, and low PRQ in both groups was high and almost equal. Hypotension occurred in one patient in each group. Although all patients were given supplementary oxygen during ERCP, transient arterial hypoxemia ($\text{SpO}_2 < 90\%$) was observed in 24 (18.5%) of 130 procedures: in 12 (16.2%) of 74

procedures in group 1 and in 12 (21.4%) of 56 in group 2 ($p = 0.596$). Episodes of desaturation were not of such severity or duration to require special intervention (such as reversal of anesthesia or unplanned intubation); the management included increasing inhaled oxygen flow, jaw thrust, and observation.

ECG changes

New ECG changes during and after ERCP occurred in 24% of all procedures performed in the older patients and in 9.3% in the younger group. In group 1, these included transient atrial fibrillation (10%), sinus bradycardia (6%), ST segment depression (10%), and ventricular premature complexes (2%). In group 2, sinus bradycardia occurred in 3.1% of procedures and ST segment elevation or depression in 6.2%. The difference in incidence of arrhythmias and ischemic changes between the two groups was not significant (Table 4).

Myocardial injury

No patient in the younger group had detectable cTnI levels before or 24 hours after the ERCP. No changes

TABLE 3. Incidence of abnormal respiratory and hemodynamic responses to ERCP

Characteristics	Group 1 (≥ 65 y, n = 74)	Group 2 (< 65 y, n = 56)	p Value
Oxygen saturation < 90%, n (%)	12 (16.2%)	12 (21.4%)	0.596
Heart rate > 100 bpm, n (%)	38 (51.4%)	47 (83.9%)	0.001
Mean arterial pressure > 110 mm Hg, n (%)	58 (78.4%)	38 (67.9%)	0.250
Mean arterial pressure < 55 mm Hg, n (%)	1 (1.4%)	1 (1.8%)	1.000
Rate pressure product > 12.0, n (%)	71 (95.9%)	56 (100%)	0.350
Rate pressure product > 20.0, n (%)	21 (28.4%)	20 (35.7%)	0.483
Pressure-rate quotient < 1.0, n (%)	16 (21.6%)	20 (36.7%)	0.114

occurred after ERCP in two older men with preexisting cTnI elevations.

New post-ERCP rises in cTnI levels (≥ 0.4 $\mu\text{g/L}$) were documented in 6 older patients. This was found 24 hours after the procedure in 5 patients and 2 days later in one. There were two deaths in this group. An 80-year-old patient on hemodialysis, with end-stage renal failure, metastatic cholangiocarcinoma, and severe aortic stenosis, developed an acute myocardial infarction after ERCP with a peak cTnI of 66.7 $\mu\text{g/L}$; he died on the 11th post-procedure day. The second death was in an 83-year-old woman with a cTnI rise to 1.3 $\mu\text{g/L}$ and CK of 211 U/L who succumbed on day 2 from a previously undiagnosed aortic dissection. In 3 patients with a mild cTnI rise (0.4–0.8 $\mu\text{g/L}$), accompanied by marked elevation of CK in 2 (669 U/L and 358 U/L), the myocardial injury was clinically asymptomatic. The sixth patient was an 85-year-old woman with hypertension, heart failure, and diabetes mellitus, who had ST depression during the procedure but no rise in cTnI in the first 24 hours after ERCP; she developed severe pancreatitis (lipase, 1844 U/L [normal: 30–300 U/L]) and myocardial injury (cTnI, 1.4 $\mu\text{g/L}$) on day 3 after ERCP and required admission to the coronary care unit.

Notably, ST segment changes were present after surgery in only 2 of the 8 patients with elevated cTnI levels. ERCP-related transient ECG changes (ST segment deviations and/or arrhythmias) were observed in 14 older patients without biochemical evidence of myocardial injury. Of 6 patients with a new cTnI elevation, 2 had episodes of hypoxemia during ERCP, 2 developed hypertension (MAP > 100 mm Hg), 2 had tachycardia (HR > 100 bpm), 1 had a very high RPP (> 20), and 2 had PRQ < 1.0.

TABLE 4. Incidence (%) of new electrocardiographic abnormalities and myocardial injury associated with ERCP in older and younger patients

Characteristics	Group 1 (≥ 65 y, n = 74)	Group 2 (< 65 y, n = 56)	p Value
Sinus tachycardia (≥ 100 bpm)	51.4	83.9	0.001
Sinus bradycardia (< 50 bpm)	6.0	3.1	0.419
Atrial fibrillation	10.0	0.0	0.170
Premature ventricular complexes	2.0	0.0	1.000
ST segment changes (depression or elevation)	10.0	3.1	0.464
Any ECG ischemia or arrhythmia	24.0	9.3	0.168
Troponin I rise (≥ 0.4 $\mu\text{g/L}$)	8.1	0.0	0.078

There were no significant differences between group 1 patients with and without a cTnI rise with respect to age, cardiovascular RFs, or type of anesthesia (in all comparisons, $p > 0.1$). Likewise, mean BP parameters and HR before and during ERCP did not differ significantly between patients with or without cTnI rise (not shown). Neither high RPP (> 20) nor low PRQ (< 1) was predictive of myocardial injury.

In patients with a new cTnI rise, the duration of ERCP was significantly longer (59.5 ± 29.0 minutes vs. 26.4 ± 14.3 minutes; $p = 0.026$). ERCP lasted longer than 30 minutes in 29 (39.2%) of 74 procedures in older group and in 12 (21.4%) of 56 procedures in the younger group ($p = 0.049$). In the older patients, a new cTnI rise occurred in 5 (17.2%) of 29 prolonged ERCPs (> 30 minutes) and only in 1 (2.2%) of 45 procedures lasting less than 30 minutes ($p = 0.060$). Linear regression analysis showed that the RR of myocardial injury detected by a cTnI rise increased by 141% for every additional 15 minutes in the duration of ERCP (RR 2.41: 95% CI [1.03, 5.65], $p = 0.043$).

Objective indications of myocardial ischemia/injury (ECG change and/or rise in cTnI) were compared with comorbid conditions, desaturation episodes, hemodynamic responses, and duration of the procedure. These revealed a significant association between myocardial ischemia or injury with a history of congestive heart failure (RR 2.6: 95% CI [1.1, 6.1], $p = 0.033$) and a longer duration of ERCP (37.7 ± 28.9 minutes vs. 24.2 ± 12.3 minutes, $p = 0.007$).

Post-ERCP pancreatitis, cardiopulmonary complication patterns, and age

Two procedures (2.7%) in group 1 and 5 (8.9%) in group 2 were associated with the development of post-

ERCP pancreatitis ($p = 0.244$). This complication was associated (regardless of age) with desaturation (RR 5.9: 95% CI [1.2, 32.0], $p = 0.027$) and myocardial ischemia or injury (RR 4.4: 95% CI [1.4, 7.8], $p = 0.009$). Every 15 additional minutes in ERCP duration also increased the RR of developing pancreatitis (RR 2.10: 95% CI [0.90, 4.89], $p = 0.085$), though not significantly.

DISCUSSION

To our knowledge, this is the first prospective study documenting that, in a substantial number of older patients, ERCP may result in symptomatic or silent structural myocardial injury, as evidenced by a rise in cTnI, a highly specific and sensitive marker for myocardial cell injury. In accordance with previous investigations, we found that ERCP was commonly associated with marked hemodynamic changes, arterial hypoxemia, and myocardial ischemia as assessed by ECG. However, in our study, the incidence and the severity of these ERCP-related cardiopulmonary responses in patients more than 65 years of age and those less than 65 years old were similar, despite the higher prevalence of comorbid conditions, including heart disease and cardiovascular RFs in the former group. Post-ERCP myocardial injury did not correlate with the type of anesthesia, the presence of comorbid disease, or the cardiac risk scores, and it occurred in one patient without any cardiovascular RFs. Nor was there any relationship with intraoperative factors, including hemodynamic stress, ECG changes, or oxygen desaturation. In our study, myocardial injury occurred only in older patients, predominantly men, and was associated with the duration of ERCP.

Cardiopulmonary complications in elderly patients have been evaluated during upper-GI endoscopy.^{12,17,22} A recent study found that complications of GI endoscopy occurred 2 to 70 times more commonly than previously recorded.¹⁰ The complication rate of ERCP has been reported to be 17 times higher than that of esophagogastroscope (21.6 vs. 1.3 per 1000 examinations).¹⁵ Older patients who undergo ERCP may be at particular risk for cardiopulmonary complications because of preexisting cardiovascular disease, sedation (with a combination of benzodiazepine and an opioid), and prolonged procedure time. In addition, they may also be more prone to silent myocardial ischemia.²³

With respect to preoperative factors, in our study, a new cTnI release was not related to any comorbid conditions or the total number of cardiovascular RFs. These findings are in agreement with observations of others, including Johnston et al,²³ who reported that over half of patients with ischemia during ERCP (defined as ST segment changes) had no previous cardiac history and had normal baseline ECG, and Kounis et al,⁹ who found that an abnormal ECG before ERCP was not predictive of ischemic changes during the procedure. Gender, however, may be a significant preoperative factor in that 5 of the 6

patients with a post-ERCP rise in cTnI were men. Male gender has been recognized as an independent RF for the occurrence of cardiovascular complications during and after GI endoscopy.¹⁰

Intraoperative factors such as transient hypoxemia, tachycardia, rise in BP, ST segment changes, and arrhythmias have previously been documented to occur during ERCPs.^{6,23} It has been suggested that both advanced age and cardiovascular disease increase the risk of these events.⁹ However, other investigators have concluded that, even in patients with severe coronary artery disease, endoscopic procedures, including ERCP, do not increase the risk of myocardial ischemia as determined by intraoperative ECG changes.^{3,16} The incidence and, most important, the clinical significance of abnormal cardiopulmonary responses to ERCP remain controversial.^{6,23,24} Several studies have, however, found that between 16.7% and 50% of all deaths related to ERCP are caused by cardiopulmonary complications.^{6,15} Whether the intraoperative derangements alluded to above are predictive of cardiopulmonary complications after ERCP is unclear. Part of this uncertainty may stem from the fact that, in previous studies, only ECG analysis was used to detect myocardial ischemia. It should be noted that, although ECG changes, including ST segment deviation, are considered reliable indicators of myocardial ischemia, the sensitivity and the specificity of ECG signs in identifying myocardial damage are limited.²⁵ Moreover, even trained observers recognize only 15% to 40% of ECG ischemic events displayed on an oscilloscope.²⁶ This may be a factor in the wide variation in reported ERCP-related myocardial ischemia evaluated by ECG monitoring (4%-50%).^{7,14,16,23}

Cardiospecific troponins, the current criterion standard of myocyte necrosis,^{18,27} have not been measured in patients undergoing ERCP. In one small study of 10 healthy patients, 2 (63 and 65 years of age) developed "true myocardial ischemia" during ERCP, as evaluated by ECG and myocardial scintigraphy.⁸ In the present study, biochemical evidence of ERCP-related myocardial injury was detected in 6 (11.3%) of 53 patients over 65 years of age (8.1% of 74 procedures), more frequently (5/6) in men.

Because an elevation of cTnI levels provides an early and highly specific and sensitive diagnosis of a myocardial lesion, our results confirm that the majority of ECG changes observed during ERCP are not predictive of subsequent myocardial injury. However, we identified one older woman with ST segment depression during the ERCP and with severe post-ERCP pancreatitis who developed an elevated cTnI level on the third day after the procedure. This observation is in agreement with previous findings that the release of pancreatic proteolytic enzymes may contribute to myocardial infarction.²⁸

With respect to intraoperative factors other than ECG changes, ERCP-related tachycardia, increases in arterial BP, or hypoxemia were not predictive of a rise in cTnI. Nevertheless, in all 6 patients with a post-ERCP rise in

cTnI, there was one abnormality and, in 4 patients, there were two of these abnormalities. The significance of these factors is controversial. Solomon et al²⁹ concluded that oxygen desaturation during endoscopy was not associated with any adverse hemodynamic effects or arrhythmias. However, other studies have linked hypoxemia to ECG manifestations of ischemia—ST segment changes and arrhythmias.³⁰ Some researchers have concluded that tachycardia associated with a metabolic stress response is the most important factor in the genesis of myocardial ischemia.^{7,23} Kounis et al⁹ reported that ECG changes occurred in 16 of 29 older persons who underwent ERCP but only if both abnormalities, hypoxemia and sinus tachycardia, were present. In our study, although in the older group of patients both tachycardia and hypoxemia were common (51.4% and 16.2%, respectively), there was no correlation between myocardial injury and either hypoxemia or tachycardia. It should be noted that, in our investigation, as in previous reports,^{8,9,31} tachycardia during ERCP was often related to the use of hyoscine butylbromide. This anticholinergic drug was administered more often to the younger patients, in accordance with the recommendation that it should be avoided in patients with cardiac disease.³¹ Only one of our older patients with myocardial injury received hyoscine butylbromide and had a maximum heart rate of 98 bpm.

In the present study, RPP and PRQ, two commonly considered indices of myocardial oxygen consumption and predictors of ischemia,^{20,21} did not show a prognostic value. Interestingly, no correlation between myocardial ischemia and either RPP, PRQ, or systolic hypertension was observed in patients who underwent general anesthesia for coronary artery bypass surgery.^{32,33}

In view of the fact that myocardial injury correlated with the duration of the procedure, it may be postulated that the duration of ischemia may be crucial in determining whether injury occurs. This “critical cutoff” period appeared to be 30 minutes in our study. Interestingly, experimental studies in pigs have revealed that the release of cTnI depends on the duration of ECG ischemia (instead of its mere presence), with a major release occurring after 30 minutes of ischemia.³⁴ In other words, an ERCP-related cTnI elevation indicating myocardial injury may occur in patients in whom prolonged (usually >30 minutes) hemodynamic stress and/or hypoxemia cause ischemia that exceeds a tolerated limit. This limit may have significant individual variation, probably being lower in elderly patients with preexisting heart failure. Indeed, when ERCP-related cTnI elevation and ECG changes were combined, the presence of either or both of these abnormalities was also associated with a history of congestive heart failure.

Although the changes in hemodynamic and respiratory parameters during ERCP in the older and younger groups in our study were similar and the cardiovascular RFs of older patients had no prognostic value in predicting cTnI elevation, it was not surprising that, in some of our

older patients, the cumulative ischemic effects (“total ischemic burden”) of ERCP appeared to result in myocardial damage during prolonged procedures. Because even slightly elevated cTnI levels reflect myocardial injury, monitoring of cTnI, especially in elderly patients undergoing ERCP, may be useful for detecting cardiac events and introducing anti-ischemic interventions early.

The pathogenic mechanisms involved in myocardial injury related to ERCP are not completely understood. They appear to be multifactorial and involve interaction between preexisting organic cardiovascular disease and acute cardiopulmonary responses. These include viscerocardio reflexes that cause coronary vasospasm, imbalance in autonomic nervous activity because of vagal withdrawal,⁸ and enhancement in sympathetic activity and endocrine stress syndrome.³⁵ Furthermore, hypercapnia, which frequently precedes the onset of hypoxemia³⁶ and induces sympathoexcitation and an increase in MAP should be taken into account, especially in older patients with heart failure and a prolonged procedure. Patient position during the procedure, increased intra-abdominal pressure, airway obstruction, and aspiration, as well as effects of drugs, may also contribute.

In our series, acute pancreatitis, the most common complication of ERCP, followed 2.8% of procedures undertaken in the older patients and 8.9% of procedures in the younger group. In the literature, the reported incidence of post-ERCP pancreatitis ranges from 1% to 18%, with a mean of 9.3%,^{37,38} and the risk of this complication is higher in younger patients.⁶ We found that post-ERCP pancreatitis was associated with myocardial ischemia or injury, as well as arterial desaturation. It could be speculated that the benefits of glyceryl trinitrate^{39,40} with respect to post-ERCP pancreatitis may be related not only to the reduction in sphincter of Oddi pressure but also to systemic vasodilatation and an improved blood supply to the pancreas. It may also be postulated that glyceryl trinitrate prevents myocardial injury not only directly by reducing cardiac preload but also indirectly by preventing pancreatitis. This may be particularly pertinent if the pancreatic process begins intraoperatively. Indirect evidence for this exists to the extent that patient discomfort during ERCP has been shown to be an independent RF for the development of post-ERCP pancreatitis.³⁷ Further studies are needed to determine the pathophysiologic relationship between these events.

In conclusion, although ERCP significantly affects hemodynamics and may cause arterial desaturation, the majority of older patients tolerate the procedure well, suggesting that older age per se does not seem to be a critical RF for cardiopulmonary complications. However, in the older population, approximately 8% of ERCP procedures, especially if prolonged (>30 minutes), result in myocardial injury as defined by the release of cTnI. Because the occurrence of such ERCP-induced complications could not be predicted on the basis of comorbid status,

cardiac RFs, ECG changes, tachycardia, hypertension, or hypoxemia during ERCP, measurement of cardiac troponins before and 24 hours after the procedure may be advisable. Desaturation and myocardial ischemia/injury are associated with post-ERCP pancreatitis.

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Received May 5, 2005. Accepted September 1, 2005.

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This work was presented, in part, as a poster presentation at the Australian Gastroenterology Week, October 4-8, 2004, Brisbane, Australia. Published as an abstract: Fisher L, Thomson A. Cardiopulmonary complications of ERCP. *J Gastroenterol Hepatol* 2004;19(Suppl):A227.

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Perioperative acute upper gastrointestinal haemorrhage in older patients with hip fracture: incidence, risk factors and prevention

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Publication data

Submitted 24 September 2006
First decision 9 October 2006
Resubmitted 21 October 2006
Accepted 30 October 2006

SUMMARY

Background

No specific preventive strategy exists for acute gastrointestinal haemorrhage in hip fracture patients.

Aims

To determine the effectiveness of prophylactic use of proton pump inhibitors in patients with risk factors for acute gastrointestinal haemorrhage.

Methods

Prospective two-stage study of 822 consecutive older (≥ 60 years) hip fracture patients.

Results

Acute gastrointestinal haemorrhage occurred in 16 (3.9%) of 407 patients and was associated with increased length of hospital stay (28.7 vs. 15.9; $P = 0.0027$) and mortality (18.8% vs. 4.3%; $P = 0.043$). Multiple analysis identified five independent risk factors for acute gastrointestinal haemorrhage: pre-existing peptic ulcer (OR 4.3; $P = 0.043$), current smoking (OR 3.1; $P = 0.023$), post-operative use of an antiplatelet agent (OR 6.5; $P = 0.046$), post-operative use of non-steroidal anti-inflammatory drug/cyclo-oxygenase-2 inhibitor (OR 4.9; $P = 0.06$) and blood group O (OR 1.7; $P = 0.046$). These risk factors were highly sensitive and had a negative predictive value of 99.8%. Prophylactic use of proton pump inhibitors in patients with risk factor for acute gastrointestinal haemorrhage significantly reduced the incidence of this complication (0.72% in treated patients vs. 13.4% in untreated; $P < 0.001$); the number needed to treat was 7.9.

Conclusions

In older hip fracture patients perioperative acute gastrointestinal haemorrhage occurs in 3.9% and is associated with poor outcome. Preventive proton pump inhibitor therapy in patients at risk of acute gastrointestinal haemorrhage is effective and safe.

Aliment Pharmacol Ther 25, 297–308

INTRODUCTION

Hip fracture (HF) in older adults is a major and growing health problem worldwide with enormous health and socio-economic consequences.¹ In older persons with HF, perioperative complications including acute gastrointestinal haemorrhage (AGIH) are the main determinants of outcomes and the leading cause of morbidity and mortality.^{2, 3} This clinical problem deserves particular attention because the number of HFs is increasing by 1–3% per year in most areas of the world and a worldwide epidemic is expected.⁴

In the perioperative setting AGIH continues to be a major source of morbidity and mortality. The reported frequency of this complication varies from 0.39%⁵ to 14%.⁶ The incidence of AGIH in older HF patients is unknown.

The risk of developing AGIH rises in older age, in patients with multiple comorbidities and physical disability, in persons with previously diagnosed peptic ulcer and in users of non-steroidal anti-inflammatory drugs (NSAIDs), anticoagulants, antiplatelet agents, selective serotonin reuptake inhibitors (SSRIs) and corticosteroids.^{7–13} HF patients are usually elderly and the majority have coexisting disease. They use a wide range of drugs associated with increased risk of AGIH and it is common practice to use anticoagulants to reduce the risk of thromboembolic disease in the perioperative setting. The relevance, magnitude and prognostic value of these factors in daily clinical management of these patients have not been evaluated.

Acid-suppressive therapy is currently considered standard care for treatment of AGIH.^{14, 15} There is a general agreement that proton pump inhibitors (PPI) are more effective than H₂-receptor antagonists in prevention and treatment of AGIH.^{14, 16–18} However, the routine use of prophylactic antisecretory medications in stress-related AGIH in critically ill patients is still debated.^{19–23} In patients with post-operative stress-induced ulcers, a recent meta-analysis found little reduction in AGIH with pharmacological prophylaxis.²¹ There are no data regarding the clinical effectiveness of PPI in AGIH prophylaxis in HF patients.

The incidence of and risk factors (RFs) for AGIH in older HF patients are unknown and no proven preventive strategy exists. Identification of possible predictors of AGIH is necessary for prognosis and may be helpful in planning appropriate management.

The aims of this study were to: (i) estimate the incidence of and RFs for AGIH in older low-trauma HF patients, (ii) determine the effectiveness of prophylactic use of PPIs in HF patients with RFs for AGIH and (iii) evaluate the diagnostic value of identified RFs.

PATIENTS AND METHODS

Study design

During a 4-year period (from 1 October 2001 to 31 August 2005) we conducted a prospective non-randomized two-stage study of 822 consecutive older (≥ 60 years) patients admitted to our hospital with a low-trauma HF (Figure 1). All patients were entered into an electronic database accumulating demographic and clinical characteristics, preoperative, intraoperative and post-operative data, comorbidities, treatment details, blood transfusions, surgical procedures and outcomes. There were no inclusion or exclusion criteria except age and type of trauma.

The first stage of the study (from 1 October 2001 to 31 July 2003, 'observational group', $n = 407$) aimed to determine the incidence of and RFs for AGIH. We identified all patients with this complication and retrieved all records to detect possible predisposing factors (medical history, preoperative peptic ulcer, presence of coagulopathy, drug use, smoking habits, alcohol intake and laboratory data), details of management and outcome. The exposure to the following medications was recorded: antiplatelet agents (aspirin, clopidogrel), NSAIDs (ibuprofen, naproxen, indomethacin, piroxicam, diclofenac), selective cyclo-oxygenase-2 (COX-2) inhibitors (celecoxib, rofecoxib, meloxicam), paracetamol, warfarin, SSRIs and bisphosphonates (alendronate, risedronate).

A single observer (LF) reviewed the hospital gastrointestinal endoscopy database and extracted the endoscopic reports of all HF patients who had developed significant AGIH.

Based on the results obtained in the first stage of the study we identified RFs for AGIH and developed and implemented an AGIH prophylaxis protocol.

In the second stage of the study (from 1 December 2003 to 31 August 2005, 'interventional group', $n = 415$) all older HF patients with identified RFs for AGIH were treated with prophylactic PPIs. The records of all patients and the endoscopy database were reviewed in the same manner as in the first stage. The effect of implementing the AGIH prophylaxis

guidelines on the incidence of this complication was determined.

The study was approved by the local Human Research Ethics Committee. The prophylactic use of PPIs was discussed with patients and/or their relatives and informed consent was obtained.

Definitions

Acute gastrointestinal haemorrhage was defined as overt bleeding (haematemesis and/or melaena) associated with haemodynamic compromise and/or need for blood transfusion. Presence of hypotension [systolic blood pressure (SBP) < 100 mmHg] and/or orthostatic hypotension (fall of SBP $\geq$ 20 mmHg or diastolic $\geq$ 10 mmHg from lying to sitting position within 3 min), tachycardia (pulse rate >100/min) and/or fall in haemoglobin of more than 20 g/L was defined as significant AGIH.

Patients were defined as taking the drug under analysis preoperatively if they had taken it regularly for at least 1 month before hospitalization. On admission HF patients were taken off anticoagulants, antiplatelet agents, NSAIDs and these drugs were restarted post-operatively only in patients with thromboembolic disease, atrial fibrillation, mechanical heart valves or for prevention of mural thrombus formation after myocardial infarction or for pain control. AGIH was attributed to therapy if a patient used the drug within the 7 days prior to the episode of bleeding.

Preventive antisecretory therapy with a PPI (in the interventional stage of the study) was defined as oral treatment with pantoprazole (40 mg daily) or omeprazole (20 mg daily) or esomeprazole (20 mg daily). This was usually started on the second post-operative day, but in eight patients, in whom surgery was delayed (>48 h), on the second hospital day. Equipotent effects have been observed when these PPIs were used in the above doses.²⁴ Mortality was defined as death within the hospitalization period.

Statistical analysis

Results on continuous variables were expressed as mean $\pm$ s.d. Chi-square test with Yates continuity correction or Fisher's exact test were used to compare categorical variables and Student's *t*-test was used for comparison of continuous variables. To determine RFs for AGIH, HF patients who developed gastrointestinal bleeding during hospitalization were compared with

those who did not. The association of potential RFs with AGIH was determined by calculating odds ratio (OR) and 95% confidence intervals (CI). To identify independent predictors of AGIH in HF patients, multivariate analysis was performed using a logistic regression model. Factors associated with dependent variables at $P \leq 0.10$ were included in the multivariate model. Two-tailed *P*-values of <0.05 were considered statistically significant.

All analyses were conducted by using statistical software (Stata Corp., Version 7, College Station, TX, USA).

In the interventional phase of the study, at least 44 patients with RFs for AGIH would be required in each group to detect a 25% difference in AGIH in favour of PPI use with an α of 0.05 and a power ($1 - \beta$) of 0.80 (two-sided test). The size of our study groups was sufficient to detect a 10% benefit with the prophylactic use of PPI.

RESULTS

Baseline characteristics

The characteristics of HF patients enrolled during the two study periods are summarized in Table 1. The majority (84.3%) of patients were 75 years of age or older with gender distribution of 4 (females):1 (males). They had on average 4.5 comorbid conditions and a high proportion was using gastrotoxic drugs. The demographics and main clinical characteristics as well as residential status of the observational and interventional groups were similar. The mean patient-days of observation (follow-up data) was 5942 in the first group and 5976 in the second. Although most comorbid diseases were equally prevalent in the two groups, patients in the second period prior to admission were prescribed clopidogrel more often and a COX-2 inhibitor less often (possibly because at this time rofecoxib, because of its cardiovascular risks, was taken off the market). During hospitalization the patients in the interventional group received clopidogrel, alone or in combination with aspirin, and a bisphosphonate, more often but a COX-2 inhibitor less often. Nearly all patients received thromboembolic prophylaxis, usually subcutaneous low-molecular weight heparin (LWMH; enoxaparin, 40 mg daily) or unfractionated heparin (5000 units twice a day). Both groups were usually taking gastrotoxic drugs in addition to prophylactic thromboembolic therapy.

Characteristics	Observational group (<i>n</i> = 407)	Interventional group (<i>n</i> = 415)
Age (years, mean $\pm$ s.d.)	82.1 $\pm$ 7.9	81.7 $\pm$ 8.3
Females, <i>n</i> (%)	304 (74.7)	309 (74.5)
Site of fracture, <i>n</i> (%)		
Cervical	237 (58.2)	222 (55.5)
Intertrochanteric	152 (37.3)	172 (41.4)
Subtrochanteric	18 (4.4)	21 (5.1)
Comorbidities (mean $\pm$ s.d.)	4.5 $\pm$ 2.02	4.4 $\pm$ 2.03
History of peptic ulcer, <i>n</i> (%)	20 (4.9)	21 (5.1)
Dementia, <i>n</i> (%)	134 (32.9)	133 (32.0)
Coronary artery disease, <i>n</i> (%)	98 (24.1)	103 (24.8)
History of myocardial infarction, <i>n</i> (%)	24 (5.9)	25 (6.0)
History of stroke, <i>n</i> (%)	49 (12.0)	54 (13.0)
Atrial fibrillation, <i>n</i> (%)	65 (16.0)	62 (14.9)
Chronic obstructive pulmonary disease, <i>n</i> (%)	45 (11.1)	48 (11.6)
Ex-smokers, <i>n</i> (%)	45 (11.1)	49 (11.8)
Current smokers, <i>n</i> (%)	44 (10.8)	39 (5.4)
Hypertension, <i>n</i> (%)	166 (40.8)	178 (42.9)
Osteoarthritis, <i>n</i> (%)	144 (35.4)	139 (33.5)
Blood group O, <i>n</i> (%)	169 (41.5)	158 (38.1)
Institutionalization, <i>n</i> (%)	138 (33.9)	195 (34.9)
Medications used prior to admission, <i>n</i> (%)		
Aspirin	104 (25.6)	117 (28.2)
Clopidogrel	7 (1.7)	22 (5.3)*
COX-2 inhibitor	31 (7.6)	16 (3.9)*
NSAID	41 (10.1)	48 (11.6)
Warfarin	44 (10.8)	49 (11.8)
Corticosteroids	29 (7.1)	31 (7.5)
SSRI	59 (14.5)	63 (15.2)
Bisphosphonate	16 (3.9)	19 (4.6)
Medications used in the hospital, <i>n</i> (%)		
Paracetamol	405 (99.5)	412 (99.3)
LMWH or UFH prophylaxis	389 (95.6)	404 (97.3)
Aspirin	36 (8.8)	43 (10.4)
Clopidogrel	7 (1.7)	19 (4.6)*
Aspirin + clopidogrel	1 (0.25)	9 (2.2)*
COX-2 inhibitor	24 (5.9)	12 (2.9)
Aspirin + COX-2 inhibitor	8 (2)	6 (1.4)
Non-selective NSAID	3 (0.7)	8 (1.9)
Warfarin	5 (1.2)	8 (1.9)
Bisphosphonate	173 (42.5)	394 (94.9)*

Table 1. Demographics, main clinical characteristics and use of gastrotoxic medications by older hip fracture patients in the two study periods: in the observational and interventional groups

LMWH, low-molecular weight heparin; UFH, unfractionated heparin; NSAID, non-steroidal anti-inflammatory drug; SSRI, selective serotonin reuptake inhibitor; COX, cyclooxygenase.

* $P < 0.05$.

Incidence of and risk factors for AGIH

Of 407 patients admitted with HF during a 22-month period 16 (3.9%; 95% CI: 2.3–6.3%) had an AGIH. Details of these patients are shown in Table 2. In

patients with AGIH there was a significantly higher prevalence of a history of peptic ulcer, current tobacco smoking, blood group O, use of aspirin or a COX-2 inhibitor prior to admission when compared with the rest of the cohort. However, there was no difference

Table 2. Demographic and clinical characteristics of older hip fracture patients with and without AGIH in the observational group

Characteristics	With AGIH (<i>n</i> = 16)	Without AGIH (<i>n</i> = 391)
Age (years, mean $\pm$ s.d.)	83.1 $\pm$ 7.6	81.9 $\pm$ 8.0
Females, <i>n</i> (%)	12 (75)	292 (74.6)
Type of hip fracture, <i>n</i> (%)		
Cervical	10 (62.5)	227 (58.1)
Trochanteric	5 (31.3)	147 (37.6)
Subtrochanteric	1 (6.3)	1 (6.3)
Comorbidities (mean $\pm$ s.d.)	4.7 $\pm$ 2.02	4.5 $\pm$ 2.01
History of peptic ulcer, <i>n</i> (%)	4 (25)	17 (4.4)*
Blood group O, <i>n</i> (%)	11 (68.8)	158 (40.4)*
Current smokers, <i>n</i> (%)	5 (31.3)	39 (9.97)*
History of smoking, <i>n</i> (%)	6 (37.5)	188 (48.1)
Medications used prior to admission, <i>n</i> (%)		
Aspirin	8 (50)	96 (24.6)*
Non-selective NSAID	0	41 (10.5)
COX-2 inhibitor	4 (25)	27 (6.9)*
Clopidogrel	1 (6.3)	6 (1.53)
Warfarin	1 (6.3)	43 (11.0)
Corticosteroids	1 (6.3)	28 (7.2)
Bisphosphonate	6 (37)	14 (3.6)
SSRI	0	59 (15.1)
H ₂ -receptor antagonist	2 (12.5)	38 (9.7)
Proton-pump inhibitor	0	29 (7.4)
Medications used in hospital†, <i>n</i> (%)	15 (93.8)	366 (93.6)
Paracetamol	16 (100)	389 (99.5)
LMWH or UFH prophylaxis	13 (81.3)	356 (91.1)
Aspirin	8 (50)	28 (7.2)*
COX-2 inhibitor	4 (25)	20 (5.1)*
Clopidogrel	1 (6.3)	6 (1.5)
Bisphosphonate	6 (37)	167 (42.7)
SSRI	2 (12.5)	57 (14.6)
H ₂ -receptor antagonist	2 (12.5)	12 (3.1)
Proton-pump inhibitor	0	37 (9.5)

LMWH, low-molecular weight heparin; UFH, unfractionated heparin; AGIH, acute gastrointestinal haemorrhage; NSAID, non-steroidal anti-inflammatory drug; SSRI, selective serotonin reuptake inhibitor; COX, cyclo-oxygenase.

* $P < 0.05$.

† Prior to AGIH.

between the two groups in all other variables. During hospitalization (and prior to AGIH) the group who developed gastrointestinal bleeding was more likely to receive aspirin (100–150 mg daily) and/or a COX-2 inhibitor in combination with LMWH or unfractionated heparin prophylaxis. Of 16 patients with AGIH two were taking an H₂-antagonist and none a PPI.

Acute gastrointestinal haemorrhage occurred after a mean hospital length of stay of 11.1 ± 10.4 days and presented with haematemesis (six patients) and/or melena (12) and haemodynamic instability/shock (12). The mean lowest haemoglobin level was 89.7 ± 19.5 g/L, the peak serum urea concentration

was 15.5 ± 6.4 mm and the albumin concentration was 25.0 ± 5.45 g/L. All patients received intravenous fluids and 11 required blood transfusion. The mean number of transfused units of packed red cells was 2.4 ± 3.12 per patient. The source of the AGIH was identified in eight of 10 patients in whom endoscopy was performed: two had duodenal ulcers, one gastric, two both types of ulcers, two erosive/haemorrhagic gastritis and one Mallory-Weiss tear; endoscopy was normal in two subjects. *Helicobacter pylori* infection was not identified in any of nine tested patients. No evidence of chronic liver disease was found in any of the patients with AGIH. No surgery was performed

for AGIH. Mean hospital length of stay in patients with AGIH was significantly higher than in the rest of the cohort (28.7 ± 15.9 vs. 15.9 ± 13.8 days; $P = 0.0027$).

Acute gastrointestinal haemorrhage was associated with poor outcome: three (18.8%) patients died from acute cardiovascular or cerebrovascular events and in one patient AGIH may have contributed to the onset of sepsis, related to aspiration. In the remainder of the study population 17 of 391 patients died, indicating in-hospital mortality rate of 4.3%. In HF patients with AGIH compared with the rest of the cohort, the odds of dying in hospital were 5.1 (95% CI: 1.04–21.8; $P = 0.043$).

When multivariate regression analysis was conducted on preoperative and post-operative variables that had yielded different results by univariate analysis ($P \leq 0.10$) five independent RFs for AGIH were identified (Table 3). The significant and independent predictors of AGIH in older HF patients receiving post-operative thromboprophylaxis with LMWH or unfractionated heparin included: (i) history of peptic ulcer, (ii) current tobacco smoking, (iii) use of an antiplatelet agent (aspirin and/or clopidogrel), (iv) use of a NSAID (COX-2 inhibitor) and (v) blood group O. As subcutaneous LMWH or unfractionated heparin alone did not increase the risk of AGIH these data may indicate an additive or even synergistic effect of concomitant use of pharmacological thromboembolic prophylaxis with an antiplatelet agent or NSAID/COX-2 inhibitor, as well as with previous history of peptic ulcer, current tobacco smoking or blood group O. Other variables,

such as preadmission use of aspirin or a COX-2 inhibitor, although of significance in the univariate analysis, did not improve the predictability in the multivariate model and were not included.

Of 16 patients who developed post-operative AGIH, three had one RF, eight had two RFs, three had three RFs, and one had all five, but in one patient no RFs were identified. Two of three patients who died had two RFs each, but no RFs were found in the third. The mean length of hospital stay in patients with one, two and three or more RFs was 17.3 ± 4.2 , 19.3 ± 13.7 and 48.5 ± 19.2 days, respectively ($P = 0.013$ for trend). These data indicate a significant relationship between the number of RFs and the severity of the disease that is reflected in prolongation of hospital stay. In the presence of at least one RF, the odds of developing AGIH were 35.6 (95% CI: 4.9–727.8; $P < 0.001$).

Effect of prophylactic PPI

During the second 21-month period a total of 415 older low-impact HF patients were admitted to our hospital and constituted the intervention group. Among these patients 139 (33.5%) were identified as having at least one RF for AGIH and all of them were started on PPI prophylaxis: 111 (79.8%) patients received pantoprazole, 16 (11.5%) omeprazole and 12 (8.6%) esomeprazole. In the first stage of our study none of the patients with AGIH received a non-selective NSAID post-operatively; this may be due to chance. However, eight patients in the interventional group continued to use a NSAID post-HF repair; all of them were prescribed a PPI.

Only one (0.72%) of 139 patients taking PPIs experienced a bleeding event. However, there were three other patients with RF who developed AGIH. These were not recognized on admission and no prophylactic PPI therapy was introduced.

During hospitalization, of these four patients with AGIH, two were receiving a COX-2 inhibitor, one clopidogrel, one clopidogrel and aspirin, two corticosteroids and two alendronate. Two patients had a history of peptic ulcer and three had blood group O. Endoscopic examination revealed oesophageal ulcers in two patients, a gastric ulcer in one and oesophageal, gastric and duodenal ulcers in one. Of these four bleeding patients, two had melaena and two both haematemesis and melaena and all required blood transfusions. Overall, one patient had one RF, two had three RFs and one had four RFs. There was no

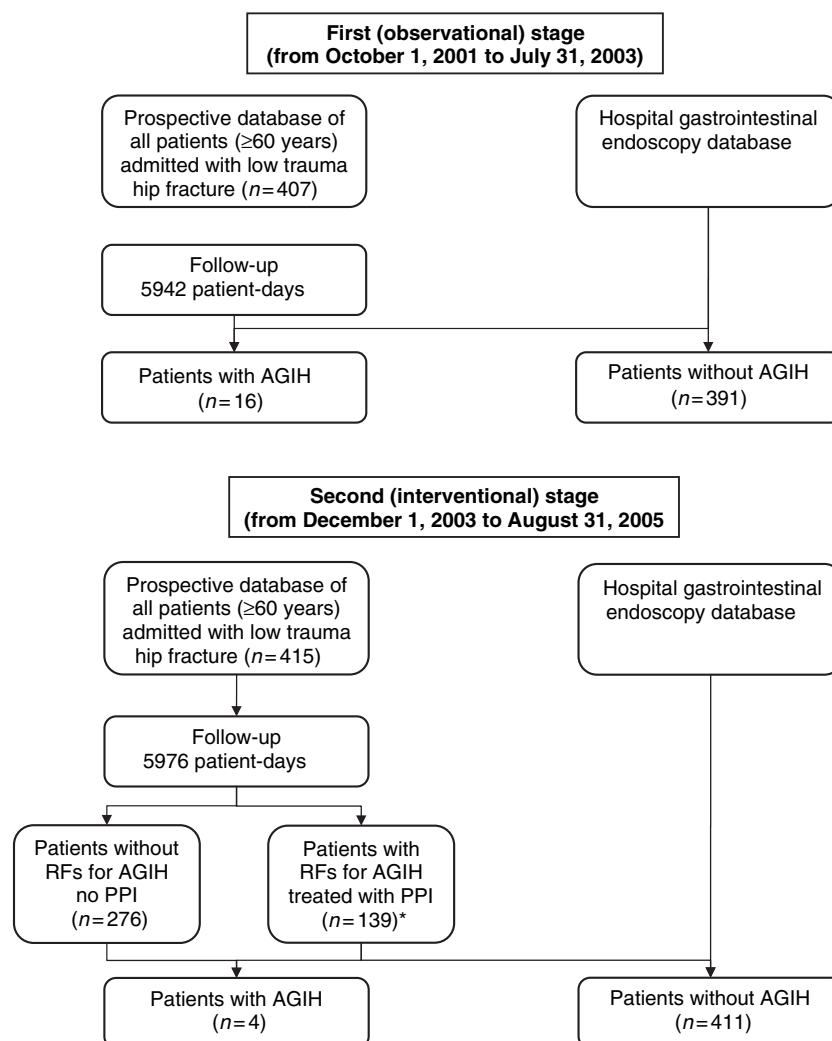
Table 3. Risk factors for acute upper gastrointestinal haemorrhage in older hip fracture patients: multivariate analysis*

Factor	OR (95% CI)	P-value
History of peptic ulcer	4.3 (1.04–12.83)	0.043
Current smoker	3.1 (1.25–6.42)	0.023
Blood group O	1.7 (1.01–2.21)	0.046
Aspirin	6.9 (3.25–11.83)	0.001
Aspirin and/or clopidogrel	6.5 (3.24–10.09)	0.001
COX-2 inhibitor	4.9 (1.48–12.19)	0.006

* Adjusted also for age, sex, type of hip fracture and number of comorbid conditions.

CI, confidence interval; COX, cyclo-oxygenase; OR, odds ratio.

Figure 1. Flow diagram and identification of older patients with hip fracture and acute gastrointestinal haemorrhage (AGIH) included into the study within a period of 4 years. Asterisk (*) in three patients risk factors (RFs) for AGIH were not recognized and they were not receiving proton pump inhibitor therapy.



in-hospital mortality. The patient who was treated with a PPI and developed AGIH had previously undergone partial gastrectomy for peptic ulcer disease, had blood group O and was taking celecoxib, prednisolone, warfarin and alendronate post-HF repair; on endoscopy oesophageal ulcers was found.

The combined data from the two study periods demonstrated that the prophylactic use of PPI in patients with a RF for AGIH significantly decreased the risk of this complication (Figure 2). In the group treated with PPIs, AGIH occurred in one of 139 patients (rate = 0.72%; 95% CI: 0.02–3.9%) compared with 18 of 134 untreated patients (rate = 13.4%; 95% CI: 8.2–20.4%), and this difference is highly significant ($P < 0.001$). The mean numbers of identified RFs in these two groups were similar (2.25 ± 0.61 RFs per patient in the treated and 2.19 ± 0.74 in the untreated group). In patients treated with a PPI compared with those not

treated, the odds of AGIH were 0.05 (95% CI: 0.002–0.338; $P < 0.001$). The number needed to treat was 7.9 (95% CI: 7.1–15.2). No adverse events or drug interactions associated with PPI therapy were observed. None of our patients given PPI developed nosocomial pneumonia or *Clostridium difficile*-associated diarrhoea, although post-operatively all patients received antibiotics for 24 h and about half for 5 days or more.

On the other hand, among 549 patients without RFs for AGIH who were not receiving prophylactic PPIs only one (0.18%) bleeding event was observed.

Diagnostic value of risk factors for acute gastrointestinal haemorrhage

At least one of the proposed RFs for AGIH was present in 273 (33.2%) of 822 patients, indicating that 1/3 of older HF patients were at risk of serious AGIH. Overall,

Table 4. Diagnostic value of risk factors for perioperative acute gastrointestinal haemorrhage in older patients with hip fracture

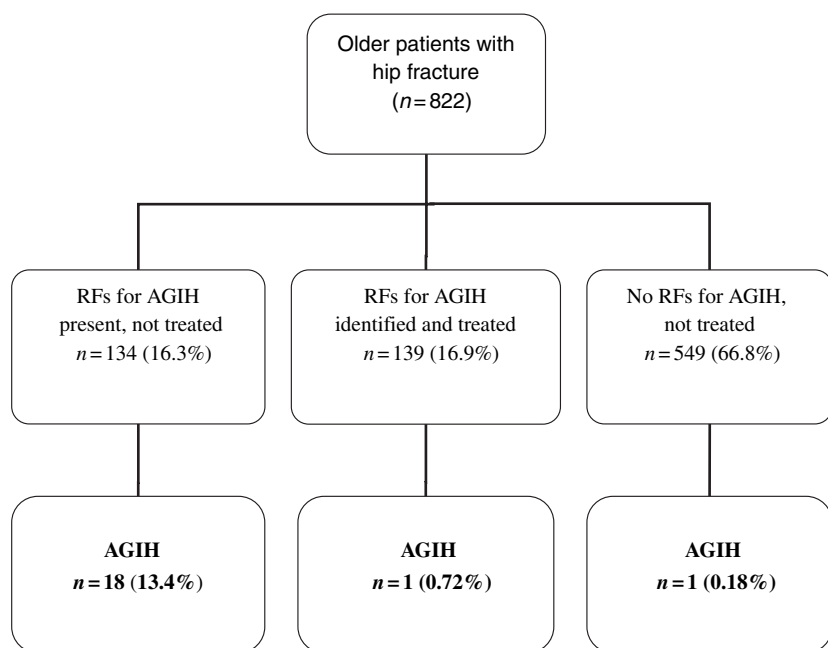
Statistic	Value	95% CI
Sensitivity	0.947	0.724–0.997
Specificity	0.825	0.819–0.827
Positive predictive value	0.134	0.103–0.141
Negative predictive value	0.998	0.990–1.000

the proposed RFs identified 19 (95%; 95% CI: 75–99.9%) of 20 patients who developed AGIH perioperatively, including 18 of 134 subjects not receiving preventative PPIs and one of 139 receiving a PPI. Among 549 patients without RFs for AGIH only one (0.18%; 95% CI: 0.005–1.013%) experienced bleeding. Table 4 shows that the sensitivity of the proposed RFs for AGIH is 94.7% and specificity is 82.5%, and they have a high negative predictive value (99.8%).

DISCUSSION

To our knowledge, this is the first systematic study of AGIH in older patients with HF. It provides estimates of the incidence and main characteristics of this perioperative complication, determines RFs for AGIH, and shows the high efficacy and safety of prophylactic use of PPIs in HF subjects with identified RFs.

The incidence of AGIH of 3.9% in our study relied on a prospectively collected database which included all older HF patients from the time of acute admission to discharge (either home or to a long-term care facility) or death, with re-examination of all hospital records and endoscopic database. Therefore, in our opinion, it provides an accurate estimation of this complication. In recent investigations the frequency of post-operative AGIH is highly variable. The incidence of overt AGIH after major surgical procedures was reported to be 0.39%,⁵ after cardiac surgery from 0.8% to 0.9%,^{25, 26} to 5.5%,²⁷ after abdominal aortic aneurysm repair 1.29%,²⁰ after hip and knee arthroplasty 4.5%,²⁸ after percutaneous coronary interventions 5.4%,²⁹ and after bone marrow transplantation 7.4%.³⁰ Patients admitted to intensive care units have an incidence of clinically important bleeding of 0.17%²⁰ to 3.7%^{31, 32} depending on severity of the illness (mechanical ventilation, coagulopathy) with a 25–50% mortality.³¹ The variability in results may reflect differences in study populations, type of surgery, study methodology and definitions of AGIH. Our result of 3.9% is comparable with data after hip and knee arthroplasty and cardiac surgery. It has been estimated that in 2050 the annual number of patients with HF worldwide will be 7.3–21.3 million.⁴ Extrapolating our results globally this translates into 285 000–830 700 HF patients at particular risk of AGIH.

**Figure 2.** Effect of preventive use of proton pump inhibitors in older patients with hip fracture and risk factors for acute upper gastrointestinal haemorrhage.

Our study suggests that AGIH occurred on average 11.1 ± 10.4 days postadmission for HF repair. The mean interval between cardiac surgery and AGIH was 9.6 days (range: 1–30),²⁶ and nosocomial AGIH occurred after 14 ± 10 days of hospital stay.³³

In accordance with other studies we found that in HF patients with AGIH the outcome was poor. Although in our series no patients died directly from AGIH, it may have contributed to multiple organ failure and death in three (15%) of 20 patients and the onset of sepsis in one. The total mortality of the cardiac surgery patients who developed AGIH was 15%²⁶ to 27.7%^{25, 34} and in patients with nosocomial AGIH 34%³³ to 42%.³⁵ Our overall mortality rate is also in agreement with the rate (14.1%) observed in older people with AGIH attributable to NSAIDs.³⁶ The clinical significance of AGIH in HF patients is further clearly indicated by our observation that this complication is associated with a substantially prolonged length of hospital stay (28.7 vs. 15.9 days).

Using a multivariate model we found that in older HF patients receiving thromboembolic prophylaxis the following five RFs are independent and significant predictors for AGIH: (i) pre-existing peptic ulcer; (ii) current smoking; (iii) post-operative antiplatelet therapy (use of aspirin or clopidogrel); (iv) use of NSAID/COX-2 inhibitors and (v) blood group O.

It is worth noting that although each of the five RFs identified in our study has been previously described in the literature, their prognostic relevance has not been evaluated in post-operative patients. Multiple regression analysis showed that only mechanical ventilation and coagulopathy were independent predictors for stress-related AGIH in the intensive care setting.³¹ None of our patients was ventilated or had evidence of coagulopathy. In one report, patients with in-hospital AGIH were less likely to have a previous history of ulcer disease, *H. pylori* infection, chronic active gastritis or the ingestion of NSAIDs than those who presented to hospital with bleeding from peptic ulcers.³³ Our findings are consistent with numerous previous studies demonstrating that history of peptic ulcer,³⁷ active smoking,^{7, 9, 25} blood group O³⁸ and the use of NSAIDs or aspirin^{10, 13, 39} are strong predictors of AGIH. However, ours is the first study to confirm the importance of these RFs in older HF patients. Our observation that AGIH occurs in users of COX-2 inhibitors and clopidogrel is in agreement with recent studies reporting that substitution of non-selective NSAIDs with COX-2 inhibitors^{16, 39, 40} and

aspirin with clopidogrel^{39, 41, 42} does not eliminate the risk of AGIH.

There were two or more RFs in 15 of 20 patients with AGIH. Because some RFs can increase the effect of others (e.g. NSAID and peptic ulcer, combination of gastrotoxic drugs, smoking and any of the other RFs) an additive or even synergistic effect is likely.^{7, 12, 43}

We did not find an association between AGIH and the total number of comorbidities, a history of smoking, the post-operative use of paracetamol or prophylaxis against thromboembolism, or the prefracture use of warfarin, aspirin or NSAID/COX-2 inhibitors (the last two variables were significant RFs for bleeding in the univariate analysis). There is a greater risk of AGIH in older people after short-term NSAID use than after long-term therapy.³⁹

Our data, as in most previous studies,⁴⁴ showed that use of paracetamol does not increase the risk of AGIH. In one report, use of high-dose paracetamol (>2 g daily) increased the risk of bleeding.⁴⁵ Nearly all our patients were receiving paracetamol for pain control but none used more than 2 g daily. In our series, use of SSRIs did not increase the perioperative risk of AGIH, although some recently published reports demonstrated a significant association between bleeding and use of SSRIs (as serotonin is an important factor in platelet aggregation), especially when used concurrently with NSAIDs or aspirin.^{46, 47} We also found that treatment with bisphosphonates (alendronate or risedronate weekly) did not cause AGIH; and this observation is in agreement with most previous reports,^{48, 49} but not with all.⁴³ Overall, the five RFs identified in our study were highly sensitive in the prognosis of AGIH and had a negative predictive value of 99.8%.

The second – and main – objective of this study was to evaluate the preventive effect of PPIs in HF patients with RFs for AGIH. We found that post-operative PPI therapy dramatically reduces the incidence of AGIH in older HF patients.

The use of antisecretory drugs in the prevention and treatment of AGIH is based on the observations that low intraluminal pH impairs clot formation and accelerates clot lysis.⁵⁰ Gastric acid suppression prevents pepsin-induced clot lysis as conversion of pepsinogen to pepsin is inhibited when pH rises above 4, and may stabilize the clot by optimizing the function of coagulation factors and platelets when pH is above 6.

A recent Cochrane Database systematic review and meta-analysis showed that PPI therapy in peptic ulcer bleeding reduced re-bleeding and the need for surgery

compared with placebo or H₂-antagonists, but did not improve all-cause mortality.¹⁸ A decline in mortality was observed in Asian patients.⁵¹ Significant decreases in ulcer re-bleeding, surgery and mortality have been reported in another meta-analysis.¹⁷ A large body of literature supports the prophylactic use of PPI for prevention of ulcers and AGIH in patients taking NSAIDs and/or aspirin.⁵²

Meta-analyses of stress ulcer prevention showed that in high-risk patients a H₂-antagonist (ranitidine) significantly reduced the rate of clinically important AGIH compared with no therapy¹⁹ or sucralfate.⁵³ These data suggest that neutralizing gastric acid has a protective role and recent studies suggest that PPIs are more effective in stress ulcer prophylaxis than H₂-antagonists.^{23, 54} However, other studies reported no significant reduction in AGIH using pharmacological stress ulcer prophylaxis in the post-operative period or in the intensive care setting.^{21, 55, 56}

The five prognostic variables identified in our study are easy to recognize in clinical practice and enable the selection of patients with and without an increased risk of perioperative AGIH.

Our results demonstrated that the preventive use of PPIs in older HF patients at risk of AGIH is an effective and safe therapeutic approach: of 139 treated patients, bleeding occurred only in one (0.72%) and none experienced adverse events or complications. Our observations are in agreement with evidence that PPI use did not increase the risk of nosocomial pneumonia in critically ill trauma patients,⁵⁷ but contrast with a recent report that PPI therapy is associated with an increased risk of *C. difficile*-associated diarrhoea in hospitalized patients receiving antibiotics.⁵⁸

Our study confirmed that PPIs are of benefit even if a non-selective NSAID, COX-2 inhibitor, aspirin and/or clopidogrel are continued post-operatively.

Our study also suggests that targeted AGIH prevention strategy is likely to be cost-effective. One or more of the RFs was present in only one-third of HF patients. The number needed to treat older HF patients with RF to prevent one bleeding event was 7.9. In two-thirds (549) of our patients without RFs and who did not receive PPI therapy, only one (0.18%) experienced a bleeding event. These results suggest that patients without RFs have a low rate of bleeding and do not need PPI therapy. AGIH prophylaxis limited to patients with RFs for bleeding may lead to decreased drug costs. Taken together, our data indicate that targeted preventive strategy can significantly improve

the quality of care and may markedly reduce high costs due to the development of AGIH complications.⁵⁹

Several potential limitations of our study should be acknowledged. In our hospital all older HF patients are managed by an orthopaedic surgeon and a geriatrician; gastrotoxic drugs, especially non-selective NSAIDs, are generally discontinued on admission. It is likely that the bleeding rate would be higher in centres where this is not routine practice. We focused on RFs that had significance after multiple regression analysis; however, other preoperative and post-operative variables may be important. Our study was not sufficiently powered to perform a subanalysis of specific comorbid conditions, use of specific gastrotoxic drugs and the effects of drug interaction. Because not all patients were tested, the role of *H. pylori* infection in development of AGIH could not be determined. *Helicobacter pylori* infection, the main factor for peptic ulcer and bleeding complications, in some studies did not potentiate the risk of NSAID use in the development of AGIH³⁸ or even had a protective effect,⁶⁰ while in other studies was found to be an independent RF which doubled the risk of bleeding in users of NSAIDs or aspirin.⁶¹

Although this study was based at a single centre, and is not a double-blind randomized trial, it is the first prospective study evaluating the effectiveness of PPI prophylaxis in older HF with RFs for AGIH. As the investigated population was predominantly Caucasian, our data may not be generalizable to other races.

In conclusion, this study has shown that in older HF patients the incidence of clinically significant AGIH is 3.9%, and that this complication is associated with prolonged length of hospital stay and increased mortality. In older HF patients receiving routine thromboembolic prophylaxis, multiple logistic regression analysis identified five independent RFs for AGIH: pre-existing peptic ulcer, current smoking, post-operative use of antiplatelet drugs or a NSAID/COX-2 inhibitor and blood group O. These RFs correctly identified patients at increased risk of AGIH. In a prospective study, preventive therapy with PPIs in patients at risk of AGIH was highly effective and safe.

ACKNOWLEDGEMENTS

We sincerely thank Natasha Clugston for help with preparation of the manuscript.

Grant support: none.

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Clinical Drug Investigation

ISSN 1173-2563

Volume 37

Number 7

Clin Drug Investig (2017) 37:587-624

DOI 10.1007/s40261-017-0519-y



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REVIEW ARTICLE

Acid-Suppressive Therapy and Risk of Infections: Pros and Cons

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Published online: 30 March 2017

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Abstract This narrative review summarises the benefits, risks and appropriate use of acid-suppressing drugs (ASDs), proton pump inhibitors and histamine-2 receptor antagonists, advocating a rationale balanced and individualised approach aimed to minimise any serious adverse consequences. It focuses on current controversies on the potential of ASDs to contribute to infections—bacterial, parasitic, fungal, protozoan and viral, particularly in the elderly, comprehensively and critically discusses the growing body of observational literature linking ASD use to a variety of enteric, respiratory, skin and systemic infectious diseases and complications (*Clostridium difficile* diarrhoea, pneumonia, spontaneous bacterial peritonitis, septicemia and other). The proposed pathogenic mechanisms of ASD-associated infections (related and unrelated to the inhibition of gastric acid secretion, alterations of the gut microbiome and immunity), and drug-drug interactions are also described. Both probiotics use and correcting vitamin D status may have a significant protective effect decreasing the incidence of ASD-associated infections, especially in the elderly. Despite the limitations of the existing data, the importance of individualised therapy and caution in long-term ASD use considering the balance of benefits and potential harms, factors that may predispose to and actions that may prevent/attenuate adverse effects is

evident. A six-step practical algorithm for ASD therapy based on the best available evidence is presented.

Key Points

Acid-suppressing drugs (ASDs), one of the most commonly prescribed and relatively safe classes of medications, through a variety of different mechanisms (alterations of important defense systems including the gut microbiome and immunity) might predispose to the development of infectious diseases, particularly in the elderly.

The existing controversies on the associations between ASDs and a variety of infections (bacterial, parasitic, fungal, protozoan and viral), proposed pathogenic mechanisms and drug-drug interactions are comprehensively reviewed.

The importance of individualized evidence-based therapy with a proper risk/benefit assessment is emphasized, actions that may prevent adverse effects (avoidance inappropriate prescribing, probiotics use and correcting vitamin D status) are discussed, and a practical algorithm for ASD therapy is presented.

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1 Introduction

Over the last decades, gastric acid-suppressing drugs (ASDs), histamine-2 receptor antagonists (H2RAs) and proton pump inhibitors (PPIs), revolutionised the treatment and prevention of acid-related diseases and currently are

among the most commonly prescribed medications worldwide. These agents are highly effective for treating acid-mediated disorders of the upper digestive tract (peptic ulcers, eradication of *Helicobacter pylori* infection, acute nonvariceal bleeding, gastro-esophageal reflux disease (GERD), erosive esophagitis), prevention of nonsteroidal anti-inflammatory drug (NSAID)-related injury and stress ulcers [1–13]. ASDs are generally well tolerated and considered to have a safe profile [14–17]. However, emerging data indicate that use of ASDs, especially in the elderly in presence of comorbidities and/or co-medications, may be associated with serious adverse health effects including bacterial infections such as *Clostridium difficile* infection (CDI), *Salmonella*, *Campylobacter*, pneumonia, and others, all of which increase morbidity and mortality.

As the development of infections depends on a plethora of factors affecting the complex balance between immune defences and flora, any medication likely to modify this balance might be involved in occurrence of infections. Therefore, clinical decision-making, assessing and balancing the efficacy, safety and tolerability [18] as well as considering ethical dilemmas [19] of prescribing medications for an elderly patient with respect to co-existing comorbidities and polypharmacy has become challenging and complex. Moreover, while some recent publications emphasised the underuse of gastroprotective agents, especially in older patients receiving NSAIDs [7, 20, 21], others highlighted the over-utilisation of PPIs [22–27]. It has been estimated that inappropriate use of PPIs, particularly in the elderly, may exceed 75% [26, 28, 29].

The absence of unambiguous guidelines, controversial reports and recommendations in the existing scientific literature leaves the prescribing physician trying to choose “the right medication for the right patient” sometimes between Scylla and Charybdis.

This narrative review attempts to provide a comprehensive insight into the main issues and uncertainties associated with the potential infectious risks of use of ASDs, especially in an elderly often multimorbid and frail patient within the context of overall clinical benefits and harms, advocating a rationale balanced and individualised approach aimed to minimise any serious adverse consequences.

2 Methods of Literature Search

We performed an updated MEDLINE/PubMed search focusing mainly on publications from 1 January 2000 to 31 December 2016, and selected appropriate articles for discussion. In order to give the reader, as much as possible, a complete review of the topic, we have widened the spectrum of clinical conditions included.

3 General Considerations

Proton pump inhibitors covalently bind with sulfhydryl groups of the cysteine residues of the H⁺/K⁺ adenosine triphosphatase (H⁺/K⁺ ATPase) on the plasma membrane of the gastric parietal cell irreversibly blocking the final step in acid secretion in response to all modes of stimulation—muscarinergic, gastrinergic and histaminergic [30–32], while H2RAs block only one—the histaminergic—of the three pathways in acid secretion. Therefore, PPIs are significantly more effective, faster and longer-acting suppressors of gastric acid secretion than the H2RAs. PPIs, despite the individual differences, are similar with respect to half-lives, time to maximum plasma concentration and safety [33]. All PPIs, except tenatoprazole, undergo hepatic metabolism via the CYP isoforms CYP2C19 and CYP3A4, and genetic polymorphism in CYP2C19 affects the metabolism and effectiveness of omeprazole and lansoprazole but not esomeprazole or rabeprazole [34–37]. Because omeprazole (but not pantoprazole) is a metabolism-dependent inhibitor of CYP2C19, it causes clinically significant interaction with clopidogrel [38].

The frequency of adverse reactions from H2RAs was reported to be similar to that for placebo [39, 40]. However, cytopenias and leukocytosis [41], nephrotoxicity and hepatotoxicity [42], as well as drug interactions [43–45] have been described.

4 Acid-Suppressing Therapy and Risk of Enteric Infections

Gastroenteritis is a common infectious disease requiring hospitalisation of approximately 1% of people ≥65 years of age annually [46]. The incidence of diarrhoea, the most common adverse effect from long-term PPI use and the most frequent indication for discontinuing PPI therapy, ranges from 3.7 to 4.1% [47–50]. In comparison, antibiotic-associated diarrhoea occurs in 5–39% [51]. The most likely causes of infectious diarrhoea include *C. difficile*, *Campylobacter jejuni*, *Salmonella* spp., *C. perfringens*, *Staphylococcus aureus*, *Klebsiella oxytoca*, enterotoxigenic *Escherichia coli*, and viruses, although in many cases no infectious agent is ever determined [52–54]. *C. difficile* infection (CDI) is the leading cause of antibiotic-associated diarrhoea and accounts for 15–39% of cases [55–57]. The potential of ASDs to increase enteric infections, particularly in the elderly, has been recognised [58, 59].

4.1 *Clostridium difficile* Infection (CDI)

In the last decade, CDI, the most common of healthcare-associated infections [60], has become increasingly

prevalent and severe [61–71]. Currently, it often (up to 75% of cases) occurs in the community or nursing homes (approximately in one-quarter of all CDI) [65, 72, 73], is not associated with healthcare/hospital exposure in up to one-third [73], and is unrelated to antibiotic use [74]. Most importantly, the incidence of CDI is disproportionately high among older patients [72, 75–78], especially among the most vulnerable and frail population of long-term residential care facilities (RCF) [73, 79–81]. The elderly with CDI often develop severe complications [82, 83] and persons aged ≥ 65 years account for up to 90% of CDI-related mortality within 30 days [65]. Current antimicrobial therapies for CDI still have relapse rates of 15–30% [84–86].

C. difficile, a Gram-positive, anaerobic, spore-forming, toxin-producing bacillus, is acquired by the ingestion of spores via the fecal-oral route. Pathogenesis of CDI involves a complex interplay of three key mechanisms: (1) *C. difficile* toxin production, (2) disruption of the gut microbiota and (3) host factors. Although antibiotics are currently recognised as a major risk factor for acquiring CDI due to their effect on the normal structure of the indigenous gut microbiota [55, 87–92], emerging data indicate that the prevalence of CDI cannot be fully explained by antimicrobial exposure, suggesting that other variables—comorbidities and medications affecting both the microbiota and the immunological status—may substantially contribute to the burden of CDI, particularly in the elderly population. Age is recognised as a major risk factor for the development of CDI with disease incidence and severity escalating as age increases [93].

Numerous epidemiological observational studies and meta-analyses [55, 74, 89, 92, 94–120] showed a statistically significant increase in both nosocomial and community-acquired CDI among patients taking PPIs or H2RAs (Table 1). In PPI users, odds ratio (OR) [or relative risk (RR)] ranges between 1.74 [46, 103]–1.90 [118]–1.96 [121]–2.15 [122]–2.90 [117]–3.3 [115] and 3.60 [97], in H2RA users between 1.40 [121]–1.44 [107] and 1.50 [123]. The pooled estimates showed a 1.3- to 3.3-fold increase in risk for CDI with ASD therapy [103, 104, 106, 115]; a lesser increase in risk with the use of H2RAs compared to PPIs as it was reported in the majority of studies may indicate a correlation with the degree of acid suppression [101, 119]. The association between ASDs and CDI appeared to be the level of acid suppression [119, 122], and is duration dependent [111]. Interestingly, in the paediatric population, CDI risk was associated with H2RAs (OR 4.6) but not PPIs use [124]. In one study, the proportion of cases of CDI among PPI users was as high as 65% [104]; others found that 31% of CDI patients without antibiotic exposure received PPIs [74]. In critically ill medical patients, risk of CDI associated with PPI therapy (OR 3.11) was comparable to the risk associated with the use of fluoroquinolones

or third-generation cephalosporins [125]. Continuous PPI use (on average observed in 40–60% of CDI patients), similar to antibiotic re-exposure, was also associated with an increased risk for recurrent CDI [96, 100, 114] with an OR of 2.51 [103]–4.17 [96]. Among patients with extra-intestinal CDI, 50% used PPIs [126].

Importantly, PPIs co-administered with an antibiotic increase the risk of CDI approximately two-fold above that observed with PPI alone [103, 127]. The absolute risk of CDI associated with H2RAs was highest in hospitalised patients receiving antibiotics with an estimated number-needed-to-harm (NNH) of 58 at 2 weeks compared to 425 not receiving antibiotics [107]. On the other hand, ASDs (taken by two-thirds of CDI in-patients) did not worsen clinical response or recurrence rate when used concurrently with vancomycin or fidaxomicin, and it is recommended to continue PPI or H2RA treatment in CDI patients with risk of gastrointestinal bleed or GERD [128].

The US Food and Drug Administration (FDA) required that the package insert for PPIs contain a warning that PPIs may increase the risk of CDI. Noteworthy, even in healthy subjects, daily acid suppression affects gut microbiota composition [129], and these microbiota shifts are associated with functional changes that could cause bacterial overgrowth [130] and pathogen colonisation including *C. difficile* [131].

However, the subject remains controversial. An increased risk of CDI in PPI users has not been confirmed by some researchers [132–135], especially after adjusting for coexisting conditions [108, 133, 136–141]. No increase in the number of patients with CDI following total gastrectomy was reported [138]. Because in the elderly the prevalence of gastric hypochlorhydria is high, ASDs may not demonstrate an additional to antibiotic use risk of CDI [135]. A case–effect relationship between PPI use and CDI has not been supported by a meta-analysis which included 37 case–control and 14 cohort studies [108]; the authors estimated that in the general population taking PPIs the risk of CDI is very low; NNH of 3925 at 1 year. A meta-analysis on CDI in H2RA users (33 studies) by the same group reported NNH of 58 (95% CI 37–115) among patients receiving antibiotics and of 425 (95% CI 267–848) among patients not receiving antibiotics [107].

A recent review concluded that the influence of acid suppression in CDI remains uncertain [93], while an expert panel of infectious disease specialists agreed that PPIs are an important risk factor [66]. The newest position statement by the Sociedad Española de Patología Digestiva indicates that the association between PPIs and CDI is mild to moderate [142]. Obviously, for a more definitive answer a prospective randomised controlled trial is needed but it would be difficult to conduct (need of a large sample size, diagnostic suspicion bias, lack of a pharmaceutical

Table 1 Selected data on pooled estimates for CDI, SBP and respiratory infections in users of PPI and H2RA

Authors	Number of studies included	No. of participants	OR	95% CI
PPP use and CDI				
Leonard et al. (2007) [121]	11	126,999	2.05	1.47–2.85
Deshpande et al. (2012) [106]	25 case–control and 5 cohort	202,965	2.5	1.81–2.55
Tleyieh et al. (2012) [108]	37 case–control and 14 cohort		1.65	1.47–1.85
Janarthanan et al. (2012) [104]	17 case–control and 6 cohort	288,620	1.69	1.40–1.97
Kwok et al. (2012) [103]	30 case–control and 12 cohort	313,000		
		Incidental	1.74	1.47–2.85
		Recurrent	2.51	1.16–5.44
MacLaren et al. (2014) [747]	ICUs	21,873	1.29	1.04–1.64
Ro et al. (2016) [115]	ICUs	1005	3.3	1.5–7.1
Faleck et al. (2016) [141]	ICU:	18,134		
	Not receiving antibiotics		1.56	0.72–3.35
	Receiving antibiotics		0.64	0.48–0.83
H2RA use and CDI				
Leonard et al. (2007) [121]	13	17,314	1.48	1.06–2.06
Tleyieh et al. (2013) [107]	33	201,834	1.44	1.22–1.70
PPI use and SBP				
Yu et al. (2016) [266]	10 case–control, 6 cohort	8145	2.11	1.46–3.06
Trikudanathan et al. (2011) [268]	4	772	2.77	1.82–4.23
Deshpande et al. (2013) [269]	8	3815	3.15	2.09–4.74
Xu et al. (2015) [267]	17		2.17	1.46–3.23
Chang et al. (2015) [259]		947/86,418 ^a	2.20	1.60–3.02
Dam et al. (2016) [265]	3 RCTs	865	1.72	1.10–2.69
H2RA use and SBP				
Deshpande et al. (2013) [269]	4	562	1.71	0.97–3.01
PPI use and SIBO				
Lo and Chan (2013) [222]	11	3134	2.28	1.24–4.21
PPI use and pneumonia				
Chang et al. (2015) [259] ^c		86,418	1.72	1.25–2.37
Eom et al. (2011) [327]	23 RCT, 5 case–control and 3 cohort	Total	1.27	1.11–1.46
		Use ≤7 days	3.45	2.86–5.45
		Use <30 days	1.61	1.46–1.78
		Use 30–180 days	1.36	1.05–1.78
Lambert et al. (2015) [333]	26	226,769/6,351,656 ^b	1.49	1.16–1.92
Giuliano et al. (2012) [748]	9 case–control and cohort	120,863		
		Total	1.39	1.09–1.76
		Use <30 days	1.65	1.25–2.19
		Use 30–180 days	1.10	1.00–1.21
		High dose	1.50	1.33–1.68
		Low dose	1.17	1.11–1.24
Johnstone et al. (2010) [749]	6 cohort	Total	1.36	1.12–1.65
		New users	1.92	1.40–2.63
		Chronic users	1.11	0.90–1.38
		High dose	1.36	1.16–1.59
		Low dose	1.20	1.02–1.43
Sultan et al. (2008) [341]	7 RCTs	2586	1.42	0.86–2.35
Estborn and Joelsson (2015) [343]	24 RCTs	15102	0.66	0.36–1.22

Table 1 continued

Authors	Number of studies included	No. of participants	OR	95% CI
H2RA use and pneumonia				
Eom et al. (2011) [327]	8	Total	1.22	1.09–1.36
		Use ≤ 7 days	5.21	4.00–6.80
		Use < 30 days	1.49	0.82–2.72
		Use 30–180 days	1.21	0.94–1.56

Statistically non-significant associations are presented in bold

OR odds ratio, CI confidence interval, CDI *Clostridium difficile* infection, SBP spontaneous bacterial peritonitis, PP proton pump inhibitor, H2RA histamine 2 receptor antagonist, ICU intensive care unit, RCT randomised controlled trial, SIBO small intestinal bacterial overgrowth

^a Cases of SBP among patients with liver cirrhosis

^b Cases of community-acquired pneumonia among participants

^c Taiwan national database

sponsor). Meanwhile, despite the limitations of observational studies, the potential association of ASDs with CDI should not be ignored [114] and clinicians should put more attention in adhering to indications for ASDs use.

4.2 Other Enteric Bacterial Infections

Use of ASDs has also been associated with an increased risk of enteric infections [121], especially *Salmonella* spp. and *Campylobacter* spp. [101, 143–148]. Five case-controlled studies have observed an association between ASDs and *Salmonella* infection with ORs ranging from 1.84 to 11.2 [146, 147, 149–152]. In PPIs users, the OR of infection caused by *Salmonella* ranged 2.09–8.3, by *Campylobacter* 1.7–11.7 [101, 145–147, 149, 150]. During an outbreak of salmonellosis, residents of a long-term care facility treated with ASDs were eight times more likely to develop the infection [153]. An 11.7-fold increase in the risk of gastroenteritis due to *Campylobacter* spp. was reported among 211 patients (aged > 45 years) receiving omeprazole in the month before infection but not in former users, and no association with H2RAs was observed [145]. The risk of *Salmonella*- and *Campylobacter*-induced gastroenteritis ($n = 6414$ patients) was significantly associated with use of PPIs (RR 2.9) but not H2RAs [146]. In a meta-analysis (6 studies, 11280 patients) the pooled OR of enteric infections in ASD users was 2.55, with a greater association for PPIs (OR 3.33) compared to H2RAs (OR 2.03) [121]. Similarly, a recent nested case-control study of a national database on hospitalised population (14,736 case patients and 58,944 controls) reported a significant association between occurrence of nontyphoid salmonellosis and PPIs (total OR 2.09, in current users OR 5.39) or H2RAs (OR 1.84) therapy [147]. These data are in line with many previous observations of increased occurrence of non-typhoid salmonellosis in patients with reduced

gastric acid secretion [154–156] and following gastric resection for peptic ulcer disease or gastric malignancy [155, 157–160]. The protective role of gastric juice against salmonella infections was also demonstrated in mice [161]. In adult volunteers, two strains of *C. jejuni* produced a higher rate of infection and illness when ingested with sodium bicarbonate indicating the protective role of gastric acid [162].

However, a retrospective analysis of almost 2 million individuals (about 360,000 were prescribed a PPI) after adjusting for confounding factors and eliminating the effect of time intervals did not find that PPIs increased the rate of *Campylobacter* and *Salmonella* infections [163]. The authors concluded that patients prescribed PPIs had greater underlying predisposing risks for gastrointestinal infections with a 3.1–6.9 times higher rate of these infections compared to non-PPI users even before PPI treatment started [163]. Similarly, observations from the SOPRAN and LOTUS studies did not indicate any difference in the incidence of enteric infections between the treatment groups [15]. The conflicting results may be, at least partially, related to selection bias [164]. Of note, it was shown that ASDs increased the susceptibility to *Salmonella* and *Campylobacter* infections mainly in current users and within 1–3 months after therapy ended [145–147, 149], while no association was seen when the incidence of enteric infection was compared in PPIs users 12 months before and after the event [149, 163].

In the last decade, an increased incidence of human listeriosis, a rare but dangerous food-borne disease that accounts for 20–30% of food-borne deaths [165–168], has been reported among the elderly, especially with reduced immunocompetency (cancer, diabetes, immunosuppressive therapy) and/or ASD users, in UK [169], Austria [170], Denmark [171], Spain [168] and Germany [172], as well as

in North America, Japan [166] and Taiwan [173]. Moreover, in England, prescribing patterns for PPIs closely correlated with the incidence of *Listeria monocytogenes* bacteraemia [169]. Previous case-controlled studies found that H2RAs and antacid use was associated with outbreaks of hospital-acquired listeriosis [174, 175]. Other researchers demonstrated that patients on long-term H2RA therapy have an increased prevalence of *L. monocytogenes* in the feces (20 vs. 2.1% in controls), but none of them developed listeriosis [176]. Cimetidine significantly lowered the infective dose of virulent *L. monocytogenes* in rats [177], although this Gram-positive bacillus and facultative intracellular organism can survive the body's natural defences within the digestive tract, including acid conditions of the stomach and bile acids [167, 178–181].

Use of H2RAs [182] or antacids [183] has also been linked to development of acute brucellosis. Because gastric juice is lethal to *Brucella* spp. in vitro [183, 184] drug-induced hypochlorhydria may facilitate the transit of microorganisms and disease. Lowering suppressor/cytotoxic T lymphocyte counts [185] by cimetidine may contribute to this adverse effect [186].

A case of septicaemia due to *Yersinia enterocolitica* (primarily a gastrointestinal Gram-negative bacilli transmitted through consumption of contaminated food or water) in a haemodialysed patient receiving omeprazole has been reported [187]; raised intra-intestinal pH and increased intraluminal iron load were suggested as the main contributing factors for the infection.

Shigella spp. are acid-resistant organisms [188] and gastric hypochlorhydria does not influence the susceptibility to this infection [189]. However, in volunteers, pre-treatment with sodium bicarbonate increased the isolation of the vaccine strain of *Shigella flexneri* in stools three-fold [190] indicating the potential role of alteration of gastric pH and/or facilitating gastric emptying on bacterial survival.

4.3 Gastric and Small Intestinal Bacterial Overgrowth (SIBO)

Acid-suppressing drug therapy is known to be associated with gastric and duodenal bacterial overgrowth. Following both H2RAs [191–195] and PPIs [195–200] high intragastric non-*H. pylori* bacterial counts (in both the gastric juice and mucosa) and rises in potentially carcinogenic nitrite and N-nitrosamine concentrations [193, 194, 196] were observed in several studies. The bacterial overgrowth correlated with the intragastric pH [199, 201], daily duration and degree of hypochlorhydria [198, 199] and increases in the concentration of unconjugated bile acids [198, 202]. It was suggested that the reflux of toxic unconjugated bile acids [203] into the esophagus may

cause mucosal injury even in ASD users [156]. In ASD users, the overgrowth was predominantly of Gram-positive organisms, resembling that found in the mouth and oropharynx [199]. Use of PPIs or H2RAs in *H. pylori*-positive subjects resulted in higher intragastric pH, greater non-*H. pylori* bacterial colonisation, increased cytokine and N-nitrosamine levels and higher risk of atrophic gastritis [204, 205].

However, in other studies, no significant changes in intragastric bacterial counts or in bacterial species and N-nitroso-compound levels were found after cimetidine [206] and no increases in the concentration of nitrates or nitrites were noted in healthy volunteers receiving omeprazole for 2 weeks [197, 207].

Numerous reports have found an association between ASDs and SIBO. It has been documented in H2RA users [191, 208, 209] and following PPI therapy [138, 208–222]. SIBO incidence was considerably higher in patients treated with PPIs compared with H2RAs [210, 223]. These observations are in line with an increase in number of subjects with SIBO among patients with atrophic gastritis [211] and after total gastrectomy [224, 225]. The pooled (11 studies, $n = 3134$) OR for SIBO in PPI users versus nonusers was 2.28 [222]; the association was highly significant with OR of 7.59 only when the diagnosis was made by an accurate test such as duodenal or jejunal aspirate culture but not glucose hydrogen breath test (GHBT). Breath tests based on bacterial metabolism of various substances may produce false results [226], especially in the elderly [208, 211, 227]. Indeed, in two large studies PPI usage was not associated with the presence of SIBO as determined by GHBT ($n = 1191$) [228] or a positive D-xylose breath test ($n = 932$) [229], and one recent small study ($n = 94$) failed to detect an association between PPIs or H2RAs use and SIBO assessed by the lactulose hydrogen breath test [230]. The newest and largest study on this topic [231] once again confirmed that impairment of acid barrier by current PPI therapy is an important pathomechanistic pathway for the development of SIBO (OR 1.43); a potential risk of SIBO in chronic PPI users has also been observed in children [232].

ASD-related SIBO is of clinical significance, as both SIBO and reduced gastrointestinal motility (which is also an independent risk factor for development of SIBO [217, 233]), are relatively frequent, especially in older adults, may cause malabsorption and are linked to many diseases [138], including diabetes mellitus [234], non-alcoholic fatty liver disease [235, 236], liver cirrhosis [209], chronic kidney disease (CKD) [237], hypothyroidism [238], autoimmune diseases [239], obesity, irritable bowel syndrome [240, 241], gastric bypass surgery [242], cholecystectomy [243] and chronic pancreatitis [244, 245]. Because ASDs may be one of several factors contributing

to SIBO and its consequences prescribing of ASDs in individuals with these conditions needs to be carefully considered.

4.4 Spontaneous Bacterial Peritonitis (SBP)

Patients with liver cirrhosis are immunocompromised and particularly prone to developing spontaneous bacterial infections often with serious complications (acute-on-chronic liver failure, renal failure, and shock) resulting in high mortality rates (30–50%) [246–249]. Because of the high prevalence of bleeding gastroduodenal ulcers [250, 251] with high mortality [252] these patients are often prescribed ASDs, although the evidence of their protective efficacy is poor [253]. Patients with cirrhosis and ascites receiving ASDs were found to be at a higher risk of SBP, overall bacterial infection [216, 254–263] and mortality [264]. In cirrhotic patients with ascites treated with PPIs the OR for developing SBP ranged between 1.40 [258] and 4.31 [254]. Meta-analyses found pooled OR for SBP for PPIs users of 1.72 [265]–2.11 [266]–2.17 [267]–2.77 [259, 268]–3.15 [269], and for H2RA users of 1.71 [269]–2.62 [259]; an OR of 1.98 for the overall risk of bacterial infection in PPIs users was reported [267]. The risk of SBP increased significantly with longer ASD use [259, 270]. A large case–control study revealed that use of PPIs in patients with cirrhosis ($n = 1166$) increases the risk of development of hepatic encephalopathy in a dose-dependent fashion [271].

However, some researchers did not confirm an increased risk of SBP in users of PPIs [216, 272, 273] or H2RAs [263] and did not observe a link between PPIs and bacterial infections, prognosis and mortality in cirrhotic patients [216, 274, 275]. A recent meta-analysis (10 case–control and 6 cohort studies, 8145 patients) showed that the association of PPIs with SBP was significant only in case–control studies (OR 2.97, 95% CI 2.06–4.26) but not in cohort studies (OR 1.18, 95% CI 0.99–1.14) and was not associated with increases in 30-day mortality [266]. Of practical importance, clinical trials [276–278] and current guidelines [279] do not support PPI use for prophylaxis of portal hypertension-related bleeding and recommend only a short-course of PPI post-endoscopic variceal ligation if ulcer healing is a concern [276, 279]. Of note, in patients with liver cirrhosis the prevalence of peptic ulcers ranges between 5 and 28% [253, 277], while inappropriate prescription of PPIs was found in 34–60% of cirrhotic patients [256, 257, 274, 278, 280–282]. Interestingly, in patients with CKD undergoing chronic peritoneal dialysis, the association of ASDs with enteric peritonitis (RR 1.65) and infectious mortality was more pronounced in H2RA users but less consistent among those treated with PPIs [283].

4.5 Liver Abscess and Acute Cholangitis

Use of PPIs was shown to be associated with an increased risk of cryptogenic liver abscess: OR was 4.7 for current users and 2.9 for the past users 31–90 days [284]. PPI therapy was also related to a higher incidence of cholangitis associated with increased number and broader spectrum (oropharyngeal flora) of pathogens in the biliary tract [285].

4.6 Enteric Parasitic Infections

Amongst protozoan parasites, *Giardia lamblia* is one the commonest etiological agents of acute usually self-limited diarrhoea worldwide (especially in developing countries), although in some patients it may become chronic with serious long-term effects [286–289]. *G. lamblia* is acid-sensitive. Hypochlorhydria was found in 54% of patients with intestinal giardiasis [290, 291] and associated with more severe symptoms. As survival of the parasite in the stomach requires reduced acidity, the infection, not surprisingly, is associated with chronic atrophic gastritis [292, 293] but gastric infection is rare (less than 100 cases reported in the literature [294]). In case reports, giardiasis was associated with chronic use of PPIs [175, 295–297] and ranitidine [298] as well as following gastric surgery [299]. On the other hand, a recent experimental study demonstrated that in vitro omeprazole, by inhibiting giardial triosephosphate isomerase, is effective against *G. lamblia*, including drug-resistant strains [300].

Strongyloidiasis, a parasitic infection endemic in tropical and subtropical regions [301–304] and observed in immunosuppressed individuals [305, 306], is also associated with hypochlorhydria [291, 307]. Gastric strongyloidiasis has been diagnosed in patients with hypochlorhydria [156, 308] and in a woman receiving H2RAs and PPIs for 2 years [309]. Opportunistic *Strongyloides stercoralis* hyperinfection has been reported following cimetidine therapy in immunosuppressed patients [308, 310, 311] but there were no publications of this in PPI users [294]. In one study, gastric acid levels were not associated with giardiasis or strongyloidiasis [189].

Experimental studies showed that rats pretreated with cimetidine can be infected orally with *Entamoeba histolytica* [312] indicating the protective effect of gastric acid against this protozoa. Artificial gastric fluid, containing 0.6% hydrochloric acid (pH 1.8) and 0.5% pepsin, but not artificial intestinal fluid, contributes to enhancing excystation for *Entamoeba* infection [313].

Interestingly, recent studies revealed that many widespread bacteria (*Salmonella enteric*, *E. coli*, *Y. enterocolitica*, *L. monocytogenes*) survive inside cysts of the

ubiquitous amoeba *Acanthamoeba castellanii*, even when exposed to highly acidic conditions (pH 0.2) or antibiotics [314, 315]. An increase in acid tolerance of *C. jejuni* when co-incubated with amoeba was also reported [316]. These findings suggest the important role of protozoa and their cysts in the epidemiology of food-borne bacteria and the possible ways of ASDs involvement.

5 Respiratory Infections, Bacterial Pneumonia

Pneumonia, one of the most common infectious diseases, is a leading cause of morbidity and mortality in the elderly [317–325]. Evidence is accumulating on an increased risk of both community-acquired respiratory infections and nosocomial pneumonia in patients receiving ASDs, although the results are mixed. Subjects using ASDs compared to non-users, 2.3 times more often experienced respiratory infections, 3.7 times more often visited a physician for an infection and 4.2 times more often received antibiotics [326]. The OR for pneumonia ranged in patients taking PPIs between 1.27 [327]–1.3 [328]–1.5 [329]–1.89 [330], and in patients taking H2RAs between 1.22 [331]–1.30 [332]–1.63 [330]; a dose-dependent association with PPIs was reported by some [330] but not all [329] researchers. In a meta-analysis of 33 studies, which included 6,351,656 participants, the pooled OR was 1.49 and the risk was reported to be higher during the first month of PPI therapy (OR 2.10) [333]. The association was particularly strong within a week (OR 5.0 [329]–3.79 [334]) or even the first 2 days (OR 6.53) [334] after PPI initiation, but declined over time [326, 329, 334] and was not significant for longer-term therapy [334]. The risk for pneumonia in users of ASDs was higher among patients with chronic obstructive airway disease (COPD) (OR 1.76 with PPIs, OR 1.25 with H2RAs [335], CKD (OR 2.21 with PPIs [336]), stroke [337] (OR 1.44 [338]–2.07 [339]–2.7 [340]), and non-traumatic intracranial haemorrhage (OR 1.61 [338]); the association was not significant for H2RAs in stroke patients [338, 340].

Other investigators, however, reported no association between H2RAs therapy and pneumonia [329], as well as between PPIs and respiratory infections [341] (Table 1) and between PPIs and occurrence of pneumonia in COPD patients [335]. One meta-analysis (31 clinical trials) found that esomeprazole use did not increase the risk of community-acquired respiratory tract infection including pneumonia [342]; this conclusion has been recently confirmed in a report based on 24 randomised controlled trials (RCTs) by the same authors [343]. In some studies [344], ASDs have been found to significantly increase the risk of recurrent pneumonia in the elderly (OR 2.1), whereas in other reports the risk was higher among younger PPI users

[329] and not obvious in individuals >70 years old [335]. Moreover, in a study based on medical record review (vs. only administrative records in most of other studies) of community-dwelling adults aged 65–94 years and controlling for confounding factors, neither PPI nor H2RA use increased pneumonia risk [345]. In patients with acute stroke, no difference in the incidence of pneumonia between PPI and H2RA users was reported in one study [346], whereas another found that in users of PPIs compared to H2RAs the relative risk of pneumonia was 1.69 [339]. Some observational studies concluded that PPIs do not increase the risk of nosocomial pneumonia, and, in contrast, reduce the risk of aspiration pneumonia in patients with a gastric tube in place [14, 347]. Of note, usefulness of ASDs in the management of GERD-related chronic cough and asthma has been described [348–351], and no difference in the incidence of lower respiratory tract infections was seen when patients treated with PPIs or anti-reflux surgery were compared [15]. The position statement by the Canadian Association of Gastroenterology [352] emphasised that the risk-to-benefit ratio appears to be largely in favour of using ASDs for conditions in which efficacy has been demonstrated.

In the setting of stress ulcer prophylaxis, the data on ASD-related pneumonia remain conflicting and require special consideration. In an intensive care unit (ICU), stress ulcer bleeding is a rare (1–6%) but severe complication (mortality 40–50%), therefore, the majority of these patients receive H2RAs or PPIs [353–355]. In a large European study, stress ulcer prophylaxis has been recognised as an independent risk factor of ICU-acquired infections among which pneumonia accounted for more than 50% [356]. Older studies indicated that prophylaxis with H2RAs is associated with an increase in the incidence of pneumonia as compared with placebo or sucralfate treatment [354, 357–359]. The risk of developing pneumonia in H2RA-treated patients was 1.3 [332] to 2 [353, 357] times higher than in the patients receiving sucralfate, which does not raise gastric pH. No difference in the rates of ventilator-associated pneumonia was observed with these two drugs in a randomised blinded placebo-controlled trial [360]. One retrospective study showed a significant association of PPIs with pneumonia only by univariate but not by multivariate analysis [125]. A PCT found a strong increase in ventilator-associated pneumonia among the PPI users compared to those receiving placebo (36.4 vs. 14.1%) [361]. The superiority of PPIs over H2RA for stress ulcer prophylaxis in patients with severe sepsis or septic shock who require mechanical ventilation has not been supported in one study [362]. However, recent meta-analyses demonstrated that in critically ill patients, PPIs were more clinically and cost effective than H2RAs in preventing upper gastrointestinal

bleeding without affecting the rates of nosocomial pneumonia, length of ICU stay or mortality [4, 355, 363, 364]. It is evident from the existing data that the role of ASDs as a risk factor for community-acquired and nosocomial pneumonia is still unclear but remains likely.

6 Septicaemia

In the elderly, bloodstream infections are common and often fatal [365–367]. Reports implying an increased susceptibility to septicaemia associated with ASDs are scant and conflicting. In a randomised trial of critically ill trauma patients, ranitidine use compared with sucralfate was associated with a significant increase in overall infectious complications (OR 1.5), including bacteraemia (46.9%), pneumonia (25.0%) and catheter-related infections (19.8%) [332]; the number of infectious complications per patient averaged 2.6 and 1.1 in those receiving ranitidine and sucralfate, respectively. The multiple sites of infectious complications may suggest a potential immunosuppressive effect of ranitidine. Severe postoperative systemic infection after technically uncomplicated gastric resection was observed in two patients (one died) receiving prolonged omeprazole treatment preoperatively and without perioperative antibiotic prophylaxis [368]. A recent international survey (11 countries) found that most ICU units are using stress ulcer prophylaxis with PPIs (66%) or H2RAs (31%), despite the risk of infectious complications [369].

On the other hand, in animal studies, administration of PPI decreased systemic production of proinflammatory cytokines (TNF- α and IL-1 β) and protected mice with endotoxic shock from death (60% survival vs. 5% of untreated mice) [370]; PPIs were proposed as promising drugs against sepsis and severe inflammatory conditions.

7 *Mycobacterium tuberculosis* Infection

A case–control study has shown that use of ASDs increases the risk of tuberculosis infection/activation (6541 cases): OR 1.63 with PPIs and OR 1.51 with H2RAs [371]. However, in a sample of near 62,000 patients, long-term PPI therapy was not associated with increased risk of acquiring gastrointestinal tuberculosis [372].

8 Furunculosis

A case of recurrent furunculosis associated with repeated courses of omeprazole therapy was reported [373]. Six cycles of furunculosis occurred mainly on the patient's neck; each episode developed within 1–2 weeks of starting

omeprazole, continued to be present through the duration of therapy, and resolving within 1–2 weeks of discontinuation of the drug.

9 Fungal Infections

Although *Candida* spp. commonly colonise the gastrointestinal tract in healthy humans [374, 375], high levels of their presence are associated with several severe diseases [374, 376–381]. Significant *Candida* overgrowth has been detected in duodenal aspirates [382] and gastric juice of peptic ulcer patients treated with H2RAs [383, 384] or omeprazole [384]. The fungal isolation rate was higher in older patients and in subjects with post-treatment gastric pH of ≥ 4 [384]. Candidiasis of the small intestine in association with ASDs has also been reported [385]. In healthy volunteers and gastric ulcer patients, 5 weeks of omeprazole therapy resulted in a significant bacterial and *Candida albicans* overgrowth in the gastric juice and jejunum fluid [386]. Surgical interventions producing hypoacidity such as vagotomy [387] or partial gastrectomy [388] are associated with massive *C. albicans* overgrowth. Other researchers, however, reported similar positive candidal culture rates in the stomach in patients receiving PPI (17.3%) or H2RA (11.5%) and not treated with ASDs (12.5%), although PPI use was associated with higher intra-gastric bacterial infection rates (66.7, 46.2 and 28.8%, respectively) [379]. In gastric ulcer patients treated with H2Ras, *C. albicans* infection did not affect the healing rate and healing time [389], while in rats, persistent colonisation with *C. albicans* induced with ranitidine delayed ulcer healing [390].

Systemic candidiasis, a common opportunistic infection, has been observed in immunocompromised patients treated with cimetidine [391]. *Candida* esophagitis has been linked to ASDs. There are case-reports of esophageal candidiasis associated with H2RSs [392, 393] and omeprazole therapy [393–397] even if patients lacked other risk factors. A recent retrospective analysis of 55,314 Koreans who underwent a screening esophagogastroduodenoscopy revealed that ASD use is an independent risk factor (OR 5.11) for *Candida* esophagitis in addition to malignancy (OR 18.68), use of steroids (OR 6.74) and diabetes mellitus (OR 2.67). It is thought that the physiological reflux of gastric acid into the esophagus may inhibit esophageal colonization by *Candida* spp. [394]. In contrast, a large (80,219 patients) Japanese endoscopic-based study did not find a significant association of PPI use with *Candida* esophagitis [398]. A case-controlled study of adult surgical ICU patients showed that the proportion of intra-abdominal *Candida* infection among patients receiving ASDs and non-ASD users was

similar (30.3 vs. 32.1%), although higher in chronic PPI users and those with prior abdominal surgery [399]. Empiric antifungal therapy in patients with complicated intra-abdominal infection with a history of prior use of ASDs was not recommended. Of practical importance is the antagonism of PPIs and antifungal agent fluconazole [400]. Avoidance of coadministration of PPIs and antifungal posaconazole has been shown to be effective in neutropenic haematological patients [401]. On the other hand, as both voriconazole, a broad-spectrum antifungal drug used in severe fungal infections, and PPIs undergo hepatic cytochrome P450-dependent metabolism mainly through isoenzymes CYP2C19, CYP3A4, CYP2C9, their concurrent administration significantly increases total voriconazole exposure and may be used to achieve higher plasma concentrations [402, 403].

10 Parasitic Protozoan Infections

No published reports on ASD-related parasitic protozoan diseases were found. In contrast, there are data that ASDs may be beneficial, exerting antimalarial and anti-leishmanial activities (two most significant of the protozoan parasites that infect man). Astemizole, an antihistamine, has been shown to inhibit chloroquine-sensitive and multidrug-resistant *Plasmodium falciparum* parasites, and the drug was effective in two mouse models of malaria [404]. Studies in vitro demonstrated antimalarial activity of omeprazole against trophozoites, schizonts and ring forms [405]. Combination of omeprazole with quinine had a synergistic antimalarial effect, combination of omeprazole with artemisinin drugs had an additive effect, but when omeprazole was used with chloroquine an antagonistic effect was observed [406].

In regard to cutaneous leishmaniasis, it has been reported that omeprazole and rifampicin are a highly effective combination [392, 407]. Oral cimetidine or omeprazole [408] with low dose of systemic meglumine antimoniate are recommended in high-risk patients with heart, kidney, and/or liver disease.

In the context of enormous health, social, and economic impact of human parasitic protozoa diseases (about a million deaths annually), particularly in tropical and subtropical regions of the world, lack of vaccines and limited therapeutic strategies, ASDs appear as attractive adjuvants to antiparasitic therapy.

Interestingly, cimetidine has been found to enhance the protective effect of a schistosome vaccine [409] and omeprazole synergistically increased the efficiency of praziquantel against schistosomiasis [410], indicating advances that may arise from use of ASDs.

11 Viral and Prion Infections

The data on pathophysiology and clinical significance of ASDs in human viral infections are scarce. Because many viruses are sensitive to the low pH in the gastric juice [291, 411, 412], patients with hypochlorhydria may be predisposed to viral and prion infections [156]. It has been shown that influenza viruses infect and persist in gastric mucosa in patients receiving ASDs [413]. Community-acquired respiratory infections, which are mainly viral in origin, are more common in ASD users (OR 2.34) [326]. A recent review and meta-analysis found that pooled prevalence of influenza viruses in stool was 20.6%, but the occurrence of gastrointestinal symptoms among patients with influenza was inconsistent [414]. In mice, given brain homogenates contaminated with scrapie via gastric intubation, lower doses of infectious material induced disease more often when gastric acidity was reduced by adding ranitidine to the drinking water [415]. In a similar model, omeprazole-induced gastric hypoacidity more than doubled the rate of brain infection [416]. These experiments indicate the important protective role of gastric juice against orally acquired prion diseases (transmissible spongiform encephalopathies), subacute neurodegenerative disorders with an inexorably lethal outcome.

12 Colonisation by Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant *Enterococcus* (VRE)

Advanced age, healthcare contact, invasive medical interventions, chronic illnesses and antibiotic use are well-known risk factors for nosocomial antimicrobial-resistant infections, including MRSA and VRE [417–423], which have become pandemic in recent decades causing a major public health problem, serious morbidity and mortality globally [424, 425]. A high prevalence of colonisation with MRSA, VRE, penicillin-resistant pneumococci, extended spectrum beta-lactamase-producing and fluoroquinolone-resistant Gram-negative organisms (*K. pneumoniae*, *E. coli*) has been well documented in the elderly, especially among residents of long-term care facilities [426–437]. Although production of gastric acid has been recognised among factors contributing to colonisation resistance [438], the possible effect of the widely used ASDs on the dissemination of nosocomial pathogens has only been addressed in a few studies. Suppression of gastric acid with an H2RA and administration of antibiotics resulted in colonisation of MRSA in the small intestine in immunosuppressed mice [439]. Similarly, in

clindamycin-treated mice, use of a PPI tripled the rate of colonisation of the large intestine by ingested vancomycin-resistant *Enterococcus* spp. and *K. pneumoniae* [440]. An increased risk of MRSA colonisation associated with ASDs use (adjusted OR 7.12) was reported in ambulatory inflammatory bowel disease patients [441]. Use of antacids was identified as an independent risk factor for acquisition of VRE in a burn ICU (OR 24.2) [442], as well as in a cohort of hospitalised patients (OR 2.9) [443]. VRE colonisation was independently associated with PPI use in liver transplant candidates (OR 2.7) [444]. Use of H2RAs was found to be a predictor of bacteraemia and colonisation with extended-spectrum beta-lactamase-producing Enterobacteriaceae (area under receiver operator characteristic curve 0.8) [445]. Taking together, these observations suggest that gastric acid suppression by ASDs may act as a factor contributing to colonisation/infection with MRSA, VRE and other antibiotic-resistant bacilli. It should be noted that subjects who are colonised with these organisms, even when at low risk of developing clinical disease (asymptomatic carriers), may act as reservoirs for spreading the infectious agents to other persons, especially in RCFs.

13 Proposed Pathogenic Mechanisms of ASD-Associated Infections

The available data, although incomplete and conflicting, suggest that use of ASDs, especially PPIs, may predispose susceptible individuals to infections, in particular the elderly and frail persons with multiple comorbidities, who are more likely to be prescribed these medications as well as antibiotics. The underlying biological mechanisms involved in ASD-associated infections are complex and still not fully understood. The promising and plausible biological mechanisms related to pleiotropic effects of ASDs include: (1) inhibition of gastric secretion and gastric emptying [446] and suppression of gastrointestinal motility [213, 274, 447, 448], (2) immune dysfunction (reduction of the immune-mediated resilience to infections), direct effects on the activity of neutrophils, monocytes, endothelial, and epithelial cells [449, 450], (3) metabolic disorders such as vitamin (B₁₂, C) and mineral deficiencies (iron, magnesium and calcium) [5, 451–453], (4) increased mucosal permeability [216, 454–456]—all factors resulting in (5) disruption of the natural gut microbial flora and predisposing to infectious diseases. These ASD-associated conditions may exert their effects separately but usually in concert; and in each infection type, despite differences in biology, predisposing and initiating events, the above-mentioned pathophysiological factors are linked and overlap.

The elderly, understandably, are potentially at higher risk of ASD-associated infections due to age-related decreased gastric acidity, often esophageal and gastro-intestinal motility disorders, impaired phagocytosis, decreased antibody production and compromised immune system—immunosenescence [457–461]. In addition, impaired drug metabolism (e.g. in advanced cirrhosis), especially of PPIs (except rabeprazole), may result in higher exposure to PPIs [33, 257].

13.1 Inhibition of Gastric Secretion

High gastric acidity, in combination with pepsin, lipase and mucus is a fundamental, though non-specific, natural physiological barrier against a variety of ingested bacterial and parasitic pathogens [307, 462–469]. Not surprisingly, alteration in this defence system—both acquired and iatrogenic gastric hypochlorhydria/achlorhydria—increases susceptibility to and severity of enteric infections [147, 156, 191, 195, 294, 307, 470–472] caused by bacteria such as *Salmonella*, *Cholera*, *E. coli*, *Campylobacter* and *Yersinia* species [156, 473], parasites and viruses; the strongest evidence is on non-typhoid salmonellosis and cholera [156, 307, 463, 474]. In mice with hypochlorhydria caused by mutation in a gastric H⁺/K⁺-ATPase (proton pump) gene significantly greater numbers of *Salmonella*, *Yersinia*, and *Citrobacter* cells and *Clostridium* spores survived, resulting in reduced median infectious doses [475]. In general, the risk of clinical infection is higher in PPIs users compared to persons receiving H2RAs because of greater gastric acid suppression with PPIs. On the other hand, some bacteria and fungi, known to inhabit the human body, contain proton pumps which belong to the family of P-type ATPases which includes the human gastric H⁺/K⁺-ATPase [476–478]. Therefore, potentially PPIs may directly target the proton pumps of these bacteria and fungi, for example, in *H. pylori* [479, 480], some *Streptococcus* spp. [481] and fungi [477, 482].

It should also be recognised that bacteria, viruses and parasites have multifaceted repertoires of strategies to evade host's defence systems including the ability to reduce gastric secretion of acid [156, 463] and mucus production [468] as well as affect immune responses.

Loss of this major non-specific defensive mechanism—the increase in gastric pH by ASDs—allows pharyngeal commensals and ingested environmental organisms (most of which, except *H. pylori*, are not adapted to low pH) to survive, proliferate and colonise in the stomach, to pass into the duodenum and further along the gastrointestinal tract causing gastrointestinal dysbiosis. The normal gastrointestinal flora maintains the histological structure of the gut mucosa and is an extremely important host defence mechanism, highly effective in protecting against

colonisation by potentially pathogenic invaders. The qualitative and quantitative alterations in the gut microbiota may result in a number of intestinal and extra-intestinal infections.

13.2 Effects of ASDs Unrelated to the Inhibition of Gastric Acid Secretion

The ability of a microorganism to cause infectious disease is a function of both its intrinsic virulence and the host's defence barriers, which include immunological competence. The ASDs in addition to the inhibition of gastric acid secretion directly and indirectly influence multiple functions related to the immunological defence status [450]. PPIs reduce different neutrophil functions [483–486], including phagocytosis and acidification of phagolysosomes [487], adhesion of neutrophils to endothelial cells [488, 489], exhibit anti-oxidant properties [449, 490–497], suppress the expression of tumour necrosis factor- α , interleukins (IL-6, IL-8, IL-1 β), intracellular and vascular adhesion molecules [488, 498–501], inhibit the nitric oxide synthase [501, 502] and lysosomal enzymes [503], dose-dependently decrease production of pro-inflammatory/profibrotic cytokines by epithelial and endothelial cells [450, 499, 504, 505], decrease natural killer cell cytotoxic activity in a dose-dependent manner [506], impair neutrophil migration from vessels to inflammatory sites by preventing the activation of heparanase [486], mitigate neutrophil adherence to endothelial cell [500], affect neutrophil chemotaxis and phagocytosis of micro-organisms [216, 483, 487, 507]. These mechanisms, although some of them remain hypothetical, might compromise immunity, contribute to bacterial colonisation and a variety of inflammatory and infectious disorders. Noteworthy, the rise of intralysosomal pH by PPIs is considered the major mode of the direct antileishmanial in vivo [392, 508] and anti-malarial in vitro activities [405] and in the inhibition of the rhinovirus infection in cultured human epithelial cells [504]. PPIs might also be potentially beneficial in inflammatory diseases, in which the role of acid and pepsin is minimal (e.g. eosinophilic esophagitis, idiopathic pulmonary fibrosis) [450], as well as for antineoplastic therapeutic regimens [500, 509–512].

Immunomodulation effects of H2RAs have also been documented [332, 513]. Histamine plays an important role in the regulation of neutrophil-dominant inflammatory reactions mediating an oxidative burst, one of the most important defence mechanisms for the elimination of invading microorganisms [514–517]. H2 receptors (as well as H1 and H4 receptors) are expressed in neutrophils and other immune cells [518–521]. The results from experimental studies on the effects of H2RAs on neutrophils, monocytes and other cells are controversial

[515–518, 522–526]. Clinical observations indicate that H2RAs may improve postoperative immunosuppression [523, 527], modulate IL-6 signal transduction and reduce CRP levels [528]. A potential beneficial impact of H2RAs in the treatment of multiple myeloma [522], gastrointestinal and breast cancers has also been reported [529, 530].

Altogether, simultaneous suppression of gastric secretion and modulation of multiple factors involved in the pathogenesis of infection and cell homeostasis by ASDs might affect the complex host-infective agent relationship and explain the increased incidence of infectious diseases among some ASD users, but beneficial effects in other patients (e.g. postoperative, with cancer, some parasitic infections, idiopathic pulmonary fibrosis, etc.). This suggests that the resulting effect should be considered in each patient individually taking into account the appropriateness of ASD use, underlying comorbid conditions, their severity and other medications prescribed. There is a need for a paradigm shift in the ASD use from disease-oriented to an individual patient-oriented. The risk of ASD-associated infections is usually the result of a complex interaction between multiple mechanisms, including ASD exposure-induced dysbiosis and altered immunity, as well as the patient's underlying immunological status, which may be affected by the different conditions and diseases (age, CVDs, DM, COPD, oropharyngeal dysphagia, CLD, CKD, cancer, polypharmacy, immunosuppressive drugs, indwelling devices, etc.) (Fig. 1).

13.3 ASDs and CDI

With regard to the association of ASDs with CDI, it should be noted that *C. difficile* vegetative forms (not spores which are acid-resistant [531]) are normally killed by gastric acid but survive when the pH >5, and the stool samples of infected individuals contain 10-fold more vegetative cells than spores [532]. Bile salts, as has been shown in the Syrian hamster, stimulate the transition of *C. difficile* spores to vegetative cells in the duodenum and small intestine [533]. Therefore, reduced gastric acidity together with presence of bile salts in gastric contents [e.g. in gastrointestinal reflux disease (GERD)] may affect *C. difficile* spore germination, facilitate the survival of the vegetative forms [534] and allow them to move down causing gut dysbiosis [199, 535] and predisposing to CDI [536, 537]. ASD-associated bacterial overgrowth increases levels of unconjugated bile acids [294] and might further facilitate CDI. However, the relationship between bile acids and CDI is complex [538, 539], no change in any of ten dominant human primary and secondary bile acids has been observed in healthy volunteers receiving high doses of PPIs (40 mg omeprazole, twice daily) [129].

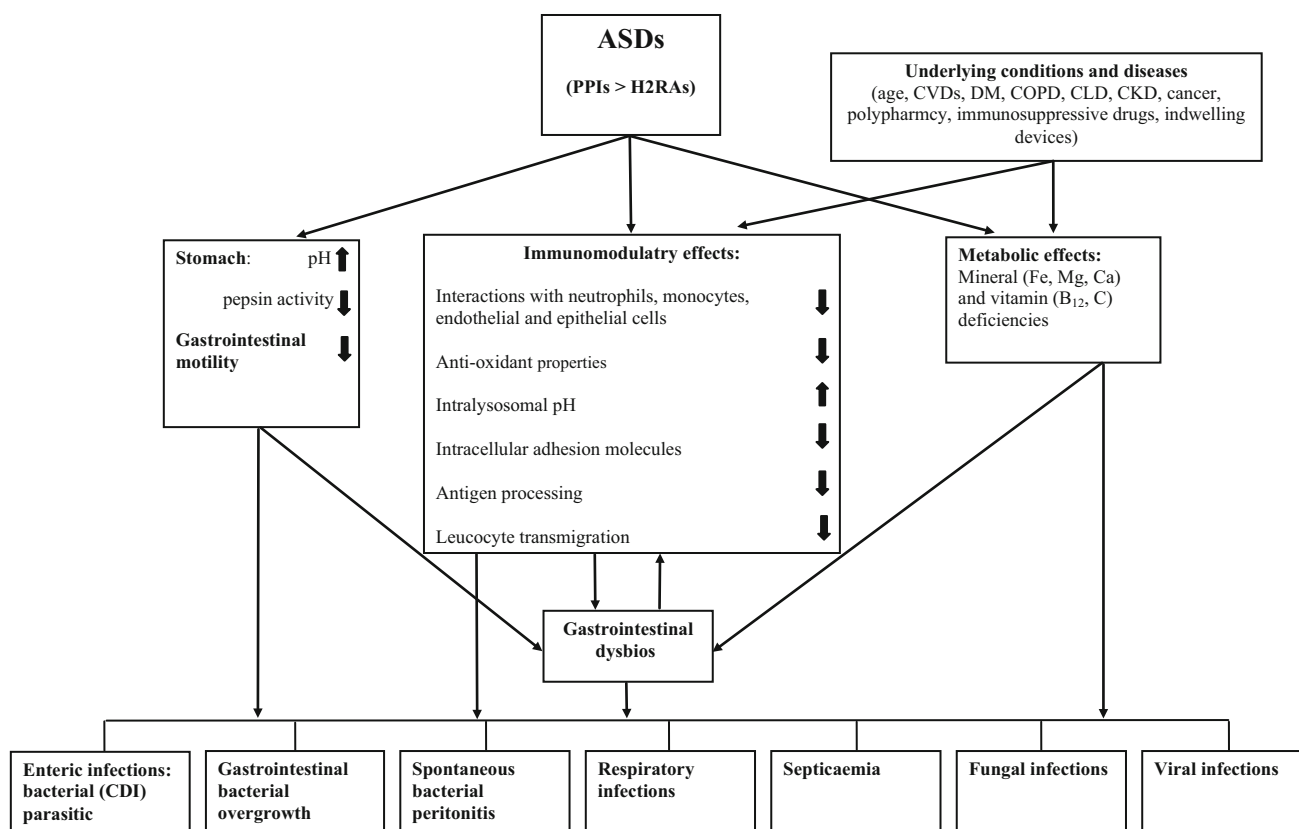


Fig. 1 Possible mechanisms linking ASD use to infections. *ASD* acid-suppressing drug, *PPI* proton pump inhibitor, *H2RA* histamine-2 receptor antagonist, *CDI* *Clostridium difficile* infection, *CKD* chronic kidney disease, *CLD* chronic liver disease, *COPD* chronic obstructive airway disease, *CVDs* cardio-vascular diseases, *DM* diabetes mellitus, *Fe* iron, *Mg* magnesium, *Ca* calcium. ASD exposure can induce dysbiosis and compromise the immunological status predisposing to and precipitating infectious diseases. Of note, the interaction between

microbiota and immunity is bidirectional, and the underlying conditions, diseases and used pharmacotherapeutic agents can alter defence mechanisms. Thus, the risk of infection is usually the result of a complex interaction between multiple mechanisms. The elderly are at a higher risk of infections because of the number of comorbidities and more pronounced impairment of defences against infections

Recent studies indicate that ASDs, especially PPIs, may increase risk of CDI by (1) altering composition of the gut microbiota [537] (towards a less healthy one), in particular the taxa involved in colonisation resistance to *C. difficile* (increased Enterococcaceae and Streptococcae, decreased Clostridiales) and taxa associated with gastrointestinal bacterial overgrowth (increased Micrococcaceae and Staphylococcaceae) [129, 130, 535, 537, 538, 540, 541], (2) affecting *C. difficile* toxin gene expression [542], (3) increasing the pathways corresponding to genes for bacterial invasion of epithelial cells and for renin-angiotensin system [129], and decreasing the expression of genes responsible for colonocyte integrity [543], and (4) directly acting on specific bacterial taxa (e.g. some Streptococcae species), which contain proton pumps belonging to the same enzyme family as the human H^+/K^+ -ATPase [478, 481]. Taking together, the available clinical and animal [70] data indicate that ASDs may directly and indirectly affect the gut microbiome and, therefore,

increase the risk of CDI. Importantly, on the population level, PPIs more than antibiotics or other used drugs are associated with profound gut microbial alterations [535].

13.4 ASDs and Pneumonia

With regard to bacterial pneumonia in patients treated with ASDs, the possible explanations and the consequence of events include (1) rise in gastric pH, promoting the proliferation of bacteria, the upper gastrointestinal tract and tracheobronchial colonisation [544–546], (2) pulmonary micro-aspiration and bacterial exchanges between the gastric and lung fluids [547], bacterial passage into the lungs and overgrowth/colonisation [198, 200, 223, 330] together (3) with impaired immune and neutrophil functions [449, 488]. In a large healthy twin cohort, PPI users demonstrated a significant increase in Streptococcaceae family [537], whereas an increased risk of community-acquired pneumonia has been reported specifically for

Streptococcus-derived pneumonia [548]. These observations support the view that in PPI users the gut is likely to become a reservoir for potential pathogens [537]. Furthermore, it has been shown that depletion of the gut microbiota reduces immune-mediated resilience to pneumococcal pneumonia in mice [549]. The postulated biological mechanisms for higher pneumonia risk in ASD users, however, do not fully explain why in some studies the correlation was weaker with the longer medication use [326, 329, 330]. Although protopathic bias (when a pharmaceutical agent is prescribed for an early manifestation of the disease that has not yet been diagnosed) could not be excluded, it is possible that susceptibility to pneumonia individuals (comorbid conditions, advanced age, poor health and immunocompromised status) might develop the disease sooner after starting ASDs [294, 334, 550, 551]. In H2RAs users, this phenomenon may also be related, at least partially, to tolerance to H2RAs (usually within the first 2 weeks) which causes decline in acid suppression.

13.5 ASDs and SBP

In immunodeficient liver cirrhosis patients, the development of ASD-associated infections, including SBP, might be related to the effects of these medications on the balance between immune defences and intestinal flora. The decreased granulocyte and monocyte oxidative burst by PPIs [552], ASD-induced dysbiosis [209] with increased intestinal permeability [456] and impaired liver drug metabolism have been suggested as important pathophysiological factors for explanation of the risk of SBP in ASD users.

14 Practical Implications

The reviewed scientific literature demonstrates that ASDs, which, undoubtedly, are of great value for treatment and prophylaxis of acid-related diseases, may be associated with an increased risk of infections, especially in the elderly. Although extensive associations between ASDs and a number of infections have been reported, it should be emphasised that most of the information was derived from observational retrospective cohort or case-control studies, and systematic reviews evaluating these studies revealed bias in selection, misclassification, interpretation and residual confounding; the observed associations may be confounded by multiple coexisting conditions and do not prove causality. It should also be recognised that prospective randomised controlled studies which are often considered the “gold standard” would not only be difficult to perform (e.g. recruitment of frail elderly patients, costs, etc.) and ethically questionable, but may not provide

absolute certainty [553]. Despite the limitations of the available data, current lack of conclusive evidence of causality, the existing reports on possible adverse effects of ASD therapy should not be ignored, but adequately interpreted and properly applied into everyday clinical practice.

Accumulating evidence suggests that adverse effects of ASDs (mainly of PPIs) in addition to infections may include poorer cardiovascular outcomes [554], an increased risk of CKD [555], fractures, especially of the hip [123, 556–573], vitamin and mineral deficiencies [574–576], altered mental status and delirium [577, 578], development of enterochromaffin-like cell hyperplasia [579], risk of gastric neuroendocrine tumours [580], and fundic gland polyps [581]. The complexity of therapeutic ASD use is further increased by drug-drug interactions (pharmacokinetic and pharmacodynamic), which, not surprisingly, are particularly common in the elderly due to polytherapy [582]. Clinically relevant interactions were reported for PPIs with clopidogrel [583–586], dabigatran [587], bisphosphonates [559, 568], metformin [588, 589], methotrexate [590–593], antidepressants and antipsychotics [38], fluconazole [400, 594], immunosuppressants (e.g. mycophenolate) [595–597], magnesium oxide [598], different anti-cancer and antiviral medications [599, 600]. In addition, all ASDs by increasing gastric pH can affect the bioavailability of several other drugs (e.g. iron salts, ampicillin, ketoconazole). H2RAs decrease absorption of magnesium oxide [598] and dasatinib [601, 602].

Although the reports on adverse drug-drug interactions with PPIs are conflicting and when evaluated in systemic reviews such events were found to be rare and the increased risk, for the most part, mild-modest [600], a careful individualised judgement in patients taking above-mentioned medications before prescribing ASDs is needed. For example, use of ASDs was shown to be associated with an increased risk of hip fracture only among persons with at least one risk factor for osteoporosis [603]; *H. pylori* positivity was found to be a significant independent risk factor for osteoporosis but its eradication was not [604].

When choosing an ASD, in general a relatively safe medication [605, 606], to manage gastric acid-related disorders the potential for possible spectrum of adverse events, drug-drug, drug-nutrient interactions and agent-specific side effects should be considered. For each individual patient, the balance of risk and benefits, agent of choice, the possible dose, and duration of use, requires careful attention.

Unnecessary or inappropriate use of ASDs, especially of PPIs, has been consistently documented in the adult population (ranged from 34.2 to 63%) [22, 29, 294, 607–614], including hospitalised patients (26.8–73.9%) [23, 25, 26, 440, 609, 615–622] and after discharge (>50–80.2%) [616, 618–620, 623–625] as well as among

nursing home residents (24–93%) [626–630]. Long-term use of ASDs, which are now available over-the-counter in many countries, may represent a prescribing cascade [630] with minimal therapeutic benefits and excess costs [606, 618, 621, 623]; unfortunately, the inappropriate use of ASDs is increasing [631], especially among patients of a lower socio-demographic status [611, 631].

On the other hand, there are reports that ASDs are underused. For example, only 31.7% of patients with Barrett's esophagus or GERD were prescribed either PPI or H2RA [632], adequate gastroprotection was not provided to more than 50% of short-term users of NSAIDs who were at an increased risk for upper gastrointestinal complications [633], and only 3.5% of low-dose aspirin users received PPIs, H2RAs or mucoprotective drugs [634]. Because the world population is ageing and age is an important risk factor for CVDs, arthritis and chronic pain, as well as for antiplatelet- and NSAID-associated ulcers and bleeding [635–638], in the coming years the prophylactic ASD use is likely to increase significantly. A recent meta-analysis demonstrated that the combination of selective COX-2 inhibitors with PPIs provides the best gastrointestinal protection, followed by selective COX-2 inhibitors, whereas co-administration of H2RAs with nonselective NSAIDs did not significantly reduce the risk of clinical gastrointestinal events [639]. PPI therapy is needed in 10 high-risk and 268 moderate-risk patients to prevent one NSAID-related ulcer [640].

The data presented above raise serious challenges and the following steps may be helpful in clinical practice when considering ASD treatment: (1) evaluate for evidence-based indications for ASD therapy, (2) weigh the risks for adverse effects of ASDs in the individual patient (*primum non nocere*, do not harm), (3) assess potential drug-drug interactions, (4) choose an adequate drug, dose (minimal effective) and duration (consider discontinuation if indications are not certain) tailored to the specific constellation of a patient's conditions and preferences, (5) consider interventions that may prevent/reduce infectious and other adverse effects associated with ASD use, and (6) monitor the patient carefully after therapy starts and decide when it could be ceased (Fig. 2). While basic sanitation, hand-washing and monitoring of antibiotics use remain central to preventing and limiting nosocomial infections, appropriate use of ASDs, especially in elderly, chronically ill and immunosuppressed patients, is also important.

ASD-associated infections (and other adverse effects) are not a class effect. Differences of clinical relevance exist between PPIs and H2RAs as well as in the pharmacokinetic profiles of individual PPIs and H2RAs. These include (1) absorption of H2RAs is not affected by food, while meal-related dosing is necessary with PPIs especially when treating GERD; (2) tolerance to H2RAs, though of minor

clinical significance, may develop after 2–7 days of therapy [641, 642], but tolerance does not occur with PPIs [643]; (3) in patients with renal impairment, the doses of H2RAs, but not of PPIs, should be reduced [644–648]; (4) although PPIs in general are more effective than H2RAs in preventing upper GI bleeding, the increased risk of CDI, pneumonia and other infections as well as fracture is lower in patients taking H2RAs compared with patients taking PPIs; (5) the risk of drug interactions mediated by cytochrome P450 enzymes should be considered for both omeprazole and cimetidine (because of their high affinity for CYP2C19 concomitant use of these medications with clopidogrel affects the biotransformation of clopidogrel by competitive inhibition of CYP2C19 [649–652]); in contrast, other PPIs (pantoprazole, lansoprazole, rabeprazole, esomeprazole and dexlansoprazole [600]) and H2RAs (famotidine, nizatidine or ranitidine, rabeprazole [653, 654]) have lower potential for drug-drug interactions; however this suggestion has not been fully confirmed in a recent meta-analysis [655]. Omeprazole when used concomitantly with protease inhibitors can cause different adverse effects [503] because of its potent pH alteration and inhibition of p-glycoprotein pathway [590]. These potential benefits and harms of PPIs and H2RAs should be considered before initiating ASDs to manage gastric acid-related disorders, especially if treatment includes polypharmacy, which for vulnerable geriatric patients can be unavoidable [656, 657].

As PPIs which are associated with a variety of adverse events do not reduce the number of reflux events and do not provide long-term cure for GERD, use of H2RAs and prokinetics and non-medical interventions should also be considered [658].

Although ASD prophylaxis against stress ulceration in critically ill patients has been part of routine clinical practice for several decades, the data on its benefits are controversial [353, 354, 359, 363, 364, 659–663]. It was concluded that the quantity and quality of evidence supporting the use of ASDs in ICU is low [664, 665]; PPIs might reduce the rate of gastrointestinal bleeding, but increase rates of nosocomial infections (especially pneumonia and CDI), myocardial ischemia [666] and mortality (e.g. patients with liver cirrhosis) [667]. The recommended alternatives to PPI prophylaxis are H2RAs [667] and sucralfate [332, 353, 358, 667], an agent which does not alter gastric pH and exerts its topical effect by binding to proteins of the ulcer site [332, 353, 358, 667]. In contrast, in three other meta-analyses, PPIs were superior to H2RAs in preventing gastrointestinal bleeding without significantly increasing the risk of pneumonia or mortality [363, 364].

Finally, strategies for reducing potential ASD-associated infections in addition to avoiding inappropriate prescribing, implementation of standardised guidelines, antibiotic

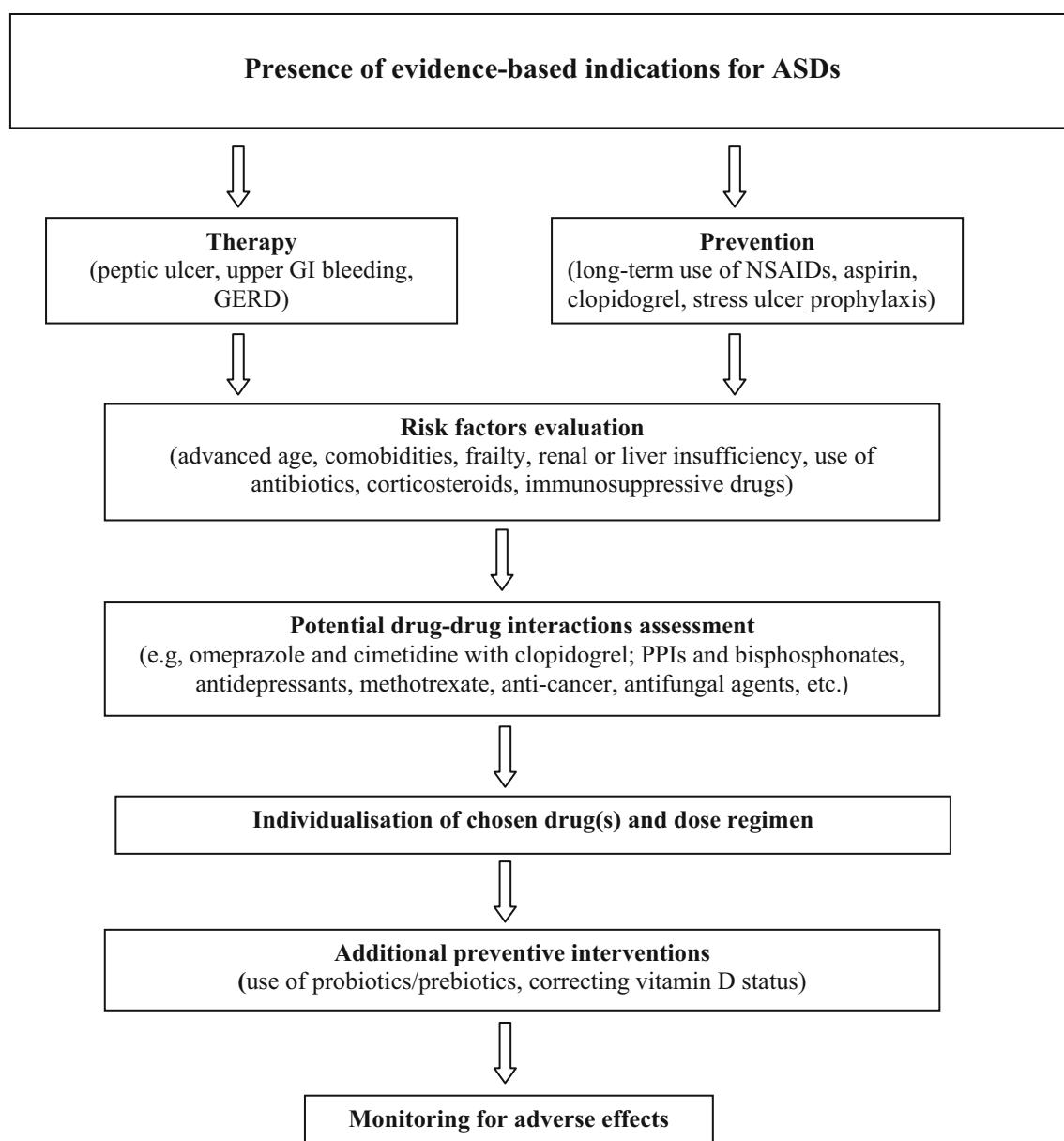


Fig. 2 Suggested algorithm for an optimised acid-suppressing drug (ASD) therapy. *GI* gastro-intestinal, *GERD* gastro-esophageal reflux disease, *NSAID* non-steroidal anti-inflammatory drug, *PPI* proton pump inhibitor

stewardship programmes and sound infection control practices, should, as adjuvant therapy, consider: (1) use of probiotics (live microorganisms with beneficial physiologic or therapeutic properties) or prebiotics (non-digestible dietary components that beneficially affects the host by stimulating the growth and/or activity of beneficial bacteria in the colon) and (2) correction of the vitamin D status. Implications of such potentially effective interventions have been, unfortunately, largely overlooked.

Currently, probiotics are recommended and used (with varying success) to protect against infections [668, 669], particularly for reduction of antibiotic-induced primary CDI [66, 670–681], travel-related diarrhoea associated with

antibiotic use [682], especially in the elderly [683], and as adjuvant therapy for *H. pylori* eradication therapy [8, 684, 685], as well as for acute upper respiratory tract infections [686, 687], ventilator-associated pneumonia in critically ill patients [688, 689], pneumonia caused by *K. pneumonia* (in mice) [690], urogenital infections [691], postoperative infections [692] and oral candidiasis [693] protection. Because ASD-related alterations in composition and function of the microbiota are associated with the development of infections, use of pro- and prebiotics may provide a simple preventive measure in ASD users. It is well documented that the gut microbiome plays a significant role in the regulation of host metabolism and

immunity [694–710], and a wide variety of systemic diseases and conditions are associated with gut dysbiosis. Increased intake of yogurt with a sufficient number of viable probiotic bacteria (mainly *Lactobacillus* and *Bifidobacterium* spp.) positively modulates gut microbiota, prevents dysbiosis, improves immune status [705, 711–713], the barrier function of the gut [697, 714] and possess antagonistic activity against *C. difficile*, *S. aureus*, *Salmonella* spp., *E. coli*, *P. aeruginosa*, *Enterobacter*, *L. monocytogenes*, *C. perfringens* and other bacterial agents [705, 715–721]. Also reduces gastrointestinal carriage of VRE [722, 723], displays antiviral, detoxifying, cholesterol-lowering, anti-diabetic and antioxidant properties [705]. Therefore, it is likely that probiotic use, a diet rich in yogurt and fibre may have significant potential health and nutritional benefits, including a protective effect on decreasing the incidence of ASD-associated infections, especially in the elderly [724, 725]. Such an approach can be considered a promising add-on therapy to counteract the effects of ASDs on gut dysbiosis. However, because of existing gaps in the understanding the human microbiota and the multiple mechanisms by which probiotics modulate various physiological functions, challenges and concerns persist regarding appropriate treatment regimens (specific probiotic strain(-s), most effective combinations, optimum dosing, use of nutrients), and even safety (mainly the potential of opportunistic infection in immunocompromised patients) [705, 714, 726–728] and genetic stability.

Recent research demonstrated the critical role of vitamin D in human innate and adaptive immunity [729–731]. Vitamin D insufficiency, which is highly prevalent (40–60% in the healthy general adult population), may lead to dysregulation of immune responses and increases susceptibility for infections and mortality in different settings [732–737]. Vitamin D deficiency is also reported to be associated with increased risk [738] and greater severity of CDI in some [739–741] but not all [742] studies. Animal studies found that dietary-induced vitamin D deficiency increases susceptibility to *Citrobacter rodentium*-induced colitis and exacerbates intestinal inflammatory response impairing mucosal defence [743]. Although results of several trials assessing the effects of vitamin D supplementation on infections were mixed [732, 736, 744–746], the currently available balance of evidence supports such intervention as a promising one for prevention the risk of infectious diseases, including ASD-related diseases. Potential benefits of correction of vitamin D insufficiency and maintaining vitamin D levels in the normal range also include reduced risk of many common bone, immune, cardiovascular, renal, liver, metabolic, malignant, and mental disorders, as well as mortality; these have been shown in numerous observational studies and meta-analyses, but adequate randomised trials are still lacking.

In view of current evidence and the underlying biological plausibility adding probiotics (e.g. yoghurt) and correcting vitamin D status may have beneficial consequences. However, given the limited and conflicting reports, further well-designed studies are needed to determine the effects of both probiotics and vitamin D supplementation as adjunctive therapies in infection prevention.

We hope that this review will help clinicians cope with information overload, enable them to individualise decisions regarding the use of ASDs, selection of a specific drug, and clearly discuss the balance between benefits and potential harms with the patient before starting long-term treatment.

15 Conclusions

Development and use of ASDs (H2RAs and PPIs), one of the most commonly prescribed classes of medications, has been revolutionary. Their therapeutic and prophylactic benefits are well recognised and the absolute risk of the complications including ASD-associated infectious diseases is relatively low. Accumulating data, however, indicate the complexity of ASD effects involving important defence systems, non-immunological and immunological, resulting in dysbiosis and increased risk for enteric, including CDI, and other infections, particularly in the elderly. Despite the limitations of the existing data, the importance of individualised therapy and caution in long-term ASD use considering the balance of benefits and potential harms, factors that may predispose to and actions that may prevent/attenuate adverse effects is evident. A six-step practical algorithm for ASD therapy based on the best available evidence is presented.

Compliance with ethical standards

Funding The present review article was not funded.

Conflicts of interest Leon Fisher and Alexander Fisher declare that they have no conflicts of interest to declare; they are not current or former employees of any pharmaceutical company and did not receive any financial support with regard to the content of this article.

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GASTROENTEROLOGY

Endoscopic ultrasound guided fine needle aspiration of solid pancreatic lesions: Performance and outcomes

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Key words

endoscopic ultrasound, fine needle aspiration, pancreas.

Accepted for publication 16 June 2008.

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Abstract

Background and Aim: We report our single-centre experience with endoscopic ultrasound guided fine needle aspiration (EUS-FNA) of solid pancreatic lesions with regard to clinical utility, diagnostic accuracy and safety.

Methods: We prospectively reviewed data on 100 consecutive EUS-FNA procedures performed in 93 patients (54 men, mean age 60.6 ± 12.9 years) for evaluation of solid pancreatic lesions. Final diagnosis was based on a composite standard: histologic evidence at surgery, or non-equivocal malignant cytology on FNA and follow-up. The operating characteristics of EUS-FNA were determined.

Results: The location of the lesions was pancreatic head in 73% of cases, the body in 20% and the tail in 7%. Mean lesion size was 35.1 ± 12.9 mm. The final diagnosis revealed malignancy in 87 cases, including adenocarcinomas (80.5%), neuroendocrine tumours (11.5%), lymphomas (3.4%) and other types (4.6%). The FNA findings were: 82% interpreted as malignant cytology, 1% as suspicious for neoplasia, 1% as atypical, 7% as benign process and 9% as non-diagnostic. No false-positive results were observed. There was a false-negative rate of 5%. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy were 94.3%, 100%, 100%, 72.2% and 95%, respectively. In 23 (88.5%) of 26 aspirated lymph nodes malignancy was found. Minor complications occurred in two patients.

Conclusions: Our experience confirms that EUS-FNA in patients with suspected solid pancreatic lesions is safe and has a high diagnostic accuracy. This technique should be considered the preferred test when a cytological diagnosis of a pancreatic mass lesion is required.

Introduction

Accurate and rapid diagnosis of pancreatic mass lesions is central to clinical management. Clinical and imaging characteristics often do not distinguish benign from malignant lesions or define the histological subtype of a malignant lesion. Thus a tissue based diagnosis is paramount.^{1–3} Whilst the necessity of tissue acquisition is debated in patients who are candidates for surgical resection, we believe it should be mandatory in both surgical and non-surgical patients as it is central to appropriate therapy. The main reason for this belief is that not all masses are malignant and not all malignancies are best treated surgically.

Options for tissue acquisition include computed tomography (CT) (or ultrasound) guided percutaneous biopsy, brush cytology at endoscopic retrograde cholangio-pancreatograph (ERCP), open biopsy at surgery or endoscopic ultrasound (EUS) guided fine needle aspiration (FNA). Of these techniques EUS-FNA has emerged as the most accurate.⁴ Additionally, EUS-FNA has an excellent safety record and, for curable tumours in the head,

eliminates the issues of tumour seeding.⁵ However until recently, this has been a technology confined to large centres of expertise and it is not clear if the early excellent results would be reproducible elsewhere in newly established units as the technology disseminates. Indeed, despite the growing use of EUS in Australia and the Asia-Pacific,^{6,7} there are few reports of the tests performance from these regions.⁸ The aim of this study was to report the diagnostic accuracy and safety of EUS-FNA of solid pancreatic lesions in a newly established unit and to detail the histological subtypes of tumours diagnosed.

Methods

The study was approved by the Hospital Ethics Committee of the Sir Charles Gairdner Hospital. The study centre is a University affiliated tertiary referral centre in Perth, Western Australia. Endoscopic data was collected prospectively and recorded in an electronic database for consecutive patients who underwent EUS-FNA of solid pancreatic masses. Data collection commenced when this

service was first introduced to the hospital. Data collection continued from March 2003 to November 2006 for 100 consecutive procedures performed in 93 patients with solid intrapancreatic lesions.

Data collection

Patient demographics, procedural data (including sedation technique, site of puncture, number of needle passes), imaging findings (lesions characteristics, lesion location, EUS stage), sample adequacy and immediate complications were prospectively recorded. Delayed complications were detected by the hospitals readmissions records (all patients were specifically advised to return to the study centre). Preliminary in-room cytology results were recorded in the final 71 cases once the service was well established.

Study definitions

Only solid mass lesions that appeared intrapancreatic were included in the study. Lesions classified as malignant by EUS-FNA with a final diagnosis by criterion standard of malignancy (see below) were considered true positive. Similarly lesions classified as benign by EUS-FNA which matched the final diagnosis of benign process were regarded true negative. Those with malignancy on final diagnosis were considered false-negative. Patients with benign, non-diagnostic, atypical or suspicious EUS-FNA readings were considered false negative if the final diagnosis was malignancy.

Procedure/technique

All EUS-FNA were performed by a gastroenterologist (IY) or supervised EUS fellow. Patients were sedated by the proceduralist using midazolam and fentanyl ($n = 26$) or with anaesthetic assistance using propofol ($n = 74$). The examinations were performed using a curvilinear echoendoscope (UC-140P; Olympus, Tokyo, Japan) and a 22 G aspiration needle (Wilson-Cook GI Endoscopy, Winston-Salem, NC, USA). A transgastric ($n = 27$) or transduodenal approach ($n = 73$) was used depending on lesion location. In 26 cases lymph nodes were also biopsied using a separate new needle. If more than one area was biopsied (e.g. a pancreatic mass and mediastinal lymph nodes) a new needle was used for each region; one to three needles were used per procedure. Unless the patient was already an inpatient all procedures were performed on an outpatient basis.

Cytology and histology

A cytopathologist was present in the endoscopy room for all procedures. Material obtained at each FNA pass was prepared on-site as air-dried smears and alcohol-fixed smears, and any remaining material was collected in normal saline for subsequent preparation of a cell block. Air-dried smears were stained with hematoxylin and eosin (H&E) and reviewed immediately. A specimen was considered subjectively adequate if there was sufficient number of representative cells from the lesion. Passes were performed until an adequate cytological specimen was obtained. A formal

preliminary diagnosis was recorded in all cases in the second part of the study and classified as malignant, benign or indeterminate.

The final cytologic report was classified as diagnostic or non-diagnostic. Diagnostic reports were sub-classified into: malignant, suspicious for malignancy, atypia present or benign.

The final diagnosis of pancreatic malignancy was based on a composite gold standard.^{9,10} Either of the following was sufficient for a diagnosis of malignancy: (i) histologic evidence (from surgery or trucut biopsy) of malignancy reviewed by two expert pathologists; (ii) unequivocal malignant cytology (reviewed by two expert cytopathologists) with clinical progression compatible with the diagnosis or death from malignancy. Patients determined to have a benign mass by EUS-FNA that was not confirmed by surgery/histology were monitored for at least 6 months. Absence of disease progression or resolution of the imaging/clinical changes (based on patient review, case record review or contact with the referring physician) in association with clinical well-being was required for the diagnosis of benign disease.

Statistical analysis

Continuous variables were expressed as mean and standard deviation (SD) or median (range) values and categorical variables were expressed as frequency (percentages) with 95% confidence intervals (CI) where appropriate. Student's *t*-test and chi-square with Fisher's two-tailed exact tests were used to evaluate statistical differences in comparisons between continuous and categorical variables, respectively. Each procedure was regarded as a separate data point. For all tests, a *P*-value less than 0.05 was considered statistically significant. For EUS-FNA diagnosis of pancreatic malignancy sensitivity, specificity, diagnostic accuracy, positive predictive value (PPV) and negative predictive value (NPV) were calculated with 95% CI. Diagnostic accuracy was defined as the ratio between the sum of the true positive and true negative values divided by the number of lesions. All statistical calculations were performed using Stata version 7 (Stata Corp., College Station, TX, USA).

Results

General characteristics

During the study period 1331 EUS examinations were performed, of which 342 were EUS-FNA procedures. One hundred EUS-FNA procedures were performed on 93 individuals for the assessment of solid pancreatic mass lesions. There were 39 (46.2%) women and 54 (53.8%) men who underwent 43 and 57 procedures, respectively. Mean age of the patients was 60.6 ± 12.9 (SD) years with a median of 63.0 years (range 15–83 years). Patient demographics, findings and outcomes are detailed in Table 1.

The masses were located in pancreatic head in 73% of cases, in the body in 20% and in the tail in 7%. The mean size of the mass lesion was 35.1 ± 12.9 mm (range 9–100 mm). The number of passes performed in our series was between one and six with a mean of 2.7 ± 1.1 and median 2.0. The mean follow-up was 402.5 ± 288.3 days. The final diagnosis established by the composite criteria was malignant in 87 cases and benign disease in 13 cases. The diagnosis of malignancy was confirmed by histology obtained at surgery ($n = 15$) or core biopsy ($n = 2$) and bone

Table 1 Demographic, sonographic and follow-up data on patients undergoing endoscopic ultrasound guided fine needle aspiration (EUS-FNA) for evaluation of solid pancreatic mass lesion

	All procedures (<i>n</i> = 100)	Final diagnosis		<i>P</i> -value
		Malignant (<i>n</i> = 87)	Benign (<i>n</i> = 13)	
Age, years				
Range	15–83	15–83	25–70	0.000
Mean $\pm$ SD	60.6 $\pm$ 12.9	62.1 $\pm$ 12.4	50.6 $\pm$ 11.9	
Gender (female/male)	43/57	39/48	4/9	0.512
Type of sedation, <i>n</i> (%)				
Fentanyl and Midazolam	26	23 (26.4%)	3 (23.1%)	1.000
Propofol	74	64 (73.6%)	10 (76.9%)	
Tumour location, <i>n</i> (%)				
Head	73	62 (71.3%)	11 (84.6%)	0.499
Body	20	19 (21.8%)	1 (7.7%)	
Tail	7	6 (6.9%)	1 (7.7%)	
Tumour size, mm				
Range	9–100	9–100	20–40	
Mean $\pm$ SD	35.1 $\pm$ 12.9	31.0 $\pm$ 13.4	29.3 $\pm$ 6.5	0.086
Number of passes, <i>n</i>				
Range	1–6	1–6	1–5	
Mean $\pm$ SD	2.7 $\pm$ 1.1	2.7 $\pm$ 1.1	2.7 $\pm$ 1.1	1.000
No of lymph nodes biopsied, <i>n</i> (%)	26	23 (26.4%)	3 (23.1%)	1.000
Number of complications, <i>n</i> (%)	2	2 (2.3%)	0	1.000
Follow-up duration, days				
Range	2–1345	2–1345	164–1120	
Mean $\pm$ SD	402.5 $\pm$ 288.3	380.8 $\pm$ 287.7	547.5 $\pm$ 257.4	0.051
Final diagnosis, <i>n</i> (%)				
Adenocarcinoma	70	70 (80.5%)	0	
Neuroendocrine	10	10 (11.5%)	0	
Lymphoma	3	3 (3.5%)	0	
Other malignancies [†]	4	4 (4.6%)	0	
Benign	13	0	13 (100%)	
Number of deaths, <i>n</i> (%)	36	36 (%)	0	0.010

[†]Other malignancies included: metastatic liposarcoma (1), papillary tumour (1), solid pseudopapillary tumour (1) and metastatic renal cell carcinoma (1).

marrow FNA (*n* = 1), non-equivocal FNA (*n* = 82), or death from malignancy (*n* = 35). Out of 13 non-malignant masses three were confirmed on repeat EUS FNA and 10 were considered benign on clinical follow-up. Most of the malignant masses were primary pancreatic adenocarcinomas (80.5%). Other types of neoplasia included neuroendocrine tumours (11.5%), lymphoma (3.4%), metastatic renal cell carcinoma, liposarcoma, papillary tumour and solid pseudopapillary tumour (one of each type, 1.1%).

Indications for EUS did not differ significantly in patients with and without neoplastic diagnoses. There was no statistically significant difference between these two groups with regard to gender, type of sedation used, tumour location, mass size, number of needle passes and number of lymph nodes biopsied. Patients with neoplastic processes were older (62.1 $\pm$ 12.4 *vs* 50.6 $\pm$ 11.9 years; *P* < 0.001) and had a shorter follow-up period (380.8 *vs* 547.5 days; *P* = 0.0513).

EUS-FNA findings

The FNA diagnoses were as follows: 82% had malignant cytology, 1% was interpreted as suspicious for neoplasia, 1% as atypical,

Table 2 Endoscopic ultrasound fine needle aspiration (EUS-FNA) cytological findings and final diagnoses of targeted pancreatic lesions

Cytological diagnosis	Final diagnosis		Final diagnosis for malignancy
	Benign	Malignant	
Benign (<i>n</i> = 7)	5	2	Adenocarcinoma, cholangiocarcinoma
Atypical (<i>n</i> = 1)	1	0	
Suspicious (<i>n</i> = 1)	0	1	Adenocarcinoma
Non-diagnostic (<i>n</i> = 9)	7	2	Adenocarcinoma
Malignant (<i>n</i> = 82)	0	82	(See Table 1)
Total (<i>n</i> = 100)	13	87	

and 7% had a benign process. In 9% EUS-FNA was non-diagnostic (lack of adequate cellularity). The test performance is detailed in Table 2. The overall frequency of indeterminate results (non-diagnostic + atypical + suspicious) was 11% (95% CI, 5.6–18.8). Three of these cases were later found to be malignant. In accordance with study definitions 82 cases were classified as true

Table 3 Diagnostic value of endoscopic ultrasound guided fine needle aspiration (EUS-FNA) for evaluation of pancreatic malignancy

	<i>n</i> = 100 [†]		<i>n</i> = 91 [‡]	
		95% CI		95% CI
Sensitivity, %	94.3	91–94	97.6	95–99
Specificity, %	100.0	76–100	83.3	41–99
Positive predictive value, %	100.0	96–100	98.8	96–99.9
Negative predictive value, %	72.2	55–72	71.4	35–85
False positives, <i>n</i>	0	0–3.6	1	0.03–6.0
False negative, <i>n</i>	5	1.6–11.3	2	0.3–7.7
Accuracy, %	95.0	89–98	96.7	91–99
Prevalence of neoplasm	87.0	79–93	93.4	86–98

[†]All patients; definite cytological diagnoses.

[‡]Non-diagnostic cytology (*n* = 9) excluded, 'suspicious' cytology was considered true-positive if the final diagnosis was malignancy and 'atypical' cytology was considered false-positive if the final diagnosis was benign process.

positive and 13 as true negative. There were no false positive cases. One patient with atypia on FNA was found to have a benign disease (chronic pancreatitis) on clinical follow-up. He also had a repeat EUS-FNA 3 weeks later which revealed reactive inflammatory changes only and was therefore classified as benign. There were five false negative cytological cases finally confirmed as ductal adenocarcinoma (*n* = 4) or cholangiocarcinoma (*n* = 1). These were classified cytologically as benign (2), suspicious (1) and non-diagnostic (2). In all cases the EUS appearances were suggestive of malignancy. In three cases EUS was technically difficult (uncinate lesions) and patients were referred for an alternative form or tissue acquisition (one CT guided biopsy and two surgical pathology). In 2 cases (one suspicious and one non-diagnostic) there were no technical problems so the EUS was repeated and subsequent result was positive for malignancy. In comparison between true-positive and false-negative lesions by EUS-FNA examination no statistically significant differences were found between tumour location, lesion size, and number of passes, as well as age and gender of patients.

Diagnostic value of EUS-FNA

To further determine the accuracy of EUS-FNA method we recalculated our data and excluded all non-diagnostic specimens (*n* = 9), while the only person with 'suspicious' cytology was considered true-positive as his final diagnosis revealed malignancy, and the patient with 'atypical' cytological aspirate whose final diagnosis was chronic pancreatitis was considered false-positive. These results are summarized in Table 3. Both calculating approaches demonstrated that EUS-FNA has high accuracy for diagnosis of pancreatic malignancy (95.0–96.7%), with sensitivity of 94.3–97.6%, specificity of 83.3–100.0% and positive predictive value of 98.8–100.0%. Importantly, the likelihood ratio for a negative result (the possibility of a negative test result in persons with the disease versus a negative test result in subjects without disease) was 0.06 and 0.03 in the first and second calculations, respectively. On the other hand, the positive likelihood ratio (the probability of a positive test result in patients with the disease versus a positive

test result in patients without the disease) was at least 200-times higher than the negative likelihood ratio.

Repeat procedures

Five patients had two procedures and one had three. In three patients the FNA was repeated due to non-diagnostic initial cytology. In two of these patients there were intra-procedural difficulties: one with sedation and one had mild self-limiting bleeding. In one case the initial cytology was atypical, with repeat FNA showing benign changes only (see above). One patient had two EUS-FNA procedures almost 18 months apart for two separate lesions—the first was benign and the second showed adenocarcinoma. The patient who had three procedures also had two separate pancreatic lesions: in the head (two FNA procedures) and the tail (one procedure) of the pancreas. The lesion in the pancreatic tail was found to be benign, whereas the cytology of head lesion was reported as suspicious on first FNA and confirmed as adenocarcinoma on second FNA.

Lymph node sampling

Twenty-six patients had peripancreatic, periduodenal, coeliac, porta-caval or mediastinal lymph nodes biopsied. Suspicious lymph nodes were biopsied if nodal metastases in the area of adenopathy would preclude surgery. In these circumstances accessible lymph nodes were sampled if any of the following characteristics were displayed: size > 1 cm, hypoechoic, distinct margins or round shape. In three EUS-FNA procedures patients had peripancreatic nodes biopsied only as pancreatic mass was not easily accessible. For masses in the head of the pancreas peritumoural nodes were not biopsied routinely as these were potentially resectable. No gold standard was available to calculate accuracy however malignancy was detected in 23 cases (88.5%).

Complications

Minor complications occurred in two patients. In one patient mucosal bleeding was noted during the procedure and required adrenaline injection at the FNA site. One patient was re-admitted at 24 hrs due to pain. In four other patients mild self-limiting mucosal bleeding was noted during the procedure which stopped spontaneously and required no intervention. In one EUS-FNA procedure the superior mesenteric vein was punctured. The patient was admitted overnight for observation and discharged the following day with no sequelae. No other procedure-related complications were documented; specifically there were no episodes of perforation, pancreatitis or sepsis.

Follow-up after EUS-FNA

The median follow-up for all patients was 366.0 days. No subjects were lost to follow-up. All patients with benign lesions were followed-up clinically for more than 163 days, with a mean of 547.5 days. The diagnosis did not change in any of 82 patients with malignant cytology by EUS-FNA of the pancreas. Of these 82 patients 32 died, including 31 (97%) with pancreatic adenocarcinoma, and one with a neuroendocrine tumour. The mean time to death after EUS-FNA was 276.94 ± 208.95 days (range 13–914).

Five of 18 patients with benign, non-diagnostic or inconclusive EUS-FNA results were found to have adenocarcinomas; four of these five died, with a mean time of 432.50 ± 241.16 days (range 121–699).

In-room results

In 71 cases, preliminary in-room cytological diagnosis was available at the end of the procedure. Notably, of these 71 cases 55 were correctly classified (accuracy 77.5%; 95% CI 66–86.5), and there were no false-positive conclusions. The preliminary cytological diagnosis of pancreatic malignancy with EUS-FNA was highly specific (100%) and had a PPV of 100%. However, in 13 cases malignancy was not definitely recognized on the preliminary examination: seven cases were defined as non-diagnostic, four as suspicious, one as atypical and one as a benign lesion. The overall kappa statistics was 0.535 (95% CI 0.210–0.752), indicating moderate agreement.

EUS-FNA and lesion size

In 41 cases (32 malignant and 9 benign) the lesion was smaller than 3 cm and in 59 cases (55 malignant and 4 benign) the lesion was 3 cm or larger. Evaluation of the diagnostic value of EUS-FNA for pancreatic malignancy in lesions of different size revealed sensitivity, specificity, accuracy, PPV and NPV as follows: 93.8, 100, 95.1, 100, 81.8 for lesions < 3 cm, respectively, and 94.5, 100, 94.9, 100, 51.1 for lesions $\geq$ 3 cm, respectively. However, the number of passes per procedure in the group with lesions < 3 cm compared with the group with larger lesion ($\geq$ 3 cm) was higher (3.03 ± 1.05 vs 2.58 ± 1.04 ; $P = 0.037$).

Predictors of a positive EUS-FNA

No technical features predicted a positive EUS-FNA. Specifically, lesion size, location and pathology were not independently associated with positive EUS-FNA. Results from the first 50 patients did not differ to the second 50.

Discussion

This study confirms that the high accuracy and safety of EUS-FNA for solid pancreatic lesions described at pioneering centres is achievable in newly established services.^{9,11–19} To our knowledge this is one of a handful of such series from the Asia-Pacific region and it confirms the spread of EUS in this region.⁸

Looking at the results in depth reveals a high sensitivity (94.3%) and specificity (100%). Importantly, in our series, there were no false-positive EUS-FNA specimens among definite cytological diagnoses, and only one case with 'atypical' cytology and final diagnosis of chronic pancreatitis could be considered as a false-positive. These observations which indicate high specificity and PPV are in keeping with many previous studies.^{17,20–23}

In the current study 11% of pancreatic aspirates were not definitive (non-diagnostic 9, atypical 1, suspicious 1). The suspicious case was confirmed subsequently to be malignant (adenocarcinoma), and the atypical case was determined to be chronic pancreatitis (both had repeat EUS-FNA). These data compare favourably with 12–14% rate of inconclusive EUS-FNA results

in other experienced centres.^{9,24} Technical failure resulting in unsatisfactory aspirates has been reported in 1.5–9%^{10,12,17,23,24} of cases, while atypical or suspicious diagnosis was reported in 6–10.9%.^{12,24–26} In our series there were five false negative cytological reports (two cases were interpreted as benign, two as non-diagnostic and one as suspicious). Two of five patients with false-negative EUS-FNA, had underlying chronic pancreatitis, a condition known to increase technical and interpretational difficulties significantly.^{10,15} Importantly, in all five patients the EUS itself was highly suspicious for malignancy and the patients were directed for further investigation in all cases. In the two cases where repeat EUS was technically feasible this was performed and subsequent results were positive for malignancy. Once again it must be emphasized that patients with a high clinical suspicion of resectable pancreatic malignancy should be considered for further investigations including exploratory surgery despite negative EUS-FNA cytology.^{9,27}

The clinical rationale for obtaining a histological diagnosis in patients with a solid pancreatic mass is to guide therapy appropriately irrespective of resectability as the histological diagnosis is varied. In non-operative candidates this is the agreed approach⁵ however in patients with a resectable lesion the need for this is debated.²⁸ We argue that an attempt at tissue acquisition should be made in all potentially resectable lesions for several reasons. First, the diagnosis may obviate the need for surgery. Indeed in this series 13% of lesions were benign. Furthermore, of the malignant lesions 20% were not adenocarcinomas. Of the 32 potentially resectable malignancies (on imaging grounds), 16 (50%) were not adenocarcinoma and these would not necessarily benefit from surgery. This data would not have been available had the patients not undergone FNA.

An important but underemphasized aspect of EUS-FNA is that it is the only accurate non-invasive method of staging local nodes in pancreatic cancer. This is particularly important as patients with nodal disease are essentially incurable.^{29,30} In line with previous studies,¹⁵ our data also showed that EUS-FNA of lymph nodes increases the accuracy of the procedure. We found malignancy in 23 of 26 lymph nodes aspirated suggesting that accurate staging may help prevent unnecessary surgical exploration.

For pancreatic neuroendocrine tumours compared to adenocarcinomas it has been suggested that preoperative diagnostic accuracy is lower,²³ as the tumours are often small and the obtained specimens may be haemorrhagic. However in the present series, all 10 neuroendocrine tumours were correctly diagnosed with EUS-FNA. Indeed, these data are in line with high sensitivity and specificity of EUS-FNA in detection of neuroendocrine tumours and distinguishing malignant from benign lesions reported by others.^{14,31–33}

It has been shown that EUS has a higher diagnostic value than ultrasound (US), CT and magnetic resonance imaging (MRI) in evaluating lesions smaller than 3 cm.^{17,34–36} Of note, in our series, lesions were smaller than 3 cm in 32 (36.8%) of 87 malignant cases and in 5 (38.7%) of 13 benign cases. Some authors found that the sensitivity and accuracy of EUS-FNA decreases when pancreatic lesions are smaller than 3 cm, compared with lesion $\geq$ 3 cm.²⁷ Our data do not confirm this conclusion. In agreement with others^{17,37} we found that mass size (< 3 cm or $\geq$ 3 cm) did not influence the operating characteristics of EUS-FNA.

The total complication rate of EUS-FNA in published series ranged from 0–13%.^{23,26,27,37–39} The risk of pancreatitis after EUS-FNA of solid pancreatic masses in a multicentre US study was 0.28%⁴⁰ and in a recent prospective study was 0.85%,⁴¹ while percutaneous FNA of the pancreas was associated with a 3–4% rate of pancreatitis and a mortality rate of 1%.^{42–44} No definite association was found between occurrence of a complication and the type and size of the pancreatic lesion, number of passes or history of chronic pancreatitis.⁴¹ We did not observe any major complications such as perforation, pancreatitis, infection or clinically significant bleeding.

The mean number of passes performed for diagnosis of solid pancreatic lesions in our series (2.7 ± 1.1 , range 1–6) is slightly lower than previously reported (median or mean 3–7).^{9,15,18,19,37,45} There was no difference between the number of passes required for the diagnosis of benign or malignant lesions or any specific type of tumours. The presence of a cytopathologist on-site with immediate interpretation available contributed significantly, we believe, to the efficiency and accuracy of the procedures in our study. In fact, immediate on-site evaluation was relatively accurate (77.5%) and highly specific for malignancy (100%). Previous studies have shown that without an in room cytopathologist the number of passes and procedure time increase and the rate of definitive cytological diagnosis falls by 10–15%.^{19,41,46}

In conclusion, our experience, although limited to one tertiary centre, confirms that EUS-FNA in patients with suspected solid pancreatic lesions is safe and has a high diagnostic accuracy. This technique could be easily performed in outpatients and should be considered the preferred test when a cytological diagnosis of a pancreatic mass lesion is required or when other modalities have failed. We believe that EUS-FNA should be seriously considered in all patients with a solid pancreatic mass lesion to guide therapy irrespective of surgical stage and symptoms.

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Changing trends in colorectal cancer: possible cause and clinical implications

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Received 18 April 2010; revised 4 May 2010; accepted 6 May 2010.

ABSTRACT

OBJECTIVES: The aims of this study were to determine whether pattern of patients presenting with colorectal cancer (CRC) in the last few years differs significantly from that previously reported in Australia, and to relate the trends, if present, to use of hormone replacement therapy (HRT). **METHODS:** We examined demographic and pathological characteristics of 145 consecutive CRC patients (65 females) treated in our institution in calendar years 2006-2007. Comparisons were made with data on 12536 CRC patients obtained from the Australian Association on Cancer Registries (AACR) for the year 2003, most recent available. Prescribing data for HRT were obtained from the Australian Commonwealth Department of Health and Ageing. **RESULTS:** The distribution of colon, sigmoid and rectal cancers in our series was 40%, 24.8% and 35.2%, respectively, which differs significantly from 65%, 8.1% and 26.9% in the AACR data ($p < 0.01$). Our cohort was significantly younger (65.4 ± 12.1 vs. 69.5 ± 12.3 years), especially females (63.0 ± 12.7 vs. 70.3 ± 13.0 years; $p < 0.001$). The proportion of female patients aged < 55 and < 60 years was significantly higher (30.8% vs. 13.8% and 41.5% vs. 21.4%, respectively). Younger patients have more aggressive and advanced cancers. In Australia HRT use declined since 2001 and fell by a half in 2006. **CONCLUSIONS:** In the changing CRC pattern of greatest concern is a significantly higher proportion of younger patients, especially females, with higher prevalence of more advanced and aggressive cancers, coincident with decreased prescribing of HRT. These findings may have important implications for refining

screening and preventive strategies and on demand for radiotherapy services.

Keywords: Colorectal Cancer; Age; Gender; Hormone Replacement Therapy; Trends

1. INTRODUCTION

Colorectal cancer (CRC) is the most prevalent non-skin malignancy and second highest cause of cancer-related death in Australia [1] as in other industrialized countries. The incidence and mortality increase with age and the risk of being diagnosed with CRC by age 85 years is one in 10 for males and one in 14 for females [1]. In the last two decades a decline in incidence and mortality rates for CRC has been observed in most developed countries including Australia [2-9]. The reasons for this trend may include risk factors modification, introduction of screening and improvements in medical intervention.

The characteristics of CRC vary significantly with age, gender, race/ethnicity and region of residence [7,10-12]. The causes for these differences, genetic, environmental or acquired, are not fully understood. Numerous epidemiological studies have suggested a protective effect of estrogens (alone or in combination with progestins) against CRC [13-18]. A meta-analysis of 18 observational studies of CRC and use of hormone replacement therapy (HRT) indicated a 34% reduction among current users and a 20% reduction among ever users [19]. Similar data were reported in a large randomised controlled trial [20]. However, since the Women's Health Initiative (WHI) hormone trial demonstrated the risks of HRT (coronary heart disease, stroke, breast cancer, venous thromboembolism, cholecystitis), a sharp decline in the use of HRT has been seen over the last few years [21]. No study to date has addressed possible effects of reduced HRT use on CRC trends.

In Australia, following a pilot program, national CRC screening program for people between 55 and 74 years of age is currently being phased in. However, the evidence in relation to target age is insufficient.

Better understanding of risk factors and regional trends, especially in relation to age, gender and anatomical site may prove invaluable in fine-tuning screening, providing better services and, perhaps, contribute to development of new preventative strategies. The purposes of the present study were 1) to determine whether pattern of patients presented with CRC to our institution in the last 2 years differs significantly from that previously reported in Australia in regard to age, gender, anatomical site, histopathology and TNM stage, and 2) to relate the trends, if present, to use of HRT.

2. METHODS

Study population consisted of 145 consecutive CRC patients (age ranged from 34 to 85 years, mean 65.4 ± 12.13 years) treated in calendar years 2006 and 2007 in the Dandenong Hospital, a major public teaching hospital. The patients were admitted from a catchment area of approximately 360,000 inhabitants. The information collected included patient demographics, stage, grade and anatomical site of the cancer. The sites of CRC were determined from the surgical description and the pathology report and classified according to the International Statistical Classification of Diseases (ICD) 10th revision. In order to compare results with previous reports three subsites were defined and analyzed: colon (the caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure and descending colon), sigmoid colon (sigmoid and rectosigmoid junction) and rectum. We used a broad definition to categorize the proximal (descending colon and above), and distal segments of colon because current data indicate that subsites within sigmoid colon have characteristics similar to rectal-type cancers rather than proximal-colon cancers.

TNM staging was based on the operative findings and the histopathological report. Histological grade was recorded as well, moderately, or poorly differentiated.

Comparison data for Australia-wide patterns of CRC were obtained from the Australian Association of Cancer Registries (AACR) for the year 2003, most recent available. These included data on 12,536 CRC patients analyzed on the same lines as ours. Unfortunately, AACR does not provide data concerning TNM staging. Therefore, such analysis was performed for our study population only.

To examine trends in HRT use data were obtained from the Pharmaceutical Benefits Scheme and Repatriation Pharmaceutical Benefits Scheme databases (Medi-

care Australia PBS Statistics), as well as from the annual Australian statistics on medicines reports. The latter use a combination of PBS data and survey data from a sample of community pharmacies.

For statistical evaluation data were presented as a number of cases and percentages with 95% confidence intervals (CI), chi-square and Fisher exact test were used for statistical analysis of these. Quantitative normally distributed data were expressed as means and standard deviation (SD) and Student's t-test was used for comparing mean values. Two-tailed P value was considered significantly at < 0.05 level.

3. RESULTS

Of 145 patients with CRC admitted to our hospital in the 2 year period there were 65 (44.8%) females with a mean age of 63.0 ± 12.7 years and 80 (55.2%) males with a mean age of 67.3 ± 11.4 years. Colon cancer was diagnosed in 58 patients (32 females), sigmoid in 36 (16 females) and rectal in 51 (17 females). In our series there was a more distal distribution of CRC comparing with that seen previously in the Australian population: the proportion of rectal (35.2% vs 26.9%, $p = 0.032$) and sigmoid cancers (24.8% vs 8.1%; $p = 0.003$) was significantly higher and the proportion of colon cancers (40% vs 65%, $p = 0.001$) was significantly lower. The distal colon (sigmoid and rectal) was the most common site of carcinomas contributing 55.9% of all cases (50.8% in females and 67.5% in males). The ratio of proximal to distal cancers was 0.67 in our series and 1.85 in the previous report.

The age-distribution pattern was similar in both studied periods with CRC rare before the age of 30, significant increase after the age of 45 until 75-79 and decline thereafter. But the 2006-2007 data revealed a marked shift to the younger age most pronounced in females. Our cohort overall was on average 4 years younger ($p = 0.001$), and the females were 7.3 years younger than previously reported ($p < 0.001$) (Table 1). The difference was statistically significant in females with colon and rectal cancers, while no differences were observed in males for any cancer site. In our series females with rectal cancer were the youngest, they were on average 7.9 years younger than previously reported and 3.8 years younger than males with rectal cancer.

The total male/female (M/F) ratio in our series was the same as reported previously (1.23 and 1.21, respectively). As indicated in Table 1, in both studied periods the M/F ratio increased markedly from colon through the rectum site. However, our series demonstrated that, comparing with the previously described, the M/F ratio was significantly lower in colon cancer patients (0.81 vs

Table 1. Comparison of mean age (years $\pm$ SD) of CRC patients in two studied periods by gender and site distribution of carcinomas.

Period	Gender	All Patients	Cancer Site		
			Colon	Sigmoid	Rectal
2003	Females	70.3 $\pm$ 13.01	71.4 $\pm$ 12.56	67.7 $\pm$ 13.09	67.8 $\pm$ 13.83
2006-2007		63.0 $\pm$ 12.68***	63.9 $\pm$ 14.16***	64.4 $\pm$ 12.63	59.9 $\pm$ 9.54***
2003-2004	Males	68.8 $\pm$ 11.56	69.9 $\pm$ 11.27	67.7 $\pm$ 10.92	66.9 $\pm$ 11.42
2006-2007		67.3 $\pm$ 11.38	72.1 $\pm$ 9.37	67.3 $\pm$ 9.24	63.7 $\pm$ 12.74
2003	Both	69.5 $\pm$ 12.26	70.6 $\pm$ 11.97	67.7 $\pm$ 11.91	67.2 $\pm$ 12.68
2006-2007		65.4 $\pm$ 12.13***	67.6 $\pm$ 12.82*	66.0 $\pm$ 10.81	62.4 $\pm$ 11.81***
2003	Male/Female	1.21	1.06	1.30	1.64
2006-2007	Ratio	1.23	0.81	1.25	2.00

*** $p < 0.001$, * $p < 0.05$

1.06) and higher in rectal cancer patients (2.00 vs 1.64), indicating that the proportion of males with distal cancers is increasing. The ratio of proximal to distal cancers in females was 0.97 in our series and 2.30 in the previous report and in males 0.48 and 1.57 respectively, again suggesting a pronounced shift to distal cancer sites especially in males.

In our cohort, patients < 50 years of age comprised 12.4% (18.5% females and 7.5% males), < 55 years of age 20.0% (30.8% and 11.3% respectively), < 60 years of age 33.1% (41.5% and 26.3% respectively). In 2003 the corresponding figures were 6.9% (7.8% and 6.2%), 12.9% (13.8% and 12.3%) and 21.4% (21.2% and 21.7%). The proportions of younger patients with CRC by gender and cancer site are given in **Figure 1**. In the recent series the proportion of patients with CRC younger than 50, 55 or 60 years of age was significantly higher than previously reported due to a 2-fold increase in the proportion of younger females with cancers at all sites except sigmoid. In our cohort, among females with colon cancer 31.3% were aged < 55 years and 37.5% were aged < 60 years. Corresponding figures in the previous report were 11.6% and 17.9%. Similarly in our series, among females with rectal cancer 35.3% were < 55 years of age and 64.7% were < 60 years of age, while previously this has been observed only on 19.1% and 28.9, respectively.

The prevalence of advanced clinicopathological features of CRC in our younger patients is presented in **Table 2**. A significant proportion of patients with locally advanced T3 and T4 cancers were younger than 55 years of age (25% females and 13.3% males). Importantly, most of patients < 55 years of age were in T3 and T4 categories (12 of 20 females and 8 of 9 men). This age group contributed to 1/3 of all females and 1/5 of all males with lymph nodes metastasis and/or poor differentiation. The frequency of node metastasis was significantly higher in females than in males. ($p = 0.018$). Subjects younger than 60 years of age comprised from 1/3 to

about 1/2 of all patients with advanced and/or unfavorable CRC.

Figure 2 presents the proportion of females with CRC in 5 year age groups in the recent series and in Australia in 2000 and 2003. The age-distribution patterns in 2000 and 2003 were similar, while in 2006-2007 there was a significant increase in proportion of females aged 45 to 59. This age group comprises the majority of women who use HRT.

Figure 3 displays the combined number of prescriptions for Premarin and Depo-premarin (most frequently prescribed HRT drugs) in Australia and Victoria over a 10-year period (financial years 1997/8-2006/7). The number of prescriptions peaked in 1999 and has declined since 2001. In Australia the total number of prescriptions for these drugs dropped in 2002 by 30.7% compared with the previous year and by 54.1% in 2006; in Victoria the corresponding figures were 32.4% and 55.4% respectively. These data are comparable with 40% drop in the number of total HRT prescriptions among conces-

Table 2. Proportion (%; 95% confidence interval) of younger subjects among patients with advanced CRC (2006-2007).

Age (years)	Total	Females	Males
T3/T4 stage			
< 55	18.5 (11.7-27.1)	25.0 (13.6-39.6)	13.3 (5.9-24.6)
< 60	32.4 (23.7-42.1)	35.4 (22.2-50.5)	30.0 (18.8-43.2)
N1/N2			
< 55	25.0 (14.4-38.4)	34.6 (17.2-55.7)	16.7 (5.6-34.7)
< 60	35.7 (23.4-49.6)	42.3 (23.4-63.1)	30.0 (14.7-49.4)
Poor Differentiation			
< 55	29.0 (14.2-48.0)	33.3 (13.3-59.0)	23.1 (5.0-53.8)
< 60	38.7 (21.8-57.8)	44.4 (21.5-69.2)	30.8 (9.1-61.4)

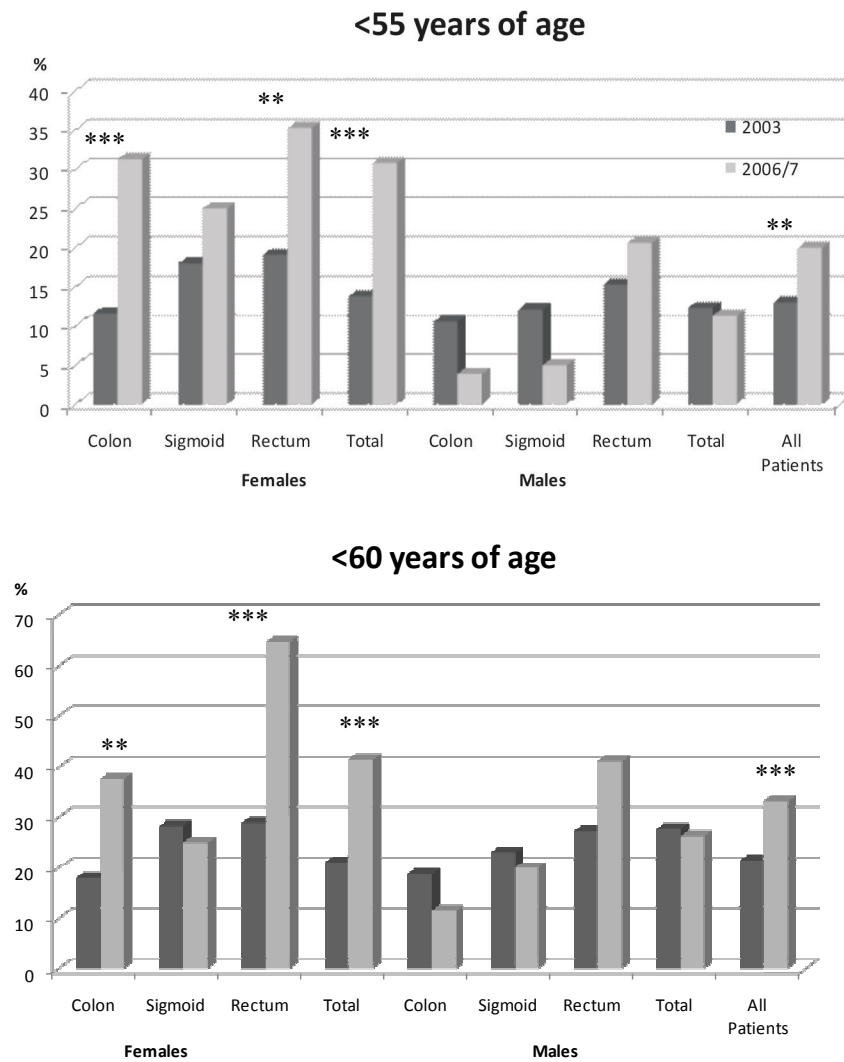


Figure 1. Proportion (%) of younger patients with CRC in two study periods by gender and cancer site. *** $p < 0.001$, ** $p < 0.01$.

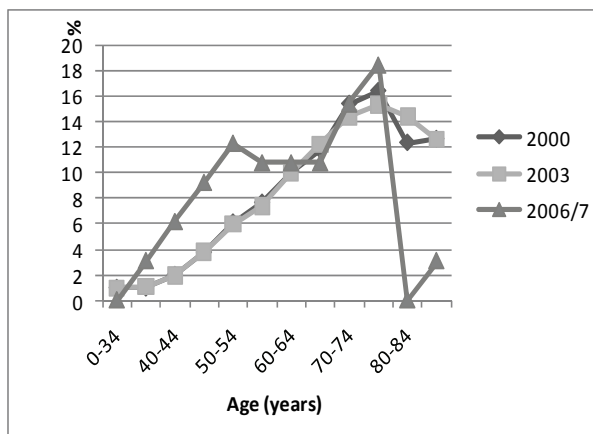


Figure 2. Age-distribution patterns of CRC in females in Australia in 2000 and 2003 and in our series 2006-2007.

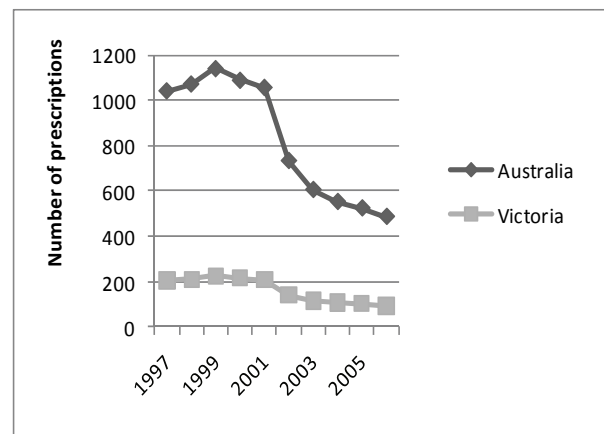


Figure 3. Prescriptions for Premarin and Depo-provera (combined data) in Australia and Victoria, 1997-2006.

sion cardholders in Australia from 2001 to 2003. In this period a similar decline was observed in prescriptions for estrogen-only preparations (39% decrease), and combined estrogen-progestogen preparations (-41%) [22].

4. DISCUSSION

Our study indicates a change in pattern of CRC occurrence in 2006-2007. Compared with Australian data for 2003 our experience is of more distal cancers, greater proportion of younger patients, especially females, with high prevalence of more advanced and aggressive tumors. Several potential explanations for the changes in CRC characteristics observed in our study should be considered.

Cancers of proximal and distal colon might differ in their genetic nature, oncofetal antigen expression and evolve through different pathways and may be associated with distinct risk factors [6,23-29]. Proximal tumors compared to distal ones have a higher proportion of DNA microsatellite instability cancers, are more common in women and older patients. Risk factors for these include high intake of red meat and animal fat, low consumption of vegetables and fiber, sedentary life style, obesity and lower socioeconomic status. Alcohol consumption and smoking [30-32], use of aspirin and non-steroidal anti-inflammatory drugs (NSAID) [33-35] and especially postmenopausal HRT use [15,19,20,36-40] have also an important influence on CRC risk. Although we cannot fully evaluate the possible effects of all the mentioned risk factors for CRC, it seems unlikely that considerable changes in dietary and lifestyle habits occurred in our population between 2003 and 2006-2007. However, one variable which may be relevant, namely, a substantial reduction in HRT use, is obvious. In Australia and Victoria after 2001 prescriptions of Premarin and Depo-provera fell by a third in 2002 and by a half in 2006. A similar rapid and substantial drop in total HRT prescriptions from 2001 to 2003 was reported in Australia and New Zealand [22]. Age and gender-related changes in anatomical subsite CRC distribution observed in our study may be attributed, at least in part, to reduced HRT use after 2001. If hormonal factors protect against CRC then the reduced use of HRT should be accompanied by an increase in colon cancers in women during early postmenopausal period, as HRT use is associated mainly with reduced risk of colon cancer [14,15,20,37,41]. Our experience is consistent with such expectations.

This study comparing with previous Australian data clearly demonstrated three points: only females with CRC were significantly younger (on average 7.5 years), there was a substantial increase in proportion of females

aged 45 to 59 years who comprise the majority of HRT users and the male predominance in colon cancer was reversed (M/F ratio for colon cancer was 0.81 in 2006-2007 whereas 1.06 in 2003). Notably, the majority of our younger patients had aggressive and advanced CRC. Previous studies indicated that decrease in estrogen level after menopause increases the risk of a colon cancer with poorer differentiation [11,42]. Our findings are in line with the assumption of an approximate 3-5 year time lag between decrease in HRT use and its impact on CRC incidence rate [21,43,44]. In the Women's Health Initiative (WHI) trial the 38% lower risk of CRC observed in women prescribed HRT during the trial phase disappeared within 3 years of discontinuing HRT [43].

Numerous epidemiological and experimental studies suggested a favorable influence of HRT on CRC risk. It was estimated that estrogens alone or in combination with progestins reduce colon risk by 20-44% in postmenopausal women, and the duration of HRT did not influence risk estimates [19-21]. In the present study a sharp drop in the HRT use after 2001 was associated with 20.1% increase in the proportion of females < 60 years of age with CRC compared to the 2003 data. If this association is causal a further increase in CRC female patients should be expected. Of interest, the substantial decrease in HRT use was associated with a decline of breast cancer incidence in Australia, US and other countries [22,45,46]. The protective effects of estrogen on colonic carcinogenesis are mediated predominately through estrogen receptor β (ER- β) involving both genomic and non-genomic mechanisms [40]. ER- β -knock-out mice demonstrate hyperproliferation, loss of differentiation and disordered apoptosis of colonic mucosa, as well as disorganization of mucin localization, reduction in the number of hemidesmosomes and loss of tight junctions of colonic epithelium [47], indicating that ER- β plays a pivotal role in maintenance of cellular homeostasis in the colon. ER- β is the dominant receptor in human colonic mucosa and seems to be essential for preventing malignant transformation of colonic epithelial cells. A significant reduction of ER- β expression has been shown in colon adenocarcinoma cells and this correlated with tumor dedifferentiation, stage and grade [42,48,49]. Moreover, in the pre-malignant phase of colon carcinogenesis ER- β expression is also reduced and inversely correlates with increase of proliferative activity in the adenomatous tissue [50]. It has been shown previously that methylation associated inactivation of estrogen receptor gene in ageing colorectal mucosa is one of the earliest events predisposing to cancerogenesis [51]. Other postulated mechanisms by which HRT use might reduce CRC risk include the influence of estrogens on bile acids [52,53], insulin and glucose control [54].

Our findings may have important practical implications for present and future screening strategies, treatment and prevention of CRC. Early diagnosis of CRC could improve survival. Currently CRC screening in Australia is advocated for people aged 55 and older. However, in our series patients younger than 55 years comprise 20% (females 31%, males 11%), suggesting that the optimal cut-off age for screening needs to be reviewed. Although we observed a distal shift of CRCs, in 40% of our patients (in 49.8% of females and in 30.6% of males) the cancers were located above the sigmoid colon. These tumors will be missed if sigmoidoscopy (not total colonoscopy) is chosen as the screening technique. The significant and rising number of rectal cancers (35.2% in total, 26.2% in females and 42.5% in males) is likely to increase demand for radiotherapy services. The observed association between the changing age and subsite patterns of CRC in females and the drop in HRT use in the context of current data on estrogen effects on growth, differentiation and functioning of epithelial cells in the colon and the protective role of estrogen in CRC may stimulate research of novel preventive and therapeutic approaches such as development of selective ER- β agonists. At present, the well established risks of traditional HRT [21] preclude its use to reduce the CRC in postmenopausal women [20]. However consumption of soy products and dietary fiber which are high in phyto-estrogens demonstrate a protective effect in CRC [55,56] and are not associated with breast cancer [57]. Of note, the re-analysis of the WHI data showed that women who started estrogen therapy at the age of 50-59 years and continued it for 6-7 years did not have an increased risk of coronary heart disease [58], and have a decreased risk of early-stage breast cancer and a decreased risk of ductal carcinomas when compared with placebo treated women, although the risk of stroke in the estrogen group was non-significantly elevated [59]. These data together with significantly decreased incidence of colon cancer and fractures with long-term HRT use indicate the potential benefits of HRT. However, additional large controlled studies are needed to find definitive criteria for HRT use—time of initiation (? menopause) and duration (? 5-6 years) of therapy—to have beneficial effects on women's health, including protection against CRC and to avoid adverse effect.

Some limitations of this study warrant consideration. Our study was a single hospital-based. We were not able to calculate true incidence rates and therefore the comparisons with previous report were performed using proportions of CRC patients in age and subsite categories. The observation that sharp decrease in HRT use was followed by changes in CRC patterns in females does not establish causal relationship between the two, but provides a logical explanation and is of importance to

public health.

5. CONCLUSIONS

Our series of CRCs in 2006-2007 show important age-, gender- and subsite-related changes in CRC patterns compared with those seen in the Australian population previously (2003). Of greatest concern is a significantly higher proportion of patients aged < 55 and < 60 years of age (20% and 33% respectively), especially females (31% and 41.5%). Furthermore, there were more female patients with colon cancer and younger patients were more likely to have more advanced and aggressive cancers. These findings seem to be temporally, and possibly causatively, related to decrease in HRT use and may have significant implications for effective screening strategies, provision of radiotherapy services and further research in CRC pathogenesis and prevention.

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