

Assessment of mineral metabolism in Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD)

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Abstract

Patients with chronic kidney disease (CKD) experience significantly poorer outcomes compared to the general population, including greater all-cause and cardiovascular-related mortality. Increased morbidity and mortality is partly attributable to the propensity for patients with CKD to be older and have an elevated burden of co-morbidities and cardiovascular risk factors. However, it is also clear that additional pathological processes specific to CKD play a role. Decline in kidney function is paralleled by progressive abnormalities in mineral and bone metabolism. This multi-system process is collectively referred to as Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD), and is almost universally present in advanced stages of CKD. CKD-MBD is thought to be a significant driver of adverse outcomes in CKD. This thesis presents a series of related works that explore the application of markers of mineral metabolism in CKD-MBD, including their association with clinical outcomes. Novel markers are utilised to examine mineral metabolism in health and also disease states, including in response to potentially therapeutic interventions.

Altered phosphate homeostasis is considered to be central to the conventional understanding of CKD-MBD. **Chapter 2** re-examines the use of serum phosphate as a marker of adverse risk in a large contemporary cohort of incident dialysis patients. Both high and low levels of serum phosphate were found to be independently associated with increased mortality, regardless of dialysis modality (haemodialysis and peritoneal dialysis), suggesting that serum phosphate remains a robust marker of patient risk.

Over the past decade calciprotein particles (CPP) have emerged as a novel marker of mineral metabolism and have been suggested to explain some of the association between phosphate and pathological events in CKD. CPP are endogenous mineral-protein colloids that are thought to be a critical protection against ectopic mineralisation in normal mineral physiology, but may become injurious in CKD due to altered levels and composition. Notably though our understanding of CPP metabolism in health and in CKD is limited.

Chapter 3 and 4 focus on the role that CPP may play in normal mineral handling. **Chapter 3** provides evidence that CPP may be formed as part of the normal response to nutritional

mineral loading, but that post-prandial metabolism of CPP precursors, calciprotein monomers (CPM), appears to be perturbed in CKD. **Chapter 4** found that serum levels of CPP are dynamically linked to bone turnover, consistent with the hypothesis that bone may also be an important determinant of serum levels of CPP.

Chapter 5 and 6 utilise samples from two recently completed interventional trials to examine potential therapeutic strategies to modify serum levels of CPP in individuals with CKD. Despite theoretical benefits, lanthanum carbonate, a non-calcium based intestinal phosphate binder, did not lower levels of CPP in individuals with non-dialysis requiring CKD (**Chapter 5**). In a cohort of patients with kidney failure being treated with haemodialysis, changing from a standard high flux dialyser to a novel mid cut-off dialyser did not appear to be associated with any change in CPP (**Chapter 6**).

Finally, in **Chapter 7** the potential role of CPM in the progression of CKD was studied. In an observational cohort, higher levels of CPM were associated with increased risk of progression of CKD. This association was independent of a range of traditional risk factors and raises the possibility that altered CPM metabolism in CKD may contribute to the progression of CKD itself.

In summary, this thesis explores the use of conventional and novel markers of mineral metabolism. It is expected that an enhanced capacity to assess the evolution of disordered homeostasis in CKD will provide new avenues to better risk assess patients with the ultimate goal of developing new therapeutic strategies in this population.

Declaration

This is to confirm that:

- i. this thesis comprises only my original work towards Doctor of Philosophy except where indicated in the preface;
- ii. due acknowledgement has been made in the text to all other material used;
and
- iii. the thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies and appendices.

Mark K. Tiong

Preface

The work contained in this thesis was performed by the candidate except where due credit is given. Contributions, funding sources and publication status of all chapters is outlined below:

Chapter 2: The study reported in this chapter involved analysis of deidentified data from the Australia and New Zealand Dialysis and Transplant Registry (ANZDATA). I would like to acknowledge the patients and treating units who voluntarily contribute data to ANZDATA, as well as Dr Matthew Sypek (Department of Nephrology, The Royal Melbourne Hospital) for his advice about performing data analysis for this study. This work was published in Nephrology (Carlton) on 28 May 2021.

For this chapter I was involved in the conceptualisation and design of the study. I performed the data analysis and wrote the manuscript. The extent of my contribution was approximately 85%. The following co-authors contributed to the work:

Name	Nature of contribution
Shahid Ullah	Commented on and revised the manuscript
Stephen McDonald	Provided advice about data analysis, commented on and revised the manuscript
Sven-Jean Tan	Commented on and revised the manuscript
Nicole Lioufas	Commented on and revised the manuscript
Matthew Roberts	Commented on and revised the manuscript
Nigel Toussaint	Conceptualisation, study design, commented on and revised the manuscript

Chapter 3: For this chapter I was involved in the conceptualisation and design of the study. I recruited all participants, collected all biological samples and clinical data, and performed basic science work. I performed the data analysis and wrote the manuscript. The extent of my contribution was approximately 85%.

This work was supported by a Royal Melbourne Hospital Research Funding Program Grant in Aid which was awarded to Dr Michael Cai. This work was published in Scientific Reports on 5 May 2022.

The following co-authors contributed to the work:

Name	Nature of contribution
Michael Cai	Conceptualisation, study design and funding acquisition. Commented on and revised the manuscript.
Nigel Toussaint	Conceptualisation, study design, commented on and revised the manuscript.
Sven-Jean Tan	Advice about recruitment and collection of biological samples. Commented on and revised the manuscript.
Andreas Pasch	Technical assistance with the CPM and T50 assay. Contributed to interpretation of the data. Commented on and revised the manuscript.
Edward Smith	Conceptualisation and study design. Supervised basic science work. Contributed to interpretation of the data and advice about statistical analysis. Commented on and revised the manuscript.

Chapter 4: This chapter involved analysis of data that was generated as part of the Fetuin-A Levels in Systemic disease and Kidney Impairment (FLEKSI) study, which was funded by a Royal Melbourne Hospital Home Lottery Research Project grant to Edward Smith and Stephen Holt.

I was involved in conceptualisation and design of this sub-study of FLEKSI. I performed the data analysis and wrote the manuscript. The extent of my contribution was approximately 60%. This work was published in JBMR Plus on 30 March 2021.

The following co-authors contributed to the work:

Name	Nature of contribution
Edward Smith	Conceptualisation and design of the original FLEKSI study as well as this sub-study. Advice about data analysis and assisted with data interpretation. Commented on and revised the manuscript.
Nigel Toussaint	Commented on and revised the manuscript.
Hasan Al-Khayyat	Participant recruitment.
Stephen Holt	Conceptualisation and design of the original FLEKSI study. Commented on and revised the manuscript.

Chapter 5: This chapter involved analysis of data and serum samples from the IMPact of Phosphate Reduction On Vascular Endpoints in CKD (IMPROVE-CKD) trial. The IMPROVE-CKD trial was supported by National Health and Medical Research Council of Australia research grants and by a Shire (a member of the Takeda group of companies) research grant. Funding support for basic science work conducted for this sub-study of IMPROVE-CKD was provided by a Royal Melbourne Hospital Home Lottery Project Grant. I would like to acknowledge Belinda Wigg (Department of Nephrology, The Royal Melbourne Hospital) for technical assistance with the CPP assay.

I was involved in conceptualisation and design of this sub-study of IMPROVE-CKD. I contributed to data analysis and interpretation and wrote the manuscript. The extent of my contribution was approximately 70%. This work was published in Nephrology Dialysis Transplantation on 25 February 2022.

The following co-authors contributed to the work:

Name	Nature of contribution
Edward Smith	Conceptualisation and design of this sub-study of the IMPROVE-CKD trial. Contributed to data interpretation. Commented on and revised the manuscript.

Elaine Pascoe	Contributed to data analysis. Commented on and revised the manuscript.
Grahame Elder	Participant recruitment in the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Nicole Lioufas	Participant recruitment in the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Eugenia Pedagogos	Conceptualisation and design of the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Carmel Hawley	Participant recruitment in the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Andrea Valks	Project manager of the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Stephen Holt	Conceptualisation and design of the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Tim Hewitson	Conceptualisation and design of the original IMPROVE-CKD trial. Commented on and revised the manuscript.
Nigel Toussaint	Conceptualisation and design of the original IMPROVE-CKD trial and this sub-study. Participant recruitment. Contributed to data interpretation. Commented on and revised the manuscript.

Chapter 6: This chapter involved analysis of data and serum samples from The tRial Evaluating Mid cut-Off Value membrane clearance of Albumin and Light chains in Hemodialysis patients (REMOVAL-HD). REMOVAL-HD was supported by a Baxter Investigator Initiated Research Grant and also by research funding from Kidney Health Australia via the Australasian Kidney Trials Network (AKTN). Funding for basic science work performed for this sub-study of REMOVAL-HD was provided by a Royal Melbourne Hospital Home Lottery Project Grant to Edward Smith and Tim Hewitson. I would like to acknowledge Belinda Wigg and Shoni Bruell (Department of Nephrology, The Royal Melbourne Hospital) for technical assistance with the FGF23 and CPP assays.

I was involved in conceptualisation and design of this sub-study of REMOVAL-HD. I contributed to data analysis and interpretation and wrote the manuscript. The extent of my contribution was approximately 70%. This work was published in Hemodialysis International on 28 March 2021.

The following co-authors contributed to the work:

Name	Nature of contribution
Rathika Krishnasamy	Conceptualisation and design of the original REMOVAL-HD trial. Participant recruitment. Commented on and revised the manuscript.
Edward Smith	Contributed to data interpretation. Commented on and revised the manuscript.
Elizabeth Ryan	Contributed to data analysis. Commented on and revised the manuscript.
Elaine Pascoe	Contributed to data analysis. Commented on and revised the manuscript.
David Johnson	Participant recruitment in the original REMOVAL-HD trial. Commented on and revised the manuscript.
Nigel Toussaint	Participant recruitment in the original REMOVAL-HD trial. Contributed to data interpretation. Commented on and revised the manuscript.

In addition to the co-authors listed above, Colin Hutchison, Carmel Hawley, Tim Hewitson, Meg Jardine, Matthew Roberts, Yeoungjee Cho, Muh Geot Wong, Anne Heath, Craig Nelson, Shaundee Sen, Peter Mount, Liza Vergara and Peta-Anne Paul-Brent were also included as co-authors of this publication in acknowledgement of their contribution to the original REMOVAL-HD study.

Chapter 7: This chapter involved analysis of data and serum samples from the ACADEMIC Study. Funding for basic science work performed in this sub-study was provided by a Royal Melbourne Hospital Home Lottery Project Grant to Edward Smith and Stephen Holt.

I was involved in conceptualisation and design of this sub-study of the ACADEMIC Study. I performed the data analysis and wrote the manuscript. The extent of my contribution was approximately 80%. This work was published in American Journal of Nephrology on 6 December 2022.

The following co-authors contributed to the work:

Name	Nature of contribution
Stephen Holt	Conceptualisation and design of the original ACADEMIC study and participant recruitment. Commented on and revised the manuscript.
Martin Ford	Conceptualisation and design of the original ACADEMIC study and participant recruitment. Commented on and revised the manuscript.
Edward Smith	Conceptualisation and design of the original ACADEMIC study. Conceptualisation and design of this sub-study. Contributed to data interpretation. Commented on and revised the manuscript.

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I would also like to acknowledge the patients with CKD and other volunteers who have participated in or contributed their data to the studies contained in this thesis.

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I have always admired Nigel's can-do attitude but learnt to appreciate it even more when the COVID-19 pandemic emerged and threatened to derail my PhD just as it was supposed to be getting started early in 2020. Nigel remained incredibly supportive and positive throughout and helped me to continually adapt my research program in what seemed like an ever-evolving environment.

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Chris is always a ready source of support and advice. Unfortunately, COVID-19 meant that our intended suite of studies that he had helped to develop faced multiple delays and

ultimately haven't formed part of this thesis, but I look forward to our ongoing collaboration on these studies in my post-PhD life.

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COVID-19 related disruptions

This PhD was commenced on 1 April 2020 and was completed in Melbourne, Australia. During this time, Melbourne was subject to multiple COVID-19 pandemic related government-imposed activity restrictions (“lockdowns”) during the following dates:

- 31 March 2020 – 12 May 2020
- 9 July 2020 – 27 October 2020
- 13 February 2021 – 17 February 2021
- 28 May 2021 – 10 June 2021
- 16 July 2021 – 27 July 2021
- 5 August 2021 – 21 October 2021

In addition to government restrictions during the above dates, The University of Melbourne and The Royal Melbourne Hospital applied numerous other restrictions during, and in between, these dates. These additional restrictions affected normal research activity through a variety of ways, including:

- Deferral of consideration of new ethics applications that were not directly related to COVID-19 research (mainly during 2020).
- Restrictions on patient recruitment and on-site patient visits for non-essential research.
- Reduced hospital resources (e.g., radiology appointments and clinical space) available to conduct research related investigations and participant study visits.

The above restrictions led to significant delays in conducting research that involved on-site patient visits. As a result, recruitment for participants in the study presented in Chapter 3 was stopped after 30 participants (instead of the planned 40) due to recruitment challenges. This thesis was also originally planned to include three studies that utilised novel bone imaging technologies to measure bone changes in CKD-MBD. However, over the course of 2020 and 2021 these studies were progressively wound back due to recruitment restrictions, and alternative studies were instead conceived to utilise pre-existing datasets and/or stored biological samples. As a result, the scope of this thesis now exclusively focusses on the assessment of mineral metabolism in CKD-MBD, instead of the initial intent of also including chapters examining the assessment of bone.

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List of abbreviations

1,25[OH] ₂ D	1,25-dihydroxyvitamin D
24-hydroxylase	25-hydroxyvitamin D 24-hydroxylase
25[OH]D	25-hydroxyvitamin D
1- α hydroxylase	25-hydroxyvitamin D 1- α hydroxylase
α KI	α Klotho
AAC	abdominal aortic calcification
ACADEMIC	Arterial Compliance and Oxidant Stress as predictors of loss of renal function, morbidity and mortality in Chronic Kidney Disease Study
ACR	albumin-to-creatinine ratio
Ahsg	α 2-Heremans-Schmid glycoprotein
AIC	Akaike information criterion
AKTN	Australasian Kidney Trials Network
ALP	alkaline phosphatase
ANZDATA	Australia and New Zealand Dialysis and Transplant Registry
AUC	area under the curve
BMI	body mass index
BMP	bone morphogenetic protein
bsALP	bone specific alkaline phosphatase
BTM	bone turnover markers
CaSR	calcium sensing receptors
cFGF23	C-terminal fibroblast growth factor-23
CI	95% confidence interval
CID	chronic inflammatory diseases
CKD	chronic kidney disease

CKD-MBD	Chronic Kidney Disease – Mineral and Bone Disorder
CPM	calciprotein monomers
CPP	calciprotein particles
CPP-I	primary calciprotein particles
CPP-II	secondary calciprotein particles
CRIC	Chronic Renal Insufficiency Cohort
CRP	C-reactive protein
CT	computer tomography
CTx	C-terminal telopeptide
CVD	cardiovascular disease
CV	coefficient of variation
eGFR	estimated glomerular filtration rate
EVOLVE	Evaluation of Cinacalcet HCl Therapy to Lower Cardiovascular Events trial
FGF23	fibroblast growth factor-23
FGFR	fibroblast growth factor receptor
FLEKSI	Fetuin-A Levels in Systemic disease and Kidney Impairment study
GFR	glomerular filtration rate
GIT	gastrointestinal tract
HD	haemodialysis
HF	high flux
HR	hazard ratio
hsCRP	high-sensitivity C-reactive protein
HU	Hounsfield unit
iFGF23	intact fibroblast growth factor-23
IL	interleukin

IMPROVE-CKD trial	Impact of Phosphate Reduction On Vascular Endpoints in CKD
IQR	interquartile range
KDIGO	Kidney Disease: Improving Global Outcomes
LMM	linear mixed-effects model
MCO	medium cut-off
MGP	matrix Gla protein
MICE	multivariate imputation by chained equations
NaPi	sodium-dependent phosphate cotransporter protein
NGAL	neutrophil gelatinase-associated lipocalin
P1NP	procollagen type 1 N-terminal propeptide
PD	peritoneal dialysis
PTFp	proximal tubular fluid
PTH	parathyroid hormone
PTH1R	parathyroid hormone primarily type 1 receptor
PWV	pulse wave velocity
REMOVAL-HD	The tRial Evaluating Mid cut-Off Value membrane clearance of Albumin and Light chains in Hemodialysis patients
SD	standard deviation
TNF	tumour necrosis factor
TRAcP 5b	tartrate-resistant acid phosphatase 5b
VSMC	vascular smooth muscle cells

List of publications

Manuscripts resulting directly from work encompassed in this thesis:

Tiong MK, Smith ER, Pascoe EM, Elder GJ, Lioufas NM, Pedagogos E, Hawley CM, Valks A, Holt SG, Hewitson TD, Toussaint ND. Effect of lanthanum carbonate on serum calciprotein particles in patients with stage 3-4 CKD - results from a placebo-controlled randomised trial. *Nephrol Dial Transplant*. 2023 Feb 13;38(2):344-351.

Tiong MK, Holt SG, Ford ML, Smith ER. Serum calciprotein monomers and Chronic Kidney Disease progression. *Am J Nephrol*. 2022 Dec 6:1-10. doi: 10.1159/000526609. Epub ahead of print. PMID: 36473431.

Tiong MK, Cai MMX, Toussaint ND, Tan SJ, Pasch A, Smith ER. Effect of nutritional calcium and phosphate loading on calciprotein particle kinetics in adults with normal and impaired kidney function. *Sci Rep*. 2022 May 5;12(1):7358.

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Tiong MK, Smith ER, Toussaint ND, Al-Khayyat HF, Holt SG. Reduction of Calciprotein Particles in Adults Receiving Infliximab for Chronic Inflammatory Disease. *JBMR Plus*. 2021;5(6):e10497.

Tiong MK, Krishnasamy R, Smith ER, Hutchison CA, Ryan EG, Pascoe EM, Hawley CM, Hewitson TD, Jardine MJ, Roberts MA, Cho Y, Wong MG, Heath A Nelson CL, Sen S, Mount PF, Vergara LA, Paul-Brent PA, Johnson DW, Toussaint ND. Effects of a medium cut-off dialyser on protein-bound uraemic toxins and mineral metabolism markers in patients on haemodialysis. *Hemodial Int*. 2021;25(3):322-32.

List of conference presentations

The following conference abstracts have been presented by Mark Tiong during his PhD candidature directly related to work encompassed in this thesis:

Tiong M, Cai M, Toussaint N, Tan S, Pasch A, Smith E. Effect of nutritional calcium and phosphate loading on calciprotein particle kinetics in adults with normal and impaired kidney function (oral). Australian New Zealand Society of Nephrology ASM 2022.

Tiong M, Cai M, Toussaint N, Tan S, Smith E. Effect of nutritional calcium and phosphate loading on calciprotein particles (poster). ESA-SRB-ANZBMS 2021.

Tiong M, Holt S, Toussaint N, Al-Khayyat H, Smith E. Reduction of calciprotein particles in adults receiving infliximab for chronic inflammatory disease (oral). Australian New Zealand Society of Nephrology ASM 2021.

Tiong M, Smith E, Lioufas N, Pedagogos E, Pascoe E, Hawley C, Valks A, Toussaint N. Effect of lanthanum carbonate on endogenous calciprotein particles in CKD patients—results from a placebo-controlled randomized trial (oral). Australian New Zealand Society of Nephrology ASM 2020.

Tiong M, Toussaint D, Smith E, Hutchison C, Pascoe E, Ryan E, Hawley C, Krishnasamy R. Effects of a medium cut-off dialyser on protein-bound solutes and mineral metabolism markers—a sub-study of the REMOVAL-HD trial (poster). Australian New Zealand Society of Nephrology ASM 2020.

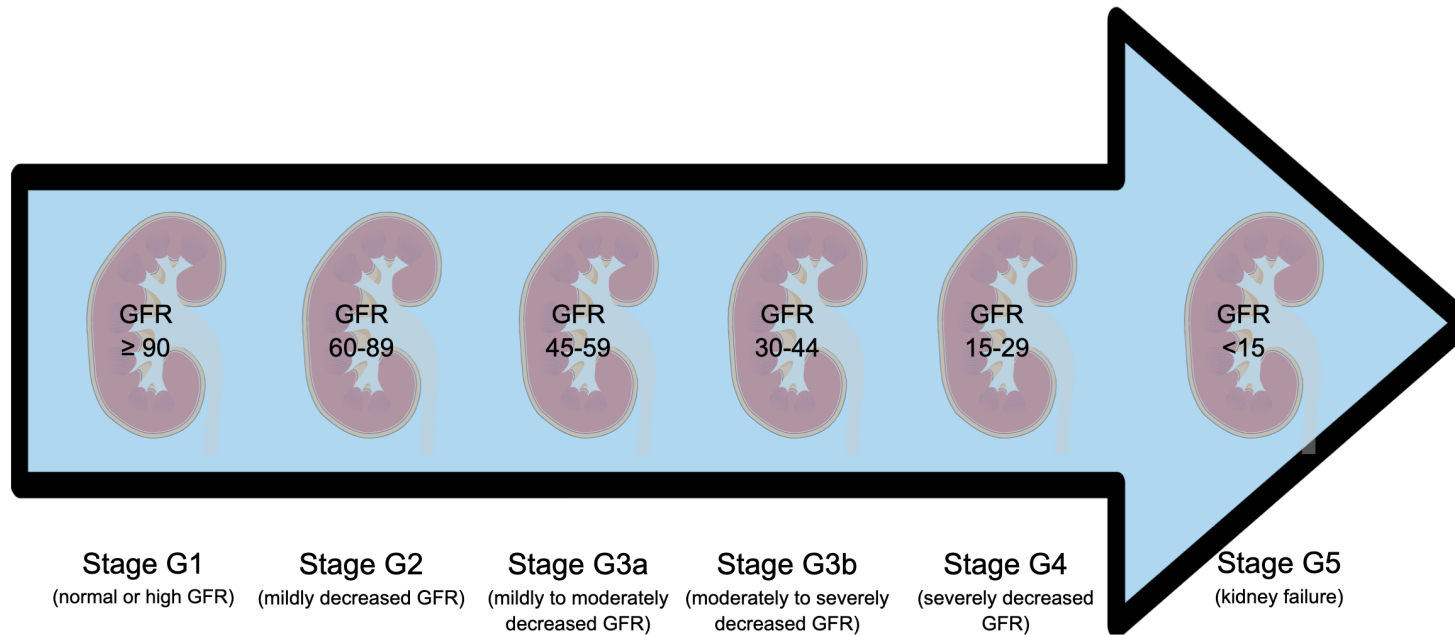
Chapter 1 General Introduction

1.1 Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD)

Chronic kidney disease (CKD) is a highly prevalent and progressive condition. Current estimates suggest that more than 10% of the global adult population is affected by CKD, and that CKD is a major driver of morbidity and mortality.¹ The risk of adverse events is present in the early stages of CKD and progressively increases as kidney function declines (Figure 1.1).^{2, 3} Despite the increasing availability of dialysis and kidney transplantation for those with the most advanced stages of kidney disease (kidney failure), the major cause of death in the CKD population is cardiovascular related, rather than kidney failure itself.¹

High rates of cardiovascular disease and death may in part be attributable to the propensity for individuals with CKD to be older and have increased prevalence of conventional cardiovascular risk factors, such as hypertension and diabetes, which are shared risk factors for CKD.¹ However, in large, multinational epidemiological studies, an increased risk of adverse cardiovascular events persists in individuals with CKD, even after controlling for age and other potential confounders.² In addition, among young individuals with advanced CKD but low burden of co-morbidities, risk of cardiovascular disease remains high.^{4, 5} Further, in those with advanced CKD the efficacy of conventional cardiovascular treatments, such as lipid⁶⁻⁸ and blood pressure⁹ lowering therapies, have been underwhelming, particularly for mortality outcomes. Taken together, CKD is considered to be a significant independent contributor to cardiovascular outcomes in this population.¹⁰

Figure 1.1: Stages of Chronic Kidney Disease (CKD)

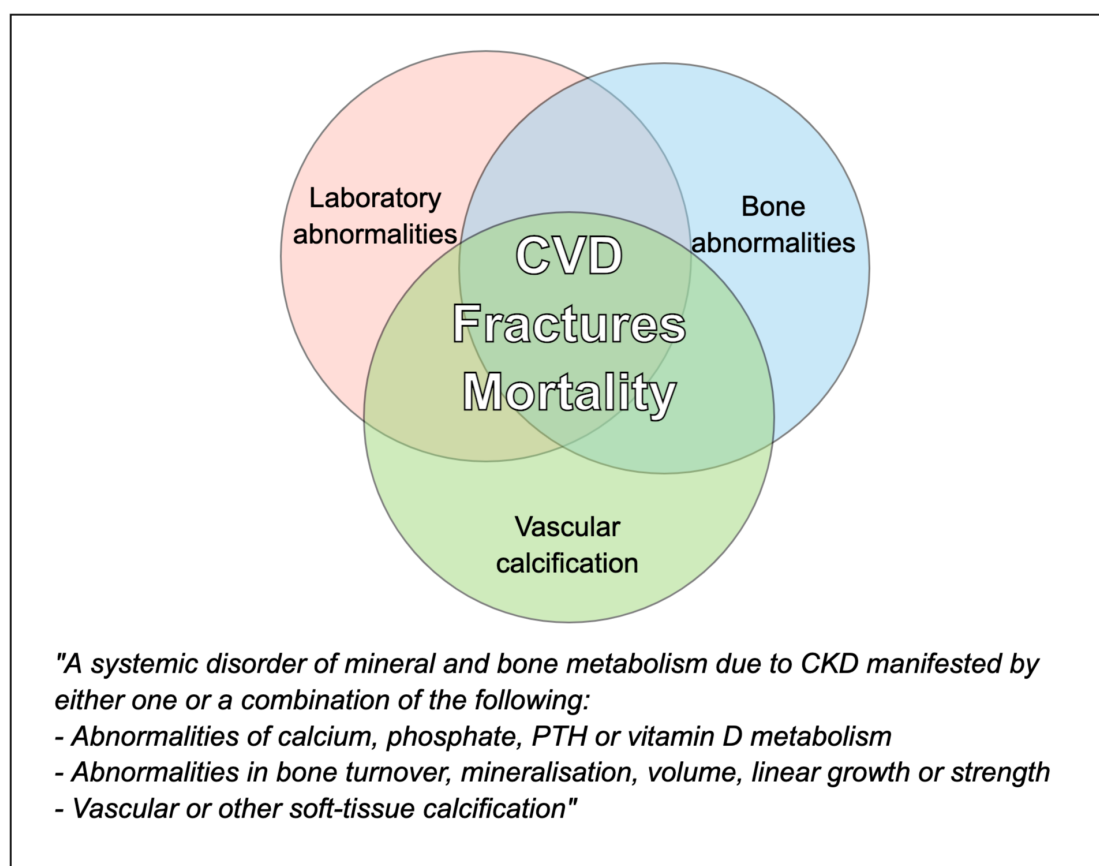


Current KDIGO classification of CKD¹¹ based on glomerular filtration rate (GFR) category (measured in mL/min/1.73m²)

Abnormalities in mineral metabolism are intrinsic to CKD, and disturbances in serum phosphate, parathyroid hormone (PTH) as well as vitamin D metabolism are almost universally present by the time individuals with CKD reach kidney failure.^{12, 13} Mineral metabolism disorders in CKD are intricately linked to abnormalities in bone metabolism. Kidney related bone disease, or “renal osteodystrophy”, may manifest as abnormal bone turnover and mineralisation (bone quality) and bone volume (bone quantity).¹⁴ Renal osteodystrophy is associated with loss of bone strength, and affected individuals have a markedly increased risk of fracture.^{15, 16} There is increasing recognition that the emergence of mineral and bone metabolism abnormalities in CKD is also paralleled by an increase in cardiovascular and other soft tissue calcification, which is thought to be an important contributor to poor cardiovascular outcomes in this population. Since 2009, the clinical syndrome of mineral and bone metabolism disorders and accelerated calcification in CKD have been collectively referred to as Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD).¹⁷ Part of the rationale for this change in definition was growing evidence that these processes are pathophysiologically inter-related (Figure 1.2).

CKD-MBD is a highly complex entity that remains incompletely understood. This thesis primarily focusses on the mineral metabolism domain of CKD-MBD and its association with cardiovascular complications, however it is acknowledged that these entities remain closely linked with bone metabolism. The following sections outline the “classical” mechanisms of mineral metabolism in health and in CKD, including the central role of phosphate in the development of CKD-MBD. The emerging area of mineral-protein colloidal physiology is then discussed including its role in understanding normal mineral homeostasis, and its possible applications in interrogating the presence of disordered mineral metabolism and its associated complications.

Figure 1.2: Chronic Kidney Disease-Mineral and Bone Disorder



Kidney Disease: Improving Global Outcomes (KDIGO) 2009 definition of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD)¹⁷

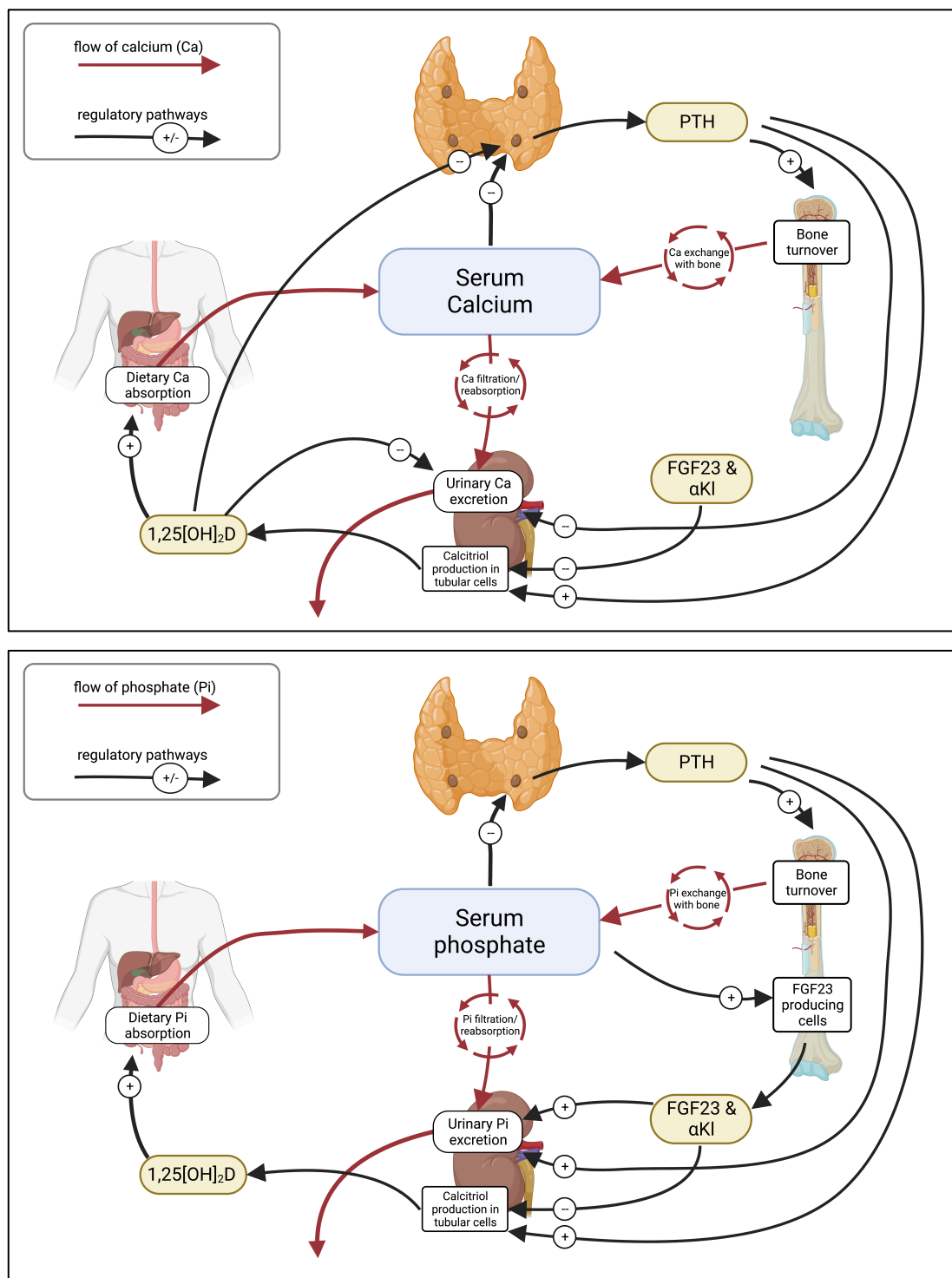
Abbreviations: CKD, chronic kidney disease; CVD, cardiovascular disease; PTH, parathyroid hormone

1.2 Normal calcium and phosphate metabolism

Calcium and phosphate are both essential minerals that are required for a wide range of normal physiological functions. Among other things, calcium is required for muscle contraction, nerve conduction, hormone secretion and blood clotting, while phosphate is required for adenosine triphosphate generation, intracellular signalling, and is an essential component of phospholipids and nucleic acids.^{18, 19} In biological fluids, calcium and phosphate can also combine to form various calcium phosphate complexes which form an indispensable component of calcified tissues in humans, such as bones and teeth. Approximately 99% of calcium and 85% of phosphate in humans is located within the bones, where they primarily exist in the form of crystalline apatite.²⁰ Not only does this rigid material provide mechanical strength to bone, but it also serves as an exchangeable pool of calcium and phosphate ions, which helps to buffer serum levels of these essential minerals. The intestines and kidneys are the other major sites of calcium and phosphate homeostasis. Large amounts of calcium and phosphate are absorbed from the diet via the intestines every day. This intake is balanced by excretion of calcium and phosphate in the urine by the kidneys. The intestines, kidneys and bone are linked by a complex network of endocrine regulatory and counter-regulatory pathways (Figure 1.3). In health, these systems maintain serum levels (as well as total body levels) of calcium and phosphate within a narrow range, despite wide variation in daily dietary intake.

Total body phosphate balance is principally regulated by the kidneys. Serum phosphate is readily filtered at the glomerulus and then reabsorbed in the proximal tubules, primarily by sodium-dependent phosphate cotransporter protein-IIa and IIc (NaPi IIa and NaPi IIc).²¹ The activity of NaPi IIa/c transporters are regulated by the phosphaturic hormones fibroblast growth factor-23 (FGF23) and parathyroid hormone (PTH). Both PTH and FGF23 cause internalisation and degradation of these transporters via proximally distinct signalling pathways.²²⁻²⁵ As a result, there is a reduction in the fraction of filtered phosphate that is reabsorbed and an increase in urinary phosphate excretion.

Figure 1.3: Classical metabolism of calcium and phosphate



Normal metabolism of calcium (Ca) and phosphate (Pi) and regulatory pathways. For clarity Ca and Pi have been separately depicted and some minor pathways have been excluded.

Abbreviations: 1,25[OH]₂D, 1,25-dihydroxyvitamin D; αKl, αKlotho; FGF23, fibroblast growth factor-23; PTH, parathyroid hormone

FGF23 is a peptide hormone that is primarily synthesised by osteocytes and osteoblasts in bone and then secreted into the circulation.²⁶ Its activity within target organs is mediated via activation of the fibroblast growth factor receptor (FGFR) with its obligate coreceptor transmembrane α Klotho (α Kl).²⁷ Dietary phosphate loading augments circulating levels of FGF23, however, the exact mechanisms through which phosphate is “sensed” by FGF23 secreting cells remains unclear.²⁸⁻³⁰

Apart from its role as a phosphatonin, PTH is the principal regulator of serum calcium, maintaining the concentration of ionised calcium within a narrow range.³¹ PTH is synthesised within the parathyroid gland and can be stored in secretory granules, or immediately released, allowing for both rapid and more sustained secretion in response to acute and chronic requirements.³² Secretion of PTH into the circulation is primarily seen in response to changes in extracellular ionic calcium concentration which is detected by transmembrane calcium sensing receptors (CaSR) located on the chief cells of the parathyroid glands.³¹ Hypocalcaemia elicits the rapid release of preformed stored PTH, as well as augmenting synthesis of new hormone.³² Conversely, restoration of normocalcaemia or hypercalcaemia inhibits secretion, resulting a rapid fall in serum PTH due to its short circulating half-life.

PTH mediates target organ effects by binding to PTH receptors, primarily type 1 receptors (PTH1R),³¹ with its most significant action as a modulator of bone metabolism. Acute release of PTH in response to hypocalcaemia is associated with increased bone turnover, thereby liberating stored calcium and phosphate.³¹ This is achieved through direct signalling to osteoblasts and osteocytes, which in turn stimulates osteoclastic cells via downstream signalling pathways. Additional calcium regulatory functions of PTH are also mediated via the kidneys. In the kidneys, PTH stimulates the distal tubular cells to increase calcium reabsorption by inducing expression of calcium exchangers,³¹ and also stimulates 1,25-dihydroxyvitamin D (1,25[OH]₂D) synthesis in the proximal tubular cells (as discussed further below).³³ Increased levels of 1,25[OH]₂D stimulate transcellular absorption of calcium in the intestines, and in addition to PTH also stimulates calcium reabsorption from the kidneys.^{31, 34} The cumulative effect of these pathways is to restore serum calcium, which suppresses further PTH synthesis and release. Increased levels of 1,25[OH]₂D also have a direct counter-regulatory suppressive effect on PTH synthesis.

Regulation of PTH secretion and its biological effects are also highly intertwined with phosphate metabolism. As discussed above, activation of PTH1R by PTH in proximal tubular cells leads to internalisation of apical and basolateral phosphate transporters, predominantly NaPi IIa, resulting in decreased reabsorption of filtered phosphate.^{22, 35} Phosphate also modulates PTH synthesis and release, with hyperphosphataemia and hypophosphatemia inducing stimulatory and inhibitory pathways respectively,³⁶ perhaps via non-competitive inhibition of the CaSR by phosphate ions.³⁷ In addition, FGF23 also has a downregulatory effect on PTH synthesis and secretion.³⁸

The vitamin D hormones are a family of steroid compounds with wide ranging biological effects. However, their chief role is in mineral homeostasis and bone metabolism.³¹ Calcitriol or 1,25[OH]₂D is the active form of vitamin D and is synthesised in the kidneys by the proximal tubular cells, where 25-hydroxyvitamin D (25[OH]D) is converted to 1,25[OH]₂D by 25-hydroxyvitamin D 1- α hydroxylase (1- α hydroxylase).³¹ 1,25[OH]₂D is required for normal bone turnover. Immature and mature bone cells express vitamin D receptors, and 1,25[OH]₂D promotes normal cellular maturation and activity.³⁹ As described above, 1,25[OH]₂D increases calcium absorption from the intestines and reabsorption from the kidneys by stimulating expression of calcium transporters.⁴⁰ 1,25[OH]₂D is also classically considered to enhance phosphate absorption in the intestine via increased expression of phosphate transporters, primarily sodium dependent phosphate cotransporter protein-IIb (NaPi IIb).³¹

As well as 1- α hydroxylase, circulating levels of 1,25[OH]₂D are also determined by 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase), which converts both 25[OH]D and 1,25[OH]₂D into inactive metabolites. PTH and FGF23 are the main regulators of 1,25[OH]₂D levels by modifying the activity of these enzymes. PTH increases circulating levels of 1,25[OH]₂D by enhancing activity of 1- α hydroxylase and inhibiting 24-hydroxylase,⁴¹ while FGF23 decreases 1,25[OH]₂D by mediating the opposite enzymatic effects.^{42, 43} PTH synthesis is in turn inhibited by 1,25[OH]₂D.^{44, 45} Further to this, there is also evidence to suggest that FGF23 synthesis is enhanced by 1,25[OH]₂D.⁴⁶

1.3 Altered mineral metabolism in CKD

Disordered mineral metabolism is an almost universal feature of advanced stages of CKD. However, evidence of altered mineral physiology is detectable much earlier in the course of CKD, and progression of CKD is often accompanied by a classical sequence of biochemical manifestations. The first measurable change is usually increased serum levels of FGF23 and decrease in αKl .^{12, 47} This is conventionally considered to be a compensatory response to maintain normal phosphate balance by augmenting urinary phosphate excretion as the number of functioning nephrons declines, however the exact molecular signal/s linking these events remain incompletely understood. As kidney function continues to decline, serum PTH begins to increase. This appears to be predominantly driven by decreased levels of $1,25[\text{OH}]_2\text{D}$ secondary to increased FGF23, however decreased levels of $1,25[\text{OH}]_2\text{D}$ may also indirectly stimulate PTH synthesis and release via alterations in calcium homeostasis.⁴⁸

Elevations of FGF23 and PTH initially allow the maintenance of normal levels of serum phosphate and calcium. However, frank hyperphosphataemia is a common feature of advanced CKD, and develops after compensatory mechanisms become overwhelmed.¹² Similarly, abnormalities in serum calcium may be seen as a relatively late feature of advanced CKD.¹³ Dietary phosphate is absorbed from the intestinal tract via both passive (paracellular) and active (transcellular) routes. The transcellular pathway is primarily via enterocytic NaPi IIb. NaPi IIb expression is regulated by $1,25[\text{OH}]_2\text{D}$, and *in vitro* enterocytic uptake of phosphate is heavily $1,25[\text{OH}]_2\text{D}$ dependent. However, the *in vivo* significance of this regulatory mechanism has recently been questioned. Contemporary evidence now suggests that the transcellular pathway saturates at relatively low luminal phosphate levels, meaning that the paracellular route facilitates the bulk uptake of dietary phosphate. Consistent with this, even in states of low $1,25[\text{OH}]_2\text{D}$, such as in CKD, total phosphate absorption is maintained,⁴⁹ likely due to the unaffected paracellular route.⁵⁰ Therefore, even though reduction of $1,25\text{D}$ by FGF23 is conventionally considered part of the hormonal counter-regulatory response in CKD, the true impact on overall intestinal phosphate absorption is unclear.

1.4 Pathological ectopic calcification in CKD

The pathophysiology of cardiovascular disease in individuals with CKD is complex and incompletely understood. In addition to the clustering of traditional cardiovascular risk factors within the CKD population, such as diabetes and hypertension, CKD is also associated with additional factors, that may contribute to poor cardiovascular outcomes, such as anaemia and chronic inflammation.¹⁰ Individuals who have kidney failure treated with haemodialysis are also subjected to repetitive intra-dialytic volume and electrolyte shifts as well as myocardial stunning.^{51, 52} In addition to these factors, development of pathological cardiovascular *calcification* is considered to be a key event. Ectopic calcification of the cardiac valves and vasculature are a common manifestation of CKD-MBD.⁵³ While pathological calcification of the vasculature and cardiac valves are each distinct entities, that independently contribute to the cardiovascular disease burden in CKD, they are considered to be interrelated and likely share some common aetiological mechanisms.

1.4.1 Vascular calcification

It is important to acknowledge that vascular calcification is an umbrella term for two distinct (but overlapping) pathologies – calcific atherosclerosis and calcific arteriosclerosis.⁵³⁻⁵⁵

Calcific atherosclerosis describes calcification deposits within atheromatous plaques of the vascular intimal layer.⁵³ This may be observed in both the CKD and non-CKD populations. Calcification within the atheroma begins as focal microcalcifications. Multiple microcalcifications may develop within the same lesion, and then grow and coalesce into larger calcifications over time.⁵⁶ The presence of any calcification is generally considered to be a feature of severity of atherosclerotic disease.⁵⁷ However, among calcified coronary atheromatous lesions, microcalcification deposits may be more vulnerable to rupture than lesions with heavier calcification.^{58, 59} It should be noted that most of this data is from the non-CKD population, and it is unclear whether these observations are also applicable to those with CKD.

The processes driving calcification within atheroma are incompletely understood, but inflammatory infiltrate within the plaque appears to be the central factor.^{53, 55} Mechanistic

studies have suggested that atheromatous lesions may be invaded by macrophages and dedifferentiated vascular smooth muscle cells (VSMC) in response to the atherosclerotic injury of the intima.⁶⁰ Inflammatory signals from macrophages induce VSMC to express bone morphogenetic protein (BMP)-2, which act in a paracrine fashion, leading to transdifferentiation of VSMC into osteoblast-like cells.⁶⁰ This process appears to be a pre-requisite step for atherosclerotic calcification in transgenic mouse models.⁶¹ Inflammatory signalling from the macrophage also downregulates VSMC expression of matrix Gla protein (MGP).⁶⁰ MGP is potent local inhibitor of calcification, and so this may further promote localised calcification. Finally, activated macrophages may also release extracellular matrix vesicles, which may act as a nidus for calcification.⁶² Micro-calcifications within intimal lesions may themselves be pro-inflammatory,⁶³ and oxidised lipids, other pro-inflammatory cytokines,⁵³ and biomechanical stress⁶⁴ may further exacerbate these processes.

While the degree of calcification within atheroma is highly variable in both CKD and non-CKD patients, previous post-mortem studies have suggested that more severe calcification may be seen in those with CKD.^{65, 66} It is not clear to what extent this may be due to clustering of traditional atherosclerotic risk factors in CKD, versus CKD specific risk factors. However, dysregulated mineral metabolism, enhanced inflammatory stimuli, CKD associated anaemia and retention of uraemic toxins have all been proposed to be significant.^{10, 55, 67}

Calcific arteriosclerosis is calcification of the arterial medial layer. It is considered one of the hallmarks of CKD, but is also seen with ageing^{68, 69} and diabetes.^{70, 71} Similar to calcification of atheromatous plaques, calcific arteriosclerosis may initially be focal within the affected vessel. However, unlike intimal calcification, medial calcification has a greater potential to become more confluent with time, and in its most advanced form, medial calcification in CKD is classically concentric.⁵⁴ In addition to calcification of the media, VSMC hypertrophy and increased collagen formation within the vascular wall are also typically seen.⁵⁴ The cumulative effect of these processes is that the arterial wall becomes thickened and loses elasticity. As a result, calcific arteriosclerosis is associated with vessel stiffening (clinically signified by increased pulse pressure and pulse wave velocity) and increased cardiac afterload, which are both thought to contribute to left ventricular hypertrophy and dysfunction.^{54, 72}

The development of calcific arteriosclerosis in CKD is thought to involve both biochemical (passive) and cell mediated (active) mechanisms. Disturbances in calcium and phosphate metabolism have long been recognised as a risk factor, and much attention has been focussed on the possible role that this may play in inducing the transdifferentiation of VSMC into osteocyte-like mineralising cells.²⁰ CKD model mice fed a high phosphate diet develop extensive arterial medial calcification. In these animals, analysis of medial smooth muscle cells from affected vessels show significant upregulation of the osteocytic lineage marker osteopontin, and corresponding downregulation of smooth muscle cell contractile markers.⁷³ Similarly, increased bone related proteins are found at sites of medial calcification in arterial samples obtained from individuals with kidney failure at the time of transplantation.⁷⁴ Evidence of VSMC undergoing an osteocytic phenotype switch appear to precede the development of detectable calcification.^{5, 75} *In vitro* exposure of VSMC to high levels of phosphate induces changes in gene expression that are consistent with conversion of cells to an osteogenic phenotype.⁷⁶⁻⁷⁹ These changes are also associated with downregulation of mineralisation inhibitors such as pyrophosphate, via expression of alkaline phosphatase (ALP).^{76, 80} ALP inhibits the action of pyrophosphate by converting it into free phosphate, perhaps further enhancing the pro-calcific micro-environment.²⁰ Similar findings have been reported in arterial samples from children with advanced CKD.⁵

Arterial elastin degradation has also been implicated in the development of calcific arteriosclerosis in CKD.²⁰ As well as contributing to loss of vessel elasticity, fragmentation of elastin fibres is thought to contribute to the extracellular matrix on which mineral can deposit.⁷⁵ Similarly, as well as undergoing phenotypic transdifferentiation, in CKD, VSMC may also release extracellular vesicles or undergo apoptosis, which may create additional nucleation sites for calcification.^{5, 81} Inflammatory infiltrate is a less prominent feature of medial calcification than intimal calcification, however activation of inflammatory pathways are also evident.⁵³ Apart from alterations in calcium and phosphate homeostasis, other biochemical abnormalities have also been implicated as important drivers of vascular calcification in CKD, including impairment of endogenous calcification inhibitors. In addition to pyrophosphate, the network of extracellular calcification inhibitors, including MGP,⁸² fetuin-A⁸³ and magnesium⁸⁴ may also be impaired in CKD, contributing to a pro-calcific microenvironment.⁸⁵

It is important to acknowledge that while calcific atherosclerosis and arteriosclerosis are distinct entities, our current ability to differentiate these using non-invasive investigations in the clinical environment is limited.⁸⁶ The anatomical location of vascular calcification may provide some indication of the likely underlying histopathology. For instance, in autopsy studies from CKD cohorts, calcification of the coronary arteries tends to be intimal rather than medial.^{65, 66, 87} However, differentiation based on anatomical distribution is not absolute and intimal and medial vascular calcification may even coexist within the same vessel segment.^{66, 87} Despite these limitations, it is important to acknowledge that both types of vascular calcification are associated with adverse patient outcomes.^{88, 89} Similarly, while vascular calcification is a diffuse disease, within an individual patient the severity of calcification is not uniform across anatomical sites. While there may be some theoretical and epidemiological differences in the prognostic significance of calcification at different locations,^{90, 91} the presence of any vascular calcification at various anatomic sites has been consistently associated with adverse patient outcomes within the CKD population.⁹²⁻⁹⁹

1.4.2 Cardiac valvular calcification

As well as vascular intimal and medial calcification, the prevalence of calcification of the cardiac valves also increases as glomerular filtration rate (GFR) declines, and is highest in those with kidney failure treated with dialysis.^{100, 101} Although any cardiac valve can be affected, most research has been focussed on calcification of the aortic and mitral valves, which are classically associated with aortic stenosis and mitral regurgitation respectively.^{102, 103} Calcification is one component of the often mixed cardiac valvular pathologies that may be seen in CKD, but calcification is consistently linked to a range of complications, including valvular dysfunction, conduction abnormalities, valvular endocarditis and cardiac mortality.¹⁰⁴

Damage to the underlying cardiac valves may be seen as a manifestation of specific aetiologies of CKD, such as systemic lupus erythematosus.¹⁰⁵ Similarly, individuals with kidney failure who are being treated with haemodialysis are at increased risk of bacterial endocarditis, owing to repetitive intravascular access.¹⁰⁶ These conditions may cause structural damage to the affected valve, which may then predispose to secondary

calcification. However, like vascular calcification, the current pathophysiological paradigm is that valvular calcification is predominantly the culmination of a complex interplay of passive and active processes.^{102, 103} Valvular calcification is often seen in conjunction with vascular calcification and these two entities also share a number of risk factors, implying a likely significant overlap in pathomechanistic pathways.^{107, 108} Elevated levels of serum calcium and phosphate have been consistently linked to valvular calcification.^{109, 110} Similarly, low vitamin D and hyperparathyroidism are also associated with increased risk.¹¹¹ As well as CKD, valvular inflammation is also seen in other pro-inflammatory conditions, such as diabetes mellitus, and histopathological studies have implicated the role of activation of inflammatory pathways.¹¹² Fluid retention in CKD may lead to intravascular volume expansion and cardiac chamber enlargement, which may further exacerbate valvular dysfunction.¹¹³

1.5 Serum phosphate – a prototypical biochemical marker in CKD-MBD

Given the prevalence of CKD-MBD and its association with adverse patient outcomes, there is natural interest in evaluating candidate biomarkers that can be utilised to assess patient risk, but also act as a modifiable target for therapeutic interventions. Serum phosphate has long been debated as one potential metric to fulfil these functions, especially given that abnormal phosphate homeostasis is central to our current understanding of CKD-MBD and serum phosphate is readily available in current clinical practice.¹¹⁴⁻¹¹⁶

Elevated levels of serum phosphate are common in those with advanced CKD and have consistently been associated with adverse patient outcomes.¹² In patients with kidney failure treated with dialysis, epidemiological studies have reported that high levels of serum phosphate are independently associated with increased risk of all-cause mortality,¹¹⁷⁻¹²⁹ and with cardiovascular related events or cardiovascular mortality.^{118, 127, 130} Similar associations are also seen in those with non-dialysis requiring CKD¹³¹⁻¹³⁴ and even in those with relatively normal kidney function.¹³⁵⁻¹³⁹ Interestingly in some of these studies, a positive gradient of risk between phosphate and outcome was still evident when serum phosphate remained within the nominal “normal” range.^{131, 134, 135, 139} Whilst all of the above epidemiological studies are observational by nature, in experimental studies, phosphate supplementation ostensibly induces vascular cell dysfunction¹⁴⁰ and calcification,¹⁴¹ and such studies have been used to support the notion that excess phosphate could be a direct cellular toxin in CKD. In addition to this purported direct toxicity, it has been suggested that phosphate may also induce pathology indirectly via intermediate pathways, such as through elevated FGF23, which has also been epidemiologically¹⁴²⁻¹⁴⁴ and experimentally^{145, 146} associated with adverse cardiovascular outcomes.

Apart from cardiovascular outcomes, there is emerging interest in the role that disordered phosphate metabolism may contribute to the progression of CKD itself. In epidemiological studies elevated levels of phosphate¹⁴⁷⁻¹⁴⁹ and FGF23^{143, 150, 151} have been associated with increased risk of kidney function decline. It has been suggested in small clinical studies^{152, 153} and in CKD animal models,¹⁵⁴⁻¹⁵⁶ that the association between

phosphate exposure and progression of CKD may be specifically related to a proportionate increase in filtered phosphate load in the kidney, relative to the reduced number of functional nephrons.

Taken together, phosphate may be an important metric not only to assess the presence of mineral metabolism disorders but also patient risk, and current international guidelines suggest therapeutic interventions to treat elevated serum phosphate in patients with kidney failure treated with dialysis.¹⁵⁷ There are however a number of important caveats in the utility of serum phosphate in clinical practice. As outlined in Section 1.2, serum phosphate represents a small fraction of total body phosphate in humans. As such, serum phosphate may be poorly representative of total body phosphate balance. In line with this, frank elevations occur relatively late in the course of CKD.¹² Further, while exposure to excess phosphate has been hypothesised to be a central driver of adverse outcomes in CKD, in several studies low serum phosphate has also been associated with increased risk.^{122, 123, 125-128, 133, 158, 159} This “U-shaped” relationship between serum phosphate and outcomes highlights that measurement of serum phosphate may be confounded by a number of clinically significant conditions, such as older age, infection, inflammation and malnutrition, which are all associated with hypophosphataemia.^{118, 121, 126, 158}

Biological plausibility of high phosphate being a direct cellular toxin has also been questioned, given that several experimental effects that have been attributed to phosphate appear to also be dependent on the presence of calcium (described further in Section 1.8).^{116, 160} Perhaps the most important limitation on the current use of serum phosphate in clinical practice is that while a number of therapeutic options are available to lower serum phosphate, including dietary phosphate restriction, intestinal based phosphate binders, and dialysis therapies, there is a paucity of convincing evidence to show that reducing phosphate improves patient outcomes.¹⁵⁷

Despite the limitations of serum phosphate, it remains a commonly used biochemical marker in current clinical practice to assess the presence of mineral metabolism disorders in CKD and to evaluate patient risk. Notably though, many of the original epidemiological studies were performed using data sets that are now decades old. Further, the demographic and comorbidity profile of the CKD population continues to change,¹⁶¹ and for patients with kidney failure treated with dialysis, dialysis therapies have evolved

over time and across different geographical regions.¹⁶² As such there may be value in periodically re-examining the relationship between serum phosphate and patient outcomes, to ensure that assumed epidemiological relationships remain relevant in current clinical practice. In addition, many previous studies examining dialysis populations have largely examined patients being treated with haemodialysis (HD). By comparison, there have been few studies that have included individuals being treated with peritoneal dialysis (PD), and previously available studies are generally small and mostly now quite old.^{158, 163-167} From an Australian perspective, there has not previously been an analysis of the relationship between serum phosphate and patient outcomes specifically from this geographical region.

1.6 Fetuin-A and calciprotein particles in mineral metabolism

Calciprotein particles (CPP) are nanoscale mineral protein colloids that are thought to have a physiological role in normal mineral trafficking, but have also emerged as potential novel markers of mineral dysregulation and cardiovascular pathology in CKD. CPP are primarily composed of calcium, phosphate and the mineral binding protein fetuin-A.

Fetuin-A is a liver derived glycoprotein which acts as a potent systemic inhibitor of ectopic mineralisation. Its name specifically references its initial discovery as the major globulin in foetal calf serum,¹⁶⁸ however fetuin-A homologues are ubiquitous in all vertebrates. Its human homologue is sometime referred to as α 2-Heremans-Schmid glycoprotein (Ahsg),¹⁶⁹ after its two independent discoverers.^{170, 171} Since initial isolation, fetuin-A has been associated with multiple biochemical effects *in vitro* and *in vivo*. It is considered an acute negative phase reactant,¹⁷² and to have possible roles in the regulation of insulin sensitivity¹⁷³ and development of the immature skeleton.¹⁷⁴ The focus of the following sections will be on the role of fetuin-A in mineral regulation.

Fetuin-A has a central role in mineralisation inhibition. This is highlighted in mice with deletion of the fetuin-A gene (Ahsg^{-/-}), albeit the effects of this are dependent on the genetic background of the knockout models. Ahsg^{-/-} mice that are bred on a DBA/2 genetic background develop severe multiorgan calcification from an early age,¹⁷⁵ and mineral-containing complexes precipitate directly in the lumen of the microvasculature leading to occlusion, ischaemia, necrosis and fibrosis.¹⁷⁶ In contrast, Ahsg^{-/-} mice bred on a C57BL/6 background appear more calcification resistant.¹⁷⁷ This latter strain of mice have been found to have higher levels of serum magnesium and pyrophosphate than their DBA/2 Ahsg^{-/-} counterparts, highlighting that fetuin-A is not the only circulating calcification regulator.¹⁷⁸ However, C57BL/6 Ahsg^{-/-} mice still demonstrate exaggerated calcification when challenged with additional pro-calcific events, such as a mineral and vitamin D rich diet¹⁷⁵ or CKD combined with a high phosphate diet, suggesting that the actions of fetuin-A are pivotal.^{179, 180}

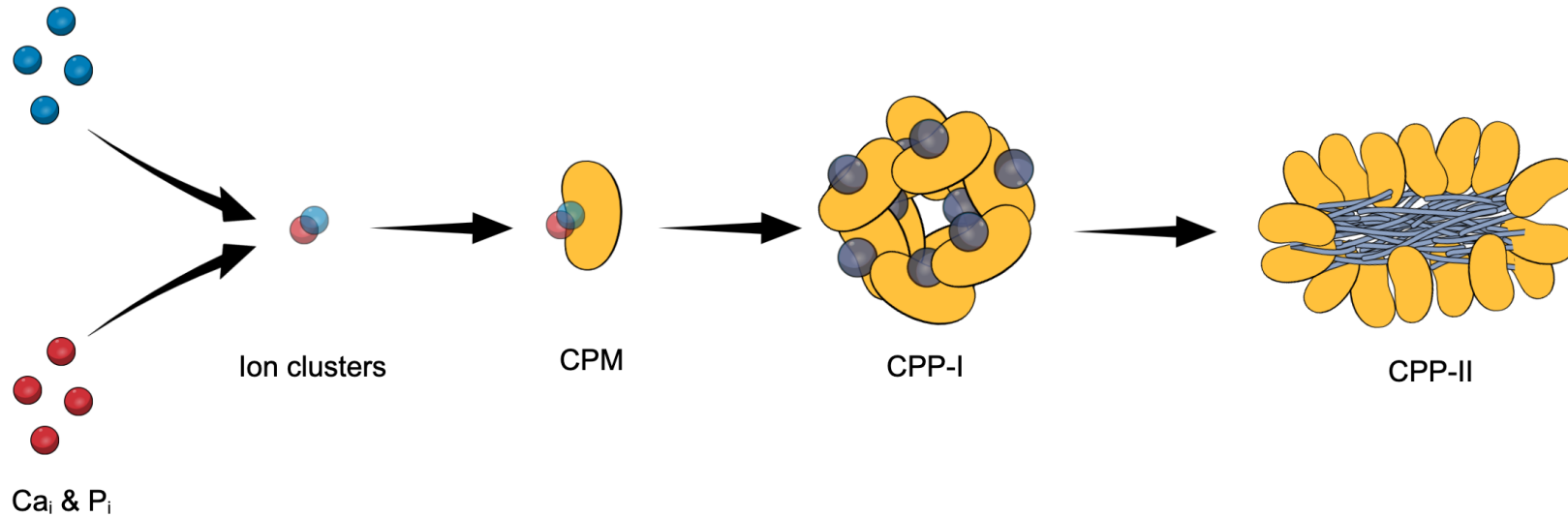
In adults, fetuin-A is primarily synthesised and secreted by hepatocytes into the serum where it circulates in high concentrations. Extra-hepatic synthesis appears minimal,¹⁸¹ however, fetuin-A may accumulate in other, mainly mineralised, tissues. This is typified

in bone, where fetuin-A is one of the most abundant of the non-collagenous proteins.^{182, 183} This observation highlights the high affinity of fetuin-A for solid phase calcium phosphates, but also that it does not dissolve mineral once already formed.¹⁸¹ Instead, the main mineral regulating action of fetuin-A appears to be via its capacity to stabilise nascent mineral clusters within biological fluids and inhibit precipitation. Fetuin-A accounts for approximately half of serum's capacity to resist mineral precipitation when challenged with supersaturating mineral ion loads.¹⁸¹ Detailed *in vitro* studies have revealed that this is achieved by the sequential formation of a series of mineral protein colloids, collectively referred to as CPP.

The process of CPP formation is depicted in Figure 1.4. Within biological fluids, calcium and phosphate ions spontaneously combine to form ion clusters. These ion clusters are then tightly bound by fetuin-A monomers, via its amino-terminal cystatin-like domain D1, forming calciprotein monomers (CPM).^{181, 184} CPM (~8 nm) then serve as the building blocks for larger polymeric structures.¹⁸⁴ First, CPM coalesce to form spherical primary CPP (CPP-I). CPP-I are 30-100 nm in diameter and contain solid-phase amorphous calcium phosphate. CPP-I may then transform into larger and denser ellipsoidal secondary CPP (CPP-II) with diameters between 100-250 nm.¹⁸⁵ As part of this transformative step, the mineral content of the particle “ripens” into crystalline apatite phase with fetuin-A rearranging to the surface to form an outer “shell”.

The name “calciprotein particles” was coined in reference to apolipoproteins, which facilitate the transport of their otherwise insoluble lipid cargo.¹⁸⁵ Similarly, fetuin-A stabilises poorly soluble calcium phosphates, preventing crystal growth and precipitation. *In vitro*, in biologically simulated solutions that are deficient of fetuin-A, mineral will spontaneously sediment within minutes.¹⁸⁴ By comparison, restoration of fetuin-A to physiological concentrations stabilises these solutions, such that sedimentation does not occur for days. It is therefore thought that the formation of CPP is critical in facilitating the bulk transport of mineral to sites of uptake in tissues for utilisation or clearance.¹⁸⁶⁻¹⁸⁸

Figure 1.4: Formation of calciprotein particles



Calcium (Ca_i) and phosphate (P_i) ions spontaneously combine to form ion clusters. Ion clusters may be bound by the mineral binding, liver-derived glycoprotein fetuin-A, forming calciprotein monomers (CPM). If present in sufficient quantities, CPM then self-aggregate into rounded structures, termed primary calciprotein particles (CPP-I). CPP-I contain amorphous, solid-phase calcium phosphates. Under permissive conditions, this mineral content transforms into thermodynamically stable crystalline phases, creating elongated secondary calciprotein particles (CPP-II). As part of this transformation, fetuin-A rearranges towards the outer surface of the molecule forming a “shell”.

1.7 Clinical relevance of fetuin-A and CPP in disordered mineral metabolism

Fetuin-A received increased clinical interest after epidemiological studies reported associations between serum levels and significant clinical outcomes in CKD cohorts.¹⁸⁹ Fetuin-A was shown to be inversely associated with vascular calcification,^{83, 190, 191} aortic stiffness,¹⁹² cardiovascular events¹⁹³ and mortality.^{189, 191, 193, 194} Nevertheless, these associations were not uniform.¹⁹⁵⁻¹⁹⁹ One explanation for this variability was that the *mineral bound fraction* of fetuin-A (i.e. CPP), may be more relevant as a metric of “mineral stress” than *total* fetuin-A serum levels.²⁰⁰

In an adenine induced CKD rat model, Matsui *et al* found that serum CPP showed a temporal association with vascular medial calcification, while total fetuin-A did not.²⁰¹ The same group, then demonstrated in a human CKD cohort that serum levels of CPP increased with severity of kidney impairment and showed a positive association with coronary artery calcification.²⁰⁰ Similar findings were reported by Smith *et al* who also found that serum CPP correlated with aortic stiffening in a CKD cohort, even after adjustment for potential confounders, including age and estimated glomerular filtration rate (eGFR).²⁰² Notably, in both of these studies it was found that serum CPP, but not total fetuin-A, was correlated with the surrogate cardiovascular marker of pulse wave velocity.^{200, 202}

Subsequent studies, predominantly in CKD populations, have also suggested that serum CPP is positively associated with cardiovascular outcomes. This included a cross-sectional study of 71 patients undergoing percutaneous coronary artery intervention, where it was reported that CPP were associated with arterial plaque volume.²⁰³ In prospective studies, Smith *et al* found that serum CPP were positively associated with increased risk of all-cause mortality in a cohort of 184 individuals with CKD stage 3-4,²⁰⁴ while Gatate *et al* found that higher levels of serum CPP were associated with increased risk of cardiovascular events in a maintenance haemodialysis population.²⁰⁵

It is notable that in the above studies (where it was reported), serum levels of CPP were higher with declining kidney function.^{200, 202, 204} This has also been shown in additional human cohorts.²⁰⁶⁻²⁰⁸ Given that CPP are notionally protective in stabilising mineral in solution, this raises the question of whether elevated levels of CPP in CKD are merely a

marker of progressively disturbed mineral metabolism, or alternatively, whether CPP may be more directly involved in the development of pathological events in CKD. It is notable that in animal studies, elevated levels of CPP precede the development of vascular calcification.²⁰¹ Further, in several of the human studies, the association between CPP and outcomes appeared to be independent of eGFR, age and various conventional cardiovascular risk factors.²⁰²⁻²⁰⁵ Nevertheless, all of these studies were observational in nature, limiting the direct assessment of causality. However, as discussed below, mechanistic studies have suggested that CPP may not be completely inert, but may induce a range of pathological effects, particularly when present in elevated concentrations.

1.8 The (patho)physiology of CPP

In vitro work initially suggested that high phosphate was able to induce a range of cellular effects reminiscent of clinical pathology observed in CKD-MBD, such as calcification of VSMC. However in some of these experiments this apparent effect may actually be dependent on the presence of calcium, which triggers the formation of hydroxyapatite microcrystals within the cell culture medium.²⁰⁹ VSMC endocytose these microcrystals, which are then dissolved by lysosomes, leading to an intracellular calcium burst.²¹⁰ This process can result in a vicious cycle of calcification²⁰⁹ and cell death.²¹⁰ Notably, if microcrystals are removed from the culture medium by centrifugation,²⁰⁹ or if their initial formation is inhibited by pyrophosphate,^{209, 211} the apparent calcification effect of phosphate is diminished. Similarly, the intracellular calcium burst and subsequent cell death can be blocked by the inhibition of lysosomal proton pumps with bafilomycin A1.²¹⁰

The ability of culture medium with high calcium and phosphate content to induce calcification of VSMC is attenuated by the addition of fetuin-A.²¹² Intuitively, this may at least in part be due to the capacity of fetuin-A to regulate crystal formation. Notably though, a similar attenuating effect is seen when pre-formed apatite microcrystals are bathed in fetuin-A rich foetal calf serum.²¹⁰ This latter observation highlights that fetuin-A may not just regulate the formation of solid phase mineral, but also has a modifying effect on its biological activity. CPP, like protein-free calcium phosphate microcrystals, are readily taken up by macrophages. In both situations, internalisation of each particle type leads to the expression of inflammatory mediators and apoptotic pathways.²¹³ However, when compared for equivalent calcium content, the pro-inflammatory and pro-apoptotic potential of CPP is significantly less than protein-free micro-crystals.²¹³ This disparity appears to be due to differences in intracellular handling, given that the uptake of each particle type by macrophages appears comparable.²¹³ While internalisation of CPP triggers an intracellular calcium spike,²¹⁴ it is thought that fetuin-A may moderate this, resulting in a lower surge than protein-free nanocrystal equivalents.²¹³

As well as macrophage activation, CPP also induce inflammatory and calcification pathways in human²¹⁴ and murine²¹⁵ VSMC *in vitro*. While *in vivo*, CPP would seemingly have limited direct contact with the vascular medial layer, as discussed in Section 1.1, many individuals with CKD have additional risk factors for atherosclerotic disease, which

may disrupt endothelial integrity. Further, CPP themselves have been shown to induce injury and hyperplasia of the vascular endothelial layer when administered to otherwise normal rats.²¹⁶ *In vitro*, vascular endothelial cells display a range of cytotoxic effects when exposed to CPP, including the expression of inflammatory and cellular transdifferentiation mediators, as well as cell death.^{216, 217}

Toxic effects of CPP often appear to be dose dependent, suggesting that their activity may have a threshold effect.^{213, 215} While this implies that total particle concentration may be of particular relevance, the composition of CPP subtypes (CPM, CPP-I and CPP-II) also likely determines biological activity. Initial studies suggested that toxic effects of CPP may be predominantly seen with CPP-II, however more recent works have suggested that CPP-I also have significant cytotoxic and pro-inflammatory effects when matched for calcium content.^{218, 219} By comparison, *in vitro*, CPM appear to be the most inert.²¹⁹

As well as considering CPP particle numbers and particle composition, it is possible that the biological activity of CPP may also be related to their capacity to act as vehicles for other bioactive compounds, particularly in uraemia. Endogenous CPP isolated from uraemic individuals are enriched with various compounds, including lipids, proteins, DNA and RNA fragments, as well as bacterial endotoxins and genetic material.²²⁰ A significant proportion of this molecular complexity is unique to uraemia. It follows that as CKD progresses, CPP may not only be increased in number, but that their biological activity may also become enhanced by these additional compounds. Taken together, it is plausible that CPP formation could ordinarily fulfill a physiological role in calcification regulation and mineral trafficking. However, in situations of disturbed mineral metabolism, such as in CKD, levels of CPP may become chronically elevated and begin to act as mediators of pathology in this patient population.

1.9 CPP metabolism

Part of the interest in CPP as a potential mediator of the pathophysiology of CKD-MBD is whether clinical measurement of CPP could serve as a metric to i) interrogate the presence of disturbed mineral homeostasis, ii) assess patient risk, and iii) ultimately act as a modifiable novel target for therapeutic interventions. These potential applications of CPP first require a better understanding of normal CPP metabolism and how this may become disturbed in CKD. As such, it is notable that the precise origins of CPP that are measured in the serum, and the drivers of elevated levels in pathological conditions are incompletely understood.

A recurrent issue in the study of CPP metabolism is that CPM and CPP-I are inherently unstable. The formation of CPM followed by sequential assembly of CPP-I, then transformation to CPP-II occurs spontaneously in a cell free environment due to the tendency of calcium phosphates to transform into more stable crystalline forms. CPP-II are the most stable of the CPP phases, in part owing to their crystalline apatite mineral core. As such, in the field of CPP research, many initial studies focussed on CPP-II physiology and pathophysiology, before advancements in the handling of biological samples, as well as the preparation of synthetic particles, subsequently facilitated additional study into CPM and CPP-I. Because of this, and in recognition that CPM, CPP-I and CPP-II are highly interrelated, the term “CPP” has been adopted as a collective term in this thesis. However, in recognition that each CPP subtype has distinctive physiological and pathophysiological properties, these are called out when relevant and discernible from the referenced work.

1.9.1 Clearance of serum CPP

Initial work in murine models elucidating the clearance pathways of serum CPP by Herrmann *et al* found that fluorescently labelled synthetic CPP-II are almost exclusively cleared by resident macrophages in the liver and spleen, via scavenger receptor-AI/II dependent endocytosis.²²¹ Subsequent work by the same group later found that, in contrast to CPP-II, CPP-I are predominantly cleared by liver sinusoidal endothelial cells, with only minor uptake by liver macrophages.²¹⁸ More recently, it was also shown that serum CPM are readily filtered by the kidneys. Koeppert *et al* proposed that while fetuin-A has a diameter small enough to cross the glomerular filtration barrier, it is usually

prevented from doing so by its negative charge.²¹⁹ The negative charge of free fetuin-A appears to be neutralised by calcium phosphate binding, as occurs during formation of CPM. Because of this effect, CPM is able to readily pass into the glomerular filtrate. As part of this work, Koeppert *et al* also showed that filtered fetuin-A was then largely reabsorbed and degraded by proximal tubular epithelial cells. Given that in these experiments particles were tracked via fluorescent labelling of fetuin-A, it was not possible to infer whether this tubular reabsorption was of CPM, or of free fetuin-A which had disassociated from its mineral cargo in the tubular fluid.

1.9.2 Sources of endogenous CPP

Notably in the clearance studies of CPM, CPP-I and CPP-II, clearance of each particle after injection occurred rapidly (in minutes rather than hours), and in each case was near complete.^{218, 219, 221} In contrast, the time taken for *in vitro* formation of CPP-I and then transition to CPP-II at physiological conditions is substantially longer. As such, doubts have been raised about whether significant amounts of CPP are formed within the circulation *in vivo*.¹⁸⁶ Instead it is proposed that CPP may be primarily formed in extravascular, extracellular fluid compartments, before entering the circulation. Two of the hypothesised sites for this are the bones and the gastrointestinal tract (GIT), given both are sites of large and repetitive mineral ion fluxes.

Evidence of bone as a source of serum CPP

Early work by Price *et al* found that acute arrest of bone formation in rats using etidronate resulted in the appearance of a high molecular weight, mineral fetuin-A complex (equivalent to CPP-II).²²² Generation of this complex was prevented when animals were pre-treated with inhibitors of bone resorption, including calcitonin, osteoprotegerin and alendronate.²²³ In combination, these experiments quite convincingly showed that bone metabolism can be manipulated to produce high levels of circulating CPP. Notably though, this effect was transient, and was produced using supratherapeutic doses of etidronate (~1000 times the minimum active dose) in skeletally immature rats, and so the direct applicability of this phenomenon to more commonly encountered scenarios is questionable. Data from more physiologically relevant situations have however also suggested that serum levels of CPP may be linked to bone metabolism. These studies have predominantly been conducted in the setting of CKD.

In humans with non-dialysis dependent CKD, Smith *et al* found that serum CPP are significantly correlated with markers of bone resorption, independent of eGFR.²⁰² In a dialysis cohort with secondary hyperparathyroidism, Ruderman *et al* found that longitudinal changes in serum CPP-I were significantly correlated with changes in serum PTH, after treatment with the calcimimetic drug cinacalcet was stopped.²²⁴ Similar correlations between CPP and PTH have been found in other dialysis cohorts.²⁰⁶ Serum PTH is itself not considered a direct marker of bone turnover, however, in a *post hoc* analysis of a randomised trial of haemodialysis patients comparing standard to high dialysate magnesium, Bressendorff *et al* found that serum CPP were inversely correlated with bone specific alkaline phosphatase (bsALP), a marker of bone formation, and positively with tartrate-resistant acid phosphatase 5b (TRAcP 5b) a marker of bone resorption. Further, in CKD model rats, inhibition of bone resorption with alendronate is also associated with large reductions in serum CPP.²⁰¹ While the above studies collectively suggest that bone metabolism and serum CPP are closely linked, the evidence remains limited, particularly in humans, where available data is almost exclusively from individuals with CKD.

Evidence for the gastrointestinal tract as a source of serum CPP

Given that large amounts of calcium and phosphate are absorbed from the diet and trafficked to sites of use and excretion, the GIT is another logical source of endogenous CPP. A number of observations support this notion however the most convincing evidence comes from animal studies. Smith *et al* demonstrated that high phosphate feeding is associated with increased serum levels of CPP in rats with normal kidney function, and also in adenine-induced CKD model rats.²⁰⁶ Interestingly, in animals with normal kidney function the increase in CPP was seen despite no change in serum phosphate. Akiyama *et al* also showed that a phosphate bolus given by oral gavage resulted in a temporary increase in serum CPP in mice, while sustained increases in dietary phosphate leads to prolonged increases in serum CPP levels.²²⁵ Notably in this latter study, phosphate supplementation was large, and as a result, concurrent changes in serum phosphate and calcium concentrations were also induced.

Outside of animal studies, evidence for a gastrointestinal source of CPP in humans is more indirect. Multiple studies of cohorts of individuals with CKD on dialysis have shown that manipulation of intestinal phosphate binders is associated with observable

changes in serum CPP. In an open-label randomised control trial of 31 haemodialysis patients, Smith *et al* found that sevelamer carbonate was associated with significant reductions in CPP-I compared to calcium carbonate at 24 weeks.²²⁶ In a separate study measuring CPM, Nakamura *et al* demonstrated that changing patients from calcium carbonate to lanthanum carbonate resulted in lower levels of CPM in a 16-week cross-over study of 24 haemodialysis patients.²²⁷ In these studies, however, it was not clear if reductions in CPM and CPP were predominantly related to a reduction in intestinal phosphate absorption *per se*, or the avoidance of exogenous calcium loading associated with calcium-based binders. Further, all patients in both studies had advanced, haemodialysis dependent, kidney failure, making it unclear what effect CKD had on these observations.^{226, 227} Similarly, Yamada *et al* observed diurnal variation in serum CPP, with peak levels coinciding with post-prandial times, in a small study of 20 patients.²²⁸ However, in this study all patients were hospitalised for management of unstable diabetes, again making it challenging to isolate the role of the GIT in normal CPP physiology. In summary, while available evidence strongly suggests that the GIT may be an important source of endogenous CPP, definitive observation of this in humans is lacking, particularly in healthy individuals with normal kidney function. This latter group is particularly relevant, given the ostensible role of formation of CPP as a physiological function in normal mineral trafficking, but lack of empirical data of CPP kinetics in healthy subjects.

1.10 Other biological associations and functions of CPP

The primary focus of the study of CPP has centred on their purported role in mineral homeostasis and in the setting of CKD. CPP have however been linked to several other (possibly overlapping) biological pathways.

1.10.1 Inflammation

Inflammation is a feature of many chronic medical conditions, including CKD. CKD is considered to be a state of chronic inflammation, and as discussed in Section 1.4, inflammation is implicated as an important pathway in the development of cardiovascular disease.^{229, 230} In CKD cohorts, serum levels of CPP tend to correlate with systemic markers of inflammation.^{202, 204, 206, 207} Notably, in one longitudinal study, the association between CPP and all-cause mortality lost significance after adjustment for high-sensitivity C-reactive protein (hsCRP).²⁰⁴ The exact nature of this association is incompletely understood, and it is possible that this association may be mediated by an intermediate pathway, such as increased inflammatory mediated bone turnover.^{231, 232} Consistent with this, individuals with chronic systemic inflammatory conditions, but normal kidney function have elevated serum CPP.²⁰⁷ However, *in vitro* evidence suggests that CPP can themselves induce inflammatory pathways. CPP cause NLRP3 inflammasome activation and release of inflammatory mediators in macrophages,^{213, 218, 219} as well as expression of inflammatory mediators in vascular endothelial, and also, smooth muscle cells.^{214, 216, 217} Indeed, the calcifying effect of CPP on cultured human VSMC appears to be blocked by TNF-alpha inhibition, suggesting an inflammation dependent pathway.²¹⁴ In addition, fetuin-A itself is considered a negative acute phase reactant.^{172, 189, 194} In summary, CPP has a number of intriguing links to inflammation, but the exact nature and direction(s) of these associations remain to be fully understood.

1.10.2 Fibroblast growth factor-23 regulation

Regulation of FGF23 is another area with intriguing links to CPP metabolism. As the major phosphatonin, serum levels of FGF23 begin to rise early in the course of CKD.¹² FGF23 ostensibly augments urinary phosphate excretion, maintaining total body phosphate balance in the face of decreasing functional nephron mass. In keeping with this central role in phosphate homeostasis, dietary phosphate loading in healthy adults induces increased serum FGF23.²³³ The actual biological trigger/s for this reflexive response, and

for the sustained increases in FGF23 observed in CKD, have however remained elusive. Intriguingly, the normal response of FGF23 to phosphate loading is blunted in hypocalcaemia, suggesting that calcium may also have a direct, or at least permissive, role in FGF23 regulation.^{234, 235} Further, several studies have also suggested strong correlation between serum CPP and FGF23 in animals and humans.^{202, 203, 206, 208, 228} It is possible that all of these above observations can be reconciled if CPP play a direct role in FGF23 regulation, as has recently been proposed by Akiyama *et al.*²²⁵

Studying cultured rat osteoblastic cells, Akiyama *et al* showed that increasing calcium or phosphate in the culture medium resulted in both increased transcription and secretion of FGF23.²²⁵ This process however, appeared to be dependent on the formation of CPP in the culture medium, given that it was blocked by the addition of citrate. Further, synthetic CPP induced this same response in a dose-dependent manner and this occurred in the absence of any change in the concentration of ionic calcium or phosphate in the culture medium. Further substantiating this concept, the authors demonstrated in mice that acute oral phosphate gavage is associated with a transient rise in serum CPP and FGF23, and that this response is larger and is sustained with ongoing dietary phosphate loading. Using live imaging of exposed calvarium, the authors showed that injected synthetic CPP labelled with a fluorescently conjugated bisphosphonate were able to access the bone marrow. This adds to the plausibility that *in vivo*, circulating CPP could serve as a signalling molecule to FGF23 secreting osteocytic cells.

Interestingly, in the work by Akiyama *et al* the *in vitro* osteoblastic FGF23 response was augmented with the addition of alendronate.²²⁵ Using small angle X-ray scattering the authors observed that alendronate resulted in a predominance of smaller CPP species, with a diameter (~9 nm) and appearance suggestive of CPM. This implies that CPM may be the most potent FGF23 secretagogue. It is notable that these cell culture experiments used doses of CPP substantially higher than levels typically encountered in observational studies, while the animal studies used large phosphate loads that were sufficient to induce frank hyperphosphataemia. Therefore, whether these effects are replicated in more physiologically typical settings is yet to be confirmed. Additionally, the precise cellular mechanism for how CPP induce FGF23 transcription and expression has yet to be demonstrated. Nevertheless, this work provides a compelling paradigm to view CPP and FGF23 as integrated components of phosphate handling.

1.11 Measurement of endogenous calciprotein particles

It is important to note that much of our current understanding about the biophysical formation of CPP is derived from *in vitro* experiments. While these experiments often use “simulated” biological fluids, often supra-physiological amounts of calcium and phosphate are utilised to generate CPP within pragmatic timescales and also to produce sufficient quantities to permit detailed analyses. In contrast, endogenous levels of serum CPP in biological fluid samples are substantially lower in concentration, making their detection and quantification more challenging. Further, the thermodynamic drive of mineral to form more stable species, means that CPM and CPP-I are inherently unstable *ex vivo*. These factors hampered early efforts to develop reliably methods to measure CPP in their endogenous form.

Initial methods to quantitate endogenous CPP in biological samples utilised properties described by Price *et al*, who identified that “fetuin mineral complex” (CPP) could be sedimented from experimental rat serum with high-speed centrifugation (>16,000 g).²²² Adapting this process, Hamano *et al*²⁰⁰ proposed measuring fetuin-A before and after this high molecular weight component was removed in order to indirectly estimate and quantify the proportion of fetuin-A incorporated into CPP as the “reduction ratio”, defined as:

$$\text{Fetuin-A reduction ratio} = (\text{total fetuin-A} - \text{supernatant fetuin-A}) / \text{total fetuin-A}$$

This initial “fetuin-A method” of quantifying CPP had several important limitations. First, this approach was based on human specific fetuin-A ELISA, limiting its ability to be directly applied to animal models. Second, sensitivity was limited in situations of low CPP abundance, which could result in overlap between the proportion of fetuin-A sedimented by centrifugation and the measurement error. Third, the inability to analyse the mineral composition meant that there was no discrimination between CPP species. Given emerging evidence of different clearance pathways and biological effects between CPM, CPP-I and CPP-II, this latter limitation was of particular importance.^{218, 219, 221}

Subsequent work led to the parallel development of two assays using OsteoSense 680EX (Perkin Elmer), a fluorescently-labelled bisphosphonate derivative with a high affinity for solid-phase calcium phosphates. The first of these next-generation assays detects

CPP-I and CPP-II particles by flow-cytometry equipped with high sensitivity fluidics to detect small particles (down to 40 nm).^{206, 236} Particles within the sample are quantified as OsteoSense positive (mineral containing) events. Given CPP-II contain crystalline rather than the amorphous mineral present in CPP-I, CPP-II generate more light scatter and so can be differentiated by their side scatter profile. This assay also distinguishes other, non-CPP, membrane-bound mineral containing particles that are present in serum, such as extracellular vesicles, using a membrane label (PKH67 green fluorescent cell linker midi kit from Sigma Aldrich or phosphatidyl serine-binding lactadherin-FITC).^{206, 226}

For the other current CPP assay, OsteoSense is incubated with serum samples, before unbound OsteoSense is removed using a gel filtration spin column.²⁰⁸ In this “gel filtration” method, the dye present in the flow-through reflects mineral-bound Osteosense, and the fluorescence intensity is measured by infrared fluorescence scanner and denoted as “total” CPP. This assay can also be performed after high-speed centrifugation to remove high density particles (equivalent to CPP-I and CPP-II). The creators of this method refer to the fluorescence intensity measured after this step in the supernatant as “low density” CPP. Given that particles in this fraction were too small to be measured by nanoparticle tracking analysis (<50nm in diameter), low density CPP measured by this method are thought to equate to CPM, as was originally described by Heiss *et al.*¹⁸⁴

1.12 T50 – an *ex-vivo* assessment of serum calcification propensity

As well as measurement of endogenous CPP levels in serum, a related test measures the capacity of serum to resist *ex vivo* formation of CPP-II, when challenged with supraphysiological additions of calcium and phosphate. The process results in near immediate *de novo* formation of CPP-I, followed by a slower transition to CPP-II. This predictable sequence can be monitored using changes in light scattering of the sample, owing to the distinct refractory properties of the mineral component of each CPP phase.¹⁸⁵ Specifically, the transition of CPP-I to CPP-II results in characteristic changes in laser light scatter, which can be measured using time resolved nephelometry, turbidimetry or dynamic light scattering.²³⁷ The time taken to reach maximum transition to CPP-II is recorded, and the time taken to reach half of this maximum transition is denoted as the sample “T50” in minutes.

The T50, or “calcification propensity”, test provides a composite measure of the various known (and unknown) promoters and inhibitors of *ex vivo* CPP formation that are present in serum.²³⁷ Shorter T50 times indicate a higher propensity of the serum sample to form CPP-II. Important factors that are associated with a shorter (higher calcification propensity) T50 include phosphate, and to a lesser extent calcium.²³⁷ The major factors that are known to prolong T50 include fetuin-A, albumin and magnesium, however other small molecules, such as pyrophosphate and citrate, also have an inhibitory effect.^{237, 238} CPP formation in the T50 assay is also highly dependent on assay conditions, including temperature and pH, necessitating standardisation of these parameters between samples.²³⁷

In clinical studies, lower serum T50 has been consistently associated with increased risk of vascular pathology and mortality in various, although predominantly CKD, cohorts. In a study of 184 individuals with non-dialysis dependent CKD, Smith *et al* showed that lower T50 was independently associated with increased risk of all-cause mortality.²⁰⁴ The authors reported that T50 was also cross-sectionally associated with aortic pulse wave velocity, and that those with a lower T50 were more likely to show increased aortic stiffening over 30 months. In two additional studies of non-dialysis dependent CKD participants, both derived from the Chronic Renal Insufficiency Cohort (CRIC) study, T50 was found to be associated with coronary artery calcification severity and progression,²³⁹ as well as risk of a composite outcome of cardiovascular events, heart

failure, progression to kidney failure and mortality.²⁴⁰ Notably in each of these three studies, eGFR was a consistent and strong predictor of T50.^{204, 239, 240} In the first two studies the association between T50 and outcome was independent of eGFR,^{204, 239} however in the latter study the association was lost after eGFR was accounted for.²⁴⁰

Lower T50 has also been associated with adverse outcomes in other patient populations. In a sub-cohort of haemodialysis patients with secondary hyperparathyroidism from the Evaluation of Cinacalcet HCl Therapy to Lower Cardiovascular Events (EVOLVE) trial, T50 was independently associated with increased risk of a composite outcome of all-cause mortality and non-fatal cardiovascular events.²⁴¹ In kidney transplant recipients, T50 has been associated with increased risk of cardiovascular events,²⁴² as well as all-cause and cardiac related mortality in prevalent²⁴³ and incident²⁴⁴ transplant recipients. In a cohort of more than 6,000 participants with relatively normal kidney function, T50 was associated with increased risk of cardiovascular mortality, independent of eGFR, conventional cardiovascular risk factors, and serum phosphate.²⁴⁵

In individuals with non-dialysis dependent CKD, T50 appears to be relatively stable over time, and has less intra-individual variability than serum phosphate over 2 years.²⁰⁴ However, this longitudinal stability does not appear to universally apply to all stages of CKD. In the first three months after initiation of dialysis (HD or PD), a significant increase in T50 is observable.²⁴⁶ This is proposed to be attributable to parallel increases in serum fetuin-A and magnesium, as well as decreases in phosphate after dialysis initiation.²⁴⁶ In contrast, in longer term dialysis patients T50 may decrease over time. In a study of 188 prevalent haemodialysis patients, Lorenz *et al* found that the majority (77%) of participants had T50 values that significantly decreased over 24 months.²⁴⁷ This study also suggested that monitoring longitudinal changes in T50 may also enhance risk prediction, given that individuals who had larger declines in T50 also had increased mortality risk in the subsequent observational period. In comparison, in this cohort, absolute T50 values at baseline and 24 months did not predict mortality.²⁴⁷

As well as measuring the kinetic properties of CPP-II formation (the T50 time), the size of the generated CPP-II can also be estimated. This method uses dynamic light scattering, which is directly related to the hydrodynamic radius of the formed particles.^{220, 237} Many of the factors that are known to accelerate or delay CPP-II formation in the T50 assay

tend to also produce larger or smaller CPP-II respectively.²³⁷ However, correlation between these effects is not always proportionate. In limited studies it has been suggested that CPP-II radius may have a stronger association than T50 time with vascular calcification in individuals with non-dialysis dependent CKD,²⁴⁸ and with all-cause mortality in a cohort of incident dialysis patients.²⁴⁹ However, the utility of measuring the radius of *ex vivo* generated CPP-II is yet to be more fully established.

With a growing body of data to characterise the T50 test as a marker of risk, there has also been interest in interventions targeted at modifying T50. Given that the T50 is a composite, functional assessment of the serum's propensity to form CPP when challenged with supraphysiological amounts of calcium and phosphate, it is unsurprising that molecular shifts that occur during haemodialysis are associated with acute changes in T50 when measured immediately before and after a haemodialysis or haemodiafiltration treatment.²⁵⁰ More sustained changes in T50 are seen with modifications to the dialysis treatment itself. Increasing dialysate magnesium to 1 mmol/L (from a standard concentration of 0.5 mmol/L) is associated with a significant increase in pre-dialysis T50.²⁵¹ This effect is seen rapidly and is likely attributable to parallel increases in serum magnesium. Consistent with this, T50 and serum magnesium quickly return to baseline if the dialysis prescription is reverted. Manipulation of other aspects of the dialysate composition have also been shown to increase pre-dialysis T50, including reduction of the dialysate calcium concentration, or the use of citrate instead of acetate as the dialysate acidifier.²³⁸ Outside of the dialysis therapies, use of sucroferric oxyhydroxide,²⁵² an intestinal phosphate binder, and oral magnesium supplementation²⁵³ have also been suggested to increase serum T50 in small randomised trials. Importantly, while each of these dialysis and medication manoeuvres convincingly increase T50, it remains to be determined whether therapeutic targeting of T50 using any, or a combination, of these strategies is associated with meaningful improvements in patient outcomes.

1.13 Thesis aims and chapter outline

Mineral metabolism disorders are near ubiquitous in CKD and almost certainly contribute to the high cardiovascular burden in this patient population. Utilising conventional and novel mineral metabolism markers may assist in furthering our understanding of the evolution of disordered mineral homeostasis, as well as provide avenues to better risk assess patients and ultimately to develop novel therapeutic strategies in this patient population.

Through a series of separate, but interrelated works, this thesis aims to address a number of questions throughout the following chapters:

Chapter 2 focusses on serum phosphate as the prototypical marker of mineral metabolism disorders in CKD-MBD and aims to address the questions:

- Is serum phosphate still a robust marker of risk in the current dialysis era?
- Are previously described relationships between serum phosphate and patient outcomes applicable to the Australia and New Zealand region and are they consistent across those being treated with HD and PD?

Chapter 3 and Chapter 4 focus on the physiology of CPP metabolism, including the contributions of the intestines, kidneys, and bone, and aim to address the questions:

- Are CPP formed in response to dietary mineral loading, and how does kidney function affect post-prandial CPP kinetics?
- Are CPP levels dynamically associated with bone metabolism in those with normal kidney function?

Chapter 5 and Chapter 6 focus on possible interventions that could be used to therapeutically target levels of CPP in CKD and aim to address the questions:

- Can intestinal phosphate binders be used to reduce serum levels of CPP in patients with CKD?
- Do newer haemodialysis membranes have any effect on novel markers of mineral metabolism compared to standard membranes?

Chapter 7 examines a novel possible association between CPP and progression of CKD, and aims to address the question:

- Could CPM help explain the association between mineral metabolism disorders and the progression of CKD?

Chapter 2 Serum phosphate and mortality in incident dialysis patients in Australia and New Zealand

2.1 Introduction

As outlined in Chapter 1, abnormalities of phosphate metabolism are a key feature of CKD-MBD. In patients with kidney failure treated by dialysis, abnormalities in serum phosphate are highly prevalent and have been consistently linked to a range of adverse outcomes.²⁵⁴ In particular, hyperphosphataemia has been consistently associated with increased risk of all-cause and cardiovascular mortality.²⁵⁴ As a result, treatment of hyperphosphataemia has been a focus of international clinical guidelines for patients requiring dialysis.¹⁵⁷ As discussed in Section 1.5, there are theoretical and technical limitations in the application of serum phosphate for clinical risk assessment. For instance, low levels of serum phosphate have also been reported to correlate with poor outcomes, in some, but not all studies, suggesting that serum phosphate may have a “U-shaped” association with mortality.^{123-126, 128, 158, 159}

Evidence for the association of high and low serum phosphate with poor outcomes is predominantly based on observational studies and there is a lack of high quality trial evidence to show that treatment of abnormalities in phosphate improves patient-centred outcomes.¹¹⁶ Interventional trials are currently being conducted to clarify the utility of therapeutic manoeuvres in response to abnormal levels of serum phosphate, specifically the treatment of hyperphosphataemia in dialysis patients.^{255, 256} Nevertheless, in the meantime, clinicians require up-to-date data to help guide their interpretation of biochemical markers encountered in everyday practice. Internationally, the demographic and comorbidity profile of the dialysis population continues to change,¹⁶¹ and given that dialysis and other aspects of clinical care also continue to evolve,¹⁶² it is conceivable that the relationship between phosphate and outcomes may not be stagnant over time. Therefore, there may be value in re-examining this association using up-to-date data that reflects current and real-world practices, including in different global regions. Furthermore, previous studies have predominantly focussed on patients being treated with haemodialysis (HD), while there is much less data relating to associations with serum phosphate in patients being treated with peritoneal dialysis (PD).

The objective of this study was to investigate the association between serum phosphate and all-cause and cardiovascular mortality, in a large and modern cohort of incident HD and PD patients from Australia and New Zealand.

2.2 Methods

2.2.1 Study population

The Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry was utilised for this study. ANZDATA collects demographic and clinical information about all patients who are resident in Australia or New Zealand and are receiving long-term treatment for kidney failure with either dialysis or kidney transplantation.²⁵⁷ Patient data (including serum phosphate) is provided by the patient's treating unit through an annual survey. Additional data is also provided in "real-time", for pre-specified events, such as change in dialysis modality or patient death. This analysis of the ANZDATA Registry was approved by the local ethics committee (Melbourne Health Human Research Ethics Committee reference number QA2020033).

2.2.2 Patient inclusion criteria and follow up

Patients commencing dialysis between 2008 until 2018, who were at least 18 years of age at the time of first dialysis, and who had at least one measurement of serum phosphate were included. For each participant, follow up time was accrued from initiation of dialysis until date of death. Patients were censored at the time of receiving a kidney transplant, loss of follow up, or end of the study (31 December 2018), whichever occurred first. Given the objective of this analysis was to describe the long-term relationship between phosphate and mortality, participants were excluded if they died or were censored within 30 days of first dialysis. Similarly, because the initial period after commencement of dialysis is associated with significant biochemical instability, biochemical measurements were excluded if they were taken within 30 days of first dialysis.

2.2.3 Study exposure

The primary exposure variable was serum phosphate concentration. ANZDATA instructs participating units to provide serum phosphate and other biochemical values which are measured midweek and, for HD patients, taken immediately pre-dialysis. Blood samples are analysed at local accredited laboratories, and units report values taken as close to the end of the annual survey period as are available.

2.2.4 Study outcomes

The primary outcome of interest was all-cause mortality. Given phosphate has also been associated with cardiovascular disease and mortality, cardiovascular-related death was also analysed in a secondary analysis. The classification used by ANZDATA of categories of death, as well as a breakdown of cardiovascular-related death, is provided in Table 2.1.

2.2.5 Study covariates

A range of demographic and clinical covariates that are collected by ANZDATA were included to adjust for potential confounding. The final fully adjusted model included the “fixed” covariates: age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer). The following “time-updated” covariates, which are updated with each annual survey were also included: dialysis modality (updated in real time), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

Table 2.1: Death by subtypes and breakdown of cardiovascular death

	Overall	Haemodialysis	Peritoneal dialysis
Death category – n (%)	n = 12,713	n = 9,784	n = 2,929
Cardiovascular	4,030 (31.7)	3,019 (30.9)	1,011 (34.5)
Withdrawal	4,057 (31.9)	3,248 (33.2)	809 (27.6)
Cancer	588 (4.6)	462 (4.7)	126 (4.3)
Infection	1,195 (9.4)	898 (9.2)	297 (10.1)
Other	2,724 (21.4)	2,048 (20.9)	676 (23.1)
Not reported	119 (0.9)	109 (1.1)	10 (0.3)
Cardiovascular deaths – n (%)	n = 4,030	n = 3,019	n = 1,011
Myocardial ischaemia and infarction	652 (16.2)	479 (15.9)	173 (17.1)
Pulmonary oedema	77 (1.9)	58 (1.9)	19 (1.9)
Hyperkalaemia	49 (1.2)	45 (1.5)	4 (0.4)
Haemorrhagic pericarditis	5 (0.1)	4 (0.1)	1 (0.1)
Hypertensive cardiac failure	57 (1.4)	36 (1.2)	21 (2.1)
Cardiac arrest-cause uncertain	1,914 (47.5)	1,410 (46.7)	504 (49.9)
Other causes cardiac failure	394 (9.8)	313 (10.4)	81 (8.0)
Pulmonary embolus	29 (0.7)	24 (0.8)	5 (0.5)
Cerebrovascular accident	474 (11.8)	354 (11.7)	120 (11.9)
Gastrointestinal haemorrhage	91 (2.3)	70 (2.3)	21 (2.1)
Haemorrhage from dialysis access site	12 (0.3)	12 (0.4)	-
Aortic aneurysm-rupture	33 (0.8)	20 (0.7)	13 (1.3)
Haemorrhage from elsewhere	105 (2.6)	81 (2.7)	24 (2.4)
Bowel infarction	138 (3.4)	113 (3.7)	25 (2.5)

Table shows subtypes of death, and subtypes of cardiovascular death, as categorised by the Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry.

2.2.6 Statistical analysis

Summary characteristics of the cohort were reported. Continuous variables with a normal distribution were expressed as mean and standard deviation (SD), and those with skewed distributions were expressed as median and interquartile range (IQR). Categorical data were presented as numbers and percentages.

Cox proportional hazards models were used to model the relationship between serum phosphate and mortality. Initially, the relationship between deciles of serum phosphate and all-cause mortality was analysed in a univariate model. Consistent with previous studies, the observed relationship between serum phosphate and mortality was non-linear. As a result, serum phosphate was categorised into 0.25 mmol/L intervals from <1.0 mmol/L to >2.75 mmol/L. These phosphate categories were empirically chosen to represent the distribution of phosphate observations. Serum phosphate between 1.50 – 1.74 mmol/L appeared to have the lowest hazard ratio (HR) in unadjusted analyses, and so was set as the reference category.

Unadjusted and multivariate adjusted models were fitted. As for serum phosphate, continuous covariates used in the adjusted models were categorised if they did not have a linear association with mortality. Patient sex and dialysis modality (HD or PD) were forced into the multivariate model, while other covariates were considered for inclusion if they were significant on univariate analysis ($p < 0.2$). Significant covariates were then entered into the main model using a manual forward stepwise procedure, where covariates were retained if they improved model goodness of fit, as assessed by Akaike information criterion (AIC). Current use of an erythropoiesis stimulating agent, and index of socioeconomic score were available as potential covariates, but were not selected in the final, fully adjusted, model. All other covariates that were selected for inclusion had p values equal to or less than 0.001 on univariate analysis. All models were stratified by treatment centre. The proportional hazards assumption was checked by visual inspection of Kaplan Meier curves for categorical variables and by examining Schoenfeld residuals for continuous variables. For simplicity, only the unadjusted, age-adjusted and fully adjusted models are presented.

Sub-analyses

To analyse the secondary outcome of cardiovascular-related death, each model was refitted for cardiovascular- and also non-cardiovascular-related mortality. To examine the effects of dialysis modality, models were also separately refitted after restricting data to patients receiving HD and PD. The fully adjusted model for all-cause and also for cardiovascular mortality was also repeated with an interaction term between serum phosphate and dialysis modality.

Missing data and sensitivity analyses

Multiple imputation was used to assess for the effect of missing data for the fully adjusted model of all-cause mortality. For this analysis, twenty datasets were generated using the multivariate imputation by chained equations (MICE) approach, with the assumption that the data was missing at random.

For each model, serum phosphate and time-updated variables were included as time-dependent covariates. To account for potential bias introduced through the use of time-dependent covariates, the main, fully adjusted model was refitted for baseline and also for mean values of serum phosphate.²⁵⁸ For the baseline model, other time-updated variables were also entered using their baseline values. In the mean model, the mean of continuous time-updated variables was used, while categorical variables were left as time-dependent predictors.

A model was also fitted using baseline values of the comorbidities of lung disease, coronary artery disease, peripheral vascular disease and cerebrovascular disease, while leaving other time-updated variables as time dependent variables. Given that high serum phosphate has been associated with the development of cardiovascular disease, this final model was included to account for the possibility of over-adjustment bias, due to adjusting for events that are potentially involved in the causal pathway.²⁵⁹

Because of the potential to introduce bias through censoring patients who receive a kidney transplant in the Cox proportional hazard models,²⁶⁰ a competing risk analysis was also performed for all-cause mortality, where kidney transplant was a competing event. For this model the Fine and Gray method was used,²⁶¹ and adjustments were made for the same covariates as in the main, fully adjusted model.

P values <0.05 were considered statistically significant. All data were analysed using Stata version 16.1 (StataCorp, College Station, USA).

2.3 Results

There was a total of 32,227 adult incident dialysis patients who were registered with ANZDATA between 2008 and 2018 and had at least one determination of serum phosphate. Of these, 238 died or were censored within 30 days of first dialysis and were excluded, leaving 31,989 patients in the analysis. Baseline demographic and clinical details of these patients are displayed in Table 2.2. Mean age at first dialysis was 61 years and 38% were female. The most common primary aetiology of kidney disease was diabetes (40%) and the initial dialysis modality was HD for 67% of patients.

Table 2.2: Participant demographics and characteristics at commencement of dialysis (by dialysis modality)

Characteristic	Baseline dialysis modality		
	Overall (n = 31,989)	Haemodialysis (n = 21,567)	Peritoneal dialysis (n = 10,422)
Age at first dialysis (years)	60.6 ± 14.9	61.3 ± 14.8	59.0 ± 15.0
No. female	12,203 (38.2)	8,175 (37.9)	4,028 (38.7)
Self-reported ethnicity			
- European or other	21,615 (68.6)	14,790 (69.8)	6,825 (66.3)
- Australian Aboriginal or Torres Strait Islander	2,854 (9.1)	2,352 (11.1)	502 (4.9)
- New Zealand Māori or Pacifica	3,929 (12.5)	2,500 (11.8)	1,429 (13.9)
- Asian	3,092 (9.8)	1,560 (7.4)	1,532 (14.9)
SEIFA (disadvantage score) quintile based on residential postcode			
- 1 st quintile	5,135 (18.9)	3,469 (18.4)	1,666 (19.9)
- 2 nd quintile	5,899 (21.7)	4,075 (21.7)	1,824 (21.7)
- 3 rd quintile	5,042 (18.5)	3,552 (18.9)	1,490 (17.8)
- 4 th quintile	5,594 (20.6)	3,902 (20.8)	1,692 (20.2)
- 5 th quintile	5,532 (20.3)	3,811 (20.3)	1,721 (20.5)
Primary aetiology of kidney disease			
- Glomerulonephritis	6,210 (19.4)	3,778 (17.5)	2,432 (23.3)
- Polycystic kidney disease or reflux	2,402 (7.5)	1,575 (7.3)	827 (7.9)
- Diabetes	12,908 (40.4)	8,935 (41.4)	3,973 (38.1)
- Other	8,460 (26.5)	5,900 (27.4)	2,560 (24.6)
- Unknown	2,009 (6.3)	1,379 (6.4)	630 (6.0)
First reviewed by nephrologist < 3 months of first dialysis	6,034 (19.1)	4,515 (21.2)	1,519 (14.7)
Comorbid conditions at first dialysis			
- Chronic lung disease	5,283 (16.6)	3,814 (17.8)	1,469 (14.1)
- Coronary artery disease	12,072 (37.9)	8,666 (40.4)	3,406 (32.8)
- Peripheral vascular disease	7,365 (23.1)	5,282 (24.6)	2,083 (20.1)
- Cerebrovascular disease	4,248 (13.3)	3,000 (14.0)	1,248 (12.0)
- Diabetes mellitus	16,569 (52.0)	11,591 (54.0)	4,978 (47.9)
BMI category at first dialysis			
- Underweight (<18.5 kg/m ²)	769 (2.5)	508 (2.4)	261 (2.6)
- Normal (18.5-24.9 kg/m ²)	8,684 (28.0)	5,505 (26.4)	3,179 (31.2)
- Overweight (25-29.9 kg/m ²)	9,728 (31.4)	6,161 (29.6)	3,567 (35.0)
- Obese (>30 kg/m ²)	11,834 (38.2)	8,655 (41.6)	3,179 (31.2)
Smoking status at first dialysis			
- Never	14,582 (46.3)	9,663 (45.6)	4,919 (47.7)
- Former	12,853 (40.8)	8,709 (41.1)	4,144 (40.1)
- Current	4,079 (12.9)	2,818 (13.3)	1,261 (12.2)
History of cancer (excluding non-melanoma skin cancer)	3,494 (10.9)	2,637 (12.2)	857 (8.2)
Erythropoiesis stimulating agent use	25,106 (81.1)	17,878 (86.3)	7,228 (70.7)
Baseline serum biochemistry/haematology (taken > 30 days after first dialysis)			
- Phosphate – mmol/L	1.64 [1.33 – 2.00]	1.63 [1.31 – 2.02]	1.65 [1.38 – 1.99]
- Calcium – mmol/L	2.24 [2.11 – 2.36]	2.24 [2.12 – 2.36]	2.23 [2.10 – 2.35]
- Haemoglobin – g/L	110 [100 – 120]	109 [98 – 119]	112 [102 – 122]
- Ferritin – µg/L	303 [155 – 521]	323 [170 – 544]	260 [129 – 467.5]
- Transferrin saturation – %	23 [17 – 30]	22 [16 – 30]	23 [18 – 30]

Mean ± standard deviation, median [interquartile range] or number (%)

Abbreviations: BMI, body mass index; SEIFA, Socio-Economic Indexes for Areas 2011²⁶²

2.3.1 Follow up and outcomes

During a median follow up time of 5.1 years (IQR 2.6 – 8.5), and a total of 97,122 person-years at risk, there were 12,713 deaths, while 6,086 patients were censored at the time of receiving a kidney transplant, and 194 were censored due to loss of follow up. Overall, the crude mortality rate was 130.9 deaths per 1,000 patient years for all-cause mortality and 41.5 deaths per 1000 patient years for cardiovascular mortality (Table 2.3).

2.3.2 Association between serum phosphate and mortality

The unadjusted, age-adjusted and fully adjusted Cox-regression models are shown in Figure 2.1. The unadjusted model showed a U-shaped association between serum phosphate and all-cause mortality. This pattern persisted after adjusting for age and other covariates, albeit the magnitude of the association between low serum phosphate and mortality was partially attenuated, while the association between high serum phosphate and mortality was accentuated, in the fully adjusted model. These associations were also seen when models were analysed by dialysis modality (Figure 2.1). There was a significant interaction between serum phosphate <1.00 mmol/L and PD (HR 1.37, 95% CI 1.10 – 1.71, $p = 0.01$), but other categories of phosphate did not have a significant interaction (Table 2.4).

The HR estimates for the fully adjusted models of all-cause mortality are displayed in Table 2.3. Overall, serum phosphate categories between 1.25 and 1.99 mmol/L conferred the lowest adjusted hazard of death, although serum phosphate of 1.75 – 1.99 mmol/L showed a trend towards increased mortality (HR 1.06, 95% CI 0.98 – 1.14, $p = 0.15$). There were incremental increases in HR observed with higher and lower serum phosphate categories outside of this range, reaching a HR of 2.13 (95% CI 1.93 – 2.36, $p < 0.001$) for phosphate ≥ 2.75 mmol/L, and a HR of 1.56 (95% CI 1.44 – 1.69, $p < 0.001$) for phosphate <1.00 mmol/L.

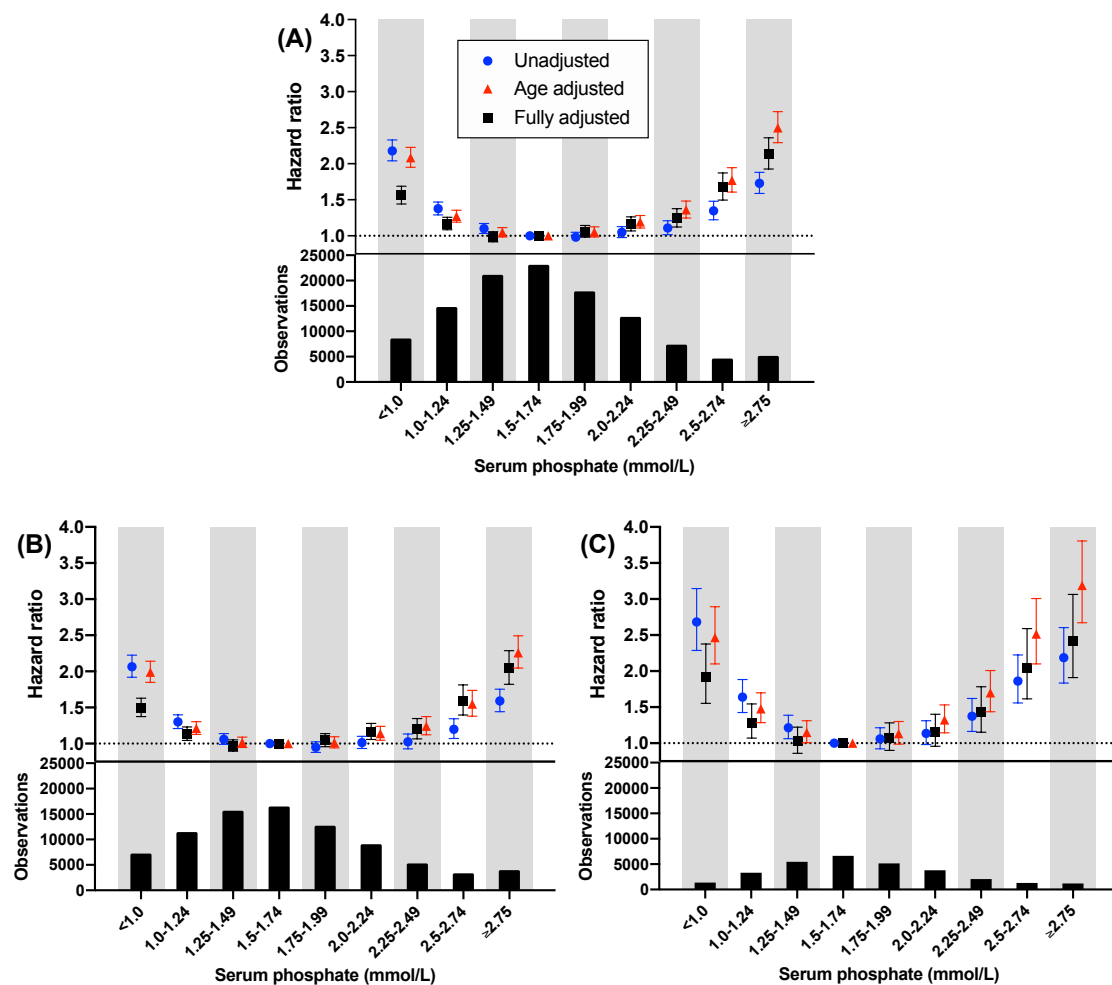
Table 2.3: Fully adjusted hazard ratios of all-cause mortality by categorical serum phosphate and dialysis modality

	Overall	Haemodialysis	Peritoneal dialysis
No. of deaths (all causes)	12,713	9,784	2,929
Person years at risk	97,122	72,824	24,298
Rate per 1000 person-years (95% CI)	130.9 (128.6 – 133.2)	134.3 (131.7 - 137.1)	120.5 (116.3 – 125.0)
Adjusted HR for serum phosphate (95% CI)			
<1.00 mmol/L	1.56*** (1.44 - 1.69)	1.50*** (1.37 - 1.63)	1.92*** (1.55 - 2.38)
1.00 – 1.24 mmol/L	1.17*** (1.08 - 1.26)	1.13** (1.05 - 1.23)	1.29** (1.07 - 1.54)
1.25 – 1.49 mmol/L	0.98 (0.92 - 1.06)	0.97 (0.90 - 1.05)	1.03 (0.86 - 1.22)
1.50 – 1.74 mmol/L	Reference	Reference	Reference
1.75 – 1.99 mmol/L	1.06 (0.98 - 1.14)	1.04 (0.96 - 1.14)	1.07 (0.90 - 1.28)
2.00 – 2.24 mmol/L	1.16*** (1.07 - 1.26)	1.16** (1.06 - 1.28)	1.16 (0.96 - 1.40)
2.25 – 2.49 mmol/L	1.24*** (1.12 - 1.38)	1.20** (1.07 - 1.35)	1.43** (1.15 - 1.78)
2.50 – 2.74 mmol/L	1.67*** (1.50 - 1.87)	1.59*** (1.40 - 1.81)	2.05*** (1.62 - 2.59)
≥2.75 mmol/L	2.13*** (1.93 - 2.36)	2.04*** (1.82 - 2.29)	2.42*** (1.91 - 3.06)

Hazard ratio (HR) estimated by Cox proportionate hazards model. Models are stratified by treatment centre and adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation. *p <0.05, **p <0.01, ***p <0.001.

Abbreviations: HR, hazard ratio, 95% CI, 95% confidence interval

Figure 2.1: Unadjusted, age-adjusted and fully adjusted[†] models of association between serum phosphate and all-cause mortality in overall cohort (A), and in models restricted to patients on haemodialysis (B) and peritoneal dialysis (C).



[†] Model adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

“Observations” are the crude number of serum phosphate observations reported to ANZDATA during study period.

Table 2.4: Adjusted hazard ratios of all-cause and cardiovascular mortality by categorical serum phosphate with adjustment for interaction between serum phosphate and dialysis modality

Variable	All-cause mortality	Cardiovascular mortality
	Adjusted HR (95% CI)	Adjusted HR (95% CI)
Serum phosphate		
<1.00 mmol/L	1.49 (1.37 - 1.63)***	1.40 (1.19 - 1.64)***
1.00 – 1.24 mmol/L	1.13 (1.04 - 1.23)**	1.07 (0.92 - 1.25)
1.25 – 1.49 mmol/L	0.97 (0.89 - 1.05)	0.95 (0.82 - 1.10)
1.50 – 1.74 mmol/L	Reference	Reference
1.75 – 1.99 mmol/L	1.05 (0.96 - 1.15)	1.15 (0.99 - 1.34)
2.00 – 2.24 mmol/L	1.17 (1.06 - 1.28)**	1.26 (1.06 - 1.48)**
2.25 – 2.49 mmol/L	1.21 (1.08 - 1.36)**	1.39 (1.15 - 1.69)**
2.50 – 2.74 mmol/L	1.60 (1.40 - 1.82)***	1.70 (1.37 - 2.11)***
≥2.75 mmol/L	2.09 (1.87 - 2.34)***	2.45 (2.04 - 2.95)***
PD : phosphate interaction		
<1.00 mmol/L	1.37 (1.10 - 1.71)**	0.99 (0.64 - 1.53)
1.00 – 1.24 mmol/L	1.18 (0.97 - 1.43)	1.14 (0.81 - 1.60)
1.25 – 1.49 mmol/L	1.09 (0.90 - 1.31)	0.99 (0.71 - 1.37)
1.50 – 1.74 mmol/L	Reference	Reference
1.75 – 1.99 mmol/L	1.05 (0.87 - 1.27)	1.03 (0.75 - 1.42)
2.00 – 2.24 mmol/L	1.00 (0.82 - 1.23)	0.87 (0.61 - 1.24)
2.25 – 2.49 mmol/L	1.14 (0.89 - 1.44)	1.18 (0.80 - 1.72)
2.50 – 2.74 mmol/L	1.23 (0.95 - 1.59)	1.14 (0.74 - 1.75)
≥2.75 mmol/L	1.10 (0.86 - 1.41)	1.41 (0.97 - 2.04)

Hazard ratio (HR) estimated by Cox proportionate hazards model. Models are stratified by treatment centre and adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

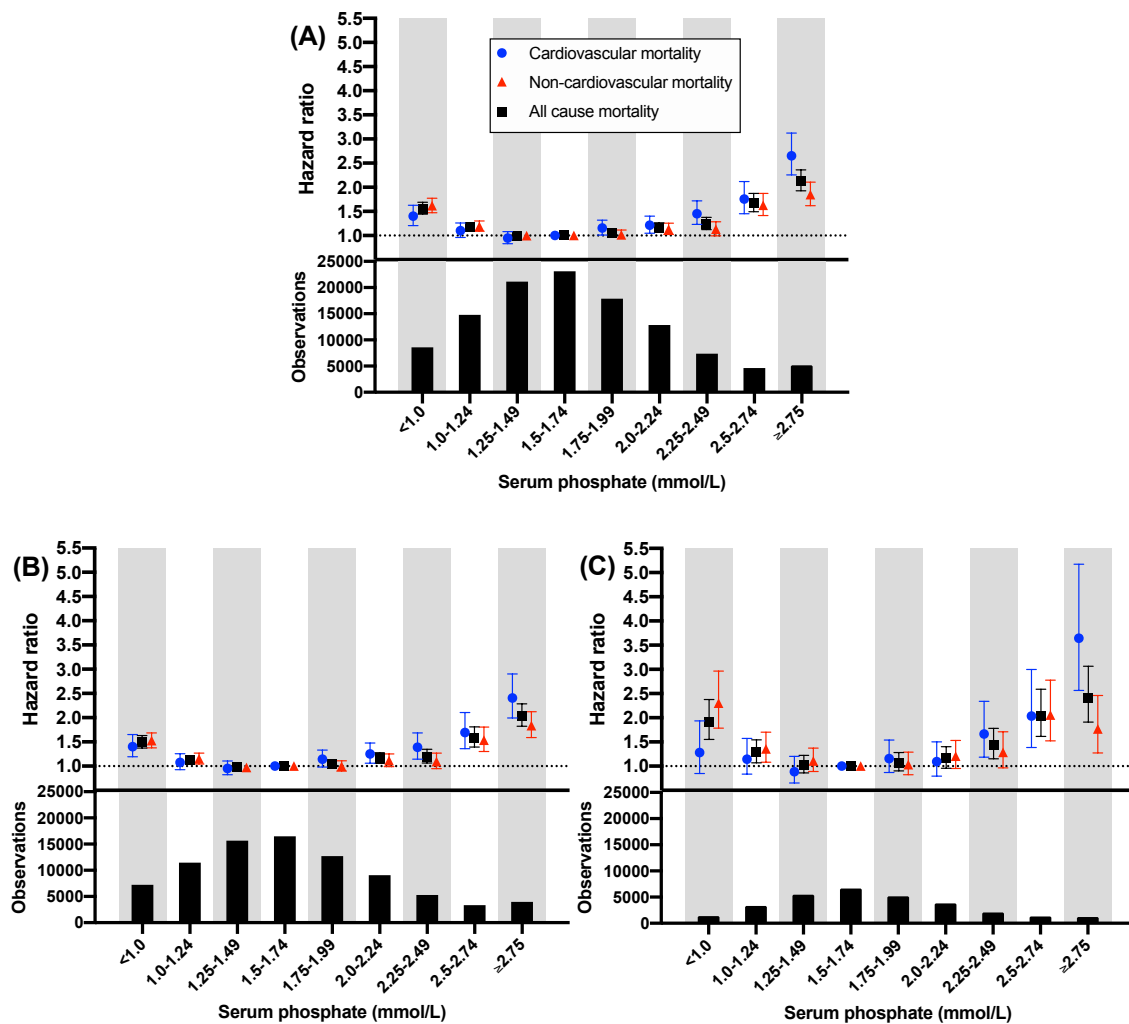
*p <0.05, **p <0.01, ***p <0.001.

Abbreviations: PD, peritoneal dialysis; 95% CI, 95% confidence interval

2.3.4 Association between serum phosphate and cardiovascular mortality

The relationship between serum phosphate and mortality subtypes, in fully-adjusted models, is shown in Figure 2.2. Similar to all-cause mortality, the HR for cardiovascular mortality increased with high and low levels of serum phosphate (Table 2.5). However, there was a much more pronounced correlation between with high levels of serum phosphate and cardiovascular mortality than low serum phosphate, resulting in a “J-shaped” rather than a “U-shaped” curve. This also appeared to be present when the models were restricted to HD and PD groups, however the confidence intervals were noticeably larger, especially for the PD group. There were no significant interactions between serum phosphate and dialysis modality for cardiovascular mortality (Table 2.4).

Figure 2.2: Fully adjusted models of association between serum phosphate and cardiovascular, non-cardiovascular and all-cause mortality in overall cohort (A), and in models restricted to patients on haemodialysis (B) and peritoneal dialysis (C).



Model adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

“Observations” are the crude number of serum phosphate observations reported to ANZDATA during study period.

Table 2.5: Fully adjusted hazard ratios of cardiovascular mortality by categorical serum phosphate and dialysis modality

	Overall	Haemodialysis	Peritoneal dialysis
No. of cardiovascular deaths	4,030	3,019	1,011
Person-years at risk	97,122	72,824	24,298
Rate per 1000 person-years (95% CI)	41.5 (40.2 – 42.8)	41.5 (40.0 – 43.0)	41.6 (39.1 – 44.2)
Adjusted HR for serum phosphate (95% CI)			
<1.00 mmol/L	1.40*** (1.21 - 1.63)	1.40*** (1.19 - 1.65)	1.28 (0.85 - 1.94)
1.00 – 1.24 mmol/L	1.10 (0.96 - 1.26)	1.08 (0.93 - 1.26)	1.14 (0.83 - 1.57)
1.25 – 1.49 mmol/L	0.95 (0.83 - 1.08)	0.95 (0.82 - 1.10)	0.88 (0.65 - 1.20)
1.50 – 1.74 mmol/L	Reference	Reference	Reference
1.75 – 1.99 mmol/L	1.16* (1.01 - 1.32)	1.14 (0.98 - 1.33)	1.15 (0.87 - 1.54)
2.00 – 2.24 mmol/L	1.21** (1.05 - 1.40)	1.25** (1.06 - 1.48)	1.09 (0.79 - 1.50)
2.25 – 2.49 mmol/L	1.45*** (1.23 - 1.72)	1.39** (1.14 - 1.69)	1.67** (1.18 - 2.34)
2.50 – 2.74 mmol/L	1.76*** (1.45 - 2.12)	1.69*** (1.36 - 2.11)	2.04*** (1.38 – 3.00)
≥2.75 mmol/L	2.65*** (2.26 - 3.12)	2.41*** (1.99 - 2.90)	3.64*** (2.57 - 5.17)

Hazard ratio (HR) estimated by Cox proportionate hazards model. Models are stratified by treatment centre and adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation. *p <0.05, **p <0.01, ***p <0.001.

Abbreviations: HR, hazard ratio, 95% CI, 95% confidence interval

2.3.5 Sensitivity analyses

Overall rates of missing values for each variable were very low (Table 2.6), and imputation of missing values for the main fully adjusted model of all-cause mortality did not significantly change the pattern or point estimates of hazard.

Models for baseline and also for mean values of serum phosphate and time-updated covariates are shown in Table 2.7. There were similar associations between serum phosphate and all-cause mortality across each model, but the time-dependent model (“main model”) had the best overall fit as judged by AIC.

The model for baseline values of comorbidities (lung disease, coronary artery disease, peripheral vascular disease and cerebrovascular disease) had a marginally improved overall fit (as judged by AIC). However, there were only negligible differences in the point estimates of hazard, compared to the main model (Table 2.7).

Sub-hazard ratios for all-cause mortality from the fully-adjusted Fine and Gray model (with kidney transplant as a competing risk) are shown in Table 2.8. While cause-specific hazard ratios from the Cox proportional hazards model and sub-hazard ratios from the Fine and Gray model are not directly comparable,²⁶³ in both models phosphate categories between 1.25 and 1.99 mmol/L were associated with the lowest risk, and each subsequent serum phosphate category above and below this range was associated with an incrementally higher risk.

Table 2.6: Missing values for each variable (number and percentage)

	Number of observations	Number missing	Percentage missing observations
Baseline variables			
Age at first dialysis	31,989	0	0%
Sex	31,989	0	0%
Self-reported ethnicity	31490	499	1.6%
Primary aetiology of renal disease	31,989	0	0%
First reviewed by nephrologist < 3 months of first dialysis	31567	422	1.3%
Smoking status at first dialysis	31514	475	1.5%
History of cancer (excluding non- melanoma skin cancer)	31,989	0	0%
Time updated variables			
Dialysis modality	119,307	0	0%
Chronic lung disease	119,307	86	0.1%
Chronic coronary artery disease	119,221	89	0.1%
Peripheral vascular disease	119,218	90	0.1%
Cerebrovascular disease	119,217	90	0.1%
Diabetes mellitus	119,217	95	0.1%
Body mass index	119,212	6,501	5.4%
Phosphate	112,806	3,834	3.2%
Calcium	115,473	4,030	3.4%
Haemoglobin	115,277	3,849	3.2%
Ferritin	115,458	10,488	8.8%
Transferrin saturation	108,819	13,546	11.4%

Table 2.7: Adjusted hazard ratios of all-cause mortality by categorical serum phosphate and dialysis modality comparing main model with baseline variable model, mean variable model, and baseline comorbidities model

Variable	Main model	Baseline variable model	Mean variable model	Baseline comorbidities
Adjusted HR for serum phosphate (95% CI)				
<1.00 mmol/L	1.56*** (1.44 - 1.69)	1.21*** (1.11 - 1.21)	2.40*** (2.16 - 2.67)	1.56*** (1.44 - 1.68)
1.00 – 1.24 mmol/L	1.17*** (1.08 - 1.26)	1.03 (0.96 - 1.10)	1.25*** (1.16 - 1.34)	1.17*** (1.08 – 1.26)
1.25 – 1.49 mmol/L	0.98 (0.92 - 1.06)	1.00 (0.95 - 1.07)	1.02 (0.96 - 1.07)	0.98 (0.91 – 1.05)
1.50 – 1.74 mmol/L	Reference	Reference	Reference	Reference
1.75 – 1.99 mmol/L	1.06 (0.98 - 1.14)	0.98 (0.91 - 1.04)	1.16*** (1.09 - 1.23)	1.06 (0.98 – 1.14)
2.00 – 2.24 mmol/L	1.16*** (1.07 - 1.26)	1.07 (1.00 - 1.15)	1.37*** (1.28 - 1.47)	1.17*** (1.07 – 1.27)
2.25 – 2.49 mmol/L	1.24*** (1.12 - 1.38)	1.15*** (1.05 – 1.26)	1.66*** (1.51 - 1.82)	1.26*** (1.14 – 1.40)
2.50 – 2.74 mmol/L	1.67*** (1.50 - 1.87)	1.28*** (1.14 - 1.42)	2.01*** (1.76 - 2.29)	1.69*** (1.51 – 1.89)
≥2.75 mmol/L	2.13*** (1.93 - 2.36)	1.47*** (1.33 – 1.67)	3.16*** (2.76 - 3.60)	2.16*** (1.95 – 2.39)
Akaike information criterion	85,432	107,010	105,313	84,997

Hazard ratio (HR) estimated by Cox proportionate hazards model. Models are stratified by treatment centre and adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

For main model, serum phosphate and time-updated variables are entered as time-dependent variables. For baseline model only baseline variable values are entered. For mean variable model, serum phosphate and other time-updated continuous variables are entered as their mean. For the baseline comorbidities model, the variables of lung disease, coronary artery disease, peripheral vascular disease and cerebrovascular disease are entered as their baseline value, while other time-updated variables are entered as time-dependent variables (as per the main model).

*p <0.05, **p <0.01, ***p <0.001.

Table 2.8: All-cause mortality and categorical serum phosphate comparing Cox proportionate hazard regression (hazard ratio) and Fine and Gray (sub-hazard ratio) models

	Cause-specific hazard ratio (95% CI)	Sub-hazard ratio (95% CI)
Serum phosphate		
<1.00 mmol/L	1.56*** (1.44 - 1.69)	1.46*** (1.35 - 1.58)
1.00 – 1.24 mmol/L	1.17*** (1.08 - 1.26)	1.15*** (1.07 - 1.23)
1.25 – 1.49 mmol/L	0.98 (0.92 - 1.06)	0.97 (0.91 - 1.04)
1.50 – 1.74 mmol/L	Reference	Reference
1.75 – 1.99 mmol/L	1.06 (0.98 - 1.14)	1.07 (0.99 - 1.15)
2.00 – 2.24 mmol/L	1.16*** (1.07 - 1.26)	1.17*** (1.07 - 1.27)
2.25 – 2.49 mmol/L	1.24*** (1.12 - 1.38)	1.28*** (1.16 - 1.42)
2.50 – 2.74 mmol/L	1.67*** (1.50 - 1.87)	1.68*** (1.50 - 1.88)
≥2.75 mmol/L	2.13*** (1.93 - 2.36)	2.16*** (1.95 - 2.39)

Cause specific hazard ratio estimated by Cox proportionate hazards model. Sub-hazard ratio estimated by Fine and Gray method. Both models are adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

*p <0.05, **p <0.01, ***p <0.001.

Abbreviations: 95% CI, 95% confidence interval

2.4 Discussion

Analysis of this large, contemporary cohort of incident dialysis patients reaffirms that both high and low levels of serum phosphate are independently associated with an increased hazard of all-cause mortality. While patients from Australia and New Zealand have been included in previous multi-national studies,¹²² to our knowledge, this is the first time an analysis of serum phosphate and mortality has been reported from this region, and utilising the ANZDATA Registry. Importantly, this work also contributes further outcome data for patients being treated with PD, who have previously been underrepresented in the literature.

After adjusting for significant covariates, high levels of serum phosphate had a positive association with all-cause mortality, with sequentially higher levels of serum phosphate conferring greater risk, in a dose-response fashion. In this analysis of mortality subtype, it was found that the association of high serum phosphate with cardiovascular mortality was more pronounced than non-cardiovascular mortality. These observations have been consistently demonstrated, and support the notion that excess phosphate is directly (or indirectly) linked to cardiovascular disease and mortality in the dialysis population.¹¹⁵ Additionally, phosphate ostensibly induces vascular cell dysfunction¹⁴⁰ and calcification,¹⁴¹ and drives elevated levels of PTH and FGF23, which themselves are associated with cardiovascular toxicity.¹¹⁵ However, as discussed in Section 1.5, it is debated whether phosphate is the direct mediator of these effects.^{116, 160} Further, while current international clinical guidelines recommend lowering elevated serum phosphate toward the normal range, there is a notable lack of definitive trial data to show that such therapeutic strategies improve patient-centred outcomes.¹⁵⁷ Large interventional trials are currently underway, and will hopefully provide more clarity on this issue.^{255, 256} Nevertheless, this study affirms that in the current era of dialysis care, elevated serum phosphate remains independently associated with increased risk of all-cause and cardiovascular death in both HD and PD patients.

As has previously been described, this study also found that low levels of serum phosphate were associated with increased risk of mortality.^{123-126, 128, 158, 159} In contrast to the pattern observed between high levels of serum phosphate and subtypes of mortality, low levels of serum phosphate were more strongly associated with non-cardiovascular than cardiovascular death. Hypophosphataemia has been previously linked to a number

of factors, including age,¹¹⁸ infection¹⁵⁸, inflammation and malnutrition.^{121, 126} These factors are all also important mortality risk factors in the dialysis population, and it is conceivable that serum phosphate may be a surrogate marker in these situations, rather than a direct mediator of pathology resulting in death. Whilst this analysis was able to correct for body mass index, ANZDATA does not collect other markers of inflammation or nutrition, such as serum albumin, limiting the ability to interrogate these effects further.

This study found that serum phosphate <1.00 mmol/L was associated with higher risk of all-cause mortality in PD patients than HD patients. However, dialysis modality did not otherwise appear to have a widespread modifying effect, and associations between serum phosphate and all-cause and cardiovascular mortality were overall similar when models were restricted to subgroups of HD and PD patients. Previous studies have largely focussed on HD patients, which is not unexpected, given the relative predominance of HD as a treatment for kidney failure.²⁶⁴ Additionally, the regular contact with medical services and ease of obtaining regular blood tests, also make HD patients inherently suitable for large population-based studies. By contrast, studies examining phosphate that include patients receiving PD have been limited to smaller^{130, 163, 164, 166} or older^{165, 167, 265} sets of data. There are a number of reasons why studies derived from HD cohorts may not be directly applicable to PD patients. The different kinetics of phosphate removal with each modality mean that samples taken in routine clinical practice may not be comparable in terms of differences in total phosphate exposure. It has previously been observed that HD patients tend to have higher levels of serum phosphate than patients on PD.^{130, 164} However, Evenepoel *et al* showed that despite this, PD patients may actually have less total phosphate clearance and higher overall exposure.²⁶⁶ Evenepoel *et al* postulated that this discrepancy may be an artefact caused by the practice of measuring phosphate in HD patients immediately prior to dialysis, when serum levels are at their highest. With these considerations in mind, it is reassuring to have further empirical evidence that serum phosphate levels in PD patients, as measured using routine and current collection methods, is a robust and independent marker of significant patient outcomes.

In this analysis serum phosphate was entered as a time-dependent variable in the main models of all-cause and cardiovascular mortality. As with all epidemiological studies, it is important to consider whether this may have inadvertently introduced bias by misrepresenting biological effects.²⁵⁸ For example, it has been suggested that there may

be a propensity for serum phosphate to progressively *decrease* in the immediate months preceding a significant event, such as hospitalisation.¹²⁶ In this scenario, a time-dependent Cox model would associate the most recent serum phosphate level with the event of interest, even if a patient previously had years of higher levels of serum phosphate - which ostensibly may have contributed to their eventual death through the accumulation of cardiovascular pathology. To assess for this effect, models were refitted using mean and baseline variables. These sensitivity analyses did not significantly change the associations between serum phosphate categories and mortality, suggesting that the main findings are robust. Other groups have recently suggested alternative modelling techniques, such as estimating the time that serum phosphate is above the reference range (“area under the curve”).²⁶⁷ While this method showed strong association with cardiovascular mortality, by focussing on elevated serum phosphate this method may ignore the prognostic significance of low serum phosphate, which as shown in this study also imbues significant risk.

The strength of this study is the use of the ANZDATA Registry, which allows for analysis of up-to-date data from a large patient cohort, and with substantial numbers of both HD and PD patients. Consent for participation in ANZDATA is via an “opt-out” process, meaning that participation levels are high and risk of selection bias and loss to follow-up is minimal. These factors may improve the generalisability of this data to other populations of incident dialysis patients.

It is acknowledged that this analysis has some limitations. Information about individual patients is restricted to the reporting fields collected as part of the ANZDATA Registry. This means that some potentially relevant covariates for this analysis, such as serum PTH, serum albumin, as well as medications including phosphate binders, were not available. Some of these variables have previously been associated with mortality in dialysis patients.²⁵⁴ While these associations have not been universal, it is possible that a lack of adjustment may have resulted in an overestimation of risk.

Another limitation of this study is that many of the clinical variables that were used, such as history of cardiovascular disease were categorical, and so it was not possible to further quantify the severity of these potential confounders. The nature of this registry analysis means there may be variation in the collection and reporting methods of serum phosphate

data. Information about assays used by laboratories at different sites was not available, and while ANZDATA provides instructions to standardise the timing of blood draws, it is possible that there is variation between, and even within sites. Further, measures of serum phosphate are only available on a yearly basis, which is less frequent than international clinical guideline recommend.¹⁵⁷ However, all of these limitations would be expected to bias this analysis towards the null, and arguably these study conditions reflect contemporary, real world practice, which may improve the external applicability of these results. Finally, as an observational study, an inherent limitation is that causation between serum phosphate and mortality cannot be inferred, and the possibility of residual confounding must be considered.

2.5 Conclusion

This analysis shows that high and low levels of serum phosphate are associated with increased risk of mortality in patients with kidney failure treated with dialysis. This association was independent of other covariates and of dialysis modality. As discussed in Chapter 1, whether phosphate is a direct mediator of these outcomes remains unclear, and whether phosphate is a useful therapeutic target also is yet to be definitively shown. Nevertheless, in the current era of dialysis care, serum phosphate remains a relevant marker of risk of significant patient-centred outcomes.

Chapter 3 Effect of nutritional calcium and phosphate loading on calciprotein particle kinetics in adults with normal and impaired kidney function

3.1 Introduction

Calcium and phosphate form highly insoluble salts in aqueous solution. This presents a physiological challenge, given that metabolism in all living organisms is dependent on these essential nutrients, but their coexistence in biological fluids creates an inherent mineralisation risk and the need for tight regulation.²⁶⁸ Extracellular fluids like plasma, are considered metastable or approaching metastability with respect to their calcium and phosphate ionic activities, readily sustaining crystal growth if nucleated.²⁶⁹ The tendency of calcium and phosphate to precipitate has been harnessed by vertebrates for its unique biomechanical properties during the evolution of the skeletal and dental tissues. However, as outlined in Section 1.4 ectopic mineralisation in pathological states, such as CKD, is associated with significant risk of cardiovascular events and mortality.⁵³ This mandates that mechanisms of rapid mobilisation and efficient bulk transport of mineral precursors exist to meet the demands of growth and repair of these physiologically mineralised tissues, as well as strategies of restricting mineralisation to these sites and preventing unwanted calcification in soft tissues.²⁷⁰

As outlined in Section 1.2, large amounts of calcium and phosphate are absorbed from the diet every day. Given this abundance of mineral to which mammals are intermittently exposed, one might suspect the risk of ectopic mineralisation to be highest following a meal due to the anticipated surge in mineral ions absorbed from the intestine. Yet, contrary to this expectation, plasma calcium and phosphate concentrations change minimally in the post-prandial period. While increased urinary excretion helps to maintain homeostasis,²⁷¹ this response is not instantaneous and lags by some hours after the ingestion of millimolar amounts of calcium and phosphate.²³³ As a solution to this physiological challenge, a blood-borne system for buffering and transporting mineral has long been suspected, and it has been proposed that the formation of CPP could help to fulfil this function.²⁷²

As outlined in Section 1.9.2, a dietary origin of CPP has been proposed, and is supported by the observation that high-phosphate feeding is associated with increased ambient levels of CPM²²⁵ and CPP-I/II²⁰⁶ in animal studies, and that reduction in serum CPM²²⁷ and CPP-I²²⁶ is seen in haemodialysis patients treated with intestinal phosphate binders. As colloidal mineral-protein complexes that stabilise nascent calcium phosphate clusters, the formation of CPP in response to dietary mineral intake could function to buffer and safely chaperone the intermittent flux of mineral through extracellular fluid to sites of utilisation or disposal.²⁷³ However, definitive observation of the effect of nutritional mineral intake on acute CPP kinetics in humans is lacking.

As outlined in Section 1.8, elevated levels of CPP are observed in CKD,²⁰⁷ and high serum levels of CPP have been linked to an increased risk of cardiovascular events^{200, 202-205} and mortality in this setting.²⁰⁴ Experimental studies have also provided biologically plausible pathways through which prolonged exposure to elevated levels of CPP could induce vascular toxicity.^{214-216, 218, 219} As such, it has been proposed that in CKD, metabolism of CPP may be disturbed, and that this may help explain the links between excess dietary mineral and poor patient outcomes.^{160, 186} Given the well described disturbances in calcium and phosphate handling seen in CKD, if CPP are formed in response to dietary mineral intake, then it is plausible that CKD could impact this post-prandial response. In addition, recent data has also suggested that CPM are directly filtered at the glomerulus,²¹⁹ implying that kidney impairment may also affect CPP metabolism through impaired CPM clearance.

Recent methodological advancements now permit direct quantification of each CPP species (CPM,²⁰⁸ CPP-I and CPP-II²⁰⁶) in biological samples. To address these key evidence gaps, a controlled study was conducted to examine the effect of standardised food intake on serum CPM, CPP-I, CPP-II. The effects of food intake on T50, and on the hydrodynamic radius of CPP-II (CPP-II size) generated in the T50 assay, was also examined given these are also novel methods for assessing mineral metabolism and vascular risk in CKD.^{204, 239-241, 248, 249}

3.2 Methods

3.2.1 Study population

Fourteen individuals with CKD and sixteen age- and gender-matched healthy controls were recruited. Each participant had to be at least 18 years of age to be eligible. Participants were excluded if they: i) had a history of mineral or bone disease, other than related to CKD; ii) were being treated with an intestinal phosphate binder or calcitriol; or iii) had a gastrointestinal disorder, history of lactose intolerance or were unwilling to consume the study meal. For the CKD group, seven individuals were recruited with an eGFR between 30-60 mL/min/1.73m² and seven with an eGFR <30 mL/min/1.73m², excluding participants who required dialysis or with a previous kidney transplant. Healthy controls had no history of chronic medical conditions and had normal kidney function (eGFR >60 mL/min/1.73m²). The study was conducted in accordance with the Declaration of Helsinki. All individuals provided written informed consent, and the study was approved by the local ethics committee (Melbourne Health Human Research Ethics Committee MH2018.363).

3.2.2 Procedure

Each participant was studied after an overnight fast and sample collection commenced between 7.30 and 9.30am. An intravenous cannula was inserted at the start of the study period. Before each blood sample was collected, an initial 5 mL draw from the cannula was discarded. Two initial fasting blood samples were taken 30 minutes apart to account for baseline variation. The mean values of these two timepoints were used as “time 0”. Immediately after collection of the second fasting sample, participants consumed a standardised meal (Sanitarium Up&Go liquid breakfast; 250 mL, vanilla flavour) containing 815 kJ energy, 300 mg calcium and 188 mg phosphate (Table 3.1). Participants were instructed to consume the entirety of the drink within 5 minutes. Serial blood samples were collected at five post-prandial timepoints (30, 60, 120, 180 and 240 minutes) from the commencement of the meal. During the study period participants were allowed to drink water but were not allowed to consume any other food or drink.

Table 3.1: Nutritional information for standardised meal (Sanitarium Up&Go liquid breakfast; 250mL, vanilla flavour)

Parameter	Contents of meal²⁷⁴	Recommended dietary intake*
Energy (kJ)	815	-
Protein (g)	8.3	13%
Fat (g)	4.3	-
Carbohydrate (g)	28.4	-
Sodium (mg)	158	17-34%
Potassium (mg)	450	45%
Calcium (mg)	300	30%
Phosphate (mg)	188	19%

*Percentage of recommended dietary intake based on Australian adult male²⁷⁵ (where generalised recommendations are available)

3.2.3 Outcome measures

Blood was collected for repeated measurement of novel markers of mineral metabolism (CPM, CPP-I, CPP-II, T50 and CPP-II size) at each timepoint. Fetuin-A was measured at each timepoint, given its role as the principal mineral-binding protein present in CPM and CPP-I/II. We also measured serum phosphate, total calcium, magnesium, albumin and bicarbonate at each timepoint, and serum intact PTH and intact fibroblast growth factor-23 (iFGF23) at three timepoints (0, 120 and 240 minutes). Serum citrate was measured at 0, 30 and 60 minutes. Serum urea, creatinine and 1,25-dihydroxyvitamin D were measured once at fasting baseline (0 minutes). Blood samples for novel markers of mineral metabolism, PTH, iFGF23, 1,25-dihydroxyvitamin D and serum citrate, were allowed to clot for 30 minutes before centrifugation, and then serum aliquots were stored at -80°C until batch-analysis. All other biochemical measurements were performed at the time of sample collection using standard laboratory methods.

3.2.4 Gel-filtration and flow cytometric assays for CPM and CPP-I/II

As outlined in Section 1.11, two complementary assays have recently been developed to quantitate different fractions of the circulating CPP pool.²³⁶ Both assays use the fluorescently-labelled bisphosphonate derivative, OsteoSense 680EX (Perkin Elmer), which binds specifically to solid-phase calcium phosphate and preferentially to crystalline phases (e.g. hydroxyapatite). The ‘gel-filtration’ method of Miura et al,²⁰⁸ was used to measure small (<50 nm diameter), low-density mineral-laden fetuin-A colloids. Briefly, frozen serum samples were thawed for 24h at 25°C to induce aggregation of CPM and phase transition to crystalline calcium phosphate. Samples were then centrifuged for 30,000 g for 2h at 4°C to remove larger CPP-I and CPP-II, leaving less dense crystal-laden fetuin-A monomer and multimers for staining with OsteoSense (0.5µM) in HEPES-buffered DMEM (pH 8.0). Unbound dye was subsequently removed by gel filtration (Micro Bio-Spin® Columns with Bio-Gel® P-30, Bio-Rad) and the resultant fluorescence measured using an infrared scanner (Odyssey CLx, LI-COR; EX 685nm, EM 700 nm). Miura and colleagues³² referred to the mineral detected as low-density (L)-CPP, however, here we refer to them as CPM to reflect the origin of this mineral fraction *in vivo*. In our hands, the mean interassay analytical coefficient of variation (CV_A) for the CPM assay was 4.9%.

For flow cytometric analysis, aliquots of frozen serum were processed using the standardised procedures described previously.²⁷⁶ CPP-I and CPP-II were measured as previously described using a BD FACSVerse flow cytometer setup to resolve particles <200nm from background and operating with fluorescence triggering on OsteoSense-positive events.^{206, 226} In this assay, CPP are distinguished from membrane-delimited mineral-containing extracellular vesicles using phosphatidyl serine-binding lactadherin-FITC (Haematologic Technologies Inc., Essex Junction, VT). CPP-I and CPP-II were distinguished by differences in side scatter (SSC) intensity (related to particle size) and OsteoSense fluorescence intensity. Interassay CV_A for CPP-I and CPP-II were <15% and <10%, respectively.

3.2.5 Other mineral markers

Serum T50 was measured by Calciscon AG, Biel, Switzerland, as previously described using a Nephelostar nephelometer (BMG Labtech, Ortenberg, Germany).²³⁷ The mean interassay CV_A for T50 was 3.4%. CPP-II hydrodynamic radius was measured by dynamic light scattering using a DynaPro Plate Reader II (Wyatt Technology, Santa Barbara, CA, USA) as described by Chen *et al.*²⁴⁸ The interassay CV_A for CPP-II size was 4%. Commercial immunoassays were used to measure iFGF23 (Kainos Laboratories, Tokyo, Japan), 1,25 dihydroxyvitamin D Immunodiagnostic Systems, Boldon, UK), and fetuin-A (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Mean interassay CV_A were 3.8%, 5.5%, and 3.2%, respectively. Serum citrate was measured using a colorimetric assay (Sigma-Aldrich, Darmstadt, Germany) with a mean interassay CV_A of 3.5%.

3.2.6 Statistical analysis

Using GLIMMPSE, a validated linear mixed model power and sample size calculator,²⁷⁷ it was estimated that 10 participants would provide >90% power to detect a doubling of CPP in the post-prandial period in with a type I error rate of 0.05. The study was not powered to detect a difference between groups.

Demographic and fasting biochemical data were compared between groups using unpaired t-test or Kruskal-Wallis test for normal and skewed continuous variables respectively, and chi-squared test for categorical variables.

The aim of this study was to describe the within and between-group post-prandial response for each repeated parameter. In order to do this, linear mixed-effects models (LMM) were fitted for each parameter, using a restricted maximum likelihood approach and with an unstructured covariance matrix.²⁷⁸ For each LMM group, categorical time and group-by-time interaction were modelled as fixed effects and a random intercept was included for each participant to account for correlation of repeated measures. The control group and time '0' were used as the reference values for group and time, respectively. Coefficient estimates for group-by-time interaction terms were used to test for differences in post-prandial response between the CKD and control group. After fitting each LMM, *post hoc* pairwise comparisons were performed to test for differences in mean values between groups at each timepoint, and to test for deviation from the fasting baseline within each group. For these pairwise comparisons Bonferroni correction method was used to adjust for multiple comparisons. CPM, CPP-I, CPP-II, CPP-II size, PTH and iFGF23 were natural log transformed before fitting LMMs to ensure normal distribution of residuals. For ease of interpretation, coefficient estimates for interaction terms from these models were then exponentiated to derive estimates of percentage change. For the LMM of CPM, group by time interaction terms suggested a significant difference in post-prandial response between groups. To further explore the effect of kidney function on post-prandial levels of CPM the area under the curve (AUC) for CPM was also calculated using the cubic spline method (time 0 to 240 minutes), and examined the relationship between eGFR, AUC and maximum concentration of CPM (using Spearman rank correlation) as well as between CKD and time of maximum CPM concentration (using chi squared test).

Several samples had undetectable levels of CPP-I (2 samples in the control group, and 6 in the CKD group) or CPP-II (5 in the control group and 10 in the CKD group). For the main analyses, the lower limit of quantification for the assay (133 particles/mL) was used for these left-censored values. To assess for potential bias from this approach, a sensitivity analysis was performed where LMMs for CPP-I and CPP-II were refitted after imputing left-censored values using multi-level Tobit regression, where time and group were entered as independent variables.²⁷⁹

Two-tailed p values <0.05 were considered significant. All data were analysed using Stata MP version 17.0 (StataCorp, College Station, USA) and figures were produced using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, USA).

3.3 Results

3.3.1 Participant demographics and clinical characteristics

The demographics and participant characteristics for each group are displayed in Table 3.2. Overall, the groups were well matched for age and sex. The mean eGFR within the CKD group was 29.2 mL/min/1.73m². Consistent with group allocation, participants within the CKD group had higher fasting levels of serum creatinine, urea, phosphate, PTH and iFGF23, and lower fasting eGFR, serum albumin, bicarbonate and 1,25-dihydroxyvitamin D. Fasting serum total calcium and magnesium were comparable between groups. Among the 14 CKD participants, one was hyperphosphataemic (>1.50 mmol/L) and 10 (71%) were hyperparathyroid (>10.0 pmol/L), while all participants in the control group had values within the respective population-based reference intervals for these two parameters. Medication use for the CKD group is shown in Table 3.3.

Table 3.2: Participant demographics and clinical characteristics

	Healthy control (n = 16)	CKD (n = 14)	p value
Participant characteristics			
Age (yrs)	44.1 ± 13.5	44.3 ± 16.0	0.917
Female sex, n (%)	9 (56.3)	8 (57.1)	0.961
CKD stage, n (%)			
Stage 3a or stage 3b	-	7 (50.0)	
Stage 4 or stage 5 (non-dialysis)	-	7 (50.0)	
Primary cause of kidney disease, n (%)			
Diabetic nephropathy	-	2 (14.3)	
Glomerulonephritis	-	6 (42.9)	
Reflux nephropathy	-	2 (14.3)	
Polycystic kidney disease	-	4 (28.6)	
Fasting biochemical parameters			
Creatinine (μmol/L)	71.6 ± 9.9	239 ± 110.3	0.001
eGFR* (mL/min/1.73m ²)	99.8 ± 10.2	29.2 ± 14.5	<0.001
Urea (mmol/L)	4.8 ± 1.4	15.2 ± 7.4	<0.001
Calcium (mmol/L)	2.29 ± 0.08	2.25 ± 0.10	0.262
Phosphate (mmol/L)	0.96 ± 0.14	1.17 ± 0.24	0.006
Magnesium (mmol/L)	0.84 ± 0.06	0.80 ± 0.08	0.132
Albumin (g/L)	40 ± 2.0	36 ± 2.9	<0.001
Bicarbonate (mmol/L)	27 ± 2.6	22 ± 3.0	<0.001
PTH (pmol/L)	5.2 (4.6 – 6.75)	16.9 (7.1 – 31.0)	0.001
1,25-dihydroxyvitamin D (pmol/L)	137 (103 – 173)	42 (31 – 56)	<0.001
Intact FGF23 (pg/mL)	48.8 (32.9 – 68.8)	132.5 (75.1 – 238.9)	0.002

P value is for between group difference, examined using unpaired t-test or Kruskal-Wallis test for normal and skewed continuous variables respectively, and chi-squared test for categorical variables.

Mean ± SD, number (percentage) or median (interquartile range)

*eGFR - calculated using CKD–Epidemiology Collaboration equation

Abbreviations: CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FGF23, fibroblast growth factor-23; PTH, intact parathyroid hormone

Table 3.3: Medications for CKD group

Medication	Number taking
Calcium carbonate	1 (7.1)
Cholecalciferol	6 (42.9)
Prednisolone	4 (28.6)
Non-steroid immune suppressant	3 (21.4)
Diuretic	7 (50.0)
ACE-I, ARB or MRA	12 (85.7)
Other anti-hypertensive agent	5 (35.7)
Statin	4 (28.6)

Medications taken at time of study for CKD group. Healthy controls were not taking regular medications. Results are presented as number (percentage).

Abbreviations: ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; MRA, mineralocorticoid receptor antagonist.

3.3.2 Novel markers of mineral metabolism

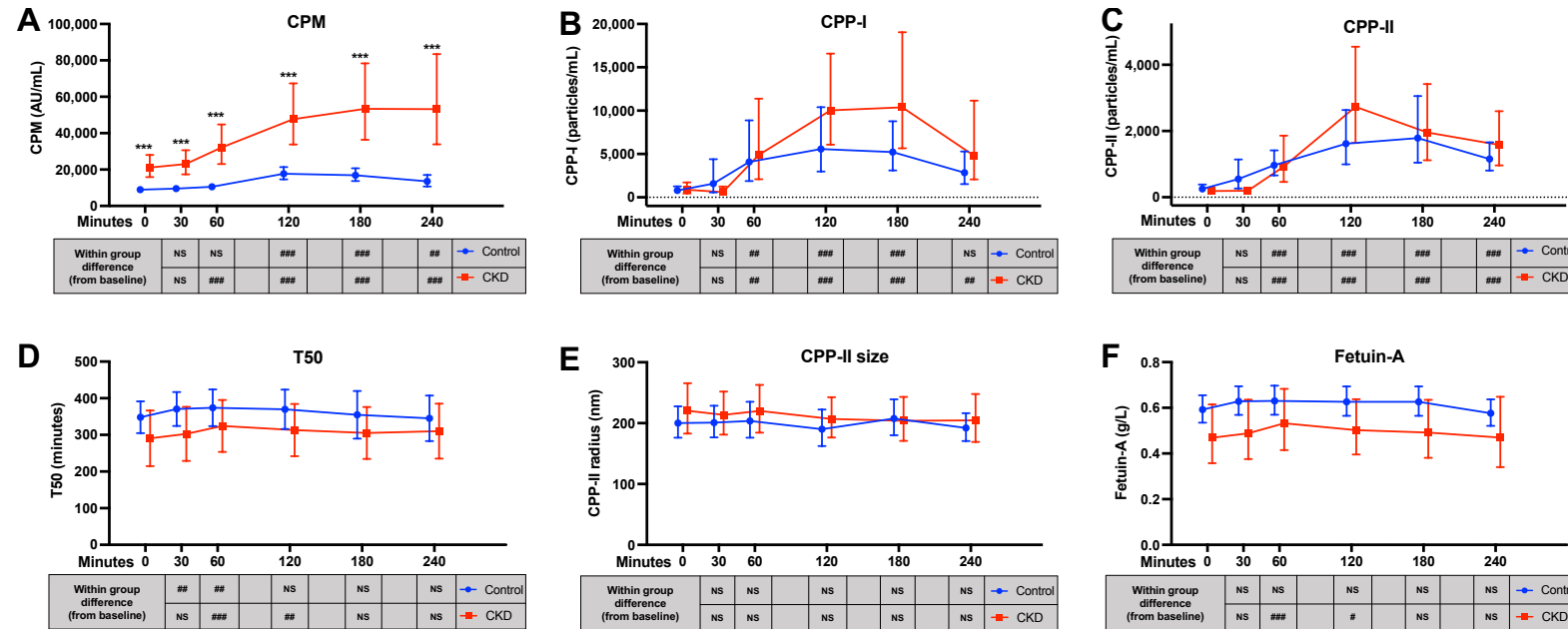
Levels of novel markers of mineral metabolism at fasting and after the standardised meal for each group are depicted in Figure 3.1. Summary data and group-by-time coefficient estimates are provided in Table 3.4.

CPM

Serum CPM was significantly higher in CKD participants than in controls when fasting, and at each post-prandial timepoint (Figure 3.1A). A significant increase in serum CPM was observed post meal within each group. This increase began at 120 minutes post meal in the control group and 60 minutes post meal in the CKD group. Levels of CPM remained above fasting levels in both groups at the end of the study period (240 minutes).

Group-by-time interaction coefficients showed significant between-group differences for CPM at multiple timepoints, indicating that the post-prandial increase in CPM was proportionately larger in the CKD group than in controls (Table 3.4). The peak difference between groups was seen at 240 minutes, where the increase from fasting levels was 73.3% higher in the CKD group than in controls (95% CI 30.8, 129.6, $p < 0.001$). To further explore the apparent effect of kidney function in post-prandial profile, CPM AUC, maximum concentration, and time of maximum concentration were also examined (Figure 3.2). There was strong, inverse correlation between eGFR and CPM AUC ($r = -0.806$, $p < 0.001$) and maximum CPM concentration ($r = -0.779$, $p < 0.001$). In addition, the time of maximum concentration for CPM occurred significantly later in the CKD group compared to controls.

Figure 3.1: Post-prandial response of novel markers of mineral metabolism after standardised meal in participants with normal and impaired kidney function



Repeated measures of novel markers of mineral metabolism depicted for control and CKD group when fasting (“0”) and then post consumption of a standardised meal. Data are presented as mean and 95% confidence interval or geometric mean and 95% confidence interval. Data points have been offset for clarity. Between-group and within-group pairwise comparisons shown for each timepoint, with Bonferroni correction for multiple comparisons.

(A) Calciprotein monomers (CPM); (B) Primary calciprotein particles (CPP-I); (C) Secondary calciprotein particles (CPP-II); (D) T50; (E) Secondary calciprotein particle size (CPP-II size); (F) Fetuin-A.

*** $p < 0.001$ for CKD group versus healthy controls. NS: $p \geq 0.05$ versus group baseline; ##: $p < 0.01$ versus group baseline; ###: $p < 0.001$ versus group baseline

Table 3.4: Linear mixed-effect models for novel markers of mineral metabolism

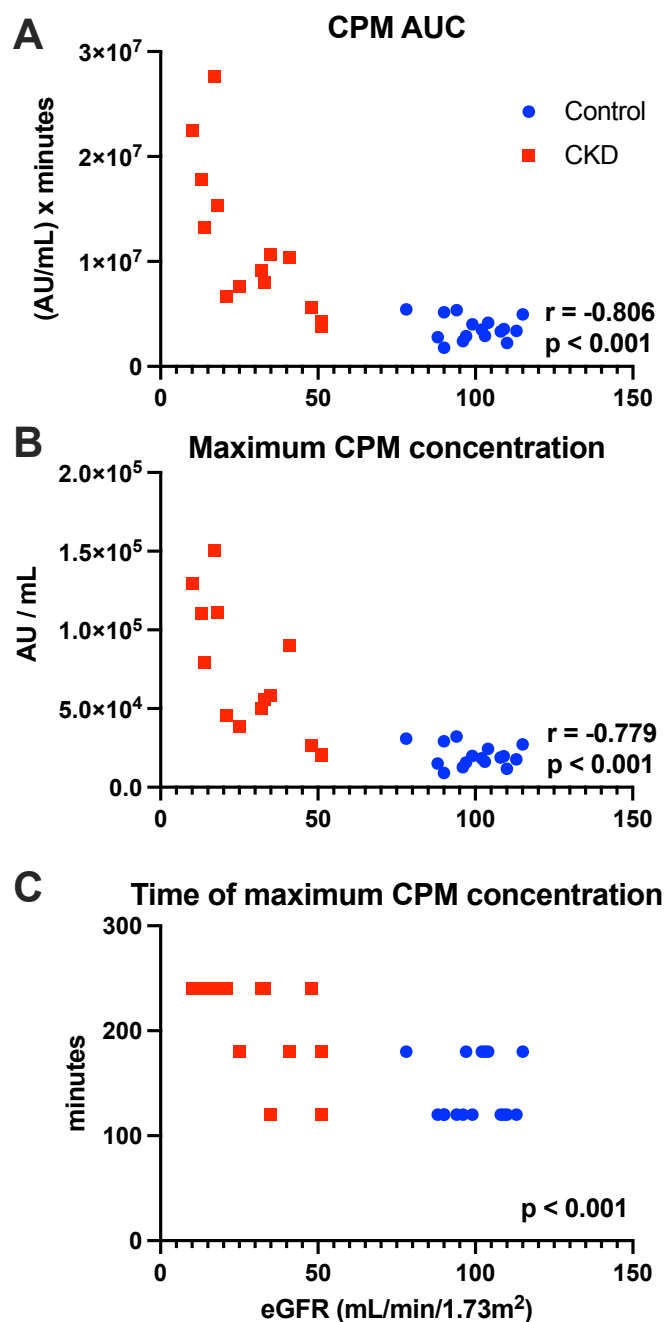
	CPM x10 ³ AU/mL	CPP-I x10 ³ /mL	CPP-II x10 ² /mL	T50 minutes	CPP-II size nm	Fetuin-A g/L
Group mean $\pm$ SD or median (IQR)						
<i>Healthy Control</i>						
Fasting	8.5 (7.8 - 11.0)	0.8 (0.4 - 1.6)	2.0 (1.3 - 4.3)	348 $\pm$ 43	204.1 (167.5 - 248.9)	0.602 $\pm$ 0.112
+30 minutes	9.7 (8.1 - 11.1)	1.1 (0.3 - 3.7)	4.7 (2.0 - 13.3)	371 $\pm$ 46	194.2 (170.7 - 261.4)	0.639 $\pm$ 0.115
+60 minutes	10.7 (8.4 - 13.2)	3.3 (1.5 - 12.0)	8.7 (5.3 - 12.7)	374 $\pm$ 50	194.0 (168.0 - 266.2)	0.641 $\pm$ 0.122
+120 minutes	18.3 (13.2 - 21.6)	5.4 (3.1 - 10.7)	13.0 (9.3 - 34.7)	370 $\pm$ 54	171.9 (163.0 - 225.8)	0.637 $\pm$ 0.125
+180 minutes	16.5 (13.5 - 23.8)	4.6 (2.9 - 8.4)	16.3 (11.3 - 41.3)	355 $\pm$ 65	199.0 (175.7 - 259.0)	0.637 $\pm$ 0.125
+240 minutes	14.3 (10.8 - 18.5)	3.7 (1.2 - 8.3)	10.7 (8.0 - 16.7)	345 $\pm$ 62	181.9 (164.2 - 233.4)	0.586 $\pm$ 0.112
<i>CKD</i>						
Fasting	21.5 (18.9 - 27.5)	1.2 (0.4 - 1.9)	1.3 (1.3 - 1.7)	291 $\pm$ 76	217.2 (180.7 - 275.1)	0.512 $\pm$ 0.199
+30 minutes	23.2 (18.7 - 30.8)	0.6 (0.2 - 1.7)	1.3 (1.3 - 2.7)	303 $\pm$ 74	216.4 (176.8 - 251.0)	0.532 $\pm$ 0.209
+60 minutes	31.9 (22.0 - 45.6)	4.8 (2.0 - 13.7)	6.3 (5.3 - 15.3)	324 $\pm$ 71	229.6 (185.4 - 246.9)	0.577 $\pm$ 0.229
+120 minutes	58.3 (29.6 - 68.7)	9.0 (4.8 - 23.3)	22.3 (17.3 - 32.7)	313 $\pm$ 71	213.0 (172.7 - 240.7)	0.538 $\pm$ 0.207
+180 minutes	49.0 (35.5 - 89.8)	8.9 (5.7 - 18.8)	17.7 (12.0 - 30.0)	305 $\pm$ 71	202.4 (157.6 - 238.0)	0.532 $\pm$ 0.214
+240 minutes	49.9 (33.5 - 110.3)	7.5 (1.9 - 14.3)	15.0 (9.3 - 36.7)	310 $\pm$ 75	215.3 (163.4 - 240.2)	0.525 $\pm$ 0.223
Coefficient estimate (95% CI) for CKD group-by-time interaction						
30 minutes	2.7% (-11.0, 18.5)	-64.2% (-87.3, 1.0)	-50.3% (-77.7, 10.5)	-10.65 (-27.47, 6.17)	-3.5% (-10.7, 4.3)	-0.02 (-0.05, 0.02)
60 minutes	28.5%** (8.7, 51.7)	9.2% (-61.7, 211.7)	30.5% (-40.9, 188.3)	7.71 (-10.83, 26.25)	-2.0% (-9.9, 6.5)	0.03 (-0.01, 0.06)
120 minutes	6.2% (-13.1, 29.8)	64.2% (-44.5, 385.5)	130.1%* (4.1, 408.6)	7.76 (-13.48, 29.01)	-8.0% (-16.3, 1.2)	0.01 (-0.02, 0.05)
180 minutes	33.9%* (5.5, 69.9)	81.2% (-41.6, 461.9)	49.2% (-32.5, 229.9)	7.67 (-16.54, 31.89)	-10.9%* (-19.5, -1.3)	0.01 (-0.03, 0.05)
240 minutes	73.3%*** (30.8, 129.6)	54.2% (-53.1, 407.1)	87.0% (-15.5, 313.8)	18.63 (-9.27, 46.52)	-2.9% (-13.4, 8.9)	0.02 (-0.02, 0.07)

Table shows group mean $\pm$ SD or median (IQR) or linear mixed-effects model coefficient (95% CI) for group-by-time interaction. For each linear mixed-effects model, time 0 (fasting) and control group were used as the reference category for time and group, respectively. CPM, CPP-I, CPP-II and CPP-II radius were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to give a percentage change from the referent category.

*p <0.05, **p <0.01, *** p <0.001.

Abbreviations: CPM, calciprotein monomers; CPP-I, primary calciprotein particles; CPP-II, secondary calciprotein particles.

Figure 3.2: Pharmacokinetics of calciprotein monomers in relation to kidney function (eGFR)



Pharmacokinetics of calciprotein monomers (CPM) in relation to kidney function (estimated glomerular filtration rate [eGFR]). (A) Area under the curve calculated using cubic spline method for time 0 until 240 minutes. (B) Maximum CPM concentration. (C) Time of maximum CPM concentration.

P value for Pearson correlation coefficient (“r”) shown for AUC and maximum concentration. P value for time to maximum concentration calculated by chi squared test, using group and categorical time.

CPP-I and CPP-II

Significant post-prandial excursions were observed within both groups for CPP-I and for CPP-II (Figure 3.1B & 3.1C). For CPP-I, serum levels were significantly increased from fasting baseline in both groups at 60 minutes. At the end of the observation period, CPP-I remained significantly higher than fasting values in the CKD group but were comparable to baseline in the control group. For CPP-II, significant post-prandial increases were evident in both groups at 60 minutes, and levels remained above fasting values at the end of the study period.

There was no significant difference between group means in pairwise comparisons at any timepoint for CPP-I or CPP-II (Figure 3.1B & 3.1C). However, there was a significant group-by-time interaction for CPP-II at 120 minutes (+130.1% for the CKD group [95% CI 4.1, 408.6], $p = 0.039$) (Table 3.4). Refitting LMMs for CPP-I and CPP-II after re-imputing left censored values using Tobit regression did not materially alter coefficient estimates, or *post hoc* between group or within group pairwise comparisons (Table 3.5 and 3.6). In contrast to CPM, we found no association between eGFR and the AUC, maximal concentration or time to maximum concentration for serum CPP-I or CPP-II (Figure 3.3).

Table 3.5: Linear mixed-effect model and post hoc pairwise comparisons for serum CPP-I after re-imputation of left censored values using Tobit regression

Timepoint	CPP-I (x10 ³ /mL)		
<i>Healthy Control</i>	Group median (IQR)	Between group difference	Within group difference
Fasting	0.8 (0.4 – 1.6)	-	-
+30 minutes	1.1 (0.5 – 3.7)	-	NS
+60 minutes	3.3 (1.5 – 12.0)	-	##
+120 minutes	5.4 (3.1 – 10.7)	-	###
+180 minutes	4.6 (2.9 – 8.4)	-	###
+240 minutes	3.7 (1.2 – 8.3)	-	NS
<i>CKD</i>			
Fasting	1.2 (0.5 – 1.9)	NS	-
+30 minutes	0.9 (0.4 – 1.7)	NS	NS
+60 minutes	4.8 (2.0 – 13.7)	NS	##
+120 minutes	9.0 (4.8 – 23.3)	NS	###
+180 minutes	8.9 (5.7 – 18.8)	NS	###
+240 minutes	7.5 (1.9 – 14.3)	NS	##
	Between group coefficient estimate (95% CI)		
+30 minutes	-56.0% (-83.8, 19.1)		
+60 minutes	6.9% (-60.4, 188.7)		
+120 minutes	60.7% (-41.1, 338.3)		
+180 minutes	77.3% (-35.9, 390.2)		
+240 minutes	71.4% (-39.0, 381.9)		

Linear mixed-effect model and *post hoc* pairwise comparisons repeated after 8 left censored values for CPP-I were re-imputed using multi-level Tobit regression. Table shows median (IQR), linear mixed-effects model coefficient (95% CI) for group-time interaction, and *post hoc* pairwise comparisons (with Bonferroni correction for multiple comparisons). Values were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to derive estimates of percentage change. *Post hoc* pairwise comparisons test for differences in mean values between groups at each timepoint, and to test for deviation from own fasting baseline within each group.

NS: $p \geq 0.05$; ##: $p < 0.01$; ###: $p < 0.001$.

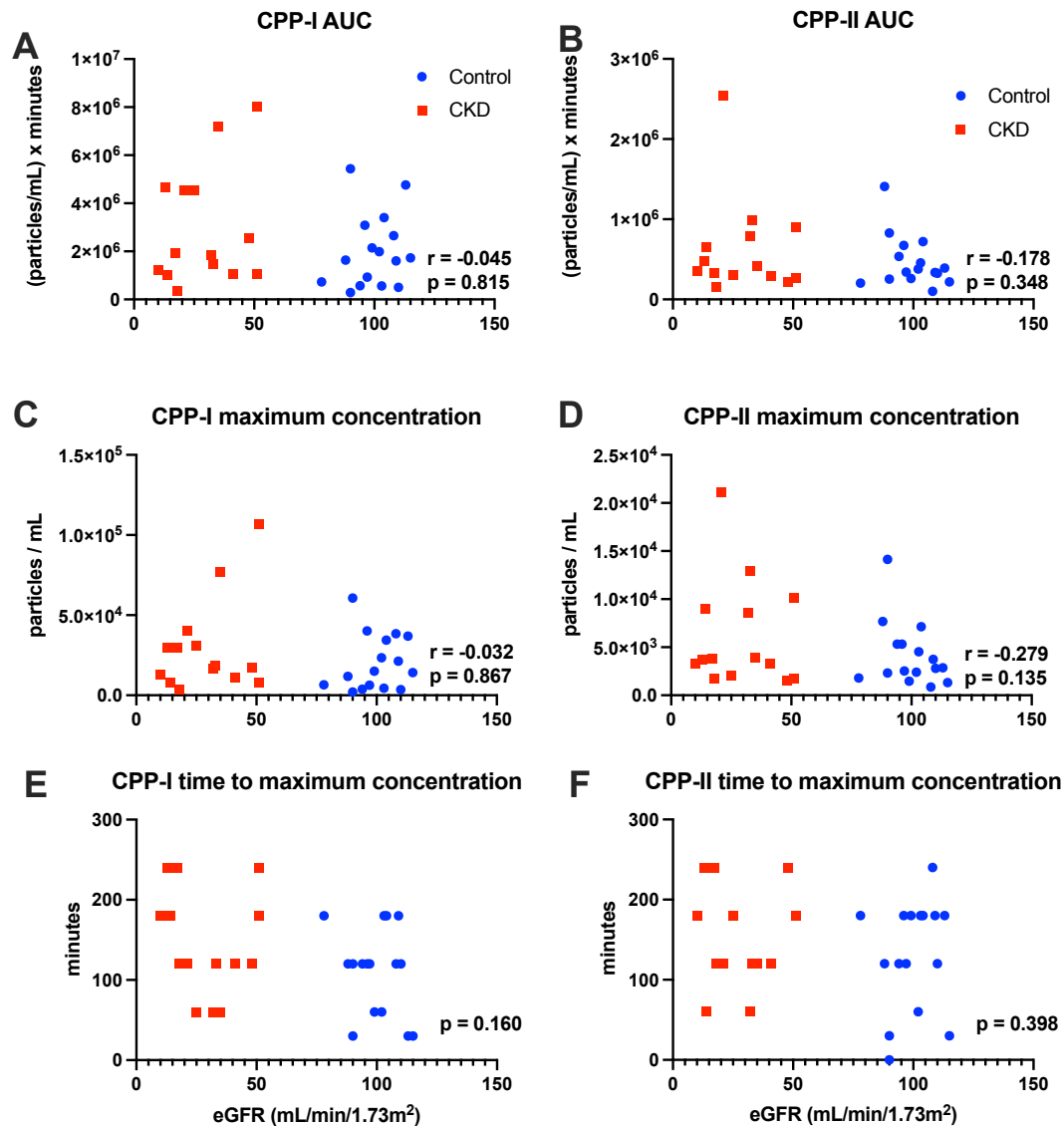
Table 3.6: Linear mixed-effect model and post hoc pairwise comparisons for serum CPP-II after re-imputation of left censored values using Tobit regression

Timepoint	CPP-II (x10 ² /mL)		
<i>Healthy Control</i>	Group median (IQR)	Between group difference	Within group difference
Fasting	2.0 (1.5 – 4.3)	-	-
+30 minutes	4.7 (2.8 – 13.3)	-	NS
+60 minutes	8.7 (5.3 – 12.7)	-	###
+120 minutes	13.0 (9.3 – 34.7)	-	###
+180 minutes	16.3 (12.0 – 41.3)	-	###
+240 minutes	10.7 (8.0 – 16.7)	-	###
<i>CKD</i>			
Fasting	1.3 (1.3 – 1.7)	NS	-
+30 minutes	2.4 (2.4 – 2.7)	NS	NS
+60 minutes	8.0 (5.3 – 15.3)	NS	###
+120 minutes	22.3 (17.3 – 32.7)	NS	###
+180 minutes	17.7 (12.0 – 30.0)	NS	###
+240 minutes	15.0 (9.3 – 36.7)	NS	###
	Between group coefficient estimate (95% CI)		
+30 minutes	-35.8% (-69.7, 36.1)		
+60 minutes	59.6% (-24.2, 236.1)		
+120 minutes	146.4%[#] (16.9, 419.5)		
+180 minutes	34.9% (-36.1, 184.8)		
+240 minutes	100.2% (-5.3, 323.5)		

Linear mixed-effect model and *post hoc* pairwise comparisons repeated after 15 left censored values for CPP-II were re-imputed using multi-level Tobit regression. Table shows median (IQR), linear mixed-effects model coefficient (95% CI) for group-time interaction, and *post hoc* pairwise comparisons (with Bonferroni correction for multiple comparisons). Values were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to derive estimates of percentage change. *Post hoc* pairwise comparisons test for differences in mean values between groups at each timepoint, and to test for deviation from own fasting baseline within each group.

NS: $p \geq 0.05$; #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$.

Figure 3.3: Pharmacokinetics of CPP-I and CPP-II in relation to kidney function (eGFR)



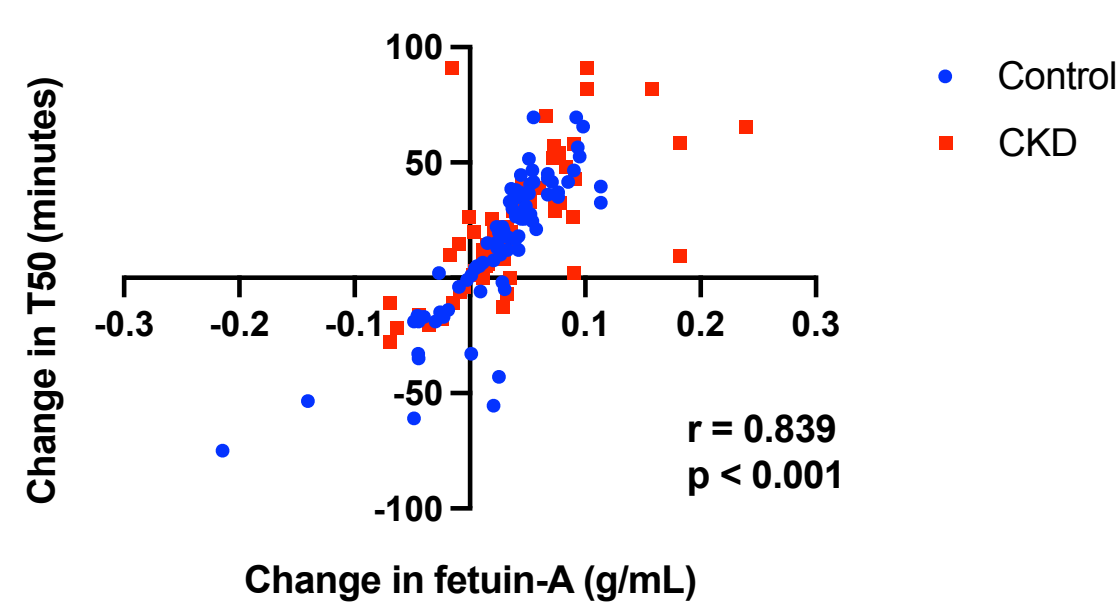
Pharmacokinetics of primary (CPP-I) and secondary (CPP-II) calciprotein particles in relation to kidney function (estimated glomerular filtration rate [eGFR]). (A & B) Area under the curve calculated using cubic spline method for time 0 until 240 minutes. (C & D) Maximum CPP-I/ CPP-II concentration. (E & F) Time of maximum CPP-I/ CPP-II concentration.

P value for Pearson correlation coefficient (“r”) shown for AUC and maximum concentration. P value for time to maximum concentration calculated by chi squared test, using group and categorical time.

T50, CPP-II size, fetuin-A and citrate

In both control and CKD groups, early but transient within-group increases in T50 from fasting levels were observed (Figure 3.1D). This was evident at 30 and 60 minutes in the control group and at 60 and 120 minutes in the CKD group. There were no between-group pairwise differences, or significant group-by-time interactions for T50 at any timepoint. Within each group, CPP-II size appeared stable over the post-prandial period, with no significant excursions from baseline values in either group (Figure 3.1E). There were no significant pairwise differences between groups at any timepoint, however at 180 minutes there was a modest, but significant, group-by-time interaction (-10.9% for CKD group [95% CI -19.5, -1.3] $p = 0.027$). For serum fetuin-A, there were no significant pairwise group differences, or group by time interactions (Figure 3.1F). However, in the CKD group, there was a transient post-prandial increase in fetuin-A, which was coincident with the increase in T50. In further *ad hoc* exploratory analysis of all participants, a strong correlation between deviation in T50 and fetuin-A from baseline was evident, irrespective of renal function ($r = 0.839$, $p < 0.001$; Figure 3.4). Since the study meal was noted to contain citrate, a known inhibitor of CPP formation and modulator of T50,²³⁸ we measured serum citrate concentrations in available stored serum at baseline, 30, and 60 minutes. Although a small increase in serum citrate was observed in the CKD group at 30 minutes (Table 3.7), this did not coincide with the observed changes in T50 in this group, nor was there any overall correlation between change in T50 and serum citrate (Figure 3.5).

Figure 3.4: Change in T50 and serum fetuin-A from baseline



Maximum increase in T50 and serum fetuin-A from baseline.

r = Pearson correlation coefficient

Table 3.7: Linear mixed-effect model and post hoc pairwise comparisons for serum citrate

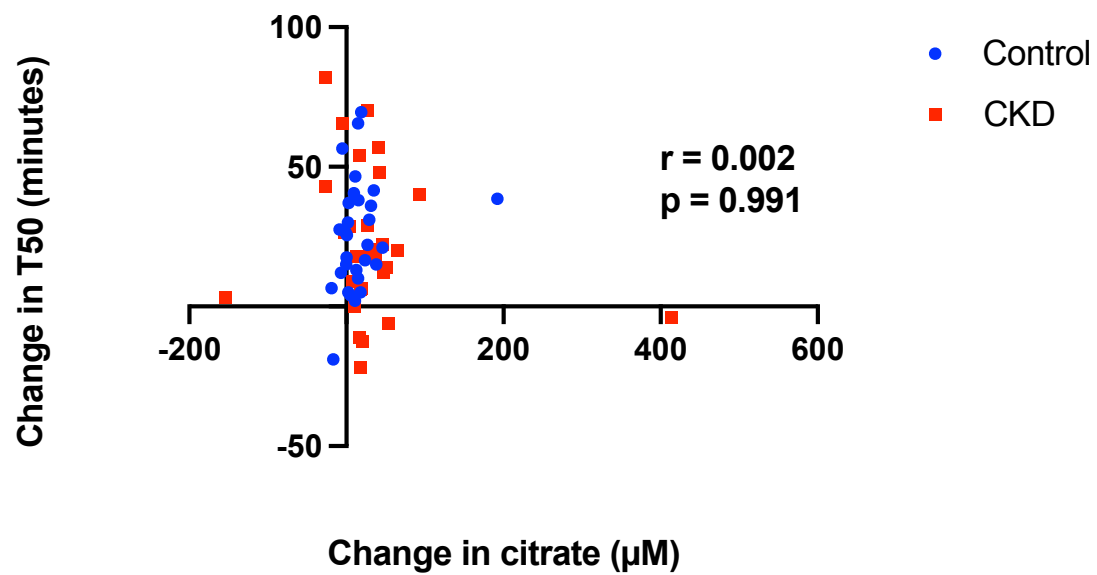
Timepoint	Citrate (μM)		
<i>Healthy Control</i>	Group median (IQR)	Between group difference	Within group difference
Fasting	77.5 (69.7 – 88.5)	-	-
+30 minutes	85.0 (76.3 – 107.1)	-	NS
+60 minutes	88.1 (82.1 – 112.5)	-	NS
<i>CKD</i>			
Fasting	65.4 (55.2 – 73.9)	NS	-
+30 minutes	83.0 (80.1 – 101.1)	NS	##
+60 minutes	84.5 (72.7 – 97.7)	NS	NS
	Between group coefficient estimate (95% CI)		
+30 minutes	21.0% (-6.1, 56.0)		
+60 minutes	8.7% (-16.7, 41.8)		

Table shows median (IQR), linear mixed-effects model coefficient (95% CI) for group-time interaction, and *post hoc* pairwise comparisons (with Bonferroni correction for multiple comparisons). Values were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to derive estimates of percentage change. *Post hoc* pairwise comparisons test for differences in mean values between groups at each timepoint, and to test for deviation from own fasting baseline within each group.

Testing of three serves of the standard meal used showed a mean of 922.2 mg of citrate per 250mL serve.

NS: $p \geq 0.05$; ##: $p < 0.01$.

Figure 3.5: Change in T50 and serum citrate from baseline



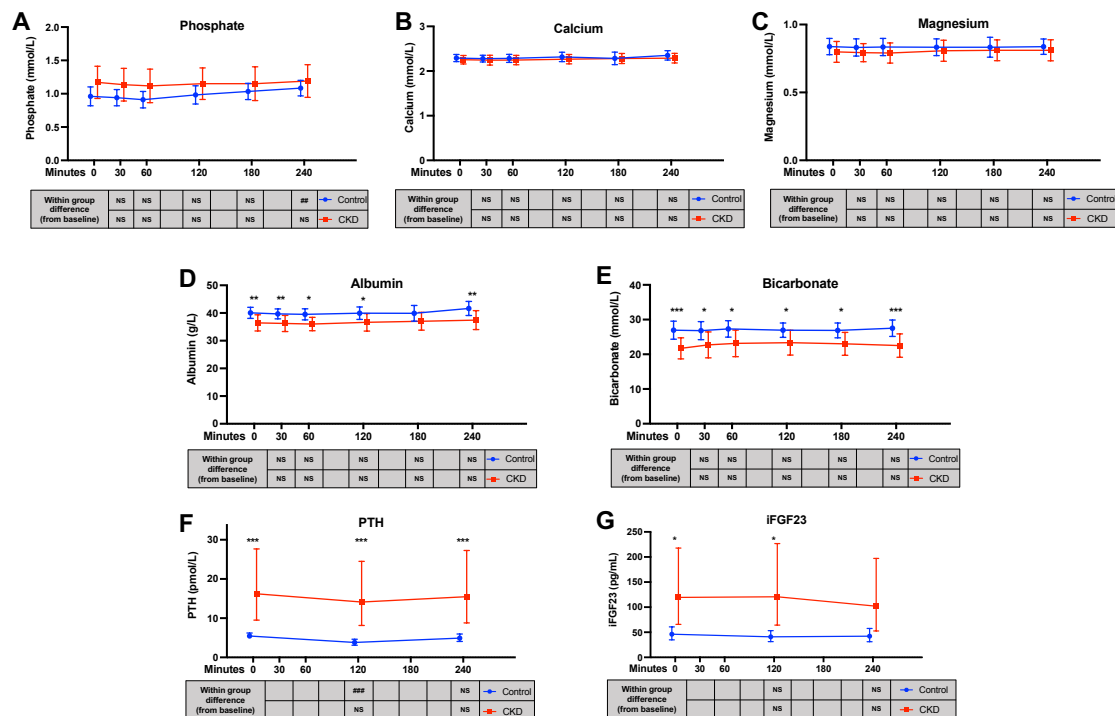
3.3.3 Conventional biochemical markers

Fasting and post-prandial levels of conventional biochemical markers are depicted in Figure 3.6, and summary data and group-by-time coefficient estimates are also shown in Table 3.8 and Table 3.9.

There was a trend for serum phosphate to increase from fasting levels post meal in the control group, which reached significance at 240 minutes (Figure 3.6A). By comparison, no within group post-prandial change was observed in the CKD group. This subtle difference in post-prandial response between groups was reflected in significant group-by-time interactions at 180 minutes (-0.09 mmol/L for CKD group [95% CI -0.17, -0.02], $p = 0.018$) and at 240 minutes (-0.10 mmol/L [95% CI -0.19, -0.02] $p = 0.019$). For serum calcium and magnesium, there were no significant between-group or within-group differences, nor significant group-by-time interactions (Figure 3.6B, 3.6C, Table 3.8). Serum albumin was lower in the CKD group compared to controls at all but one timepoint and remained unchanged from baseline levels in both groups post-prandially (Figure 3.6D). Serum bicarbonate was higher in control than in CKD participants at all timepoints (Figure 3.6E). There was no significant deviation from baseline in either group, however there was a significant group-by-time interaction at 120 minutes (+1.64 mmol/L for CKD group [95% CI 0.25, 3.04] $p = 0.021$). This coincided with the T50 peak in the CKD group, and in *ad hoc* analysis, there was correlation between the deviation in T50 and bicarbonate from baseline (Figure 3.7), albeit this was weak compared to the apparent interdependence of T50 and fetuin-A (Figure 3.4).

With respect to the major hormonal mineral regulators, serum PTH was significantly higher in the CKD group than in controls at all measured timepoints (Figure 3.6F). There was a transient drop in PTH from fasting to 120 minutes in the control group, which was not seen in the CKD group. This corresponded to a significant group-by-time interaction at 120 minutes (+25.2% for CKD group [95% 5.5, 48.5] $p = 0.01$). Serum iFGF23 was significantly higher in the CKD than control group at baseline and 120 minutes, but not at 240 minutes (Figure 3.6G). No significant post-prandial within group changes were observed.

Figure 3.6: Post-prandial response of conventional markers of mineral metabolism after standardised meal in participants with normal and impaired kidney function



Repeated measures depicted for control and CKD group when fasting (“0”) and then post consumption of a standardised meal. Data are presented as mean and 95% confidence interval or geometric mean and 95% confidence interval. Data points have been offset for clarity. Between-group and within-group pairwise comparisons shown for each timepoint, with Bonferroni correction for multiple comparisons.

(A) Phosphate; (B) Calcium; (C) Magnesium; (D) Albumin; (E) Bicarbonate; (F) Intact parathyroid hormone (PTH); (G) Intact fibroblast growth factor-23 (iFGF23).

* $p < 0.05$ for CKD group versus healthy controls; ** $p < 0.01$ for CKD group versus healthy controls; *** $p < 0.001$ for CKD group versus healthy controls. NS: $p \geq 0.05$ versus group baseline; ###: $p < 0.01$ versus group baseline; ####: $p < 0.001$ versus group baseline.

Table 3.8: Linear mixed-effect models for conventional biochemical markers

	Phosphate mmol/L	Calcium mmol/L	Magnesium mmol/L	Albumin g/L	Bicarbonate mmol/L
Group mean $\pm$ SD					
<i>Healthy Control</i>					
Fasting	0.96 $\pm$ 0.14	2.29 $\pm$ 0.08	0.84 $\pm$ 0.06	40 $\pm$ 2.0	27 $\pm$ 2.6
+30 minutes	0.94 $\pm$ 0.12	2.28 $\pm$ 0.07	0.83 $\pm$ 0.06	40 $\pm$ 1.8	27 $\pm$ 2.6
+60 minutes	0.91 $\pm$ 0.12	2.28 $\pm$ 0.09	0.83 $\pm$ 0.06	40 $\pm$ 2.0	27 $\pm$ 2.4
+120 minutes	0.98 $\pm$ 0.14	2.32 $\pm$ 0.11	0.83 $\pm$ 0.06	40 $\pm$ 2.2	27 $\pm$ 2.1
+180 minutes	1.03 $\pm$ 0.12	2.28 $\pm$ 0.14	0.83 $\pm$ 0.07	40 $\pm$ 2.8	27 $\pm$ 2.2
+240 minutes	1.08 $\pm$ 0.12	2.35 $\pm$ 0.11	0.84 $\pm$ 0.06	42 $\pm$ 2.6	28 $\pm$ 2.4
<i>CKD</i>					
Fasting	1.17 $\pm$ 0.24	2.25 $\pm$ 0.10	0.80 $\pm$ 0.08	36 $\pm$ 2.9	22 $\pm$ 3.0
+30 minutes	1.14 $\pm$ 0.25	2.24 $\pm$ 0.11	0.79 $\pm$ 0.07	36 $\pm$ 2.9	23 $\pm$ 3.7
+60 minutes	1.12 $\pm$ 0.25	2.25 $\pm$ 0.11	0.79 $\pm$ 0.07	36 $\pm$ 2.4	23 $\pm$ 3.8
+120 minutes	1.15 $\pm$ 0.24	2.27 $\pm$ 0.11	0.81 $\pm$ 0.08	37 $\pm$ 3.2	23 $\pm$ 3.6
+180 minutes	1.15 $\pm$ 0.25	2.28 $\pm$ 0.11	0.81 $\pm$ 0.08	37 $\pm$ 3.2	23 $\pm$ 3.3
+240 minutes	1.19 $\pm$ 0.25	2.29 $\pm$ 0.11	0.81 $\pm$ 0.08	37 $\pm$ 3.4	23 $\pm$ 3.4
Between group coefficient estimate (95% CI)					
+30 minutes	-0.01 (-0.07, 0.04)	0.00 (-0.05, 0.05)	0.00 (-0.02, 0.02)	0.16 (-0.84, 1.17)	1.13 (-0.09, 2.34)
+60 minutes	0.00 (-0.07, 0.06)	0.00 (-0.05, 0.05)	0.00 (-0.03, 0.02)	0.13 (-0.95, 1.22)	1.17 (-0.12, 2.47)
+120 minutes	-0.04 (-0.11, 0.03)	-0.01 (-0.06, 0.04)	0.01 (-0.01, 0.04)	0.34 (-0.86, 1.54)	1.64* (0.25, 3.04)
+180 minutes	-0.09* (-0.17, -0.02)	0.04 (-0.02, 0.09)	0.02 (-0.01, 0.05)	0.76 (-0.59, 2.11)	1.35 (-0.18, 2.88)
+240 minutes	-0.10* (-0.19, -0.02)	-0.03 (-0.09, 0.03)	0.01 (-0.02, 0.04)	-0.56 (-2.08, 0.96)	0.22 (-1.47, 1.92)

Table shows group mean $\pm$ SD or median (IQR) or linear mixed-effects model coefficient (95% CI) for group-by-time interaction. For each linear mixed-effects model, time 0 (fasting) and control group were used as the reference category for time and group respectively.

*p < 0.05

Table 3.9: Linear mixed-effect models for PTH and iFGF23

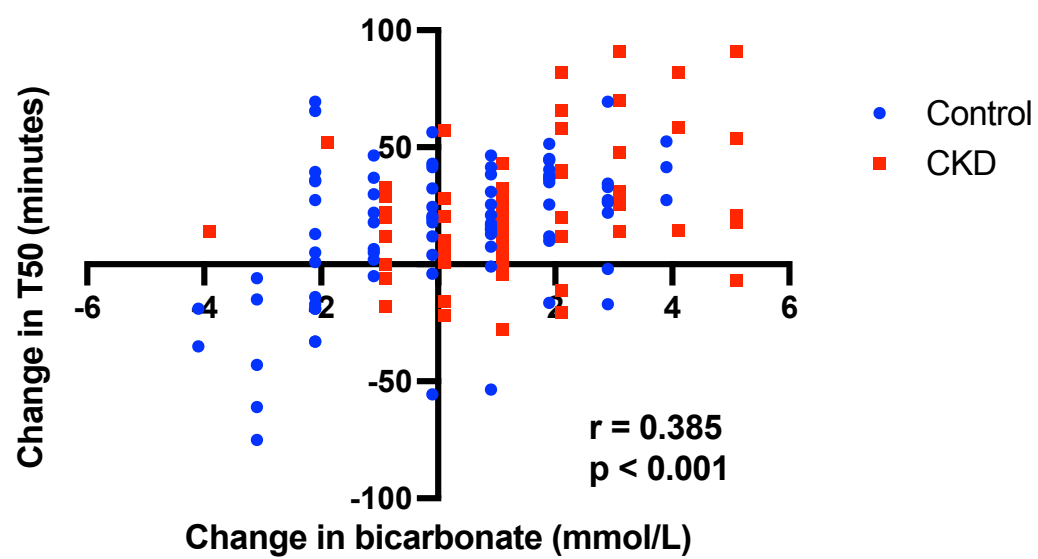
	PTH pmol/L	iFGF23 pg/mL
Group mean $\pm$ SD		
<i>Healthy Control</i>		
Fasting	5.2 (4.6 - 6.8)	48.8 (32.9 - 68.8)
+120 minutes	4.3 (3.1 - 5.0)	40.1 (29.7 - 58.5)
+240 minutes	5.3 (4.4 - 5.9)	42.9 (32.9 - 59.8)
<i>CKD</i>		
Fasting	16.9 (7.1 - 31.0)	132.5 (75.1 - 238.9)
+120 minutes	15.4 (5.3 - 30.5)	114.8 (62.7 - 240.9)
+240 minutes	18.4 (5.5 - 29.3)	99.1 (72.3 - 194.6)
Between group coefficient estimate (95% CI)		
+120 minutes	25.2%* (5.5, 48.5)	7.8% (-12.9, 33.5)
+240 minutes	6.1% (-12.3, 28.3)	-7.1% (-27.9, 19.8)

Table shows group mean $\pm$ SD or median (IQR) or linear mixed-effects model coefficient (95% CI) for group-by-time interaction. For each linear mixed-effects model, time 0 (fasting) and control group were used as the reference category for time and group, respectively. PTH and iFGF23 were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to give a percentage change from the referent category.

*p < 0.05.

Abbreviations: PTH, intact parathyroid hormone; iFGF23, intact fibroblast growth factor-23.

Figure 3.7: Change in T50 and serum bicarbonate from baseline



Maximum increase in T50 and serum bicarbonate from baseline.

r = Pearson correlation coefficient

Data points have been offset on the x-axis to improve clarity.

3.4 Discussion

To the best of our knowledge, this is the first study to report a significant post-prandial effect of nutritional intake on serum levels of CPM, CPP-I and CPP-II in humans. These effects were common to individuals with normal and impaired kidney function; however, the post-prandial excursion of serum CPM was much more pronounced in CKD participants. An early and transient post-prandial effect on T50 was also found, which was present regardless of kidney function, and accompanied by a concomitant increase in serum fetuin-A.

These findings are consistent with the notion that intestinal absorption of a dietary mineral load can directly lead to formation of circulating CPM, CPP-I and CPP-II. This has previously been proposed,^{187, 188, 273} but based largely on animal data.^{206, 225} In contrast, evidence in humans has been indirect, coming from studies of intestinal phosphate binders in haemodialysis dependent CKD patients.^{226, 227, 252} A small study by Yamada *et al* suggested diurnal variation in serum CPP with post-prandial peaks, however this study used the older fetuin-A based method, that was unable to separately quantify CPP sub-species, and participants were all hospitalised for management of unstable diabetes.²²⁸ In contrast, all patients in this study were clinically stable, and as far as we are aware, for the first time it has been demonstrated that post-prandial excursions are seen not only in individuals with CKD, but also in healthy adults, substantiating a role of CPM, CPP-I and CPP-II formation in the normal physiological response to the ingestion of food.

Surges of serum CPP were seen even after relatively modest, and physiologically relevant, nutritional mineral loads (Table 3.1). In contrast, there was minimal post-prandial variation in the more conventional markers of mineral metabolism. Previous studies have similarly shown limited post-prandial deviation in phosphate,^{49, 271} except when subjects are challenged with large pharmacological loads.^{280, 281} In health, total body phosphate is regulated, such that net intestinal absorption is matched by urinary excretion.²³³ However, this response is not instantaneous, and a lag of several hours may be seen before augmentation of urinary phosphate excretion occurs, even when the phosphate load is given intravenously.²³³ Instead, animal models have demonstrated that other, non-renal, mechanisms serve to maintain serum ionic concentrations more acutely,

via distribution to bone and other tissues.²⁸¹⁻²⁸³ It is plausible that formation of CPP may be an important additional temporary depot of phosphate (and calcium), that is able to acutely buffer local mineral loads, such as from the gastrointestinal system.¹⁸⁶ The physiochemical properties of CPP mean that potentially large quantities of otherwise insoluble mineral can exist in the circulation without risk of precipitation, which ostensibly facilitates mineral to be safely transported in bulk to sites of use or clearance.

A transient increase in serum fetuin-A was observed in the CKD group. In contrast, fetuin-A appeared stable in controls, however, when pairwise comparisons were repeated without correction for multiple comparisons, there were also significant post-prandial increases in fetuin-A in controls (Table 3.10), indicating that this study may have been underpowered to detect an underlying effect. Beyond being a negative acute phase reactant, with levels strongly suppressed in response to acute and chronic inflammation,¹⁷² little is known about other mechanisms that directly regulate the hepatic synthesis and secretion of fetuin-A, and there is a paucity of previous data about diurnal, or acute post-prandial variation in any species. Given the observed rise in serum fetuin-A after feeding, especially in the CKD group, and the requisite role of fetuin-A in CPM and CPP-I/II formation, it is intriguing to consider whether feeding may be “sensed” via a yet unknown mechanism, leading to hepatic release of fetuin-A to coincide with an influx of mineral from the intestines. Indeed, Uedono *et al* recently suggested that CPP itself, may be a trigger for fetuin-A expression in cultured hepatocytes.²⁸⁴ Thus, mechanisms controlling fetuin-A release and their response to mineral loading warrant further investigation.

Table 3.10: Post-hoc pairwise comparisons for serum fetuin-A showing with and without Bonferroni correction method to adjust for multiple comparisons

Fetuin-A		Corrected (Bonferroni)		Uncorrected	
	Difference	95% CI	p value	95% CI	p value
Between group					
Diff at baseline	-0.090	-0.295, 0.116	1.000	-0.209, 0.030	0.142
Diff at 30	-0.107	-0.313, 0.100	1.000	-0.227, 0.014	0.083
Diff at 60	-0.064	-0.272, 0.144	1.000	-0.185, 0.057	0.300
Diff at 120	-0.077	-0.288, 0.133	1.000	-0.200, 0.045	0.216
Diff at 180	-0.084	-0.297, 0.129	1.000	-0.208, 0.040	0.184
Diff at 240	-0.065	-0.281, 0.151	1.000	-0.191, 0.061	0.312
Control group					
30 vs baseline	0.037	-0.002, 0.075	0.094	0.014, 0.059	0.001
60 vs baseline	0.039	-0.001, 0.079	0.078	0.015, 0.062	0.001
120 vs baseline	0.035	-0.008, 0.079	0.423	0.010, 0.060	0.006
180 vs baseline	0.035	-0.012, 0.082	0.810	0.008, 0.063	0.012
240 vs baseline	-0.016	-0.068, 0.036	1.000	-0.046, 0.014	0.296
CKD group					
30 vs baseline	0.020	-0.021, 0.061	1.000	-0.004, 0.044	0.107
60 vs baseline	0.065	0.021, 0.108	0.000	0.039, 0.090	0.000
120 vs baseline	0.048	0.000, 0.095	0.046	0.020, 0.075	0.001
180 vs baseline	0.041	-0.011, 0.092	0.488	0.011, 0.071	0.007
240 vs baseline	0.009	-0.047, 0.065	1.000	-0.024, 0.041	0.599

Post hoc pairwise comparison was performed after fitting LMM to test for differences in mean values for fetuin-A. Differences between groups (CKD v Control) at each timepoint as well as deviation from the fasting baseline within each group were examined. Table shows confidence interval and p value with and without Bonferroni correction method to adjust for multiple comparisons.

T50 is a functional assessment of the serum's ability to resist *ex vivo* CPP-II formation, representing a composite of various potentiating (including calcium and phosphate) and inhibiting (including fetuin-A, albumin, magnesium, citrate and bicarbonate) calcification factors.²³⁷ In this study an early but transient increase in T50 was observed. This was seen in both groups, but in the CKD group, the peak was coincident with increases in serum fetuin-A. Further, among all participants, change in T50 from baseline was very closely correlated with change in serum fetuin-A (Figure 3.4), suggesting that fetuin-A may be the main factor underlying the observed changes in T50. Indeed, no other corresponding early post-prandial changes in other known modulators of T50 (serum phosphate, calcium, bicarbonate, magnesium, or albumin) were found, although this does not exclude more subtle changes in one, or a combination, of these factors. For instance, a positive group-by-time interaction was present for serum bicarbonate in the CKD group at 120 minutes, which overlapped with the increase in T50. As for fetuin-A, there was also correlation between change in bicarbonate and T50 from baseline (Figure 3.7), albeit this was relatively weak compared to the correlation with the former ($r=0.385$ vs. $r=0.839$). Nevertheless, the post-prandial “alkaline tide” is a recognised phenomenon, and it is plausible that multiple factors contributed to the observed post-prandial changes in T50.²⁸⁵ The study meal also contained a surprising amount of citrate (~16mM), a known potent inhibitor of CPP formation, however, although there was a small increase in serum citrate in the CKD group, this change did not coincide with the observed changes in T50, nor was there any overall correlation between change in T50 and serum citrate across both groups. Other previously described modulators of T50, such as pyrophosphate and zinc, were not measured in this study. Although it is possible that these, or other yet unknown, factors may also have contributed to the observed post-prandial increase in T50, it is notable that quite substantial changes in concentration of these small inorganic molecules are generally required to significantly impact T50.⁸⁶ In contrast to the post-prandial effects on T50, the hydrodynamic radius of CPP-II remained ostensibly unchanged in both groups.

While post-prandial excursions of CPM were clearly observed in both groups, CPM levels were consistently higher in the CKD group at each of the timepoints, and group-by-time interaction terms indicated that the magnitude of the post-prandial response was significantly larger in CKD participants than in controls. Accordingly, in exploratory analysis, there was strong correlation between eGFR and CPM AUC and maximum

concentration. Peak levels of CPM also tended to occur later in the CKD group. Theoretically, these differences could indicate an increased production of CPM following nutritional intake and/or an impaired capacity to clear CPM in those with CKD. The latter is supported by recent data by Koeppert *et al*, who used live two-photon microscopy to show that circulating CPM are predominantly cleared by glomerular filtration in mice.²¹⁹ A reasonable prediction therefore, may be that the capacity to clear CPM becomes impaired as GFR declines. However, alterations in other aspects of mineral homeostasis, including bone turnover,²⁸⁶ and delayed renal excretion of acute phosphate loads^{271, 281} are also commonly seen in CKD and may well have contributed to elevations in post-prandial levels. As discussed in Section 1.3, recent evidence has questioned the long-held notion that CKD affects net intestinal phosphate absorption.^{49, 287, 288} However, in this study six of the 14 individuals in the CKD group were taking cholecalciferol. Considering the potential for stimulatory effects of vitamin D on intestinal mineral absorption, we performed an *ad hoc* exploratory analysis to test whether use of nutritional vitamin D impacted the acute response of CPM, CPP-I and CPP-II to feeding (Tables 3.11-3.13). Apart from a single positive group-by-time interaction for CPP-I at 30 minutes in those taking cholecalciferol, all other interaction terms and *post hoc* pairwise comparisons for CPM, CPP-I and CPP-II were not significant, suggesting that cholecalciferol use was unlikely to have had a substantial effect on post-prandial CPM/PP kinetics. While the small sample size and potential for confounding may preclude definitive conclusions, the lack of effect may also reflect the predominance of phosphate absorption via the paracellular pathway when mineral is abundant, which is not actively regulated by the vitamin D axis.⁵⁰

Table 3.11: Linear mixed-effect model and post hoc pairwise comparisons for serum CPM in those with CKD (by use of cholecalciferol)

Timepoint	CPM (x10 ³ AU/mL)		
<i>Not taking cholecalciferol</i>	Group median (IQR)	Between group difference	Within group difference
Fasting	22.4 (13.0 - 26.4)	-	-
+30 minutes	22.2 (14.2 - 28.1)	-	NS
+60 minutes	33.7 (18.5 - 47.6)	-	###
+120 minutes	58.5 (29.6 - 79.6)	-	###
+180 minutes	57.8 (29.9 - 94.7)	-	###
+240 minutes	67.5 (25.7 - 110.6)	-	###
<i>Taking cholecalciferol</i>			
Fasting	20.1 (19.1 - 28.8)	NS	-
+30 minutes	25.3 (20.7 - 37.4)	NS	NS
+60 minutes	31.9 (22.5 - 45.6)	NS	NS
+120 minutes	48.9 (25.0 - 68.7)	NS	###
+180 minutes	49.0 (35.5 - 89.8)	NS	###
+240 minutes	48.7 (45.8 - 49.9)	NS	###
	Between group coefficient estimate (95% CI)		
+30 minutes	-1.2% (-20.4, 22.6)		
+60 minutes	-19.1% (-37.2, 4.2)		
+120 minutes	-17.7% (-39.5, 11.9)		
+180 minutes	-22.4% (-46.2, 11.9)		
+240 minutes	-17.2% (-46.5, 28.1)		

Linear mixed-effect model and *post hoc* pairwise comparisons of the CKD group by use of cholecalciferol (n=6). Table shows median (IQR), linear mixed-effects model coefficient (95% CI) for group-time interaction, and *post hoc* pairwise comparisons (with Bonferroni correction for multiple comparisons). Values were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to derive estimates of percentage change. Model was adjusted for eGFR. *Post hoc* pairwise comparisons test for differences in mean values between groups at each timepoint, and to test for deviation from own fasting baseline within each group.

NS: $p \geq 0.05$; ###: $p < 0.001$.

Table 3.12: Linear mixed-effect model and post hoc pairwise comparisons for serum CPP-I in those with CKD (by use of cholecalciferol)

Timepoint	CPP-II (x10 ³ /mL)		
<i>Not taking cholecalciferol</i>	Group median (IQR)	Between group difference	Within group difference
Fasting		-	-
+30 minutes	0.9 (0.3 - 2.1)	-	NS
+60 minutes	0.2 (0.1 - 0.6)	-	NS
+120 minutes	3.1 (1.7 - 7.5)	-	###
+180 minutes	6.4 (4.4 - 20.9)	-	###
+240 minutes	8.3 (4.3 - 21.6)	-	##
<i>Taking cholecalciferol</i>			
Fasting	1.5 (0.5 - 1.9)	NS	-
+30 minutes	1.7 (1.1 - 4.3)	NS	NS
+60 minutes	11.1 (2.5 - 16.9)	NS	#
+120 minutes	14.2 (5.1 - 24.7)	NS	###
+180 minutes	11.3 (7.8 - 18.8)	NS	##
+240 minutes	4.8 (1.9 - 8.0)	NS	NS
	Between group coefficient estimate (95% CI)		
+30 minutes	401.7%[#] (24.6, 1921.0)		
+60 minutes	56.7% (-61.2, 532.1)		
+120 minutes	9.6% (-72.9, 343.4)		
+180 minutes	-11.8% (-78.3, 258.1)		
+240 minutes	-35.9% (-84.3, 161.4)		

Linear mixed-effect model and *post hoc* pairwise comparisons of the CKD group by use of cholecalciferol (n=6). Table shows median (IQR), linear mixed-effects model coefficient (95% CI) for group-time interaction, and *post hoc* pairwise comparisons (with Bonferroni correction for multiple comparisons). Values were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to derive estimates of percentage change. Model was adjusted for eGFR. *Post hoc* pairwise comparisons test for differences in mean values between groups at each timepoint, and to test for deviation from own fasting baseline within each group.

NS: $p \geq 0.05$; #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$.

Table 3.13: Linear mixed-effect model and post hoc pairwise comparisons for serum CPP-II in those with CKD (by use of cholecalciferol)

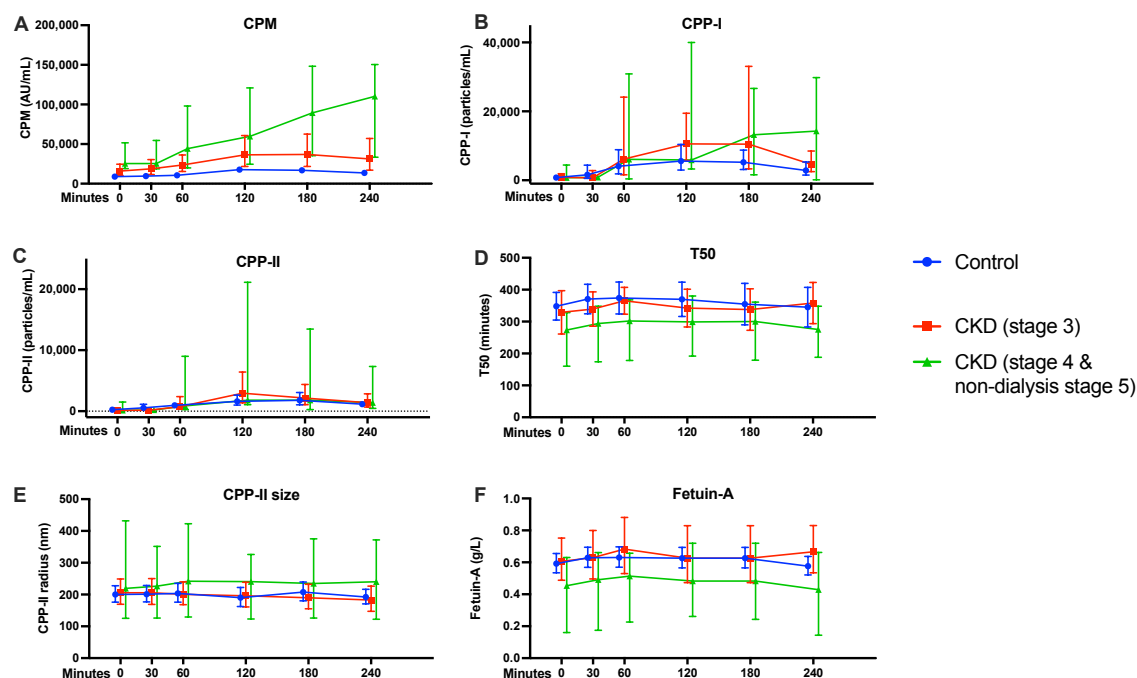
Timepoint	CPP-II (x10 ² /mL)		
<i>Not taking cholecalciferol</i>	Group median (IQR)	Between group difference	Within group difference
Fasting	1.3 (1.3 - 2.5)	-	-
+30 minutes	1.3 (1.3 - 3.0)	-	##
+60 minutes	6.3 (5.0 - 12.0)	-	###
+120 minutes	22.3 (17.7 - 30.7)	-	###
+180 minutes	14.7 (10.0 - 19.3)	-	###
+240 minutes	15.3 (9.3 - 37.7)	-	###
<i>Taking cholecalciferol</i>			
Fasting	0.1 (0.1 - 0.1)	NS	-
+30 minutes	0.2 (0.1 - 0.3)	NS	NS
+60 minutes	0.8 (0.5 - 3.1)	NS	##
+120 minutes	2.6 (1.1 - 3.9)	NS	###
+180 minutes	2.4 (1.7 - 3.3)	NS	###
+240 minutes	1.5 (0.7 - 1.8)	NS	###
	Between group coefficient estimate (95% CI)		
+30 minutes	-4.7% (-68.1, 130.7)		
+60 minutes	3.8% (-65.4, 211.4)		
+120 minutes	-8.7% (-69.8, 175.9)		
+180 minutes	74.4% (-42.9, 432.1)		
+240 minutes	-25.4% (-75.9, 130.7)		

Linear mixed-effect model and *post hoc* pairwise comparisons of the CKD group by use of cholecalciferol (n=6). Table shows median (IQR), linear mixed-effects model coefficient (95% CI) for group-time interaction, and *post hoc* pairwise comparisons (with Bonferroni correction for multiple comparisons). Values were natural log transformed before fitting regression models to ensure normal distribution of residuals. For ease of interpretation coefficient estimates for log transformed variables have been exponentiated to derive estimates of percentage change. Model was adjusted for eGFR. *Post hoc* pairwise comparisons test for differences in mean values between groups at each timepoint, and to test for deviation from own fasting baseline within each group.

NS: $p \geq 0.05$; #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$.

In contrast to CPM, kidney function did not appear to have a strong effect on either CPP-I or CPP-II. Unlike CPM, circulating CPP-I and CPP-II are primarily cleared from the circulation by non-renal mechanisms. Animal and *in vitro* models have suggested rapid clearance of CPP-I predominantly by liver sinusoidal endothelial cells,²¹⁸ and of CPP-II by resident macrophages of the liver and spleen.²²¹ It is plausible that while participants in the CKD group displayed evidence of altered CPM metabolism, that these discrete CPP-I/II clearance pathways were sufficient to maintain normal post-prandial CPP-I/II profiles. It had been anticipated that the CKD group in this study would have discerningly higher levels of CPP-I/II compared to controls based on previous studies, albeit these studies enrolled patients with more advanced CKD who were dialysis dependent,²⁰⁶ or used the older and indirect method of CPP measurement.^{200, 207} It is possible that individuals with more advanced CKD than those studied here may exhibit greater differences in fasting and post-prandial CPP-I/II levels. The lack of separation for CPP-I/II between the CKD and control group may also be due to limited participant numbers, which also limited the ability to formally test for an effect of CKD stage (Figure 3.8). Indeed, this study was powered based on examining post-prandial responses rather than between group differences. Of note, CPP-I appeared to return to fasting levels earlier in controls than CKD participants, and there was a positive group-by-time interaction for CPP-II in CKD participants at 120 minutes. Both findings potentially signal that this study may have observed more pronounced between-group effects in a larger cohort. Higher analytical imprecision for flow cytometry-based CPP-I/II measurements may have also contributed to the null findings.

Figure 3.8: Novel markers of mineral metabolism (showing individual CKD groups)



Repeated measures of novel markers of mineral metabolism depicted for control, CKD stage 3, and CKD stage 4 & non-dialysis stage 5 groups when fasting (“0”) and then post consumption of a standardised meal. Data are presented as mean and 95% confidence interval or geometric mean and 95% confidence interval. Data points have been offset for clarity.

(A) Calciprotein monomers (CPM); (B) Primary calciprotein particles (CPP-I); (C) Secondary calciprotein particles (CPP-II); (D) T50; (E) Secondary calciprotein particle size (CPP-II size); (F) Fetuin-A.

In addition to providing new insights into the physiology of CPM and CPP-I/II metabolism, this study also has direct relevance for optimising the use of these novel markers of mineral metabolism in future studies. While each of these novel assays have shown promise in early clinical work, very few studies have reported²⁴⁰ or controlled for fasting/absorptive status.^{228, 249} In this study a number of individuals had undetectable levels of CPP-I or CPP-II in the fasting and early post-prandial period, and this profound effect of fasting on serum CPP is in itself a previously unreported and notable finding. In contrast, fasting CPM levels were measurable and significantly higher in the CKD group than in controls, which suggests that sustained elevations in CPM may not necessarily manifest in elevated CPP-I/II levels in those with non-dialysis-dependent CKD.

Previous epidemiological studies have suggested links between elevated levels of CPM and CPP-I/II with a range of surrogate markers of vascular disease,^{200, 202, 203} as well as with cardiovascular events²⁰⁵ and all-cause mortality.²⁰⁴ Further, lab-based studies have provided plausible mechanisms by which CPP may themselves mediate these pathological vascular outcomes.^{214, 215, 218} However, while *in vitro* studies have suggested that many of these toxicities are induced in a dose-dependent manner, if CPP do have a normal physiological role in health then it is unclear at what point these particles may become injurious. Knowledge of the effect of nutritional intake on CPP kinetics as revealed here may prove crucial to further understanding this process. It is possible that a threshold concentration for the onset of pathological effects of CPM and CPP-I/II may (at least initially) only be reached in the post-prandial state *in vivo*. If so, dynamic testing of the post-prandial response may provide additional opportunities to evaluate earlier manifestations of dysregulated mineral metabolism, as well as the risk of associated vascular disease. Another important possibility is that, as discussed in Section 1.8, the composition and thus the intrinsic toxicity of CPP-I and CPP-II differs among health and CKD.²²⁰

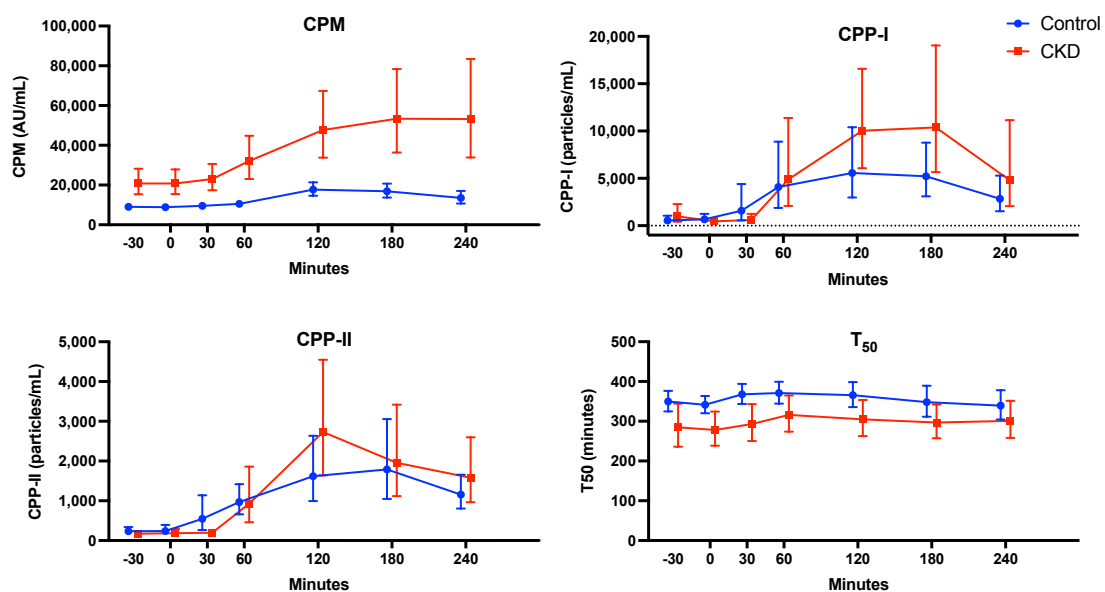
Several limitations of this study should be acknowledged, including the limited patient numbers as already discussed. Notably, we also used a standardised meal given after an overnight fast, and so cannot comment on the effect of varying meal composition or the effect of subsequent meals given throughout the rest of the day. The meal was chosen based on its commercial availability, allowing for standardisation between participants, and because it represented physiologically relevant nutritional loads (Table 3.1). It is

however possible that if participants were challenged with larger mineral loads, that further separation between those with normal and impaired kidney function may have been apparent. Similarly, given individuals were only observed for four hours post-meal, it is conceivable that the cumulative effect of subsequent meals may have also distinguished groups further.

Faeces and urine were not recovered and so no comment can be made on the total mineral absorbed or excreted during the study. Changes in each measure seen after feeding in this study are assumed to be directly related to nutritional intake. This assumption is supported indirectly by detailed imaging studies showing flux of calcium and phosphate ions following food intake,⁴⁹ as well as direct evidence from animal studies showing that acute oral gavage of mice with a buffered phosphate solution results in serum spikes in CPP.²²⁵ Nevertheless, participants were not observed over extended fasting conditions, and so non-dietary related diurnal fluctuations in any of the studied parameters cannot conclusively be ruled out. Each participant did however have two fasting samples collected, the average of which was used as “time 0”, and variability between these fasting samples was trivial compared to the magnitude of changes seen in the post-prandial period (Figure 3.9).

The Bonferroni method was used to account for the multiple *post hoc* pairwise comparisons between and within groups for each parameter. This is undoubtedly a conservative approach, and to our knowledge not one adopted by other similar feeding studies of post-prandial mineral metabolism. This approach was chosen given the large number of timepoints and comparisons that were made and with the rationale that it was preferable to focus on the most robust and significant signals. However, as a result smaller but potentially relevant effects may have been missed. No correction for multiple testing across different mineral parameters was made given the likelihood of interdependent physiologically linked changes.

Figure 3.9: Novel markers of mineral metabolism (showing repeated fasting timepoints)



Repeated measures of novel markers of mineral metabolism for control and CKD group. Depicting repeated fasting measurements (30 minutes, and immediately prior) and post standardised meal. Data are presented as mean and 95% confidence interval or geometric mean and 95% confidence interval. Data points have been offset for clarity.

Abbreviations: CPM, calciprotein monomers; CPP-I, primary calciprotein particles; CPP-II, secondary calciprotein particles.

3.4 Conclusion

This study has revealed for the first time that nutritional mineral intake leads to the formation of CPM, CPP-I and CPP-II in blood as a normal physiological response to feeding. These findings corroborate the hypothesis that CPP formation helps to buffer post-prandial mineral loads, functioning as a temporary circulating store of bulk calcium phosphates ultimately destined for utilisation/storage (e.g., mineral precursor for bone mineralisation) or elimination. Higher fasting levels of serum CPM were also observed as was a larger post-prandial response in those with impaired kidney function, suggesting that CPM metabolism is manifestly altered in CKD. Analysis of post-prandial CPP handling may provide new insights into the mechanisms linking excessive dietary calcium and phosphate intake to increased risks cardiovascular disease in patients with impaired mineral excretion. In the broader context of this thesis, these novel findings underscore the important, but often neglected, contribution of colloidal biochemistry to mineral homeostasis.

Chapter 4 Reduction of calciprotein particles in adults receiving infliximab for chronic inflammatory disease

4.1 Introduction

As described in Chapter 1, mineral metabolism abnormalities in CKD are often accompanied by accelerated bone and vascular disease, which are collectively referred to as CKD-MBD.¹⁷ The pattern of a loss of bone mineralisation that is accompanied by a parallel, and paradoxical, increase in mineralisation of blood vessels is sometimes referred to as ‘the calcification paradox’.²⁸⁹ Intriguingly, this phenomenon is seen in a range of other settings, including age-related osteoporosis,²⁹⁰ and also in chronic inflammatory diseases (CID) such as inflammatory bowel disease and inflammatory arthritis.^{291, 292}

There is mounting evidence that bone loss and vascular calcification are directly linked through a series of shared and interconnected pathways. Both processes share a number of common underlying risk factors, such as hypertension, diabetes, physical inactivity, smoking, excess alcohol consumption, as well as age and inflammation itself.²⁹³ In addition, newer insights into overlapping molecular pathways have strengthened the biological plausibility of a “bone-vascular axis”.^{294, 295}

As outlined in Section 1.8, exposure to excessive levels of CPP has been hypothesised to be a potential novel mediator of vascular pathology. This is based on epidemiological evidence that high levels of CPP are positively associated with adverse vascular related events, including vascular stiffness,²⁰² vascular calcification²⁰³ and mortality,²⁰⁵ as well as *in vitro* data showing that CPP can induce calcification and inflammatory responses in vascular smooth muscle cells.^{214, 218} Intriguingly, while CPP metabolism has been largely studied in the setting of CKD, elevated levels of circulating CPP have also been observed in CID cohorts.²⁰⁷

The precise origins of CPP in circulation and the drivers of elevated levels in pathological conditions are yet to be fully described. As outlined in Chapter 1, and supported by the

results in Chapter 3, there is highly suggestive evidence that a relationship between circulating levels of CPP and dietary intake exists.^{206, 226-228} However, there is also a line of evidence to suggest that bone may be an important determinant of serum CPP. In early animal models it was shown that CPP levels could be altered by manipulating bone turnover in rats with normal^{222, 223} and impaired²⁰¹ kidney function. There is limited data exploring the link between bone and CPP in human studies. In patients with CKD, CPP levels have been observed to correlate with serum PTH,²²⁴ and markers of bone turnover.^{202, 296} Beyond CKD, whether bone metabolism may also be linked to elevations in serum CPP in the setting of CID has not previously been explored in clinical studies. If serum levels of CPP are linked to bone metabolism in CID, then this could further substantiate the role of bone as a determinant of circulating CPP, as has been suggested in the CKD literature. Further, given CID and CKD are both associated with accelerated vascular and bone disease, this would also raise the possibility of CPP being involved in the bone-vascular axis.

Outcomes of patients with various forms of CID have been dramatically improved with the introduction of anti-inflammatory and immunomodulatory biologic therapies such as tumour necrosis factor (TNF)-alpha inhibitors, including infliximab. Exact indications are specific to individual conditions, but these agents are generally used in patients with moderate to high disease activity, often after failure to respond to conventional therapies. Their use in CID has been associated with improved bone mineral density and cardiovascular outcomes.²⁹⁷⁻³⁰⁰ Bone loss in CID is driven by both suppressed bone formation and enhanced bone resorption, and TNF-alpha inhibitor therapy has been shown to normalise markers of bone turnover.^{301, 302} Given the hypothetical links between CPP and bone turnover, we hypothesised that treatment of active CID with infliximab would lower circulating CPP levels and be temporally related to changes in markers of bone turnover.

4.2 Methods

For this study, a cohort of patients with CID, who were planned to receive treatment with the TNF-alpha inhibitor infliximab for active disease were recruited. This study was undertaken as part of the Fetuin-A Levels in Systemic disease and Kidney Impairment (FLEKSI) study.²⁰⁷

Ten patients with current active CID who were planned to receive induction therapy with infliximab were recruited. All participants had either inflammatory bowel disease (ulcerative colitis or Crohn's disease) or inflammatory arthritis (psoriatic arthritis or ankylosing spondylitis) and had disease specific indications for infliximab use (outlined in Table 4.1). For comparison, an additional group of three patients with quiescent CID who were receiving maintenance therapy with infliximab were also recruited (two with Crohn's disease and one with ulcerative colitis). Participants were recruited via the outpatient clinics at The Royal Melbourne Hospital. All participants were at least 18 years of age and gave written informed consent to participate in this study. Key exclusion criteria were participants who were unable to give informed consent, or who were pregnant or breastfeeding. This study was approved by the local ethics committee (Melbourne Health Human Research Ethics Committee reference number MH 2012.141) and conducted in accordance with the Declaration of Helsinki.

4.2.1 Participant groupings and infliximab dosing

Patients with active disease received intravenous infliximab (Remicade, Janssen Immunology) 5 mg/kg at 0, 2, 6 weeks according to local dosing guidelines for induction therapy, and in line with current international recommendations.³⁰³ The quiescent group received maintenance treatment with intravenous infliximab, at a dose of 5mg/kg on an 8-weekly schedule. Patients with active disease who received induction therapy were classified into "non-responders" and "responders" based on their clinical and biochemical response to treatment. Criteria used for response was a decrease in Mayo score of ≥ 3 or a score of 0 for participants with ulcerative colitis; a decrease in Harvey-Bradshaw Index ≥ 4 for participants with Crohn's disease; a decrease in Disease Activity Score-28 ≥ 1.2 for participants with psoriatic arthritis; or, a decrease in Ankylosing Spondylitis Disease Activity Score ≥ 1.1 for participants with ankylosing spondylitis.³⁰⁴⁻³⁰⁷ Assessment against these criteria were made by the participant's clinicians, who were otherwise not

involved in the conduct of this study or analysis of results. These clinicians were also solely responsible for decisions regarding eligibility for, and use of infliximab, however each participant met local clinical eligibility criteria which are required for government funding of infliximab therapy,³⁰⁸ and are consistent with international prescribing guidelines.³⁰⁹

4.2.2 Variables

Serial measurements of the bone formation (osteoblast activity) markers bone specific alkaline phosphatase (bsALP) and procollagen type 1 N-terminal propeptide (P1NP), bone resorption (osteoclast activity) markers tartrate-resistant acid phosphatase 5b (TRAcP 5b) and C-terminal telopeptide (CTX), CPP parameters (CPM, CPP-I, CPP-II), as well as intact and C-terminal FGF23 (iFGF23 and cFGF23, respectively) were performed.

In the participants with active disease who were receiving induction therapy, bone turnover markers (BTM), CPP and FGF23 were measured one week before treatment as well as on the day of treatment and then on week 1, week 2, week 6 and week 8 post the initial dose of infliximab were measured. To account for variation, the mean of values taken one week prior and on the day of initial treatment was used as baseline (“week 0”) for the analysis. Participants receiving maintenance infliximab for quiescent disease had BTM, CPP and FGF23 measured one week before and on the day of infliximab, as well as week 8 post dose. As for participants in the active group, for the quiescent group the values for the week prior and the day of infliximab were combined to calculate “week 0”. All participants also had blood collected at week 0 and week 8 for standard biochemistry as well as the cytokines interleukin (IL)-6, -8, -10, -12, -17A and -23 and fetuin-A.

Blood for BTM, CPP and FGF23 was collected via standard venepuncture technique, and allowed to clot before centrifugation (<1h at room temperature). Aliquots were stored at -80°C until batch analysis. Other biochemistry was performed by our institution in an accredited laboratory. Commercial immunoassay kits were used to measure IL (BioLegend, San Diego CA), fetuin-A (R&D Systems, Minneapolis, USA), BTM (Immunodiagnostic Systems, East Boldon, United Kingdom) and iFGF23 (Kainos Laboratories, Tokyo, Japan) and cFGF23 (Immutopics Inc, San Clemente, USA)

according to the manufacturer's instructions. Serum CPM, CPP-I and CPP-II were measured using the same gel-filtration²⁰⁸ and flow cytometer^{206, 226} method as described in Chapter 3.

4.2.3 Statistical analysis

Demographics, standard biochemical and cytokine data are presented using descriptive statistics. Mean and standard deviation or median and interquartile range are presented for continuous variables as appropriate, and number and percentage are presented for categorical variables.

Since there was no published data pertaining to the effect of infliximab on CPP or clinically important differences in levels, sample size calculations were based on observations from a patient with Takayasu's arteritis,³¹⁰ in whom infliximab treatment (5mg/kg at 0 and 4 weeks) resulted in reduction in total CPP of 31% ($25 \times 10^4/\text{ml}$) from baseline to 8 weeks. GLIMMPSE, a validated linear mixed model power and sample size calculator,²⁷⁷ was used to conduct *a priori* sample size calculations. Assuming a mean baseline CPP-I of $65 \times 10^4/\text{ml}$ and standard deviation of $8 \times 10^4/\text{ml}$ at each of the five timepoints, it was estimated that a total of seven patients would be required to provide >90% power in detecting a linear decline in CPP-I levels of 30% over 8 weeks at a 1% significance level (Geisser-Greenhouse corrected). Data for individual CPP phases were not available to estimate appropriate samples sizes

For patients with active disease receiving induction therapy with infliximab, the longitudinal between-group differences for the non-responder and responder groups were examined for each measure of BTM, CPP and FGF23. In order to do this while accounting for repeated measures, linear mixed effects models using a restricted maximum likelihood approach with an unstructured covariance structure were fitted. In each model response group, categorical time and time-response group interaction were modelled as fixed effects and a random intercept for each participant was included to allow for individual differences and correlation between repeated measures. In all of these models, week 0 and non-responder group were used as the reference levels for time and group respectively. For the model of bsALP, values were naturally log transformed before fitting the model to ensure normal distribution of residuals. Estimates for bsALP were

then back transformed for ease of interpretation. There was an apparent between group difference in the responder group compared to the non-responder group in multiple BTM, CPP and FGF23 variables. As a result, in further analyses linear mixed effects models were fitted, restricted to data from the responder group, and using categorical time as the fixed effect, in order to confirm whether between group differences represented changes from baseline. As this was an exploratory study, no correction for multiple testing was made. Two-tailed p values <0.05 were considered significant. All data were analysed using Stata version 16.1 (StataCorp, College Station, USA) and figures were produced using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, USA).

4.3 Results

Of the 10 participants with active CID, four were adjudged by their treating clinician using disease-specific criteria to be “non-responders” and six to be “responders” after completing the induction regimen. The demographics of these participants, as well as the additional three with quiescent disease are presented in Table 4.1. The overall mean age of the cohort was 51 years and 54% were female. Three patients had inflammatory arthritis (two in the responder group and one in the quiescent group) while the other 10 patients had inflammatory bowel disease.

Conventional biochemical measures as well as inflammatory markers at week 0 and week 8 are presented in Table 4.2. All participants had normal kidney function at both time points. Groups appeared to have comparable levels of serum calcium, phosphate, albumin, bicarbonate, magnesium, total alkaline phosphatase and PTH, while participants in the non-responder group appeared to have lower levels of 25-hydroxyvitamin D at both week 0 and week 8.

Expectedly, there were differences in the inflammatory marker C-reactive protein (CRP) and interleukin panel between sub-groups at baseline and over the 8-week study period (Table 4.2 and Figure 4.1). Participants with active disease tended to have higher levels of CRP and pro-inflammatory cytokines at week 0 compared to those with quiescent disease. In the responder group there were reductions in CRP, IL-6, IL-8 and IL-17A from baseline to week 8, while there was an increase in the regulatory cytokine IL-10. In contrast, levels of these markers appeared unchanged in non-responders and those with quiescent disease. Consistent with fetuin-A being a negative acute-phase reactant, at baseline, patients with active disease appeared to have lower levels of serum fetuin-A than those with quiescent disease, however levels appeared to rise in the responder group by week 8 (Table 4.2).

Table 4.1: Baseline demographics

		Induction therapy		Maintenance therapy
	Overall (n=13)	Non-responder (n=4)	Responder (n=6)	Quiescent (n=3)
Age (yrs)				
Mean (SD)	40.9 (11.8)	46.5 (15.4)	37.2 (11.9)	41 (4)
Sex - female (%)	7 (53.8)	4 (100)	2 (33.3)	1 (33.3)
Inflammatory arthritis - no. (%)	3 (23.1)	1 (25)	2 (33.3)	--
Diagnosis and original indication for infliximab - no. (%)				
Moderate to severe (Mayo score ≥ 6) active ulcerative colitis despite conventional therapy ³⁰⁴	4 (30.8)	1 (25)	2 (33.3)	1 (33.3)
Moderate to severe Crohn's disease (Crohn Disease Activity Index score ≥ 220) despite conventional therapy ³¹¹	6 (46.2)	2 (50)	2 (33.3)	2 (66.7)
Active ankylosing spondylitis not responding to non-steroidal anti-inflammatory drugs	1 (7.7)	--	1 (16.7)	--
Severe active psoriatic arthritis not responding to conventional therapy	2 (15.4)	1 (25)	1 (16.7)	--

Mean (standard deviation) or number (%)

Table 4.2: Biochemical and interleukin profile at Week 0 and Week 8 by subgroup

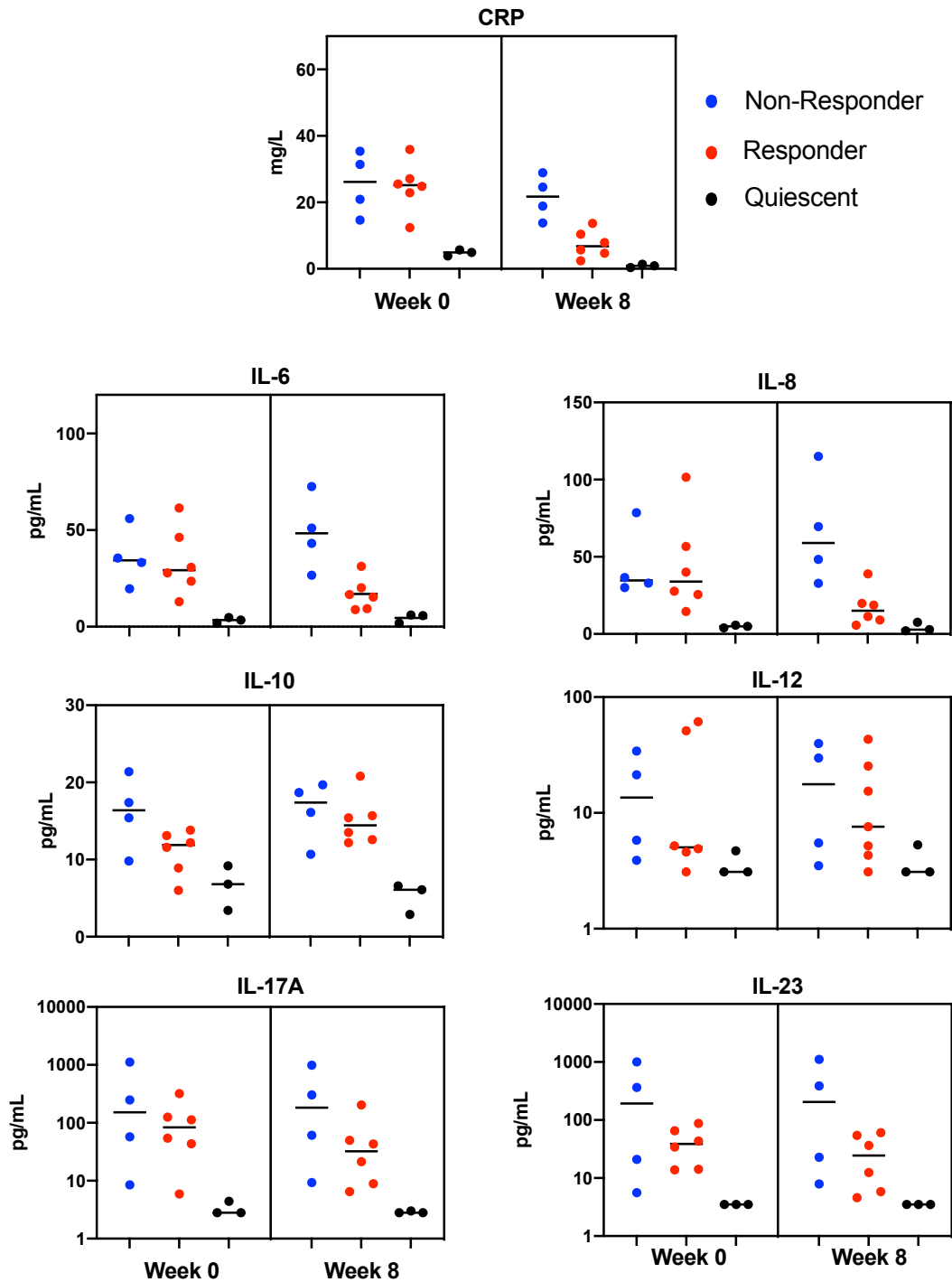
	Non Responder (n=4)	Responder (n=6)	Quiescent (n=3)
Creatinine (µmol/L)			
Week 0	66.0 (8.3)	67.2 (6.4)	69.3(5.1)
Week 8	69.8 (6.6)	65.7 (3.9)	69.9 (3.5)
Calcium (mmol/L)			
Week 0	2.28 (0.05)	2.41 (0.11)	2.23 (0.01)
Week 8	2.32 (0.10)	2.36 (0.07)	2.26 (0.05)
Phosphate (mmol/L)			
Week 0	0.81 (0.14)	0.83 (0.24)	0.80 (0.25)
Week 8	0.77 (0.23)	0.93 (0.23)	0.92 (0.24)
Albumin (g/L)			
Week 0	37 (34, 38)	38 (37, 41)	40 (39, 41)
Week 8	37 (34, 39)	39 (37, 40)	41 (39, 42)
Bicarbonate (mmol/L)			
Week 0	26.3 (1.0)	26.0 (1.8)	26.7(1.2)
Week 8	27.5 (0.6)	27.3 (1.9)	25.3 (0.6)
Magnesium (mmol/L)			
Week 0	0.78 (0.13)	0.79 (0.06)	0.82 (0.07)
Week 8	0.73 (0.05)	0.79 (0.10)	0.87 (0.07)
ALP (IU/L)			
Week 0	55.8 (20.6)	65.5 (17.0)	54.0(11.4)
Week 8	51.3 (12.6)	68.8 (16.9)	57.0 (7.5)
25-OH vitamin D (nmol/L)			
Week 0	34.5 (18.7)	62.3 (16.9)	75.0(24.3)
Week 8	36.5 (13.3)	68.8 (22.1)	71.7 (15.9)
PTH (pmol/L)			
Week 0	6.0 (3.5, 8.4)	4.4 (4.0, 5.5)	4.4 (3.8, 4.9)
Week 8	5.9 (3.7, 8.3)	4.1 (3.5, 5.7)	5.6 (4.4, 5.7)

CRP (mg/L)			
Week 0	26.2 (17.8, 33.4)	25.2 (22.9, 27.1)	0.5 (0.3, 0.7)
Week 8	21.8 (16.4, 26.8)	6.8 (4.7, 10.4)	0.9 (0.4, 1.4)
Fetuin-A (g/L)			
Week 0	0.39 (0.24)	0.35 (0.14)	0.64 (0.11)
Week 8	0.41 (0.21)	0.52 (0.09)	0.66 (0.15)
IL-6 (pg/mL)			
Week 0	36.1 (14.9)	33.8 (17.3)	3.3 (1.5)
Week 8	48.2 (19.1)	16.9 (8.3)	4.6 (2.4)
IL-8 (pg/mL)			
Week 0	34.7 (31.5, 57.5)	33.9 (25.5, 56.7)	4.9 (3.9, 5.7)
Week 8	59.0 (40.6, 92.3)	15.0 (9.1, 19.8)	2.8 (2.0, 7.5)
IL-10 (pg/mL)			
Week 0	16.0 (4.8)	10.9 (2.9)	6.5 (2.9)
Week 8	16.3 (4.0)	15.0 (3.2)	5.2 (2.0)
IL-12 (pg/mL)			
Week 0	13.6 (4.9, 27.8)	5.1 (4.6, 51.2)	3.1 (3.1, 4.7)
Week 8	17.7 (4.5, 34.8)	6.4 (4.3, 25.4)	3.1 (3.1, 5.3)
IL-17A (pg/mL)			
Week 0	152.4 (32.9, 683.9)	83.4 (43.4, 125.7)	2.8 (2.8, 4.4)
Week 8	182.7 (35.2, 645.9)	32.25 (8.9, 49.9)	2.8 (2.8, 3.0)
IL-23 (pg/mL)			
Week 0	192.8 (13.3, 684.1)	38.9 (14.2, 65.4)	3.5 (3.5, 3.5)
Week 8	205.1 (15.4, 747.9)	24.5 (5.8, 54.3)	3.5 (3.5, 3.5)

Numbers show mean (standard deviation [SD]) or median (25th, 75th percentile)

Abbreviations: ALP, total alkaline phosphatase; PTH, parathyroid hormone; CRP, C-reactive protein, IL-, interleukin-.

Figure 4.1: C-reactive protein (CRP) and interleukin (IL-) panel at baseline and week 8 by sub-group



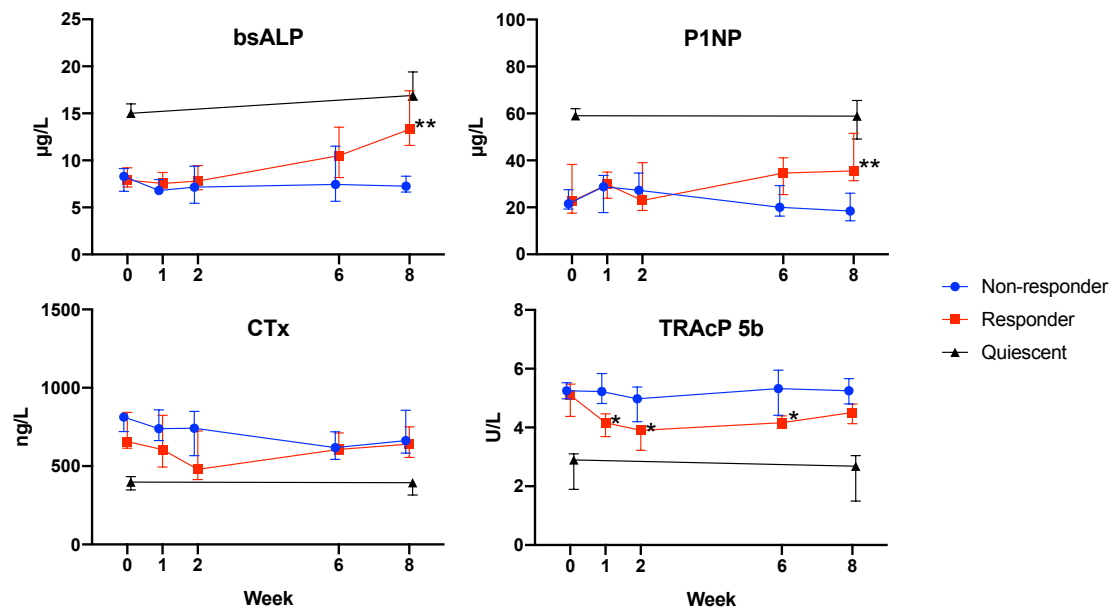
Line indicates group median. IL-12, IL-17A, IL-23 plotted on log 10 scale for clarity.

4.3.1 Bone turnover markers

Repeated measures of BTM are displayed in Figure 4.2 and Table 4.3. Coefficient estimates for linear mixed-effects models fitted to the non-responder and responder groups for each BTM are also presented in Table 4.2. At baseline, patients in the quiescent group appeared to have higher levels of the bone formation (osteoblast) markers bsALP and P1NP and lower levels of the bone resorption (osteoclast) markers CTx and TRAcP 5b than those with active disease.

In the responder group, a significant increase was observed at week 8 for both bsALP ($\beta = 6.4 \mu\text{g/L}$ [95% CI 2.5, 11.9], $p < 0.001$) and P1NP ($\beta = 16.8 \mu\text{g/L}$ [95% CI 8.1, 25.6], $p < 0.001$), whereas TRAcP 5b showed a transient reduction in levels at week 1 ($\beta = -0.93 \text{ U/L}$ [95% CI -1.66, -0.19], $p = 0.014$), week 2 ($\beta = -0.96 \text{ U/L}$ [95% CI -1.70, -0.22], $p = 0.011$) and week 6 ($\beta = -0.81 \text{ U/L}$ [95% CI -1.55, -0.06], $p = 0.011$), but not at week 8 ($\beta = -0.48 \text{ U/L}$ [95% CI -1.23, 0.27], $p = 0.207$). In contrast, no change in CTx levels was detected at any time point in either group.

Figure 4.2: Change in bone turnover markers with infliximab treatment



Comparison between “non-responder” and “responder” groups of bone formation (osteoblast) markers bsALP and P1NP as well as bone resorption (osteoclast) markers CTx and TRAcP 5b, during induction therapy with infliximab for active disease. Quiescent group also displayed for reference. Data presented as median with error bars showing interquartile range. Data points offset for clarity. *p < 0.05 for responder versus non-responder, ** p < 0.01 for responder versus non-responder.

Abbreviations: bsALP, bone specific alkaline phosphatase; P1NP, and procollagen type I N-terminal propeptide; TRAcP 5b, tartrate-resistant acid phosphatase 5b; CTx, C-terminal telopeptide)

Table 4.3: Linear mixed-effect models for bone turnover markers in patients with active CID receiving infliximab induction therapy

	bsALP[†] μg/L	PINP μg/L	CTx ng/L	TRAcP 5b U/L
Group median (IQR)				
<i>Non-responder</i>				
Baseline	8.3 (7.2, 9.0)	21.5 (19.5, 26.0)	813 (750, 824)	5.3 (5.1, 5.5)
Week 1	6.8 (6.6, 7.7)	28.8 (19.9, 33.6)	739 (686, 821)	5.2 (4.9, 5.7)
Week 2	7.2 (6.0, 8.7)	27.3 (26.4, 32.3)	742 (592, 847)	5.0 (4.5, 5.3)
Week 6	7.5 (5.9, 10.5)	20.0 (16.4, 27.4)	618 (567, 687)	5.3 (4.5, 6.0)
Week 8	7.3 (6.7, 8.2)	18.5 (15.6, 23.6)	664 (589, 814)	5.3 (4.9, 5.6)
<i>Responder</i>				
Baseline	7.9 (7.2, 8.3)	22.5 (20.0, 38.0)	657 (618, 839)	5.1 (4.4, 5.4)
Week 1	7.6 (7.2, 8.4)	29.8 (27.6, 34.5)	606 (522, 816)	4.2 (3.8, 4.4)
Week 2	7.8 (7.1, 9.3)	23.0 (20.4, 38.9)	480 (419, 680)	3.9 (3.4, 4.0)
Week 6	10.5 (8.5, 12.6)	34.7 (29.4, 40.5)	607 (584, 672)	4.2 (4.0, 4.4)
Week 8	13.3 (11.9, 17.3)	35.6 (33.8, 50.5)	640 (577, 738)	4.5 (4.2, 4.6)
Between group coefficient estimate^{††}				
Week 1	0.5 (-1.8, 3.7)	-0.8 (-9.6, 7.9)	-36.8 (-231.9, 158.4)	-0.9* (-1.7, -0.2)
Week 2	0.6 (-1.7, 3.8)	-5.5 (-14.3, 3.2)	-84.1 (-279.4, 111.2)	-1.0* (-1.7, -0.2)
Week 6	2.4 (-0.4, 6.4)	8.2 (-0.6, 16.9)	97.6 (-97.9, 293.1)	-0.8* (-1.6, -0.1)
Week 8	6.4** (2.5, 11.9)	16.8** (8.1, 25.6)	27.7 (-168.1, 223.5)	-0.5 (-1.2, 0.3)
Coefficient estimate versus baseline for responder group^{†††}				
Week 1	-0.5 (-1.9, 1.2)	3.2 (-1.5, 7.8)	-70.4 (-209.1, 68.3)	-0.9** (-1.4, -0.4)
Week 2	-0.3 (-1.8, 1.4)	1.1 (-3.6, 5.7)	-152.2* (-291.1, -13.4)	-1.3** (-1.8, -0.8)
Week 6	2.2* (0.4, 4.6)	7.3** (2.6, 12.0)	-62.8 (-201.8, 76.3)	-0.8** (-1.3, -0.3)
Week 8	5.5** (3.0, 8.5)	13.7** (9.0, 18.4)	-58.4 (-197.9, 81.1)	-0.5 (-1.0, 0.0)

Table shows group median (IQR) or mixed effects model coefficient (95% confidence interval). Week 0 and non-responder group used as reference groups for each model.

*p <0.05, **p <0.01.

[†]bsALP was naturally log transformed prior to fitting regression models to ensure normal distribution of residuals. Estimates have been back transformed for ease of interpretation. ^{††}Coefficient estimate is for responder group compared to non-responder group. ^{†††}Coefficient estimate is for categorical time compared to baseline for models fitted to responder group only

Abbreviations: IQR, interquartile range; bsALP, bone specific alkaline phosphatase; PINP, and procollagen type 1 N-terminal propeptide; TRAcP 5b, tartrate-resistant acid phosphatase 5b; CTx, C-terminal telopeptide

4.3.2 Calciprotein particles

Participants in the quiescent group appeared to have lower levels of CPM and CPP-I than the groups with active disease (Figure 4.3 and Table 4.4). Coefficient estimates for linear mixed-effects models fitted to the non-responder and responder groups for CPP and FGF23 are presented in Table 4.4. In the responder group, there was a significant reduction in CPM at week 8 ($\beta = -6.5 \times 10^3$ AU [95% CI -11.1, -1.8], $p = 0.006$), while for CPP-I, a significant reduction was evident at week 6 ($\beta = -23.8 \times 10^4$ particles/ml [95% CI -34.7, -12.8], $p < 0.001$) and was sustained at week 8 ($\beta = -23.4 \times 10^4$ particles/ml [95% CI -34.8, -11.9], $p < 0.001$).

For CPP-II, there was no clear difference between those with active and quiescent disease at baseline or serially, and with no between group difference was observed at week 8 ($\beta = 4.2 \times 10^3$ particles/ml [95% CI -2.9, 3.7], $p = 0.803$).

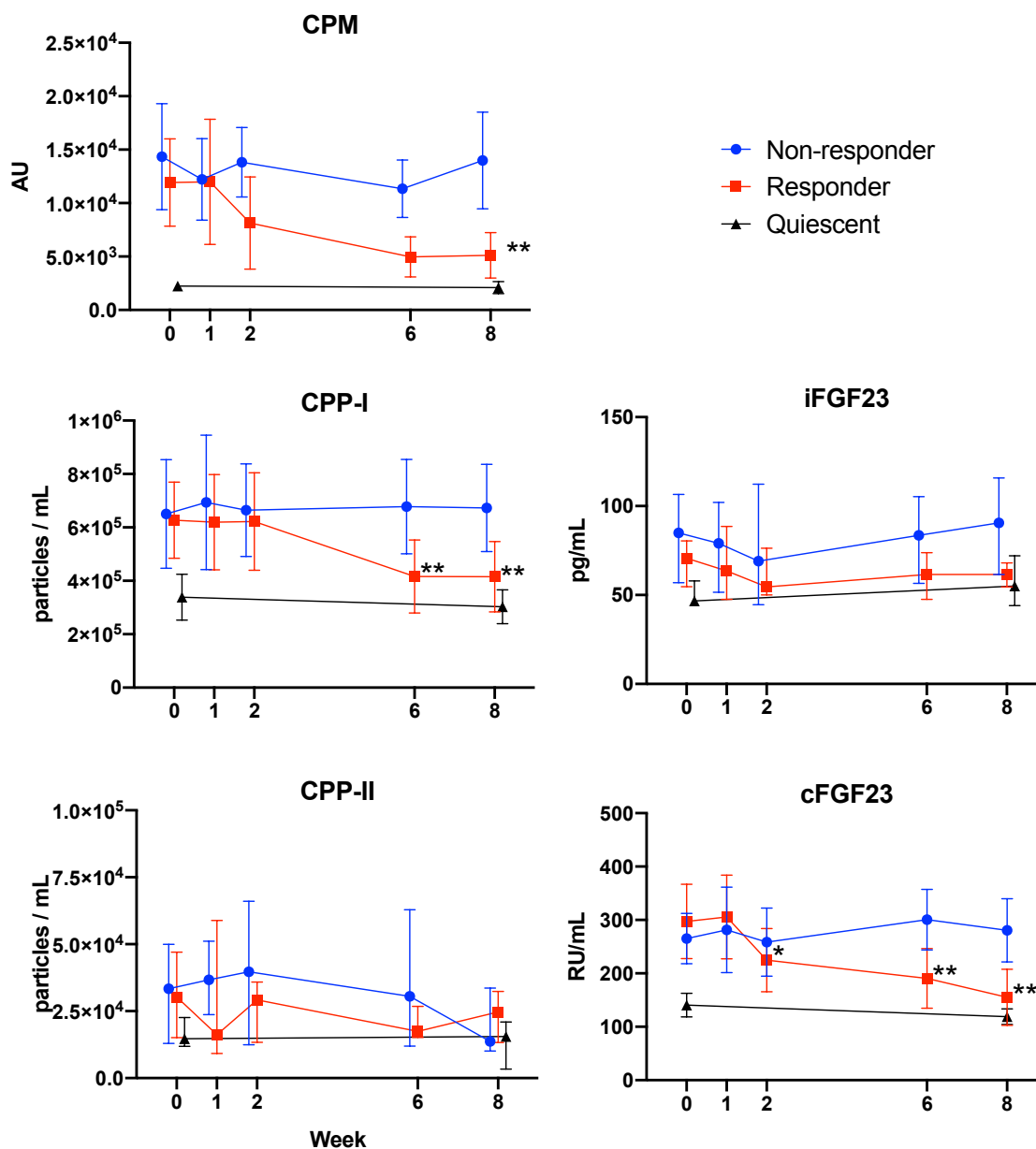
4.3.3 Fibroblast growth factor-23

The quiescent group appeared to have lower levels of cFGF23 than the non-responder and responder groups at baseline (Figure 4.3 and Table 4.4). Longitudinally, a significant reduction in cFGF23 was observed for the responder group, which was evident by week 2 ($\beta = -66.1$ RU/mL [95% CI -130.5, -1.7], $p = 0.044$) and persisted at week 6 ($\beta = -142.0$ RU/mL [95% CI -208.6, -75.4], $p < 0.001$) and at week 8 ($\beta = -157.4$ RU/mL [95% CI -227.0, -87.8], $p < 0.001$). In contrast, no significant difference in iFGF23 between the non-responder and responder groups were observed at any timepoint.

4.3.4 Linear mixed effect models restricted to responder group

Given the between group differences found in several of the above variables, linear mixed effect models were fitted to data restricted to the responder group, in order to confirm whether between group differences also represented change from baseline values (Table 4.3 and Table 4.4). These analyses confirmed that there was a significant increase at week 8 for bsALP ($\beta = 5.5$ μ g/L [95% CI 3.0, 8.5], $p < 0.001$) and P1NP ($\beta = 13.7$ μ g/L [95% CI 9.0, 18.4], $p < 0.001$) compared to baseline. There were also significant decreases for CPM ($\beta = -6.8 \times 10^3$ particles/ml [95% CI -10.7, -2.9], $p = 0.001$), CPP-I ($\beta = -21.2 \times 10^4$ particles/ml [95% CI -28.6, -13.8], $p < 0.001$) and cFGF23 ($\beta = -142.2$ RU/mL [95% CI -192.8, -91.6], $p < 0.001$) at week 8 compared to baseline.

Figure 4.3: Change in calciprotein particles and fibroblast growth factor-23 with infliximab treatment



Comparison between “non-responder” and “responder” groups of CPM, CPP-I, CPP-II, iFGF23 and cFGF23 during induction therapy with infliximab for active disease. Quiescent group also displayed for reference. Normally distributed data (CPM, CPP-I and cFGF23) presented as mean with error bars showing standard deviation. Skewed data (CPP-II and iFGF23) presented as median with error bars showing interquartile range. Data points offset for clarity.

*p < 0.05 for responder versus non-responder, ** p < 0.01 for responder versus non-responder.

Abbreviations: CPM, calciprotein monomer; CPP, calciprotein particle; FGF23, fibroblast growth factor-23

Table 4.4: Linear mixed-effect models for calciprotein particles and fibroblast growth factor-23 in patients with active CID receiving infliximab induction therapy

	CPM x10 ³ AU	CPP-I x10 ⁴ /mL	CPP-II x10 ³ /mL	iFGF23 pg/mL	cFGF23 RU/mL
Group mean (SD) or median (IQR)					
<i>Non-responder</i>					
Baseline	14.3 (5.0)	65.1 (20.4)	33.3 (15.8, 48.4)	85.0 (64.0, 101.6)	265.2 (47.2)
Week 1	12.2 (3.8)	69.4 (25.1)	36.7 (24.8, 49.5)	79.2 (58.0, 97.0)	281.4 (80.2)
Week 2	13.8 (3.3)	66.4 (17.3)	39.7 (16.5, 62.3)	69.1 (49.9, 100.7)	258.5 (63.7)
Week 6	11.3 (2.7)	67.8 (17.7)	30.5 (14.0, 56.2)	83.3 (64.9, 98.5)	300.3 (56.5)
Week 8	14.0 (4.5)	67.3 (16.3)	13.7 (10.9, 27.4)	90.7 (69.1, 109.5)	280.4 (58.8)
<i>Responder</i>					
Baseline	11.9 (4.1)	62.7 (14.2)	30.3 (15.2, 43.3)	70.7 (59.1, 77.2)	297.4 (69.7)
Week 1	12.0 (5.9)	61.9 (17.8)	16.2 (9.2, 52.0)	63.7 (51.0, 88.4)	305.8 (78.6)
Week 2	8.1 (4.3)	62.2 (18.2)	29.2 (15.0, 34.8)	54.3 (52.6, 74.7)	224.6 (59.2)
Week 6	5.0 (1.9)	41.5 (13.7)	17.4 (15.4, 26.3)	61.6 (48.2, 71.4)	190.5 (56.1)
Week 8	5.1 (2.1)	41.5 (13.1)	24.7 (15.9, 30.3)	61.4 (57.9, 65.4)	155.2 (52.4)
Between group coefficient estimate[†]					
Week 1	2.2 (-2.5, 6.8)	-5.1 (-15.5, 5.3)	-6.7 (-38.4, 24.9)	2.7 (-13.5, 18.9)	-7.9 (-70.9, 55.2)
Week 2	-3.3 (-7.9, 1.4)	-1.9 (-12.5, 8.8)	-13.3 (-45.2, 18.5)	-1.2 (-18.0, 15.5)	-66.1* (-130.5, -1.7)
Week 6	-4.0 (-8.6, 0.7)	-23.8** (-34.7, -12.8)	-15.2 (-47.3, 17.0)	-5.3 (-23.0, 12.4)	-142.0** (-208.6, -75.4)
Week 8	-6.5** (-11.1, -1.8)	-23.4** (-34.8, -11.9)	4.2 (-28.5, 36.8)	-13.6 (-32.6, 5.3)	-157.4** (-227.0, -87.8)
Coefficient estimate versus baseline for responder group^{††}					
Week 1	0.1 (-3.0, 3.1)	-0.8 (-8.0, 6.5)	-1.7 (-18.0, 14.6)	-2.6 (-13.0, 7.8)	8.4 (-36.1, 52.8)
Week 2	-3.8* (-7.0, -0.6)	-0.5 (-7.8, 6.8)	-6.0 (-22.8, 10.8)	-8.8 (-20.1, 2.6)	-72.9** (-118.6, -27.1)
Week 6	-7.0** (-10.5, -3.4)	-21.1** (-28.4, -13.7)	-12.2 (-29.8, 5.5)	-6.5 (-19.2, 6.3)	-106.9** (-154.8, -59.1)
Week 8	-6.8** (-10.7, -2.9)	-21.2** (-28.6, -13.8)	-8.8 (-27.6, 10.0)	-7.2 (-21.7, 7.4)	-142.2** (-192.8, -91.6)

Table shows group mean (SD), median (IQR) or mixed effects model coefficient (95% confidence interval).

Week 0 and non-responder group used as reference groups for each model.

*p <0.05, **p <0.01.

†Coefficient estimate is for responder group compared to non-responder group.

††Coefficient estimate is for categorical time compared to baseline for models fitted to responder group only.

Abbreviations: SD, standard deviation; IQR, interquartile range; CPM, calciprotein monomer; CPP, calciprotein particle; FGF23, fibroblast growth factor-23

4.4 Discussion

In this exploratory observational study, modulation of inflammation in patients with CID appeared to be associated with dynamic changes in bone turnover profile, and this was paralleled by changes in CPP and FGF23 metabolism. To our knowledge, this is the first study to report that interventions with anti-inflammatory and immunomodulatory anti-TNF biologics like infliximab might be useful in modifying CPP levels. At baseline patients with active inflammation were found to have evidence of suppressed bone formation (osteoblast activity), and high bone resorption (osteoclast activity), which was accompanied by high levels of CPM, CPP-I and cFGF23. Serially, patients who showed response to infliximab demonstrated improvements in bone formation markers which were accompanied by reductions in CPM, CPP-I and cFGF23. Notably, all of these longitudinal changes appeared to trend significantly towards values seen in the quiescent group.

This data adds to the accumulating evidence that alterations in bone metabolism may be a significant determinant of CPP in the circulation. While at baseline, elevated levels of CPM and CPP-I were observed alongside both suppressed bone formation and high bone resorption, it is notable that only effects on bsALP and P1NP appeared sustained at week 8 in responders, whereas changes in the osteoclast marker TRAcP 5b, occurred much earlier, were transient, and not mirrored by changes in CTx. Indeed, the increase in bone formation markers appeared to more closely parallel reductions in CPM and CPP-I. Given these observations, it could be speculated that bone formation may be the dominant driver underlying serum levels of CPP in CID. This contrasts with observations in dialysis cohorts, where CPP positively correlate with the degree of hyperparathyroidism, a condition where high turnover bone disease and enhanced osteoclastic activity is a prominent feature.²²⁴ Moreover, in a *post hoc* analysis of a randomised trial of haemodialysis patients comparing standard to high dialysate magnesium, Bressendorff *et al* found that while CPP were weakly negatively correlated with bsALP, the association with TRAcP 5b was nearly twice as strong.²⁹⁶ Taken together this suggests both formation and resorption components of remodelling may contribute to changes in circulating CPP and it is the net balance or coupling of these processes that determines effects on the serum CPP pool. Further work is needed to validate these findings against ‘gold standard’ assessment of bone turnover by histomorphometric analysis of bone biopsies. Compatible with these observations in humans, early animal models by Price *et al* found

that use of etidronate at supratherapeutic doses known to acutely inhibit bone formation was associated with the appearance of CPP in circulation.²²² In subsequent work, they found that this effect was ameliorated when the animals were first pre-treated with various inhibitors of bone resorption.²²³ A notable point of difference between these experiments and available clinical data, is that the supratherapeutic doses of etidronate also led to significant fluctuations in serum calcium and phosphate, potentially resulting in intravascular CPP formation. Nevertheless, one plausible explanation of this experimental data, as well as previous clinical studies in CKD and now in CID is that bone acts as both a source and a sink of CPP.²⁰⁶ Therefore, it may be that in situations where rates of bone formation and bone resorption are geared toward accelerated net bone loss, this leads to elevated serum CPP, especially when mineral excretion is impaired as in CKD.

This hypothesis is still lacking a complete mechanistic explanation of how alterations in bone turnover would eventually result in elevations of CPP in circulation. In lab-based experiments, under physiological conditions, the process from assembly of CPM which coalesce to form CPP-I and ultimately CPP-II takes hours.²²⁰ By comparison, injected artificial CPP particles are rapidly cleared from circulation within minutes,²¹⁸ and so doubts have previously been raised whether CPP-I/II could be assembled in significant numbers within the serum itself.²²⁰ In their early works, Price *et al* speculated that CPP could instead be assembled within the extravascular microcompartments of the bone, before spilling over into the intravascular circulation, however this requires further confirmation.²²³ Importantly, a “bone-centric” paradigm of CPP formation does not exclude the gastrointestinal tract from also being a significant contributor. As described in Chapter 1, and built on in Chapter 3, there is significant evidence that the gastrointestinal tract is also an important source of circulating CPP. It is possible that an interrelationship between the gastrointestinal tract, bone and CPP may exist.¹⁸⁶ Indeed, given that the majority of patients in this pilot study had GI disease, it is plausible that improvement in GI tract barrier function in response to infliximab may have imparted effects on the absorption of mineral.³¹²

The clinical interest in CPP stems mostly from its apparent role as a mediator of vascular toxicity. However, almost all of the human data linking CPP to clinically relevant events come from patients with CKD. While CPP have also been found to be elevated in CID,

larger studies incorporating vascular, patient relevant, outcomes are needed to clarify the significance of CPP in this setting.²⁰⁷ While in this study a reduction of CPM and CPP-I was observed after treatment of the underlying inflammatory condition, there was less clear delineation for CPP-II between those with active and quiescent disease at baseline, and no clear longitudinal change between response groups. This is potentially of significance, given that several pre-clinical studies have suggested that CPP-II is the main mediator of cellular toxicity, albeit more recent pre-clinical,²¹⁸ and clinical data has suggested that smaller CPP species are associated with important outcomes.^{203, 205} The failure to detect a change in CPP-II may reflect the small sample size of this study, however it is notable that some,^{224, 226} but not all,²⁹⁶ previous clinical studies using the same flow cytometric assay also found that only CPP-I was responsive to intervention. Given the potential link between CPP and bone, if CPP is also confirmed to be associated with vascular outcomes in CID then this may suggest a role of CPP in the “bone-vascular axis”. Importantly, given the reductions of CPM and CPP-I with treatment, it is possible that CPP could act as not only a marker, but also a modifiable risk factor for bone and/or vascular outcomes, through therapeutic interventions targeting drivers of high bone turnover, including systemic inflammation. Clearly, further work is needed to clarify the mechanistic role, and ultimately, the clinical utility of CPP measured in these processes.

Fetuin-A, an indispensable component of CPP as well as its precursors, has notable links to inflammation, as well as bone and mineral metabolism. Fetuin-A is considered a negative acute-phase reactant,¹⁷² and consistent with this, in this study lower serum levels were observed in those with active disease, which numerically increased in responders following induction therapy with infliximab. Whilst primarily secreted by hepatocytes in adults, fetuin-A extravasates from extracellular fluid to mineralised tissues because of its high affinity for hydroxyapatite and in bone represents one of the most abundant of the non-collagenous proteins.¹⁸² Fetuin-A has also previously been associated with significant pathological outcomes, with serum levels showing negative correlations with vascular calcification,¹⁹⁰ aortic stiffness,¹⁹² and mortality.¹⁸⁹ Nevertheless, these associations have not been uniform,¹⁹⁸ and one explanation for this variability is that the mineral bound component of fetuin-A (i.e. CPP), may be the more relevant metric of pathology than total serum levels.²⁰⁰

The peptide hormone FGF23 is another factor with evidence of biological toxicity, and with intriguing links to inflammation and bone.³¹³ FGF23 is itself bone-derived and primarily functions as an endocrine regulator of phosphate excretion and vitamin D metabolism in the kidney.³¹⁴ In CKD populations it has consistently been positively associated with cardiovascular events and death, although whether this is through direct causation is unclear.¹⁴⁴ While the exact mechanism(s) regulating FGF23 in health and disease remain under investigation, levels have previously been shown to positively correlate with inflammation, including in clinical studies of patients with rheumatoid arthritis and inflammatory bowel disease.^{315, 316} FGF23 has also been observed to correlate with CPP,^{206, 228} and recently, CPP have been proposed to be a direct mediator of FGF23 expression in bone cells.²²⁵

Inactivation of FGF23 by post-translational cleavage is believed to be an important step in the regulation of FGF23 bioactivity,³¹⁷ and this can be captured *ex vivo* by measurement of FGF23 in blood using intact and C-terminal assays in tandem. Whereas intact FGF23 assays exclusively measure the full-length protein, the C-terminal assay detects both the full-length hormone and cleaved C-terminal fragments. In this study, the pattern of changes in cFGF23 largely followed that of CPM and CPP-I, with patients with active CID demonstrating higher levels than the quiescent group at baseline, and those responding to infliximab therapy showing progressive reductions in levels over 8 weeks. By comparison, no evidence of differences in intact FGF23 at baseline or longitudinally were found. Interestingly, pro-inflammatory stimuli have been found to differentially effect intact and C-terminal FGF23 concentrations in osteocyte cultures and mouse models.^{318, 319} Thus it is possible that inflammation, and potentially CPP, might drive an increase FGF23 transcription in CID, but that this is accompanied by an appropriate and compensatory upregulation of cleavage in order to maintain overall bioactivity and null effects on phosphaturia and vitamin D. This could explain the elevation in cFGF23 in patients with active inflammation at baseline which “normalised” in responders, while also accounting for the lack of signal in iFGF23.

This study has a number of limitations, including the study of a small and heterogenous group of patients. Inflammatory bowel disease and inflammatory arthritis each encompass diverse disease entities with distinct pathogenesis and clinical manifestations. Nonetheless, these conditions share a number of common features, including an apparent

environmental stimulus, often in individuals with genetic susceptibility, triggering an exaggerated and sustained inflammatory response. Indeed, most CIDs also share a propensity for accelerated systemic bone loss and vascular calcification. Importantly, another shared feature of these disease entities is the efficacy of treatment with TNF-alpha inhibitors, such as infliximab, supporting the idea of shared pathological pathways.

Given the small sample size no formal comparison testing was undertaken of between group demographics, nor were adjustments made for any demographic variable in the models. For similar reasons we also considered it inappropriate to formally test for correlations between variables or to include the quiescent group in the linear mixed models, as this would have significantly added to the number of comparisons applied to the limited number of participants. Hence, formal hypothesis testing was limited to a small number of pre-defined outcomes relating to CPP and bone markers parameters. This was always intended as an exploratory study, and larger trials are needed to test the robustness of the apparent associations described. Nevertheless, considering the small heterogenous cohort, the delineation between patients with active disease and quiescent disease in several of the study variables at baseline was remarkable. Further, the tendency of multiple variables in the responder group to trend towards values seen in the quiescent group was also impressive in their uniformity. An exception to this were the bone resorption markers CTx and TRAcP 5b. However, considering the small numbers and relatively short observational period, it is possible that TRAcP 5b and/or CTx may “normalise” in larger or longer-term studies, as has previously been suggested.^{301, 302}

Given the observational nature of this study, the findings are associative, and we can only speculate on potential underlying mechanistic links. The notion of CPP being directly linked to bone turnover requires confirmation in future carefully designed studies. In particular, we speculate that inflammation drives dynamic changes in bone turnover, which sequentially results in elevations in CPP. These assumptions are largely based on the well-described effects of inflammation on bone turnover, driven by the “cross talk” between multiple cytokine and bone cell signalling pathways.²³¹ Given that the observations in this study are temporarily linked to the administration of infliximab, it seems reasonable to assume that the observations of CPP are “downstream” to the effects of inflammation modulation and bone turnover. However it is notable that in lab studies CPP itself has been found to induce cellular inflammatory responses and also to inhibit

osteoblast mineralisation.^{214, 215, 218} As such, if interactions between inflammation, bone turnover and CPP are confirmed, they may not necessarily be unidirectional. Similarly, both inflammation³¹⁸ and CPP²²⁵ have previously been proposed as modulators of FGF23 expression, and data from this study does not allow for clarification of the hierarchy of these effects.

4.5 Conclusion

This data suggests that infliximab induction therapy is associated with an increase in bone formation markers and concomitant reductions in CPM and CPP-I in patients with active CID. This raises the possibility that bone turnover may be a significant determinant of serum CPP levels, and that in situations of net bone loss commonly seen in CID and CKD, may lead to expansion of the circulating CPP pool. Given the growing evidence implicating CPP as a mediator of vascular disease, this may also suggest that elevations in CPP form part of the disturbed bone-vascular axis.

Chapter 5 Effect of lanthanum carbonate on serum calciprotein particles in patients with stage 3-4 CKD – results from a placebo-controlled randomised trial

5.1 Introduction

For patients with CKD, the risk of cardiovascular disease and mortality is highest in those with kidney failure treated with dialysis. However vascular pathology often begins much earlier in the course of CKD.² As discussed in Chapter 1, while some of this risk is attributable to a high burden of traditional risk factors for cardiovascular disease, non-traditional risk factors, such as abnormalities in mineral metabolism may also contribute. Given that mineral metabolism abnormalities are often apparent in the early stages of CKD,¹² it is conceivable that early intervention may prevent or reduce the progressive burden of cardiovascular disease as CKD advances. Given previous evidence that elevated levels of CPM and CPP-I/II may be linked to a range of intermediate,^{200, 202, 203} and hard,^{204, 205} cardiovascular endpoints, there may be a rationale to therapeutically lower serum levels of CPP in this setting.

As described in Chapter 3, there is considerable evidence to suggest that mineral from the diet is a significant source of circulating CPP. As such, reduction of gastrointestinal phosphate load has been suggested as a potential strategy to reduce elevated levels of serum CPP in CKD. In patients with kidney failure treated with haemodialysis, it has been shown that use of non-calcium-based intestinal phosphate binders are associated with lower levels of serum CPM and CPP-I compared to calcium-based binders.^{226, 227} In these studies, however, it was not clear if reductions in CPP were predominantly related to a reduction in intestinal phosphate absorption *per se*, or the avoidance of exogenous calcium loading associated with calcium-based binders. In addition, given these previous studies have been in patients requiring dialysis, it is also unclear whether phosphate binders can be used to lower serum CPP in patients with earlier stages of CKD, who notionally tend to also have less advanced cardiovascular disease, and so may ultimately benefit more from intervention.

The IMpact of Phosphate Reduction On Vascular End-points in CKD (IMPROVE-CKD) trial³²⁰ was a placebo-controlled, randomised trial assessing the effect of the non-calcium based phosphate binder lanthanum carbonate on cardiovascular markers over 96 weeks in 278 participants with stage 3b/4 CKD. In this sub-study analysis, the effect of lanthanum carbonate on serum CPP in a non-dialysis dependent CKD population was examined. All participants who had CPP data at baseline and at least one follow-up measure were included.

5.2 Methods

IMPROVE-CKD was an international study, conducted across 17 sites in Australia, New Zealand and Malaysia and coordinated by the Australasian Kidney Trials Network (AKTN).³²⁰⁻³²² Ethical approval was obtained by each local institutional ethics committee and the trial was prospectively registered (ACTRN12610000650099) and conducted in accordance with the Declaration of Helsinki.

All participants provided written informed consent, were at least 18 years of age, had stage 3b or 4 CKD (eGFR 15-44 mL/min/1.73m²), and had at least one recording of serum phosphate ≥ 1.00 mmol/L in the 6 months prior to enrolment. Potential participants were excluded if they had a serum phosphate ≤ 0.8 mmol/L at screening, or if they had a kidney transplant, a medical condition that may affect calcium and phosphate metabolism (apart from CKD), a gastrointestinal disorder, were pregnant or breast feeding, or had been hospitalised or had a cardiovascular event within one month of screening.

Participants were randomised to receive either 500 mg lanthanum carbonate or matching placebo three times a day with meals for 96 weeks. Study medication was up-titrated by local investigators if serum phosphate remained persistently > 1.60 mmol/L, to a maximum total dose of six tablets per day. Other phosphate binders were ceased for 2 weeks prior to the commencement of the study medication, however, other phosphate binders could be prescribed during the study for persistent hyperphosphataemia if study medication had already been maximally titrated.

5.2.1 Outcome measures

For this sub-study, blood samples for CPP were collected at baseline and after 24, 48, 72 and 96 weeks. Blood was collected via standard venepuncture techniques and allowed to clot for 30 minutes before centrifugation. Serum aliquots were stored at -80°C until blinded batch-analysis at our laboratory. Serum CPP-I and CPP-II were measured using the same fluorescent probe-based assay running on a BD FACSVerse flow cytometer as described in Chapter 3 and Chapter 4.^{206, 226}

5.2.2 Surrogate cardiovascular markers

In IMPROVE-CKD³²⁰⁻³²² vascular stiffness was assessed using pulse wave velocity (PWV), which was measured as the mean of two carotid-femoral measurements at each time-point using a SphygmoCor device (AtCor, PWV Inc., Sydney, Australia), while abdominal aortic calcification (AAC) was determined by Agatston score from computed tomography (CT) at baseline and 96 weeks. Agatston score was calculated from the density and total size of calcification in the abdominal aortic wall over a length of 10 cm below the upper end plate of the L2 lumbar vertebral body. As well as PWV and AAC, the binary variables of “PWV > 10 m/s” as well as “presence of AAC” were also included as variables at baseline and 96 weeks. PWV $\geq$ 10 m/s was used as it is considered as the threshold where risk of cardiovascular events is significantly increased.³²³ Presence of AAC was defined as Agatston score greater than zero.

5.2.3 Statistical analysis

The main outcome of interest was the effect of lanthanum carbonate on CPP-I and CPP-II at 96 weeks. Between group differences for both CPP-I and CPP-II were analysed by fitting linear mixed effects models. For each model, treatment group, categorical time, group-time interaction and baseline values were fitted as fixed effects, and an unstructured covariance structure was used to model within-patient correlation. Missing baseline values (6 participants for CPP-I and 7 participants for CPP-II) were replaced by mean imputation. Values for both CPP-I and CPP-II were natural log transformed before fitting each model to ensure normal distribution of residuals. To aid interpretation, the regression coefficients and 95% confidence limits were exponentiated to obtain estimates of percentage change. Models for CPP-I and CPP-II were repeated after i) adjusting for medication compliance, ii) restricting the analysis to participants with at least 80% compliance, iii) adjusting for CKD stage, age and diabetes, and iv) restricting the analysis to participants with complete CPP data for each visit. For these analyses, compliance was defined as the number of study follow-up visits that the participant was taking the randomised treatment.

In further exploratory analyses, Pearson correlation was used to examine the relationship between baseline serum CPP and demographic and biochemical variables. Correlations

between baseline CPP and both baseline and change in surrogate cardiovascular markers were also explored, as well as change in eGFR.

No adjustments for multiple comparisons were made. All statistical hypothesis testing was two-sided with p values <0.05 considered statistically significant. Analyses were performed using SAS software (version 9.4; SAS Institute Inc., Cary, NC).

5.3 Results

5.3.1 Participant characteristics and medication use

A total of 278 participants were enrolled in IMPROVE-CKD. Of these, 253 had CPP data for baseline and at least one follow-up timepoint and were included in this analysis. Participant demographic, biochemical and clinical characteristics at baseline were well-matched between groups (Table 5.1, Table 5.2 and Table 5.3). Overall, the mean age of the cohort was 62.4 ± 12.6 years, 32.0% were female, 67.2% had stage 4 CKD and 91% were normophosphataemic (serum phosphate ≤ 1.50 mmol/L). At baseline, median serum CPP-I was 14.9×10^4 particles/mL [interquartile range (IQR) 4.6 – 49.3] and median CPP-II was 3.3×10^3 particles/mL [IQR 1.4 – 5.4].

There was no significant between group difference in the median number of study medication tablets consumed per day (2.69 for placebo; 2.55 for lanthanum), and the proportion of participants using a non-study phosphate binder during the study was low in each group (8.7% for placebo; 7.9% for lanthanum). Use of cholecalciferol (22.5% overall), and calcitriol (9.1% overall) was similar in each group.

Table 5.1: Participant demographics and characteristics at baseline

	Overall (n = 253)	Placebo (n = 127)	Lanthanum (n = 126)
Age (yrs)	62.4 ± 12.6	61.8 ± 13.1	63.1 ± 12.1
Female sex, n (%)	81 (32.0)	41 (32.3)	40 (31.7)
Country of study site, n (%)			
Australia	182 (71.9)	92 (72.4)	90 (71.4)
New Zealand	30 (11.9)	15 (11.8)	15 (11.9)
Malaysia	41 (16.2)	20 (15.7)	21 (16.7)
Ethnicity			
White/Caucasoid	157 (62.1)	85 (66.9)	72 (57.1)
Australian Aboriginal or Torres Strait Islander	1 (0.4)	0	1 (0.8)
New Zealand Māori or Pasifika	6 (2.4)	1 (0.8)	5 (4.0)
Asian	62 (24.5)	29 (22.8)	33 (26.2)
Other	27 (10.7)	12 (9.4)	15 (11.9)
Body Mass Index (kg/m ²)	29.8 ± 6.0	30.1 ± 6.2	29.6 ± 5.8
Waist circumference (cm)	102.4 (15.8)	103.0 (16.0)	101.9 (15.70)
CKD stage, n (%)			
Stage 3b	83 (32.8)	42 (33.1)	41 (32.5)
Stage 4	170 (67.2)	85 (66.9)	85 (67.5)
Primary cause of kidney disease, n (%)			
Diabetic nephropathy	78 (30.8)	41 (32.3)	37 (29.4)
Hypertension/vascular	42 (16.6)	19 (15.0)	23 (18.3)
Glomerulonephritis	36 (14.2)	21 (16.5)	15 (11.9)
Reflux nephropathy	9 (3.6)	5 (3.9)	4 (3.2)
Polycystic kidney disease	12 (4.7)	4 (3.1)	8 (6.3)
Other	38 (15.0)	17 (13.4)	21 (16.7)
Unknown	38 (15.0)	20 (15.7)	18 (14.3)
Diabetes mellitus, n (%)	113 (44.7)	54 (42.5)	59 (46.8)
Hypertension, n (%)	228 (90.1)	114 (89.8)	114 (90.5)
Dyslipidaemia, n (%)	198 (78.3)	100 (78.7)	98 (77.8)
Cardiovascular disease, n (%)	75 (29.6)	35 (27.6)	40 (31.7)
Peripheral vascular disease, n (%)	21 (8.3)	10 (7.9)	11 (8.7)
Cerebrovascular disease, n (%)	16 (6.3)	9 (7.1)	7 (5.6)
Smoking status, n (%)			
Never	123 (48.6)	65 (51.2)	58 (46.0)
Former	115 (45.5)	57 (44.9)	58 (46.0)
Current	15 (5.9)	5 (3.9)	10 (7.9)

Mean ± SD or number (percentage)

Table 5.2: Biochemical and clinical parameters at baseline

	Overall (n = 253)	Placebo (n = 127)	Lanthanum (n = 126)
Haemoglobin (g/L)	124.1 ± 16.4	123.2 ± 16.4	125.1 ± 16.4
Albumin (g/L)	39.0 ± 4.4	38.7 ± 4.2	39.2 ± 4.6
Bicarbonate (mmol/L)	23.4 ± 3.4	23.5 ± 3.2	23.3 ± 3.7
Urea (mmol/L)	15.9 ± 5.1	15.6 ± 4.8	16.2 ± 5.4
Creatinine (µmol/L)	221.2 ± 59.9	218.9 ± 58.56	223.5 ± 61.4
eGFR (mL/min/1.73m ²)	26.6 ± 8.3	27.0 ± 8.3	26.2 ± 8.2
Uric acid (mmol/L)	0.5 ± 0.11	0.5 ± 0.1	0.5 ± 0.1
Glucose (mmol/L)	6.3 ± 3.0	6.3 ± 2.8	6.4 ± 3.2
Calcium (mmol/L)	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
Phosphate (mmol/L)	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
ALP (IU/L)	88.9 ± 31.3	92.4 ± 30.3	85.4 ± 32.0
Bone specific ALP (µg/L)	14.2 (10.4-18.1)	14.8 (10.9-18.2)	13.3 (9.8-17.9)
PTH (pmol/L)	12.1 (7.2-17.8)	11.8 (7.9-16.4)	12.9 (6.4-19.4)
25-hydroxyvitamin D (nmol/L)	70.3 ± 31.2	68.8 ± 30.0	71.8 ± 32.4
1,25-dihydroxyvitamin D (pmol/L)	77.8 ± 26.8	75.5 ± 26.7	80.3 ± 26.9
C-terminal FGF23 (RU/mL)	222.0 (155.7-334.1)	225.8 (161.0-328.2)	220.7 (151.7-353.1)
Intact FGF23 (pg/mL)	133.0 (89.1-202.0)	136.1 (89.1-202.0)	124.8 (88.9-208.5)
Urinary phosphate excretion (mmol/24hrs)	24.1 ± 11.6	25.8 ± 12.9	22.4 ± 9.9
Cholesterol (mmol/L)	4.5 ± 1.1	4.5 ± 1.1	4.6 ± 1.2
Triglyceride (mmol/L)	2.1 ± 1.3	2.0 ± 1.1	2.2 ± 1.5
C-reactive protein (mg/L)	4.8 ± 6.4	4.6 ± 6.3	5.0 ± 6.5
Protein excretion (mg/24h)	805 (209-1765)	920 (310-1880)	570 (120-1730)
Urine ACR (mg/mmol)	73 (20-138)	86 (23-147)	53 (16-137)
Pulse-wave velocity (m/s)	10.7 ± 3.6	10.8 ± 3.6	10.7 ± 3.5
Pulse wave velocity ≥ 10 m/s	115 (47.7)	55 (45.5)	60 (50.0)
Aortic calcification	174 (80.6%)	81 (76.4%)	93 (84.5%)
Agatston score (HU)	1288 (58-5178)	710 (8-3373)	1975 (257-5744)

Mean ± SD, number (percentage) or median (interquartile range)

eGFR - calculated using CKD–Epidemiology Collaboration equation

Abbreviations: ACR, albumin-to-creatinine ratio; ALP, alkaline phosphatase; FGF23, fibroblast growth factor-23; HU, Hounsfield unit; PTH, parathyroid hormone.

Table 5.3: Baseline dietary phosphate intake

	Overall (n = 80)	Placebo (n = 40)	Lanthanum (n = 40)
Daily phosphate intake (mg)	1538.5 ± 359.8	1463.9 ± 313.8	1613.1 ± 390.3
Animal phosphate intake (mg)	893.6 ± 321.3	888.9 ± 283.6	898.4 ± 358.6
Plant phosphate intake (mg)	400.6 ± 179.4	384.1 ± 135.5	417.2 ± 215.1
Mixed phosphate intake (mg)	244.1 ± 180.1	190.7 ± 116.0	297.5 ± 215.5

Mean ± SD

Dietary phosphate intake assessed by 7-day self-administered diet record in a subgroup of participants as previously described.^{324, 325} Assessment was made prior to baseline appointment.

5.3.2 Primary outcome

Summary data for CPP-I and CPP-II at each timepoint are displayed in Figure 5.1. After 96 weeks, lanthanum carbonate was not associated with a significant difference in CPP-I (22.8% [95% confidence interval (CI) -39.2, 36.4], $p = 0.65$) or CPP-II (-18.3% [95% CI -40.0, 11.2], $p = 0.20$) compared to placebo (Table 4.4). Adjustment for medication compliance, participants with at least 80% compliance, and for CKD stage, age and diabetes, did not significantly alter the results of these analyses. There was notable variation in the observed distribution of values of CPP-I and CPP-II between sites (Figure 5.2), however further adjustment for study site yielded similar estimates of treatment effect.

Figure 5.1: Serum primary (CPP-I) and secondary (CPP-II) calciprotein particles over 96 weeks by treatment group (geometric mean and 95% confidence interval)

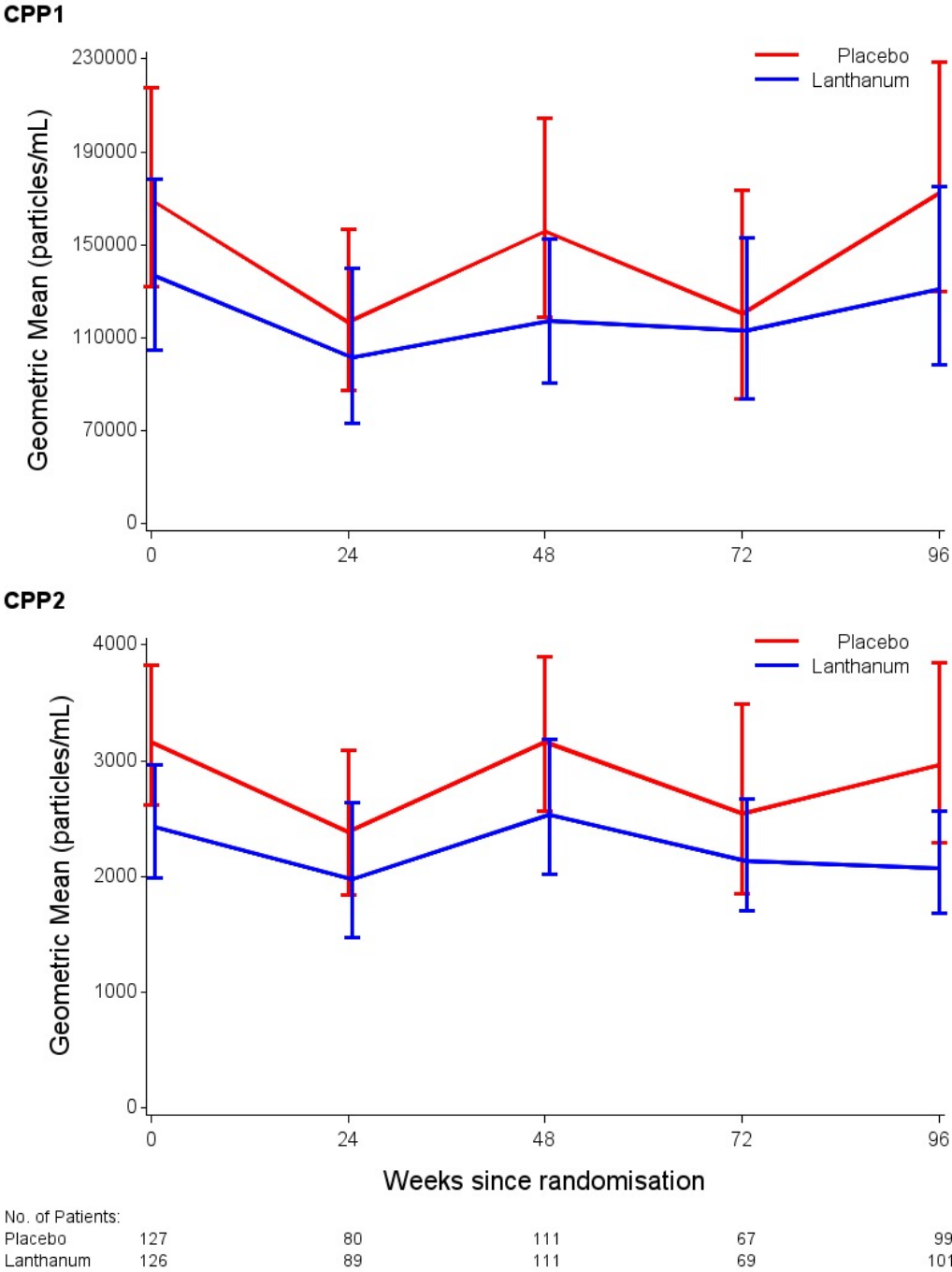
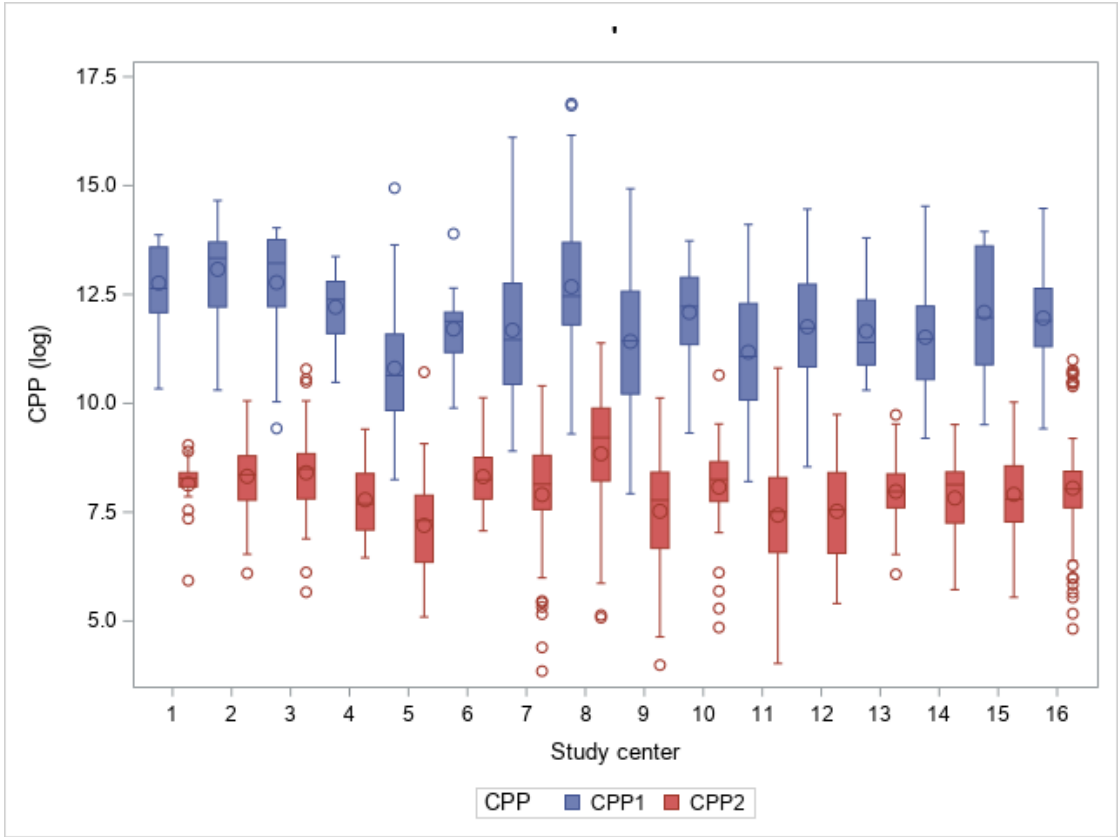


Table 5.4: Calciprotein particles (CPP) at baseline and after 96 weeks, with between group differences

	Baseline		Week 96		Treatment effect vs placebo at Week 96	p value
	N	Median (IQR)	N	Median (IQR)	% change (95% CI)	
CPP-I (x 10⁴ particles/mL)						
Placebo	127	16.3 (6.5-59.7)	99	17.6 (6.0-54.8)	-	-
Lanthanum		12.4 (3.7-48.5)		15.2 (4.6-35.8)	22.8 (-39.2, 36.4)	0.648
CPP-II (x 10³ particles/mL)						
Placebo	126	3.8 (1.5-5.8)	99	3.5 (1.3-5.8)	-	-
Lanthanum		3.0 (1.3-5.1)		2.6 (1.0-4.0)	-18.3 (-40.0, 11.2)	0.198

Change in calciprotein particles (CPP) compared to baseline, estimated from mixed effects models with repeated measurements. Models were fitted to natural log transformed data in order to ensure normal distribution of residuals; thereafter the regression coefficients have been exponentiated into percentage change to aid interpretation.

Figure 5.2: Distribution of serum primary (CPP-I) and secondary (CPP-II) calciprotein particles by study site



Distribution of observations across all timepoints and study groups. CPP-I and CPP-II are measured in particles/mL (natural log transformed values shown for clarity).

5.3.3 Correlation between CPP and other variables

There were no correlations between serum CPP-I or CPP-II and markers of vascular stiffness (PWV and $PWV > 10$ m/s) or vascular calcification (AAC Agatston score and binary presence of AAC) at baseline, nor was there any correlation between baseline levels of CPP-I or CPP-II and change in PWV, Agatston score, or eGFR at 96 weeks (Table 5.5). There were few correlations between patient baseline demographics and conventional biochemistry with serum CPP (Table 5.6).

Table 5.5: Correlations between baseline serum CPP-I and CPP-II and cardiovascular surrogate markers and eGFR

	Ln CPP-I		Ln CPP-II	
	ρ	p value	ρ	p value
Baseline cardiovascular surrogate markers				
Pulse wave velocity	-0.080	0.222	-0.041	0.535
Pulse wave velocity >10 m/s	-0.025	0.706	-0.013	0.838
Presence of abdominal aortic calcification	-0.001	0.991	0.007	0.925
Agatston score	-0.006	0.936	-0.002	0.981
Change in cardiovascular surrogate outcomes				
Δ Pulse wave velocity	-0.063	0.402	-0.089	0.240
Δ Agatston score	-0.086	0.263	0.035	0.650
Change in renal function				
Δ eGFR	-0.003	0.966	-0.048	0.481

ρ = Pearson correlation coefficient

CPP-I and CPP-II natural log transformed

Δ = value at 96 weeks – baseline value

Presence of abdominal aortic calcification defined as Agatston score greater than zero

Abbreviations: eGFR, estimated glomerular filtration rate; CPP, calciprotein particles

Table 5.6: Correlations between baseline serum CPP-I and CPP-II and other variables

	Ln CPP-I		Ln CPP-II	
	ρ	p value	ρ	p value
Demographic variables				
Age	-0.026	0.687	-0.082	0.200
Male gender (categorical)	0.131	0.040	0.120	0.060
Clinical variables				
BMI	0.067	0.293	0.060	0.351
Diabetes mellitus (categorical)	0.074	0.247	0.075	0.239
Systolic blood pressure	-0.068	0.292	-0.117	0.070
Diastolic blood pressure	-0.067	0.297	-0.004	0.946
Biochemical variables				
Albumin	0.106	0.099	0.021	0.748
Bicarbonate	0.095	0.148	0.024	0.715
Urea	0.050	0.432	0.014	0.826
Creatinine	-0.026	0.068	0.094	0.143
eGFR	-0.021	0.732	-0.131	0.041
Uric acid	-0.048	0.468	0.123	0.063
Glucose	-0.015	0.820	-0.066	0.314
Calcium	0.084	0.191	0.013	0.841
Phosphate	0.015	0.819	0.025	0.695
ALP	-0.063	0.322	-0.063	0.327
Bone-specific ALP	-0.100	0.119	-0.014	0.833
PTH	-0.090	0.193	-0.030	0.664
25-hydroxyvitamin D	-0.064	0.389	-0.103	0.166
1,25-dihydroxyvitamin D	-0.081	0.324	-0.163	0.044
C-terminal FGF23	0.009	0.896	0.014	0.831
Intact FGF23	0.052	0.439	-0.075	0.269
Urinary phosphate excretion	0.063	0.352	-0.025	0.711
Cholesterol	-0.014	0.830	0.051	0.430
Triglyceride	-0.006	0.932	-0.018	0.785
C-reactive protein	-0.070	0.279	0.038	0.557
Protein excretion	-0.006	0.929	0.016	0.806
Urine ACR	-0.035	0.611	0.067	0.328

ρ = Pearson correlation coefficient

CPP-I and CPP-II natural log-transformed

Abbreviations: ACR, albumin-to-creatinine ratio; ALP, alkaline phosphatase; BMI, body mass index; CPP, calciprotein particles; eGFR, estimated glomerular filtration rate; FGF23, fibroblast growth factor-23; PTH, parathyroid hormone.

5.4 Discussion

In this exploratory analysis of a large, international, randomised clinical trial, the use of lanthanum carbonate, a non-calcium containing phosphate binder, in patients with stage 3b and 4 CKD was not associated with any significant change in serum CPP-I or CPP-II over 96 weeks. This finding was not altered by adjusting the analysis for medication compliance, or other potential confounders, including CKD stage and diabetes. These findings suggest that intestinal phosphate binders may not be an effective strategy to lower serum CPP in patients with non-dialysis dependent CKD.

The lack of effect of lanthanum carbonate on serum CPP is perhaps not unexpected given that in the IMPROVE-CKD trial, lanthanum carbonate was also not associated with a reduction in serum phosphate when compared to placebo.³²⁰ While serum phosphate is arguably an insensitive marker of total phosphate balance, other measures of phosphate metabolism, including 24-hour urinary phosphate excretion, serum PTH and serum fibroblast growth factor-23 were also unaltered by lanthanum carbonate in IMPROVE-CKD.³²⁰ In the absence of other measurable effects of lanthanum carbonate on mineral metabolism in this cohort, the lack of a treatment effect on CPP would appear consistent with these other null observations.

These results do however contrast with previous smaller and shorter trials in haemodialysis patients, which reported that alterations of phosphate binders were associated with changes in serum CPP, as well as its precursor serum CPM, despite an absence of change in serum phosphate. In an open-label randomised control trial of 31 haemodialysis patients, Smith *et al* found that sevelamer carbonate was associated with significant reductions in CPP-I compared to calcium carbonate at 24 weeks.²²⁶ In a separate study measuring CPM by the gel-filtration method,²⁰⁸ Nakamura *et al* demonstrated that changing patients from calcium carbonate to lanthanum carbonate resulted in lower levels of CPM in a 16-week cross-over study of 24 haemodialysis patients.²²⁷

Notably though, both previous studies were in the setting of kidney failure requiring dialysis, and participants in these studies had numerically higher baseline values of conventional markers of mineral metabolism, including serum phosphate, than in the current cohort. Additionally, recent evidence has suggested that CPM appears to be

predominantly cleared by the kidneys.²¹⁹ A reasonable prediction may be that as CKD progresses, CPM, and as a result CPP-I/II, progressively accumulate. It is plausible that in more severe stages of CKD, as the homeostatic role of the kidneys is progressively lost, that gastrointestinal mineral load may become a more prominent determinant of serum CPP-I/II, and perhaps also more responsive to therapeutic interventions with phosphate binders. In line with this, in Chapter 3 post prandial metabolism of CPP-I and CPP-II appeared to be relatively unaffected in individuals with non-dialysis dependent CKD, whereas differences in CPM metabolism were prominent compared to healthy controls. This current study was conceived and undertaken before the full results of Chapter 3 were available, and in retrospect, analysing CPM instead of CPP-I and CPP-II may have been more informative given the degree of kidney impairment in this study was comparable to the CKD cohort in Chapter 3.

Another potential explanation for the differing results between IMPROVE-CKD and previous studies by Smith *et al* and Nakamura *et al*, is that the latter two studies used calcium carbonate as the comparator, rather than placebo. Given that calcium is a significant component of CPP, it is conceivable that in these studies the exogenous calcium load from calcium carbonate³²⁶ may have contributed to elevated levels of CPP, making it challenging to isolate the effects of intestinal phosphate binding in these studies. Given the divergent results of previous studies and our study, carefully designed future trials are required to separate and characterise the individual effect of phosphate binding from calcium load, as well as to clarify whether severity of CKD modifies any effect of phosphate binders on serum CPP.

It is notable that in this study there was a lack of correlation between serum CPP-I/II and two surrogate cardiovascular markers. Previous clinical studies suggested positive correlation between serum CPP and vascular endpoints in CKD, such as vascular stiffness,²⁰² coronary artery calcification,^{200, 203} the risk of cardiovascular events,²⁰⁵ and mortality.²⁰⁴ In this regard, it was unexpected that no correlation was found between CPP-I/II and markers of arterial stiffness or aortic vascular calcification at baseline, nor was baseline level of CPP-I/II associated with progression of either of these surrogate cardiovascular measures at 96 weeks. Interestingly though, in the overall study cohort, conventional markers of mineral metabolism, including serum phosphate, calcium and PTH were also not predictive of these measures.³²² It should be acknowledged that the

majority of participants in this study already had evidence of advanced vascular stiffness and calcification at baseline, given that the mean carotid-femoral PWV of the cohort was greater than 10 m/s and that 81% of participants had AAC on CT. This relative lack of heterogeneity may have reduced the ability to properly assess the association between serum CPP and arterial stiffening and calcification than in a more diverse population.

There was also a general lack of correlation between serum CPP and other demographic and biochemical markers. This study utilised the next generation flow-cytometric assay, which allows discrimination of CPP-I/II from other mineral containing components in blood, as well as separately quantifying CPP-I and CPP-II subtypes.²⁰⁶ This method uses a rapid freeze protocol and avoids prolonged incubation, minimising *ex vivo* generation of mineral precipitation artefact, and readouts have been shown to be stable after a freeze-thaw cycle.²²⁶ In addition, *post-hoc* analyses did not show any correlation between storage time of samples and levels of either CPP-I or CPP-II. This study adopted the same collection protocols with centralised batch analysis that has reliably been used in several recent single centre^{224, 226, 296} and multicentre³²⁷ studies. Unexpected variation in the distribution of CPP values between sites was observed. However, there was no suggestion of a centre effect when the main linear mixed effects models were refitted with adjustment for study site, and results were consistent when analyses were restricted to data from the largest study site, which contributed 48 participants (19%) to the overall study cohort. These additional analyses provide some reassurance that variation in sample collection and processing between sites is less likely to be the explanation for these null findings, although this is not possible to definitively exclude in retrospect. Nevertheless, future studies utilising this assay may help to clarify the relationship of serum CPP-I/II with other markers of mineral metabolism, as well as vascular risk, in patients with non-dialysis dependent CKD.

IMPROVE-CKD was a multi-national randomised controlled trial which, in comparison to other studies assessing serum CPP-I/II using the flow-cytometric assay, had large participant numbers and long follow-up. In comparison to previous trials assessing the effect of phosphate binders on serum CPM and CPP-I/II, this study was the only trial to use placebo as the comparator, and so the potential confounding from exogenous calcium loading can be excluded.

This sub-study did have several limitations. IMPROVE-CKD was designed to test the effect of lanthanum carbonate on surrogate cardiovascular markers rather than serum CPP, and so this analysis is exploratory by nature. Another permutation of the design is that trial medication was titrated against serum phosphate, rather than serum CPP, and so whether an alternative strategy to titrate lanthanum carbonate directly to serum CPP would have been more effective remains unclear. However, given that the median number of lanthanum tablets taken by participants was three per day, even if higher doses of lanthanum achieved reductions in serum CPP, it would be questionable whether such a therapeutic regimen would be feasible in routine clinical practice.

It is also important to note that blood draws were not standardised to time of day nor in relation to meals. As discussed, this current study was commenced before the full results of Chapter 3 were available, which demonstrated a significant post-prandial variation in serum CPP in those with normal or impaired kidney function. Data about last food intake and time of sample collection were not routinely collected in IMPROVE-CKD, and so it was not possible to retrospectively examine or control for this effect. Finally, surrogate cardiovascular markers of vascular stiffness (PWV) and calcification (AAC) were utilised, and it is not clear if serum CPP-I/II levels ascertained using this second-generation assay may still hold value in assessing risk of hard patient outcomes in this population, such as cardiovascular events and death.

5.5 Conclusion

This study found that use of the non-calcium containing phosphate binder lanthanum carbonate was not associated with any reduction of serum CPP-I or CPP-II in participants with stage 3b or 4 CKD over 96 weeks when compared to placebo. Further, the purported relationship between CPP and the surrogate cardiovascular markers of PWV and AAC were not able to be confirmed.

Chapter 6 Effect of a medium cut-off dialyser on novel mineral metabolism markers in patients on haemodialysis

6.1 Introduction

A hallmark of CKD is the increased concentration of inorganic and organic molecules in circulating plasma.³²⁸ This is initially related to the progressive retention of molecules that are usually excreted in the urine. However, secondary abnormalities in homeostatic controls, such as mineral metabolism, also evolve and become significant in the milieu of CKD.^{17, 329} For instance, as described in Section 1.3, retention of phosphate in CKD-MBD is classically accompanied by secondary elevations in FGF23 and PTH.

Globally, haemodialysis (HD) remains the principal therapy for kidney failure.²⁶⁴ The implicit goal of HD is to correct circulating levels of excess molecules, primarily through dialysis clearance. High flux (HF) dialysers have become standard in HD and allow rapid clearance of small (<500 Da) water soluble molecules, and some so-called *small* middle molecules (0.5-25 kDa). Medium cut-off (MCO) dialysers are a novel class of dialysis membrane with larger pore sizes than HF membranes. The putative advantage of the MCO dialyser is the permissive passage of an expanded range of molecules to include *large* middle molecules (25-60 kDa), whilst retaining essential molecules with higher molecular weights, such as albumin (66 kDa).^{330, 331} Dialysis may affect the milieu of circulating molecules directly through clearance, or indirectly by addressing the drivers of secondary metabolic abnormalities.³³² There are limited data on how MCO membranes affect specific subgroups of biologically significant molecules, such as novel markers of mineral metabolism.

As outlined in Chapter 1, FGF23 is a bone-derived phosphate and vitamin-D-regulating hormone that rises early in CKD, with the highest circulating levels seen in patients on dialysis.^{12, 144} FGF23 has been independently and positively associated with a range of adverse events, including all-cause mortality in patients with and without CKD.¹⁴²⁻¹⁴⁴ Whether FGF23 is a surrogate marker of other pathological pathways, or whether FGF23 itself has direct cytotoxic effects is debated. Nevertheless, FGF23 remains of interest as a potential therapeutic target in dialysis patients.^{330, 331}

Fetuin-A has a molecular weight of approximately 58 kDa, and so may ostensibly be lost during HD with an MCO dialyser, although this has not previously been studied. While CPP-I (30-100 nm) and CPP-II (100-250 nm) are too large to be directly cleared by an MCO membrane, given the complex and heterogeneous composition of endogenous CPP,²²⁰ it is unclear whether HF and MCO dialysers may differentially affect levels of CPP in the circulation.

The tRial Evaluating Mid cut-Off Value membrane clearance of Albumin and Light chains in HaemoDialysis patients (REMOVAL-HD) was a multi-centre device study, primarily examining the safety of MCO dialysers over 6 months in comparison to baseline use of HF dialysers.³³³ In this study, results of an exploratory analysis of REMOVAL-HD, evaluating the effects of an MCO membrane on the novel mineral metabolism markers FGF23, fetuin-A, CPP-I and CPP-II are reported.

6.2 Methods

REMOVAL-HD was a non-randomised, single-arm device study involving nine sites across Australia and New Zealand. Consenting adult patients who had been treated with HD for at least 12 weeks and had a functioning arteriovenous fistula or graft were eligible.^{333, 334} All participants were either anuric or oliguric (< 500 mL/24 hours). Key exclusion criteria were anticipated change in dialysis modality, dialysis site or kidney transplant within the study period; presence of chronic infection or inflammatory condition or currently receiving immunosuppressants; life expectancy < 12 months; and serum albumin < 30 g/L within 4 weeks of screening. Ethical approval was obtained from Institutional Ethics Committees for participating sites and REMOVAL-HD was conducted in accordance with the Declaration of Helsinki.

Participants received in-centre HD three times per week throughout the study. A standardised HF dialyser (Revaclear R400; Baxter Healthcare, Sydney, Australia) was used for a four-week wash-in period, followed by 24 weeks with an MCO dialyser (Theranova 400; Baxter Healthcare, Sydney, Australia). Dialysis prescription remained the responsibility of the patient's usual care provider throughout the study, however sites were instructed to maintain the same dialysis prescription for study duration where possible, and to maintain a target blood flow rate > 300 mL/min and a dialysate flow rate of 500 mL/min. Participants received usual care for other CKD and non-CKD related issues.

6.2.1 Outcome measures

Blood samples were collected at baseline after wash-in but before first MCO dialyser use, and then after 12 and 24 weeks of dialysis with the MCO dialyser. All blood samples were collected pre-dialysis prior to the mid-week session. Blood for FGF23, fetuin-A and CPP was centrifuged and serum aliquots were stored at -80°C until batch analysis. Other biochemistry was performed locally at an accredited laboratory.

Intact FGF23 (iFGF23) and fetuin-A were measured using commercial ELISA kits (Kainos Laboratories, Tokyo, Japan and R&D Systems, Minneapolis, USA, respectively) according to the manufacturers' instructions. Serum CPP-I and CPP-II were measured

using the same fluorescent probe-based assay running on a BD FACSVerser flow cytometer (BD Biosciences, San Jose, CA, USA) as described in Chapter 3-5.²⁰⁶

6.2.3 Statistical Analysis

Baseline characteristics and descriptive data are presented as mean ($\pm$ SD) or, when skewed, median (IQR) for continuous variables, and as number and percentage for categorical variables. Shapiro-Wilk test and distribution plots were used to assess normality, and right-skewed data were natural log transformed prior to analysis.

The main outcomes of this secondary analysis were change in pre-dialysis serum levels of iFGF23, fetuin-A, CPP-I and CPP-II after 12 and 24 weeks of dialysis with an MCO dialyser compared to baseline. For each molecule of interest, a linear mixed effects model was fitted using a restricted maximum likelihood approach. To test for longitudinal change with MCO dialyser use, time was modelled as a fixed effect factor variable and random intercepts were used for each participant to allow for individual variation and repeated measures. Dunnett's test was used when analysing each of the measures to correct for the multiple comparisons that occurred when comparing each follow-up time point to the common control baseline. For these models, iFGF23, CPP-I and CPP-II were natural log-transformed, to ensure that the residuals were normally distributed. To aid interpretation, the regression coefficients for iFGF23, CPP-I and CPP-II were exponentiated to obtain estimates of percentage change. All available data at each time point were used for the main analysis, but models were also refitted using only participants with complete data to assess for sensitivity to missing data.

In further exploratory analyses, correlation between longitudinal change (from baseline to 24 weeks) in mineral metabolism markers and other variables were examined using Pearson correlation for normally distributed and Spearman rank correlations for skewed data. For these analyses, only participants with data at baseline and 24 weeks were included and no correction for multiple testing was made. All hypothesis tests were assessed at 5% level of significance. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and R (version 4.0.2). The linear mixed models were fitted in R using the "lmerTest" package.³³⁵

6.3 Results

The full CONSORT participant flow diagram for REMOVAL-HD has been published elsewhere.³³³ Eighty-nine participants completed the four-week wash-in with the standardised HF dialyser and commenced treatment with the MCO dialyser. Participant demographics are listed in Table 6.1. The mean age was 67 ± 15 years, 38% were female and 51% were diabetic. Forty-five percent of participants were anuric, and among oliguric participants, the median urine output was 200 mL/day with an IQR of 130 – 300 mL/day.

All available samples from each of the 89 participants were included in the analysis. Baseline values iFGF23, fetuin-A and CPP are displayed in Table 6.2, and summary data at each timepoint are shown in Figure 6.1. For each analyte, previously reported serum concentrations in apparently healthy adults, and measured using the same assays as in this study, are also provided in Table 6.2 for reference.

Table 6.1: Participant demographics and baseline characteristics

Participant characteristic (n=89)	
Female sex	34 (38)
Age – yrs	67 ± 15
Ethnicity	
Caucasoid	66 (74)
Aboriginal or Torres Strait Islander	1 (1)
New Zealand Māori	8 (9)
Pacific Islander	4 (4.5)
Asian	6 (7)
Other	4 (4.5)
Body mass index – kg/m ²	28.8 ± 5.8
Primary cause of renal disease	
Diabetic nephropathy	33 (37)
Hypertension/vascular	13 (15)
Glomerulonephritis	10 (11)
Reflux nephropathy	3 (3)
Polycystic kidney disease	6 (7)
Other	19 (21)
Unknown	5 (6)
Urine output	
Anuric	40 (45)
Oliguric (<500 mL/day)	49 (55)
Residual urine volume (n=49) – mL/24hr	200 [130 – 300]
Diabetes mellitus	45 (51)
Ischemic heart disease	21 (24)
Smoking (current or former)	46 (52)

Mean ± standard deviation, median [interquartile range] or number (%)

Table 6.2: Baseline serum concentrations of study analyte

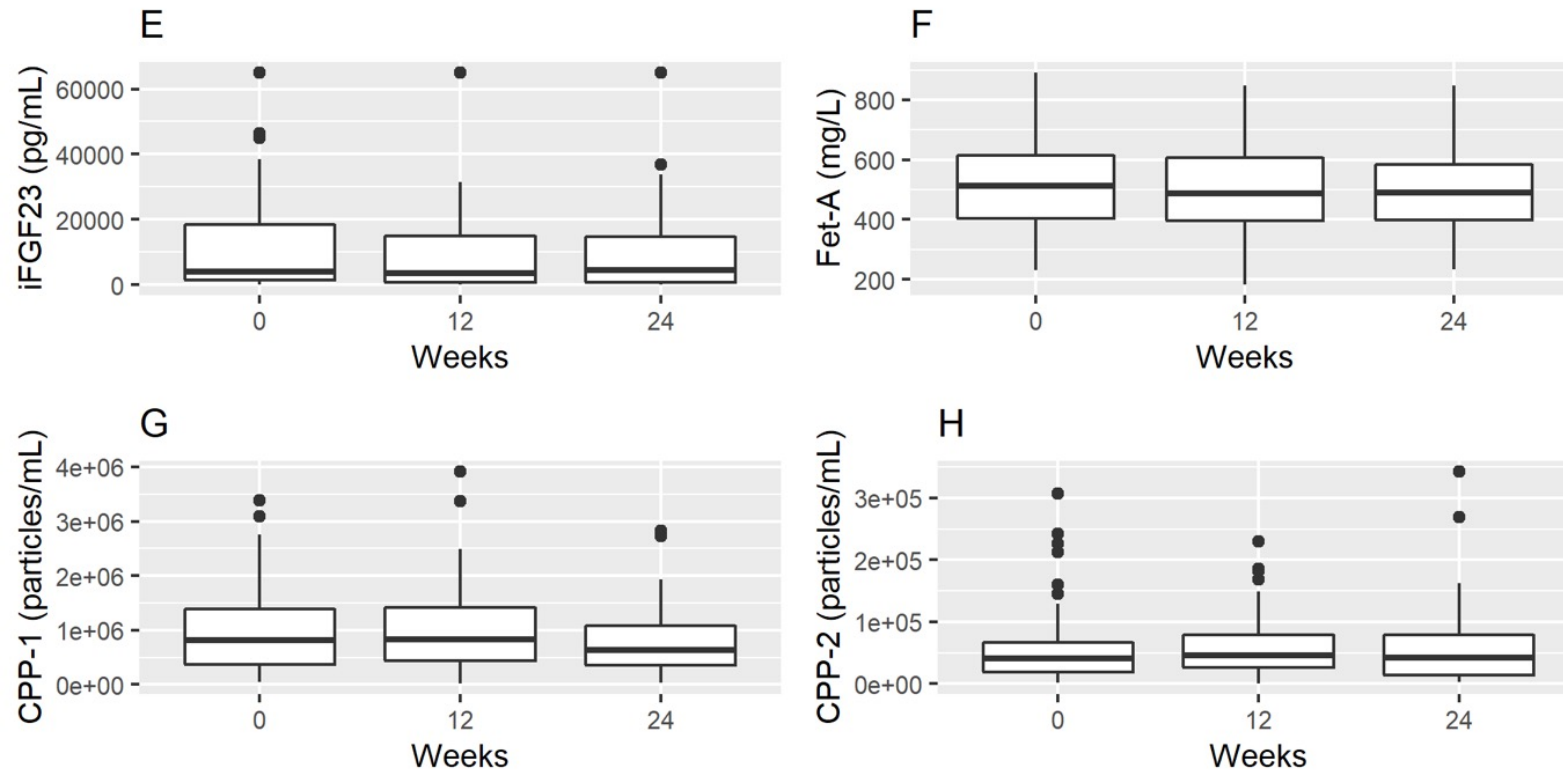
Analyte	Baseline mean or median	Previously reported values in healthy adults for assay*
Intact FGF23 (n=87) – pg/mL	3,985 [1,414 – 18,362]	42.0 ± 9.9 ³³⁶
Fetuin-A (n=86) - mg/L	511 ± 148	303-671 ³³⁷
CPP-I (n=87) – x10 ⁵ particles/mL	8.2 [3.7 – 13.9]	2.2-17.3 ²⁰⁶
CPP-II (n=87) – x10 ⁴ particles/mL	4.1 [1.9 – 6.6]	1.7-28.2 ²⁰⁶

Mean ± standard deviation, median [interquartile range], or range minimum-maximum

*Values are serum concentrations previously reported using the same assay for each analyte in apparently healthy adults

Abbreviations: CPP, calciprotein particles; FGF23, fibroblast growth factor-23

Figure 6.1: Box plot of study analytes before first MCO use and after 12 and 24 weeks



Box shows median and interquartile range

Abbreviations: CPP, calciprotein particles; Fet-A, fetuin-A; iFGF23, intact fibroblast growth factor-23

6.3.1 Intact FGF23

There was a significant reduction in pre-dialysis iFGF23 between baseline and 12 weeks (-26.8% [95% CI -39.7, -11.1], adjusted $p = 0.001$) which was sustained at 24 weeks (-21.7% [95% CI -35.7, -4.5] adjusted $p = 0.012$) (Table 6.3).

Linear mixed models were refitted with complete cases ($n=76$) to examine the effect of missing data. This sensitivity analysis restricted to complete cases did not change the main finding of reductions in serum iFGF23 levels at 12 and 24 weeks of MCO use (-27.4% [-41.0, -10.8] adjusted $p = 0.001$ and -21.8% [-36.4, -3.9] adjusted $p = 0.016$ respectively).

6.3.2 Fetuin-A and calciprotein particles

There were no significant changes in pre-dialysis serum fetuin-A, CPP-I or CPP-II concentrations between baseline and week 12 or baseline and week 24 (Table 6.3).

6.3.3 Correlations of mineral metabolism markers with selected variables

The correlations between change in novel mineral metabolism markers and other variables were examined for participants that had data available at both baseline and 24 weeks (Table 6.4). There was no longitudinal correlation between any of the novel mineral metabolism markers and pre-dialysis serum albumin, urea reduction ratio, haemoglobin or hsCRP.

There were moderate positive correlations between iFGF23 and both CPP-I ($r = 0.37$, $p = 0.001$) and CPP-II ($r = 0.33$, $p = 0.004$). Fetuin-A negatively correlated with CPP-II ($r = -0.24$, $p = 0.038$) but not CPP-I ($r = -0.07$, $p = 0.563$). There was also a moderate correlation between CPP-I and CPP-II ($r = 0.38$, $p = 0.001$).

Table 6.3: Change in mineral metabolism markers

Analyte	Baseline		Week 12				Week 24			
	<i>n</i>	Mean or median	<i>n</i>	Mean or median	Change from baseline (95% CI)*	Change at week 12 p value **	<i>n</i>	Mean or median	Change from baseline (95% CI)*	Change at week 24 p value **
iFGF23 (pg/mL)	87	3,985 [1,414 – 18,362]	83	3,594 [688 – 14,888]	-26.8% (-39.7, -11.1)	0.001	79	4,526 [844 – 14,657]	-21.7% (-35.7, -4.5)	0.012
Fetuin-A (mg/L)	86	511 ± 148	80	500 ± 155	-11.0 (-47.8, 25.8)	0.73	77	496 ± 142	-7.2 (-44.5, 30.2)	0.88
CPP-I (x10 ⁵ particles/mL)	87	8.2 [3.8 – 13.9]	83	8.2 [4.5 – 14.2]	9.1% (-14.0, 38.3)	0.63	78	6.4 [3.6 – 10.8]	-16.0% (-34.0, 7.1)	0.20
CPP-II (x10 ⁴ particles/mL)	87	4.1 [1.9 – 6.6]	83	4.6 [2.6 – 7.9]	11.4% (-17.6 - 50.7)	0.64	79	4.2 [1.3 – 7.8]	-6.4% (-31.1, 27.2)	0.85

Mean ± standard deviation or median [interquartile range]

* Change in analytes compared to baseline, estimated from linear mixed model. For iFGF23, CPP-I and CPP-II, linear models were fitted to natural log transformed data in order to ensure normal distribution of residuals; thereafter the regression coefficients have been exponentiated into percentage change to aid interpretation.

** Adjusted p value using Dunnett's post estimation test

Abbreviations: CI, confidence interval; CPP, calciprotein particles; iFGF23, intact fibroblast growth factor-23

Table 6.4: Correlation between longitudinal change (baseline to 24 weeks) in novel mineral markers and selected variables

	Change in iFGF23		Change in fetuin-A		Change in CPP-I		Change in CPP-II	
	<i>r</i>	p	<i>r</i>	p	<i>r</i>	p	<i>r</i>	p
Change in fetuin-A	-0.08	0.481	-	-	-	-	-	-
Change in CPP-I	0.37	0.001	-0.07	0.563	-	-	-	-
Change in CPP-II	0.33	0.004	-0.24	0.038	0.38	0.001	-	-
Change in albumin	0.01	0.913	0.10	0.387	0.08	0.491	-0.13	0.261
Change in urea reduction ratio	-0.17	0.182	0.07	0.603	-0.03	0.807	-0.09	0.470
Change in haemoglobin	-0.11	0.356	0.04	0.767	-0.17	0.141	0.10	0.381
Change in hsCRP	0.08	0.471	0.07	0.582	0.01	0.929	0.00	0.973

Urea reduction ratio = (pre-dialysis urea – post dialysis urea)/pre-dialysis urea x100%

Abbreviations: CPP, calciprotein particles; hsCRP, high sensitivity C-reactive protein; iFGF23, intact fibroblast growth factor-23

6.4 Discussion

In this study, use of a MCO dialyser over 24 weeks was associated with a sustained reduction in serum concentrations of intact FGF23 in patients on HD, while other novel markers of mineral metabolism were not significantly altered.

Despite advances in dialysis technology and techniques, patients requiring dialysis for kidney failure continue to experience significant excess morbidity and mortality compared to those without CKD.²⁶⁴ Part of this increased risk may be attributable to retained molecules that are incompletely cleared by current therapies, and may directly mediate toxicity or indirectly drive disordered metabolic processes, such as abnormalities in mineral metabolism. MCO dialysers offer the putative advantage over current HF dialysers of expanding the range of molecules cleared during HD. Clinical efficacy studies of MCO dialysers have mainly examined a selection of molecules which are either thought to be clinically significant, or are chosen to give a representative spread of molecule weights.^{333, 338-342} These studies are used to inform predictions about how other molecules with similar physiochemical properties may be affected by a MCO dialyser. However, given the complex, and often inter-related, milieu of abnormalities in CKD, it is important to test these predictions, particularly for molecules that may have significant biological and therapeutic implications.

In this study continuous MCO dialyser use was associated with a sustained reduction in pre-dialysis serum iFGF23 concentration. Intact FGF23 is a middle molecule, with an approximate molecular weight of 32 kDa, and would be expected to be cleared more efficiently through dialysis with a MCO than HF membrane. FGF23 was not directly measured in spent dialysate and so it is not possible to confirm that clearance was the mechanism behind this observed reduction. There are limited previous data on circulating FGF23 comparing relative effects of HD with a MCO and a standard HF dialyser. Previous studies by Belmoaz *et al* and by Kim *et al* have suggested that a MCO dialyser is associated with a higher reduction ratio of FGF23 compared to a HF dialyser, implying removal through MCO use.^{343, 344} Of note though, neither study demonstrated a reduction in pre-dialysis serum levels. In addition, the trend towards increased reduction ratio in the study by Kim *et al* did not reach statistical significance, however this was a small study (six patients) and used lower blood flow rates (250 mL/min), both of which may have contributed to the equivocal result. This study also used a C-terminal FGF23 assay, in

contrast to the intact assay in the current study. MCO membranes are designed to improve clearance of larger middle molecules (>25 kDa). Given that the C-terminal assay measures both intact FGF23 and the smaller C-terminal fragment (12 kDa), use of this assay may be less discriminatory between these membrane types, although in patients receiving dialysis virtually all circulating FGF23 is thought to be intact.^{345, 346}

Hypothetically, the observed reduction in FGF23 with MCO dialyser use could be due to mechanisms apart from direct clearance. For instance, phosphate balance is known to correlate with FGF23 production,³¹⁴ and measurement of serum phosphate or phosphate clearance was not systemically collected in this cohort. However, it is worth noting that previous studies have suggested there is minimal, if any, difference between MCO and HF dialyser clearance of small solutes, including phosphate.³⁴⁰ A correlation between FGF23 and CPP was observed, which as outlined in Section 1.10.2, has also been observed in other studies.^{205, 206, 228} It has also recently been shown that CPP induces FGF23 expression in osteoblasts.²²⁵ However, given there was no significant longitudinal change in CPP-I or CPP-II in this study, it is less likely that a link between FGF23 and CPP explains the reduction in FGF23 observed in this study.

FGF23 has consistently and positively been linked to adverse outcomes in epidemiological studies.¹⁴²⁻¹⁴⁴ Further, *in vitro* studies have demonstrated biologically plausible mechanisms of cardiovascular toxicity, such as the induction of ventricular hypertrophy.^{145, 146} Taken together, this adds weight to the assertion that elevated levels of FGF23 in CKD may directly mediate adverse outcomes and makes FGF23 a potential therapeutic target in patients on dialysis. However, previous studies have shown mixed benefits of interventions associated with FGF23 reduction,^{347, 348} and further studies are required to determine whether the reductions with MCO dialyser use in this study will result in improved patient outcomes. Indeed, the percentage reductions in intact FGF23 observed here are only modestly greater than the biological variation of this hormone in healthy adults.³⁴⁶

Pore size is a key determinant of the range of molecules that can be cleared by a dialysis membrane. MCO dialysers are specifically manufactured to have a distribution of pore sizes that allow passage of larger middle molecules (25-60 kDa) while restricting loss of albumin (66 kDa).^{330, 349} Fetuin-A (58 kDa) has a beneficial role in the prevention of

ectopic mineralisation, and given that it has a molecular weight within the upper limit of the cut-off range of a MCO dialyser, it is reassuring that pre-dialysis levels were unchanged across the study period. The large diameter of CPP-I/II (50-100 nm for CPP-I and 100-250 nm for CPP-II), mean it was unlikely that there would be direct clearance by an MCO membrane (effective pore size 3.0-3.5 nm),³⁴⁹ and so it is unsurprising that levels were unchanged. However, given the complex composition²²⁰ and metabolic pathways²¹⁸ of CPP, it is useful to have empiric evidence to confirm this prediction.

As discussed above, one limitation of this study is that no direct measure of clearance of the study molecules in spent dialysate was performed, and so we can only speculate on the mechanisms of change (or lack thereof). However, change in serum levels better reflects time-averaged exposure to the molecule of interest, as it accounts for the effects of intercompartmental sequestration and interdialytic generation.³²⁸ This arguably makes pre-dialysis concentrations a more biologically relevant metric than the kinetics of dialysis clearance in isolation. It is also important to note that this sub-study is in essence an exploratory analysis, and so caution is required when interpreting results. Specifically, in regard to the main finding of a significant reduction in intact FGF23, while adjustment for multiple hypothesis testing at week 12 and week 24 was made, no adjustment for multiple hypothesis tests across the various molecules was performed and so the possibility that this result was due to chance cannot be excluded. Finally, this was a study of surrogate markers, and while each has a body of evidence supporting biological relevance, only larger long-term outcome studies can properly examine the potential clinical benefits of HD with a MCO dialyser.

6.5 Conclusion

This study found that HD with a MCO dialyser was associated with a sustained reduction of intact FGF23 in serum compared to a HF membrane. There were no changes in other markers of mineral metabolism, including no evidence of loss of the beneficial protein fetuin-A. Further, MCO dialyser use did not appear to have a significant impact on serum concentrations of CPP-I/II. Whether the reduction in intact FGF23 is associated with improved patient outcomes requires further assessment in future trials.

Chapter 7 Serum calciprotein monomers and Chronic Kidney Disease progression

7.1 Introduction

The major focus of this thesis has been on mineral metabolism abnormalities and its associations with cardiovascular complications in CKD-MBD. There have also been consistent epidemiological signals that disturbed phosphate metabolism may contribute to the progression of CKD itself. For instance, elevated serum phosphate¹⁴⁷⁻¹⁴⁹ and FGF23^{143, 150, 151} have been positively correlated with faster decline in kidney function. Some have observed in small clinical^{152, 153} and experimental studies¹⁵⁴⁻¹⁵⁶ that this risk is highest when there is an anticipated mismatch between the residual number of functioning nephrons and the total filtered phosphate load.

During the course of this thesis, Shiizaki *et al* proposed that the above observations may be explained by the spontaneous precipitation of calcium phosphate crystals in the tubular fluid, which may then induce cell dysfunction and interstitial inflammation, culminating in tubular damage.¹⁵¹ The propensity to form tubular crystals appeared to be related to the concentration of phosphate in the proximal tubular fluid (PTFp), given that higher PTFp was found to predict progression of CKD in mice and humans.¹⁵¹ Both PTFp and tubular damage were correlated with FGF23, consistent with its action as the major stimulator of urinary phosphate excretion.²¹

Although Shiizaki *et al* suggested that calcium phosphate crystals form *in situ* in the tubular fluid, another possibility is that this mineral derives partly from circulating CPM (8.4 nm), which unlike much larger CPP-I (30-100 nm) and CPP-II (100-250 nm), are reported to be readily filtered at the glomerulus.^{186, 219} Consistent with this, in Chapter 3 the post-prandial rises in CPM were higher in CKD patients compared to age-matched controls without CKD, and the magnitude of the rise inversely correlated with eGFR. In addition, CPM have also been proposed to stimulate osteocytic FGF23 expression,²²⁵ providing a further plausible link between CPM and CKD progression.

In this chapter, to further examine the potential relationship between CPM and the progression of CKD, data from the Arterial Compliance and Oxidant Stress as predictors of loss of renal function, morbidity and mortality in Chronic Kidney Disease (ACADEMIC) study was analysed. The ACADEMIC study was a prospective observational study of 200 stable outpatients with CKD stages 3 and 4 who were followed prospectively until time of death or commencement of kidney replacement therapy.²⁰⁴ The hypothesis of this study was that higher serum levels of CPM would be associated with increased risk of a progression to dialysis and faster decline in eGFR.

7.2 Methods

7.2.1 Study population and follow up

Participants in the ACADEMIC cohort were recruited from nephrology outpatient clinics at Brighton and Sussex University Hospitals NHS Trust between March 2006 and September 2009.²⁰⁴ To be eligible, participants had to be between the age of 40 and 90 years and have CKD stages 3 and 4 at the time of recruitment. The study was approved by the West Sussex Research Ethics Committee [REC Ref.# 05/Q1911/89], and patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Follow up time was accrued from the time of recruitment until the commencement of kidney failure, death, or the end of the study (1 November 2012), whichever occurred first. Participant status was gathered prospectively via biannual study visits and hospital electronic medical records.

7.2.2 Exposure assessment

The primary exposure analysed in this study was serum CPM. Blood samples were collected at baseline study visit using standard venepuncture techniques. Samples were centrifuged after being allowed to clot, and serum samples were stored at -70°C until batched analysis. Serum CPM was measured by the gel-filtration method as previously described,²⁰⁸ and utilised in Chapter 3 and Chapter 4.

7.2.3 Covariate assessment

Assessment of demographics and baseline clinical characteristics were recorded at study entry. Blood and urine samples were collected at study entry for standard biochemical measurements, which were performed at the time of collection by the hospital's accredited pathology service. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease-Epidemiology Collaboration equation.

Serum iFGF23 was measured using a commercial ELISA from Kainos Laboratories (Tokyo, Japan), with an inter-assay CV of <5%. Serum and urine fetuin-A was measured using ELISA kits from Biovendor (Brno, Czech Republic), inter-assay CVs were <3.5% and <6.5%, respectively. Urine neutrophil gelatinase-associated lipocalin (NGAL) was

measured using the particle-enhanced turbidimetric immunoassay as previously described with an inter-assay CV of <3.5%.³⁵⁰ Urinary fetuin-A and NGAL were each normalised to the urine creatinine concentration.

ePTFp was calculated based on measured serum phosphate (sPi) and urinary fractional excretion of phosphate (FEp) using the equation ($ePTFp = sPi \times FEp \times 3.33$) proposed by Shiizaki *et al*,¹⁵¹ which assumes that 70% of the filtered fluid fraction is reabsorbed along the proximal tubule.³⁵¹

7.2.4 Study outcomes

Progression of kidney disease was examined using a composite kidney disease outcome and by analysing eGFR slope. The composite kidney disease outcome was defined as commencement of kidney replacement therapy or drop in eGFR > 30% from study entry (as suggested by Coresh *et al*³⁵²). eGFR slope was defined as average change in eGFR per year,³⁵³ using all available measures of eGFR taken after enrolment in the study, including from biannual study visits and from hospital electronic medical records (excluding those recorded during an acute illness).

7.2.5 Statistical analysis

Baseline characteristics of the cohort are summarised across CPM tertile, using mean and SD for normally distributed, or median and IQR for skewed, data respectively. Categorical data are presented as number and percentages. P value for trend in baseline characteristics across CPM tertiles was estimated using linear regression, with CPM tertile as an ordinal variable.

Cox proportionate hazards models were used to examine the relationship between the serum CPM and the composite kidney disease outcome. Serum CPM was categorised into tertiles, given visual inspection of Martingale residuals suggested that the association between serum CPM and outcome was non-linear. An unadjusted and three hierarchical multivariable adjusted models (Models 1-3) were created. The multivariate models were sequentially adjusted for participant demographics and for conventional risk factors for CKD progression which were defined *a priori*, based on known clinical associations. For Model 1, adjustment for participant age and sex was made. Model 2 adjusted for the

covariates in Model 1, plus eGFR and urinary albumin-to-creatinine ratio (uACR). Model 3 adjusted for the covariates in Model 2 plus cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medication use (use of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta-blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker). Interaction terms between CPM tertile and covariates were assessed and Schoenfeld residuals were used to assess the proportionate hazards assumption.

Univariate and multivariate linear regression models were used to examine the relationship between serum CPM and eGFR slope. The multivariate regression models were sequentially adjusted for the same covariates as the Cox models. Serum CPM, as well as other skewed covariates, were natural log-transformed before fitting these models, and robust confidence interval estimates were used to account for heteroskedasticity of residuals. For the multivariate models, collinearity was assessed using the variance inflation factor.

Additional analyses

In the unadjusted and multivariate adjusted Cox models, participants were censored at time of death or end of study follow up (whichever occurred first). Because death precludes progression of CKD, a competing risk analysis was also performed for the composite kidney disease outcome using the Fine and Grey method.²⁶¹ For this analysis, death was the competing event and the model was adjusted for the same covariates as used in Model 3.

In the main Cox and linear regression models, no adjustment for conventional markers of mineral metabolism (serum calcium, phosphate, and parathyroid hormone) were made, given that each is potentially involved in the hypothesised pathway. An additional sensitivity analysis was also performed, where these variables were included in addition to the covariates in Model 3.

In the main analyses it was found that serum CPM was associated with increased risk of the composite kidney disease outcome and also greater eGFR decline. Given the hypothesis that CPM may be related to kidney outcomes via the pathway proposed by

Shiizaki *et al* a mediation analysis was also performed to investigate whether the association between CPM and eGFR slope was explained by FGF23 and PTFp. To do this, a simplified directed acyclic graph was constructed with serum CPM as the independent variable, eGFR slope as the dependent variable, and iFGF23 and ePTFp as the mediators. The potential confounding effect of baseline eGFR on serum CPM, iFGF23, ePTFp and eGFR slope was modelled, as well as the potential indirect effect of baseline eGFR on eGFR slope via uACR. For the mediation analysis skewed variables were natural log transformed before estimating linear coefficients, and 95% confidence intervals were bias corrected using bootstrapping with 2,000 resamples. Where variables were significantly associated with eGFR slope via direct and indirect pathways, the proportion of the association that was directly mediated was also calculated.

Two-tailed p values <0.05 were considered significant. All data were analysed using Stata MP version 17.0 (StataCorp, College Station, USA). Graphics were produced using the same software.

7.3 Results

7.3.1 Baseline demographic and clinical characteristics

One hundred and eighty-nine of 200 participants in the ACADEMIC study had stored baseline serum samples available for measurement of CPM and were included in this analysis. No participants in this sub-study were lost to follow up.

Baseline demographic details and clinical characteristics are displayed in Table 7.1. Of the 189 individuals, 186 self-identified as Caucasian, 2 as Middle Eastern, and 1 as Black African. The mean age of the study cohort was 69.1 ± 11.3 years, 27.5% were female and 23.8% had a history of diabetes. At study entry, the mean eGFR was 32.7 ± 10.8 mL/min/1.73m² and median albumin-to-creatinine ratio was 5.3 mg/mmol [IQR 1.7 – 30.5]. The distribution of primary cause of CKD for the cohort is listed in Table 7.2.

Across CPM tertiles, there was no trend in age, sex, blood pressure, baseline comorbidities or medication use. However, ascending tertiles of serum CPM were associated with lower eGFR at baseline (p value for trend <0.001). Consistent with this, participants with higher serum levels of CPM also had lower haemoglobin, higher urinary albuminuria and proteinuria, and higher serum phosphate, parathyroid hormone, iFGF23, C-reactive protein levels and ePTFp. Total serum fetuin-A concentrations were lower in those with higher serum CPM, whereas urine fetuin-A levels, increased across the tertiles. Urinary NGAL, a marker of kidney injury, was also higher in those with higher serum CPM, and was itself associated with higher ePTFp ($r = 0.198$, $p = 0.007$), iFGF23 ($r = 0.418$, $p < 0.001$), fetuinuria ($r = 0.332$, $p < 0.001$).

Table 7.1: Baseline participant clinical and biochemical characteristics

Participant characteristic	Overall (n=189)	Tertile 1 (n=63)	Tertile 2 (n=63)	Tertile 3 (n=63)	p value for trend
Serum CPM – $\times 10^3$ AU/mL	26.6 [15.8 – 51.1]	12.6 [8.9 – 15.8]	26.6 [22.6 – 31.5]	66.7 [51.1 – 92.7]	–
Age – yrs	69.1 $\pm$ 11.3	69.9 $\pm$ 10.9	69.8 $\pm$ 11.3	67.5 $\pm$ 11.7	0.228
Female sex	52 (27.5)	14 (22.2)	21 (33.3)	17 (27.0)	0.552
Baseline comorbidities					
Cardiovascular disease	81 (57.1)	28 (44.4)	28 (44.4)	25 (39.7)	0.591
Diabetes mellitus	45 (23.8)	12 (19.1)	16 (25.4)	17 (27.0)	0.298
Smoking history – pack years among smokers	25 [7 – 40] (n=115)	33 [8 – 40] (n=41)	20 [7 – 40] (n=42)	26 [7 – 43] (n=32)	0.297
Systolic blood pressure – mmHg	152 $\pm$ 21.5	150 $\pm$ 20.4	154 $\pm$ 20.4	151 $\pm$ 23.8	0.782
Diastolic blood pressure – mmHg	81 $\pm$ 11.3	82 $\pm$ 11.5	82 $\pm$ 11.1	81 $\pm$ 11.3	0.777
Body mass index – kg/m ²	29.4 $\pm$ 5.6	29.7 $\pm$ 5.1	29.4 $\pm$ 6.4	29.2 $\pm$ 5.4	0.587
Baseline biochemical parameters					
eGFR [#] – mL/min/1.73m ²	32.7 $\pm$ 10.8	35.6 $\pm$ 10.4	34.9 $\pm$ 9.8	27.6 $\pm$ 10.5	<0.001
Haemoglobin – g/L	127 $\pm$ 16.8	133 $\pm$ 17.2	128 $\pm$ 16.6	120 $\pm$ 14.4	<0.001
Serum Albumin – g/L	43 $\pm$ 3.2	43 $\pm$ 3.2	43 $\pm$ 2.8	42 $\pm$ 3.6	0.105
Urine albumin-to-creatinine ratio – mg/mmol	5.3 [1.7 – 30.5]	3.1 [1.0 – 16.1]	5.1 [1.3 – 34.9]	12.5 [3.0 – 48.2]	0.001
Urine protein-to-creatinine ratio – mg/mmol	27.2 [13.2 – 64.7]	21.9 [11.4 – 42.0]	18.9 [11.3 – 47.1]	40.7 [22.1 – 101.7]	<0.001
Calcium (adjusted) ^{##} – mmol/L	2.29 $\pm$ 0.12	2.30 $\pm$ 0.11	2.30 $\pm$ 0.11	2.28 $\pm$ 0.13	0.327
Phosphate – mmol/L	1.08 $\pm$ 0.20	0.93 $\pm$ 0.14	1.09 $\pm$ 0.14	1.22 $\pm$ 0.20	<0.001
Parathyroid hormone – ng/L	76 [50 – 118]	70 [49 – 105]	67.5 [50 – 95]	103 [57 – 161]	0.002
Intact fibroblast growth factor-23 – pg/mL	53.6 [35.4 – 83.9]	40.9 [25.7 – 63.0]	46.4 [34.3 – 70.6]	71.7 [53.9 – 113.3]	<0.001
uFet-A/creat ratio – ug/mmol	106.4 [43.7 – 271.2]	66.2 [23.0 – 154.8]	98.7 [28.8 – 342.5]	168.4 [70.7 – 376.9]	0.004
Urinary fractional excretion of calcium – %	1.7 [1.2 – 2.2]	1.6 [1.2 – 2.0]	1.6 [1.1 – 2.2]	1.8 [1.3 – 2.7]	0.015
Urinary fractional excretion of phosphate – %	29.8 [21.6 – 40.3]	25.9 [19.6 – 34.7]	26.6 [20.7 – 33.8]	39.9 [25.8 – 53.0]	<0.001
ePTFp ^{###} – mmol/L	1.01 [0.74 – 1.45]	0.81 [0.56 – 1.04]	1.00 [0.80 – 1.25]	1.77 [0.99 – 2.22]	<0.001
C-reactive protein – mg/L	2.27 [0.94 – 5.63]	1.73 [0.76 – 3.56]	2.68 [1.01 – 8.01]	2.52 [1.05 – 5.91]	0.043
Serum Fetuin-A – mg/L	209 $\pm$ 63.0	226 $\pm$ 67.6	200 $\pm$ 55.8	200 $\pm$ 62.4	0.018

uNGAL/creatinine ratio – µg/mmol	1.7 [0.6 – 4.3]	1.3 [0.6 – 2.6]	1.6 [0.4 – 2.3]	4.4 [1.1 – 14.6]	<0.001
Baseline use of medications related to mineral metabolism					
Cholecalciferol	7 (3.7)	3 (4.8)	4 (6.4)	0 (0.0)	0.159
Calcitriol or alfacalcidol	15 (7.9)	6 (9.5)	1 (1.6)	8 (12.7)	0.512
Bisphosphonate	13 (6.9)	4 (6.4)	5 (7.9)	4 (6.4)	1.000
Calcium supplement or calcium-based phosphate binder	14 (7.4)	5 (7.9)	3 (4.8)	6 (9.5)	0.735
Non-calcium based phosphate binder	3 (1.6)	1 (1.6)	1 (1.6)	1 (1.6)	1.000
Baseline use of other medications					
Angiotensin-converting enzyme inhibitor or angiotensin receptor blocker	124 (65.6)	45 (71.4)	34 (54.0)	45 (71.4)	1.000
Beta-blocker	62 (32.8)	25 (39.7)	18 (28.6)	19 (30.2)	0.257
Calcium channel blocker	88 (46.6)	26 (41.3)	29 (46.0)	33 (52.4)	0.213
Diuretic	102 (54.0)	31 (49.2)	32 (50.8)	39 (61.9)	0.154
Alpha blocker	60 (31.8)	21 (33.3)	16 (25.4)	23 (36.5)	0.704
Statin	110 (58.2)	42 (66.7)	34 (54.0)	34 (54.0)	0.150
Erythropoiesis stimulating agent	11 (5.8)	3 (4.8)	3 (4.8)	5 (7.9)	0.449

Mean ± standard deviation, median [interquartile range] or number (%)

p value for linear trend determined by linear regression with tertile as an ordinal variable.

eGFR calculated according to the Chronic Kidney Disease Epidemiology Collaboration equation

serum calcium adjusted for plasma albumin concentration according to the following equation: measured calcium (mmol/L) + 0.02 (40 - albumin [g/L])

ePTFp calculated based on measured serum phosphate and urinary fractional excretion of phosphate using the equation proposed by Shiizaki *et al.*¹⁵¹

Abbreviations: CPM, calciprotein monomers; CPP-I, primary calciprotein particles; CPP-II, secondary calciprotein particles; eGFR, estimated glomerular filtration rate; ePTFp, estimated proximal tubular phosphate concentration; uNGAL, neutrophil gelatinase-associated lipocalin; uFet-A/creat ratio, urinary fetuin-A-to-creatinine ratio.

Table 7.2: Primary cause of kidney disease for the study cohort

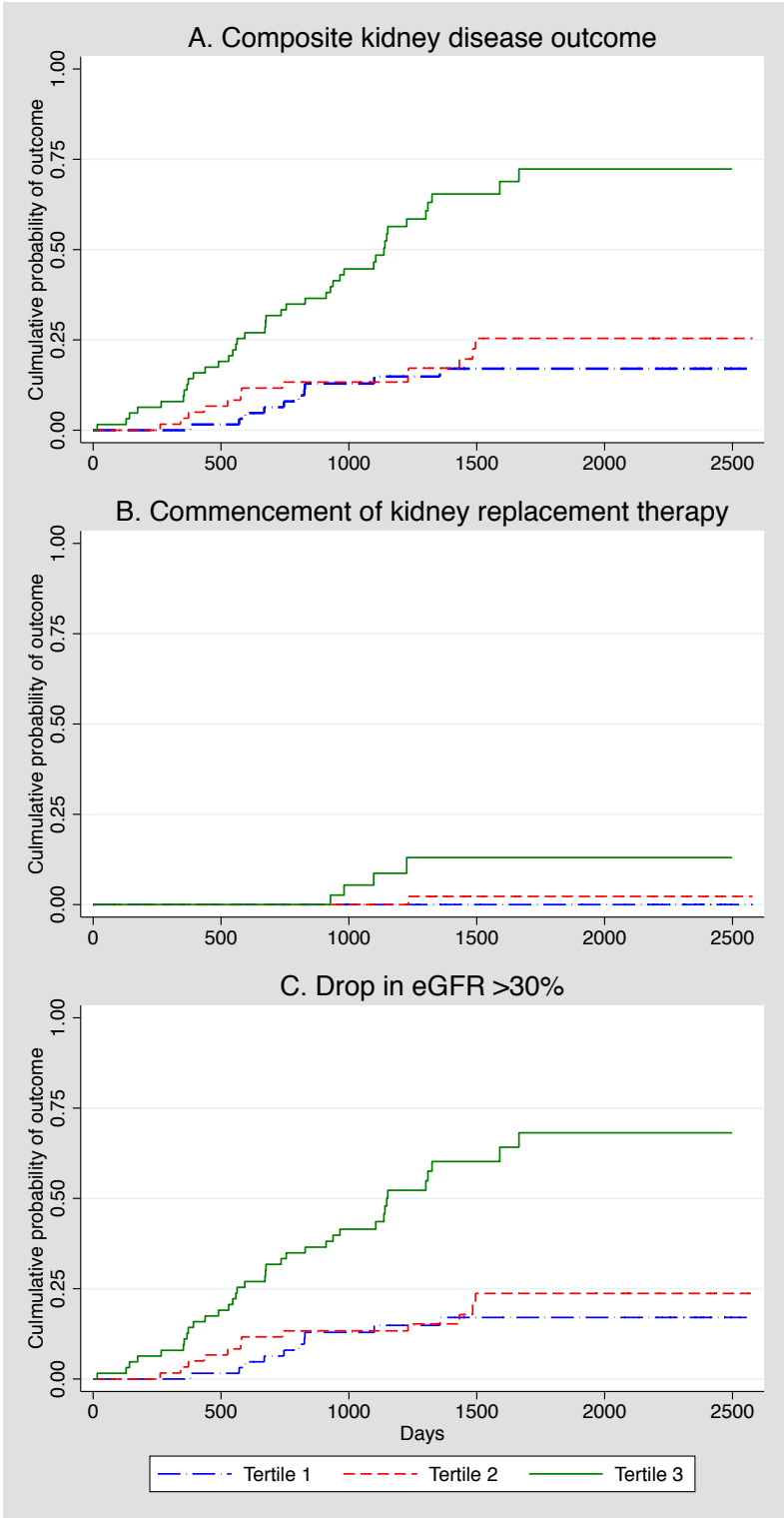
Primary cause of kidney disease	n (%)
Diabetic nephropathy	11 (5.8)
Hypertension / glomerulosclerosis	64 (33.9)
Glomerulonephritis	36 (19.0)
Chronic interstitial nephritis	11 (5.8)
Obstructive uropathy or nephrolithiasis	12 (6.3)
Reflux nephropathy or congenital	11 (5.8)
Cystic kidney disease	10 (5.3)
Nephrectomy or AKI	7 (3.7)
Unknown	27 (14.3)

7.3.2 Association between CPM and composite kidney disease outcome

During a median follow up of 3.6 years (IQR 2.5 – 5.9), and a total of 730.3 person-years, there were 63 kidney disease events, while 21 participants died, and the remaining 105 participants were censored at the conclusion of study follow up. The overall event rate was 8.6 kidney disease events per 100 person years. Of the 63 recorded composite kidney disease events, the majority (92%) were due to drop in eGFR >30%.

The unadjusted cumulative hazard function for the composite kidney disease outcome by tertile of serum CPM is depicted in Figure 7.1. The cumulative incidence curves for the lower two tertiles of serum CPM converged, however there was clear delineation of the highest tertile. Unadjusted and adjusted Cox proportionate hazards models examining the association between CPM and the composite kidney disease outcome are displayed in Table 7.3. The highest tertile of serum CPM was associated with increased risk of the composite kidney disease outcome in the unadjusted model and this association persisted in all three multivariate adjusted models, with only a modest attenuation in the hazard ratio.

Figure 7.1: Cumulative hazard function for composite kidney disease outcome and serum CPM tertile (commencement of kidney replacement therapy or drop in eGFR >30%)



Panel A shows the cumulative hazard function for the composite kidney disease outcome (commencement of kidney replacement therapy or drop in eGFR >30%). Panels B and C show the individual component hazard functions of the composite outcome.

Table 7.3: Association between CPM tertile and composite kidney disease outcome (commencement of kidney replacement therapy or drop in eGFR >30%)

	Tertile 1		Tertile 2		Tertile 3	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Serum CPM	(3.5 – 19.3 x10 ³ AU/mL)		(19.4 – 36.2 x10 ³ AU/mL)		(37.8 – 183.4 x10 ³ AU/mL)	
Unadjusted	Reference	-	1.37 (0.60 - 3.13)	0.454	5.88 (2.93 - 11.82)	<0.001
Model 1	Reference	-	1.41 (0.62 - 3.22)	0.418	6.32 (3.13 - 12.75)	<0.001
Model 2	Reference	-	1.23 (0.53 - 2.86)	0.638	4.21 (2.02 - 8.79)	<0.001
Model 3	Reference	-	1.05 (0.42 - 2.64)	0.922	4.22 (1.91 - 9.33)	<0.001

Hazard ratio (HR) estimated by Cox proportionate hazards model.

Model 1 adjusted for participant age and sex. Model 2, adjusted for the covariates in Model 1 plus eGFR and urinary albumin-to-creatinine ratio. Model 3 adjusted for the covariates in Model 2 plus cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta-blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker).

Abbreviations: CPM, calciprotein monomers; HR, hazard ratio; 95% CI, 95% confidence interval.

7.3.3 Association between CPM and eGFR slope

During the study period there were a median of 21 (IQR 14 – 33) eGFR recordings available for review per participant. The mean eGFR slope of the cohort was -0.8 ± 4.9 mL/min/1.73m²/year. Unadjusted and multivariate adjusted linear regression models examining the association between CPM and eGFR slope are shown in Table 7.4.

Consistent with Cox models for composite kidney disease outcome, higher serum CPM was associated with greater eGFR decline per year, and this association remained significant after sequential adjustment for demographics and risk factors for CKD progression, again with minimal attenuation of the coefficient.

Table 7.4: Association between CPM and eGFR slope

	β coefficient (95% CI)	p value
Ln CPM		
Unadjusted	-2.10 (-3.45, -0.74)	0.003
Model 1	-2.08 (-3.44, -0.72)	0.003
Model 2	-1.75 (-3.27, -0.23)	0.024
Model 3	-1.66 (-3.10, -0.22)	0.024

eGFR slope defined as average change in eGFR per year.³⁵³

β coefficient and 95% CI estimated by linear regression models using robust confidence interval estimates to account for heteroskedasticity of residuals. CPM as well as other skewed covariates were natural log-transformed.

Model 1 adjusted for participant age and sex. Model 2, adjusted for the covariates in Model 1 plus eGFR and urinary albumin-to-creatinine ratio. Model 3 adjusted for the covariates in Model 2 plus cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta-blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker).

Abbreviations: CPM, calciprotein monomers; eGFR, estimated glomerular filtration rate; 95% CI, 95% confidence interval.

7.3.4 Additional analyses

Adjusted Fine and Gray models using death as a competing risk are displayed in Table 7.5. Consistent with the main Cox proportionate hazards models, the highest tertile of CPM was associated with increased risk of kidney disease events. Further adjustment of our main Cox and linear regression models for conventional markers of mineral metabolism (calcium, phosphate and PTH) did not substantially change the association between serum CPM and the composite kidney disease outcome or eGFR slope (Tables 7.6 and 7.7).

The directed acyclic graph and pathway coefficients from mediation analysis are shown in Figure 7.2. The proportion of direct to total association is also displayed in Table 7.8 for serum CPM, which was found to have both significant direct and indirect associations with eGFR slope. In mediation analysis the effect of CPM on eGFR slope appeared to be partially mediated by iFGF23, however there was also a significant direct association between CPM and eGFR slope (75.6% of the total association). Notably, the association between iFGF23 and eGFR slope did not appear to be significantly mediated by ePTFp in this exploratory analysis.

Table 7.5: Association between CPM and composite kidney disease outcome (commencement of kidney replacement therapy or drop in eGFR >30%) – comparison of Cox proportionate hazard regression (hazard ratio) and Fine and Gray (sub-hazard ratio) models

	Cause-specific hazard ratio		Sub-hazard ratio	
	HR (95% CI)	p value	SHR (95% CI)	p value
Serum CPM				
Tertile 1	Reference	-	Reference	-
Tertile 2	1.05 (0.42 - 2.64)	0.922	1.06 (0.40 - 2.84)	0.901
Tertile 3	4.22 (1.91 - 9.33)	<0.001	4.34 (1.85 - 10.16)	0.001

Cause specific hazard ratio estimated by Cox proportionate hazards model. Sub-hazard ratio estimated by Fine and Gray method. Both models are adjusted for participant age, sex, eGFR, urinary albumin-to-creatinine ratio, cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta-blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker).

Abbreviations: CPM, calciprotein monomers; HR, hazard ratio; 95% CI, 95% confidence interval.

Table 7.6: Association between CPM and composite kidney disease outcome (commencement of kidney replacement therapy or drop in eGFR >30%) – additional adjustment for conventional markers of mineral metabolism

	Tertile 1		Tertile 2		Tertile 3	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Serum CPM	(3.5 – 19.3 x10 ³ AU/mL)		(19.4 – 36.2 x10 ³ AU/mL)		(37.8 – 183.4 x10 ³ AU/mL)	
Model 3	Reference	-	1.05 (0.42 - 2.64)	0.922	4.22 (1.91 - 9.33)	<0.001
Model 3 + conventional mineral markers	Reference	-	1.43 (0.54 – 3.77)	0.466	6.22 (2.48 - 15.60)	<0.001

Hazard ratio (HR) estimated by Cox proportionate hazards model. Model 3 adjusted for participant age, sex, eGFR, urinary albumin-to-creatinine ratio, cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta-blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker). Additional model adjusted for covariates in Model 3 plus serum calcium (corrected for albumin), phosphate and parathyroid hormone.

Abbreviations: CPM, calciprotein monomers; HR, hazard ratio; 95% CI, 95% confidence interval.

Table 7.7: Association between CPM and eGFR slope – additional adjustment for conventional markers of mineral metabolism

	β coefficient (95% CI)	p value
Ln CPM		
Model 3	-1.66 (-3.10, -0.22)	0.024
Model 3 + conventional mineral markers	-2.52 (-4.40, -0.63)	0.009

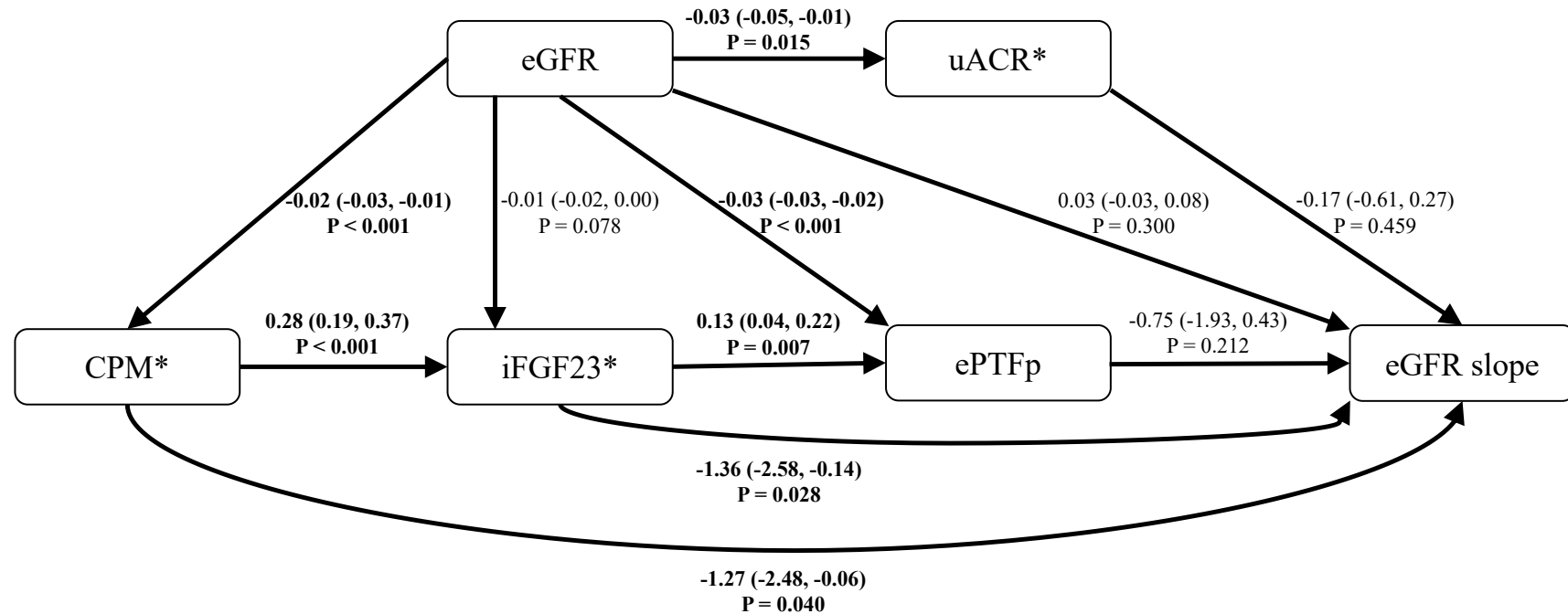
eGFR slope defined as average change in eGFR per year

β coefficient and 95% CI estimated by linear regression models using robust confidence interval estimates to account for heteroskedasticity of residuals. CPM as well as other skewed covariates were natural log transformed.

Model 3 adjusted for participant age, sex, eGFR, urinary albumin-to-creatinine ratio, cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin converting enzyme inhibitor or angiotensin receptor blocker, beta-blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker). Additional model adjusted for covariates in Model 3 plus serum calcium (corrected for albumin), phosphate and parathyroid hormone.

Abbreviations: CPM, calciprotein monomers; eGFR, estimated glomerular filtration rate; 95% CI, 95% confidence interval.

Figure 7.2: Simplified directed acyclic graph of the potential relationship between serum CPM and eGFR slope showing β coefficient (95% CI[#]) from mediation analysis



Numbers in bold indicate significant pathways in mediation analysis (p value < 0.05).

* Skewed variables were natural log transformed before estimating linear coefficients.

95% CI are bias corrected using bootstrapping with 2,000 resamples.

Abbreviations: CPM, calciprotein monomers; eGFR, estimated glomerular filtration rate; ePTFp, estimated proximal tubular phosphate concentration; iFGF23, intact fibroblast growth factor-23; uACR, urinary albumin-to-creatinine ratio; 95% CI, 95% confidence interval.

Table 7.8: Proportion of directly mediated association (with eGFR slope) in mediation analysis

Variable	Mediation analysis	β coefficient (95% CI)	Proportion directly mediated
Log transformed serum CPM	Direct association	-1.27 (-2.48, -0.06)	75.6%
	Total association	-1.68 (-3.10, -0.26)	

Only pathways with direct and indirect associations with eGFR slope are shown.

7.4 Discussion

In this analysis of 189 individuals with Stage 3 and 4 CKD, serum CPM, a novel marker of mineral metabolism was found to be associated with disease progression. Specifically, higher levels of serum CPM were significantly associated with both increased risk of kidney disease events and eGFR decline, independent of a range of traditional risk factors for progression of CKD.

Disordered mineral metabolism is near ubiquitous in the advanced stages of CKD,^{12, 13} and has long been associated with worse patient cardiovascular and mortality outcomes.³⁵⁴ The (patho)physiology of CPM/CPP-I/II metabolism have recently emerged as a novel pathway in the understanding of these processes, and have been suggested to be a possible mediator of cardiovascular outcomes in CKD.^{160, 186} While clinical studies to date have focussed on the relationship between CPM/CPP-I/II and cardiovascular complications, this is the first to suggest that measurement of CPM may also have utility in evaluating the risk of progression of CKD itself.

Previous epidemiological studies have also suggested that the presence of disordered mineral metabolism in CKD may be independently associated with loss of kidney function.^{143, 147-151} Like this current study, these previous studies have largely been observational and so do not definitively show causation. However, Shiizaki *et al* have recently suggested that the formation of calcium phosphate crystal particles in the proximal tubule lumen may initiate cytotoxic responses that result in tubular cell loss.¹⁵¹ In their study, it was proposed that this process is triggered by an initial reduction of functional nephrons, leading to a compensatory rise in FGF23 to maintain total urinary phosphate excretion. This ostensibly adaptive change results in increased per nephron phosphate excretion, which is reflected in an increased concentration of proximal tubular fluid phosphate, causing a predisposition for calcium phosphate crystal formation. The finding of the current study are largely compatible with this proposed mechanism which is further supported by several other observations: firstly that serum levels of CPM and CPP-I/II have been found to increase with reduced eGFR,^{207, 208} secondly, that CKD patients experience greater elevations in CPM following a meal compared to non-CKD controls (Chapter 3), and thirdly, that CPM has been elucidated as the molecular signal that leads to increased FGF23 expression in osteocytes in response to phosphate

loading.²²⁵ Taken together, it is plausible that as kidney function declines, elevated CPM may trigger increased FGF23 expression, which leads to vicious cycle of increased proximal tubular fluid phosphate and progressive tubular loss due to the formation of calcium phosphate crystals.

In exploratory mediation analysis, a significant proportion of the association between serum CPM and eGFR slope was explained by its association with iFGF23, notably though, the association between iFGF23 and eGFR slope did not appear to be explained by ePTFp. There are several potential explanations for this. First, it is possible that iFGF23 may be associated with CKD progression via alternative mechanisms than that proposed by Shiizaki *et al*, such as direct activation of injury primed renal fibroblasts, as has previously been suggested.^{355, 356} Second, while ePTFp did correlate with kidney disease outcomes in mice and human cohorts studied by Shiizaki *et al*,¹⁵¹ it is important to remember that this is an indirect estimation of true end proximal tubular concentration of phosphate (and precipitation potential), and it is unclear whether this estimation method is prone to diurnal or post prandial variation. Related to this, given the limited patient numbers and the availability of only single blood and urine samples, it is possible that the current study may have been underpowered to demonstrate an effect of ePTFp on eGFR slope. Third, it is likely that the propensity to form calcium phosphate crystals in the tubular fluid is dependent on multiple additional local factors that were not accounted for in this current analysis, such as pH, as well as concentrations of calcium, magnesium, and zinc, amongst others.¹⁵¹ Of note, Shiizaki *et al*, proposed a 2-segmented linear association between ePTFp and injury markers, with an inflexion point above 0.75 mmol/L. Given that the majority (75%) of participants in the current study had an ePTFp above this threshold, a single linear function was used. Repeating the analyses with restriction to individuals with an ePTFp equal to or greater than 0.75 mmol/L did not substantially change the association between ePTFp and eGFR slope in linear regression or mediation analysis.

It was notable that in mediation analysis most of the association between serum CPM and eGFR slope appeared to be direct, and independent of iFGF23. Shiizaki *et al* did not examine for the presence of fetuin-A in the calcium phosphate crystal particles they observed. However, in a separate study, Koeppert *et al* recently showed that CPM are rapidly filtered at the glomerulus using fluorescently labelled fetuin-A in a live mouse

model.²¹⁹ While in this latter study it was suggested that filtered fetuin-A is mostly reabsorbed by proximal tubular cells, it is tempting to speculate whether this resorptive capacity may become overwhelmed in situations of chronically elevated serum CPM, such as in CKD, which would presumably lead to correspondingly increased glomerular filtration of CPM by the remnant functioning nephrons. Consistent with this possibility, in the current study an association between higher CPM and higher urinary fetuin-A excretion was observed. Thus, one hypothetical explanation for the direct association between serum CPM and eGFR slope may be that under permissive conditions (i.e., high PTFp, low inhibition), filtered CPM could exacerbate the formation of calcium phosphate crystals in the tubular fluid, serving as a source of additional mineral ions, or as nuclei for *in situ* precipitation. In this context, however, it is worth acknowledging that fetuin-A may well be protective, as shown in recent models of ischaemic and fibrotic kidney injury.³⁵⁷ So, while excessive levels of CPM might ultimately potentiate injury, fetuin-A-bound mineral is certainly less toxic than naked calcium phosphate crystals,²¹³ congruent with its functions as a mineral chaperone. Indeed, Koeppert *et al* also reported that CPM by itself, did not induce any cytotoxicity in their tubular cell cultures.²¹⁹ Ultimately future studies are required to better quantify the composition of proximal tubular fluid and to elucidate how various components, including ions (such as calcium, phosphate, magnesium and zinc), proteins (such as albumin and fetuin-A), physiochemical properties (such as pH), and filtered CPM, may interact to potentiate kidney damage. Functional measures of calcification propensity in urine, akin to the T50 in serum may be highly informative in this regard.

This study did have complete participant follow up with minimal missing data, albeit the study cohort was small. Only single baseline measurements were available for serum CPM. However, the availability of only single measurements would be expected to lead to underestimation, rather than overestimation, of the true association between serum CPM and CKD outcomes. This study is observational, and so the findings are associative by nature. As outlined, recent mechanistic studies have provided biologically plausible explanations through which elevated CPM in CKD could contribute to kidney function decline. Nevertheless, it should be acknowledged that future studies are required to confirm these associations, and additionally, only controlled interventional studies will ultimately be able to determine whether targeting mineral metabolism is a useful intervention to slow progression of CKD.

7.5 Conclusion

CPP are novel markers of disordered mineral metabolism in CKD. While elevated levels of CPM/PP-II have been associated with adverse cardiovascular outcomes in individuals with CKD, this study suggests that elevated levels of CPM may also be associated with increased risk of progression of CKD itself. This adds to accumulating evidence that disordered mineral metabolism in CKD may directly contribute to increased tubular damage and loss of kidney function.

Chapter 8 General discussion and conclusions

CKD is a highly prevalent condition which is frequently complicated by the parallel development of mineral metabolism abnormalities, cardiovascular calcification and bone disease. This clinical syndrome is collectively referred to as CKD-MBD, however the exact mechanisms linking these processes remains incompletely understood. The ultimate goal of enhancing our understanding of these processes is to progress diagnostic and therapeutic opportunities in clinical practice. This thesis incorporated a series of interrelated studies on the “classical” prototypical marker of disturbed mineral metabolism in CKD, serum phosphate, and the emerging role of CPP in health and disease. Indeed, work over the last decade suggests that CPP, nanoparticles of solid-phase calcium phosphate, may better explain some of the associated pathological effects attributed to phosphate excess than free phosphate ions themselves. As such, a central focus of this thesis, explored how CPP fits into normal mineral physiology and how this may become perturbed in CKD. Samples from two recently completed interventional studies were then used to examine potential therapeutic manoeuvres to modify serum levels of CPP. Finally, the association between CPP and clinical outcomes was also explored.

8.1 Serum phosphate remains associated with mortality in dialysis patients

Although serum phosphate has long been associated with adverse patient outcomes, particularly in individuals with kidney failure being treated with dialysis, there was limited data from the current dialysis era. This may be germane, given the continual evolution of dialysis technologies and techniques,¹⁶² and the now near universal use of phosphate lowering pharmacotherapies in some jurisdictions.³⁵⁸ Given variation in global clinical practices also exists, the lack of data from the Australia and New Zealand region was also notable. The analysis of the ANZDATA registry presented in Chapter 2 aimed to address some of these gaps, and reaffirmed that in the current era, serum phosphate concentration, as measured using routine and contemporaneous methods, remains a robust and independent marker of significant patient outcomes in this patient population. There are theoretical reasons why the relationship between phosphate and patient

outcomes may vary across dialysis modalities, and so the consistency of findings across HD and PD in this analysis was also noteworthy.

Serum phosphate is a readily obtained metric in current clinical care. Yet, while the role of serum phosphate as a metric of *patient risk* was reaffirmed in Chapter 2, its utility as a *clinical target* remains debated. Current international clinical guidelines recommend “lowering elevated phosphate levels towards the normal range”, however these guidelines acknowledge that “trial data demonstrating that treatments that lower serum phosphate will improve patient-centred outcomes are still lacking.”¹⁵⁷ With this in mind, there are two large interventional studies currently being conducted that aim to examine effects of lowering serum phosphate in individuals with kidney failure being treated with dialysis. Rather than studying a singular phosphate lowering intervention, both trials instead examine the strategy of lowering phosphate to a target range compared to a more liberal approach. The Pragmatic randomised trial of High Or Standard PHosphAte Targets in End-stage kidney disease (PHOSPHATE) trial is a multinational trial of HD and PD patients, examining the effects of a liberal phosphate target (aiming serum phosphate between 2.0 to 2.5 mmol/L) compared to a more intensive target (≤ 1.50 mmol/L) on cardiovascular events.²⁵⁶ The HiLo trial is a USA based multicentre cluster-randomised trial of HD patients, comparing a phosphate target of ≥ 2.10 mmol/L with < 1.78 mmol/L on a composite outcome of all-cause mortality and all-cause hospitalisation.²⁵⁵ Critically, both trials should be well powered (target recruitment 3,600 and 4,400 participants respectively) to detect any clinically meaningful benefit of a phosphate lowering approach. Data from both studies will help to clarify whether serum phosphate can continue to be used as a therapeutic target in dialysis populations and should also provide further insight into whether phosphate is a direct mediator of adverse patient events.

8.2 CPP are formed in response to nutritional mineral intake substantiating a physiological role of CPP in normal mineral homeostasis

The biochemical properties of CPP have meant that they have long been purported to have a physiological role in mineral trafficking. This was largely based on *in vitro* and animal work, while studies in humans had mainly been conducted in those with CKD or other acute or chronic diseases. Chapter 3 added to this knowledge base by demonstrating

that circulating levels of CPM and CPP-I/II acutely rise in healthy adults following a standard meal. This novel finding adds to the plausibility of the GIT being an important site of CPP formation, but also supports the notion that these mineral-protein colloids may serve a physiological function in buffering acute fluxes of mineral from the diet. Post-prandial surges of CPM, CPP-I and CPP-II were seen despite serum levels of calcium and phosphate remaining relatively stable. This raises the possibility that CPP act as an additional pool for intermittent surges of mineral ions to be acutely dispersed in the body, before longer term adaptive mechanisms, principally enhanced urinary excretion, restore total body balance. The finding that some individuals studied in Chapter 3 showed a transient post prandial rise in serum fetuin-A deserves further exploration. If this observation is replicated in future studies, this would suggest that the physiological response to mineral surges may be more intricate than currently appreciated.

The findings in Chapter 3 also provide further insight into the incompletely understood area of phosphate homeostasis. Soon after the initial identification of FGF23 as a major regulator of urinary phosphate excretion, it was recognised that dietary mineral intake led to increased secretion of FGF23.²³³ However, how phosphate is “sensed” remains unclear. Recently, Akiyama *et al* provided compelling experimental evidence that CPM, rather than phosphate, may be the molecular signal that triggers FGF23 release.²²⁵ Taken together with the findings in Chapter 3, where a surge of CPM was observed post mineral consumption, it is plausible that CPM provide a link between dietary mineral and endocrine mineral regulation in the kidney.

8.3 Implications of post-prandial variation on utilisation of novel mineral metabolism assays

The findings in Chapter 3 also have practical implications for optimising the future use of the novel assays of mineral metabolism that were utilised throughout this thesis. Post-prandial levels of CPM, CPP-I and CPP-II were substantially higher than fasting, while there was also a transient post-prandial increase in T50. It seems logical that future testing protocols using the gel filtration CPM assay, the flow cytometric CPP-I/II assay, and the T50 assay, may benefit by controlling for this source of biological variation, or at very least recording fasting status so that these effects can be retrospectively interrogated.

The pharmacokinetic post-prandial profiles of CPM and CPP-I/II may also present an additional paradigm to study the evolution of mineral metabolism abnormalities. It is possible that different components of CPP metabolism may be more specifically reflected by the different phases of the post-prandial profile, analogous to way that insulin secretion, hepatic insulin sensitivity and gluconeogenesis, and peripheral insulin resistance differentially affect fasting and post-prandial indices in the oral glucose tolerance test.³⁵⁹⁻³⁶¹ In Chapter 3, there was an association between eGFR and high post-prandial CPM AUC. Given the recent work by Koeppert *et al* showing that CPM are primarily cleared by the kidney, it seems plausible that reduced clearance may have contributed to these findings.²¹⁹ However, reductions in GFR are also associated with additional abnormalities, such as secondary hyperparathyroidism and disturbances in bone metabolism. Indeed, in Chapter 4, it was shown that bone turnover likely influences serum levels of CPM and CPP-I in patients with CID. Thus, an interplay of effects in the kidney and bone may account for the observed differences in serum CPM kinetics. Unfortunately, the patient numbers in Chapter 3 were small and bone turnover markers were not available, so further delineation of these effects in this cohort were not possible. Therefore, a future avenue of investigation would be to further investigate whether kidney function and bone turnover separately contribute to different phases of the post-prandial profile. Related to this, it is also possible that abnormalities in CPP metabolism may be detectable via dynamic testing earlier than by fasting or random sampling.

8.4 Bone as a determinant of circulating CPP

The key finding in Chapter 4 was that serum levels of CPM and CPP-I longitudinally changed in parallel with markers of bone formation. While in itself not definitive, this data strongly supports the notion that bone turnover directly influences serum levels of CPP, and builds on a range of pre-existing findings.^{201, 202, 222-224, 296} In contrast to the findings presented in Chapter 4, Bressendorff *et al* recently described that longitudinal changes in CPP had a stronger correlation with markers of bone resorption in a post-hoc analysis of a randomised trial of increasing dialysate magnesium in HD patients.²⁹⁶ Given that the process of bone remodelling involves the coupling of formation and resorption, these findings may well be complementary, rather than contradictory.³⁶² Remodelling is necessary for repair and maintenance of normal bone, but given that bone also stores the

vast majority of calcium and phosphate, remodelling also allows bone to act as a critical exchangeable pool of mineral, by acting as both a mineral source and sink.²⁰ It is plausible that the net balance between resorption and formation in bone determines its contribution to the circulating pool of CPP. This concept is certainly supported by early animal experiments conducted by Price *et al*, which suggested that pharmacological manipulation of bone formation and resorption both influenced the appearance of CPP in serum.^{222, 223} Price *et al* also hypothesised that CPP could be formed within the bone remodelling compartment (BRC) – an extracellular microenvironment within cancellous bone that links bone formation and resorption.^{223, 363} Given the BRC is contiguous with the microvasculature,³⁶⁴ Price *et al* speculated that formed CPP could then spill over into the circulation.²²³ While plausible, confirmation of this proposed mechanism is still required.

Previous associations in humans between serum CPP and markers of bone metabolism had exclusively been from studies of individuals with various stages of CKD.^{202, 224, 296} Given that CKD is intimately associated with abnormalities in mineral *and* bone metabolism, this made it challenging to isolate the contribution of bone to CPP metabolism from other aspects of CKD. By contrast, the cohort that was studied in Chapter 4 had normal kidney function, showing that the association between bone and CPP is consistent in a range of clinical settings. The reverse of this is also true – consistency of the findings with previous animal and human studies suggests that the link between bone and CPP in the chronic inflammatory cohort studied in Chapter 4 was not merely a surrogate marker of inflammation. The implications of this are several. First, it potentially adds to the mechanistic interconnectedness of the mineral, bone and vascular domains of CKD-MBD. Given the hypothesis that elevated levels of CPP contribute to the development of vascular pathology, by extension, elevated levels of CPP could form a direct link between bone and vascular complications in CKD-MBD. Second, outside of CKD, increased levels of circulating CPP due to abnormalities in bone metabolism could further substantiate the existence of a “bone-vascular axis”.^{294, 295} The “calcification paradox”, where loss of bone mineral density is seen in parallel with a paradoxical increase in vascular calcification has been described in other settings, such as aging, diabetes, and chronic inflammatory disease.²⁸⁹⁻²⁹² Importantly though, while there is epidemiological evidence linking elevated levels of CPP with vascular outcomes in CKD,^{202, 205} comparable studies have not been conducted in any of these other patient

groups. Finally, there is evidence to suggest that CPP synthesised in or exposed to uraemic serum are enriched with additional, potentially bioactive compounds, and so it is plausible that even if CPP are confirmed to be elevated in other settings of accelerated loss of bone mineral density, that the pathogenic consequences of this may be different than in CKD.²²⁰

8.5 Intestinal phosphate binders as a strategy to lower serum CPP

Use of intestinal phosphate binders have been a common strategy to target total body phosphate exposure in CKD.³⁵⁸ Previous studies had suggested that change in intestinal phosphate binders were associated with changes in serum levels of CPP.^{226, 227} These studies had been relatively small, had exclusively been conducted in participants with kidney failure being treated with haemodialysis, and each study had compared levels of CPM or CPP-I/II using calcium containing phosphate binders with non-calcium containing phosphate binders. The IMPROVE-CKD trial provided a unique opportunity to build on these pre-existing studies and attempt to address some of their limitations, given the larger number of participants and the use of a placebo control. With this in mind, the main finding of Chapter 5, that lanthanum carbonate did not significantly reduce serum levels of CPP-I or CPP-II, brings into question the utility of intestinal phosphate binders as a strategy to lower serum CPP.

There are a number of caveats to the findings in Chapter 5 that should be acknowledged. In the main IMPROVE-CKD trial, lanthanum carbonate did not have a demonstrable effect on serum phosphate, 24-hour urinary excretion or serum FGF23 and so it is questionable whether differences in phosphate exposure were achieved.³²⁰ There are however a number of additional factors specifically relating to metabolism of CPP that also deserve consideration. The study was conceived before the full results of Chapter 3 were available, and in retrospect, the design of this study may have been revised had this otherwise been the case. Firstly, in Chapter 3, individuals with CKD (eGFR <60 ml/min/1.73m²) had marked disturbances in CPM metabolism, whereas post-prandial metabolism of CPP-I and CPP-II appeared comparable to healthy controls. The kidney function of individuals in IMPROVE-CKD was not dissimilar to those in Chapter 3, raising the possibility that focussing on CPM rather than CPP-I/II may have been a more

sensitive marker of mineral metabolism in this patient cohort. Second, the timing of sample collections was not standardised in IMPROVE-CKD in relation to fasting and post-prandial status. As was found in Chapter 3, this could have introduced a significant amount of variation in measures of CPP-I/II. Notably, there was unexpected differences in the distribution of CPP-I/II between sites (Figure 5.2), and one potential contributor to this may have been systematic differences in the timing of sample collection. A counter argument to this point, is that a lack of standardisation of the timing of sample collection has not universally precluded the demonstration of longitudinal changes in serum CPP-I/II using the same flow cytometric assay, including in Chapter 4. As such, further data would be useful to confirm or refute the findings of Chapter 5. Notably, since this work was completed, Thiem *et al* found that sucroferric oxyhydroxide, another non-calcium-based phosphate binder reduced serum levels of CPM, CPP-I and CPP-II compared to placebo in a randomised cross-over trial. The use of a placebo comparator suggests that the findings of the two previously cited trials^{226, 227} may not have been entirely related to the theoretical effect of calcium carbonate on serum CPP. However, like these previous trials, the study by Thiem *et al* was relatively small ($n = 28$) and studied HD patients. As such, this new data does not directly assist in the further interpretation of Chapter 5.

8.6 Medium cut-off haemodialysis membranes lower serum FGF23 but not CPP

The most notable finding in Chapter 6 was that use of a MCO HD membrane was associated with lower levels of serum iFGF23 compared to the use of a standard HF membrane. Like serum phosphate, FGF23 progressively increases as kidney function declines, and has been consistently linked to adverse patient outcomes in epidemiological studies.¹⁴²⁻¹⁴⁴ In experimental studies, FGF23 induces left ventricular hypertrophy, suggesting that there are biologically plausible pathways through which elevated FGF23 could directly contribute to cardiovascular pathology in CKD.^{145, 146} The degree of reduction associated with the MCO membrane was statistically significant, but modest in magnitude (22% reduction at 24 weeks). It is worth pointing out that these results were based on pre-dialysis blood collections, and it is plausible that this under-estimates the time-averaged exposure in between dialysis sessions.³⁶⁵ Nevertheless, the biggest

question remaining is about the clinical significance of this finding, which cannot be addressed by this study alone.

The other main findings in Chapter 6 were that use of a MCO membrane did not significantly affect levels of pre-dialysis fetuin-A, CPP-I or CPP-II. The absence of evidence of significant loss of fetuin-A is important, given its critical role as a mineralisation inhibitor, and theoretical loss given that its molecular weight (58 kDa), coincides with the “cut-off” of MCO dialysers. It has previously been reported that serum levels of CPP (using the older fetuin-A reduction ratio) are lower immediately following a HD treatment compared to pre-dialysis levels.²⁰⁷ However, the lack of difference in serum CPP between MCO and HF membranes in this study is perhaps not unexpected given that fetuin-A levels were unaltered and previous evidence that MCO and HF membranes are comparable in terms of clearance of small solutes (including calcium and phosphate).³⁴⁰ Further, the size of CPP-I and CPP-II are significantly larger than the average pore size of an MCO membrane (3.0-3.5 nm).³⁴⁹ It is unlikely that further alterations to HD membrane pore size will be a viable strategy to augment the removal of CPP, given that “high cut-off” HD membranes have a larger maximum effective pore size than MCO membranes, but their clinical use is limited by significant albumin loss.^{366, 367} Plasma exchange is an additional extracorporeal technique that has previously been reported to reduce serum levels of CPP further than HD treatment alone.³⁶⁸ However, plasma exchange carries a risk of haemodynamic, coagulopathic, allergic and infection related complications which, as well as logistical and financial constraints, would likely preclude this from being a long-term therapy for the purposes of serum CPP reduction.³⁶⁹⁻

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8.7 Measurement of CPP and clinical outcomes

Some of the interest in CPP is based on previous epidemiological evidence of independent associations with adverse events in CKD, as well as biologically plausible pathways elucidated from experimental studies through which elevated levels of CPP may directly contribute to these events. While this thesis did examine the epidemiological associations between serum phosphate and patient outcomes in Chapter 2, the direct examination between serum CPP and cardiovascular outcomes was not a major focus in this collection

of works. However, while the main aim of Chapter 5 was to examine the effect of lanthanum carbonate on serum CPP-I and CPP-II, the availability of two surrogate vascular outcomes (PWV and abdominal aortic calcification) in the IMPROVE-CKD study did allow for some examination of the links between CPP and these endpoints. Given previous findings that CPP were linked to aortic stiffening²⁰² and coronary artery calcification²⁰⁰ in CKD populations, the lack of an association between CPP-I/II and PWV or aortic calcification in Chapter 5 was somewhat unexpected. As discussed above, there were a number of reasons why the measurement of CPP in this cohort may not have been optimised in terms of the lack of standardisation of timing of sample collection, as well as the theoretical disadvantage of the use of CPP-I/II in this cohort with non-dialysis dependent CKD rather than CPM. Further, the majority (>80%) of participants in this cohort already had evidence of aortic calcification at baseline and the median baseline PWV was >10 m/s, which may have limited the ability to discriminate between serum CPP-I/II and these surrogate endpoints. Given these considerations, and the discrepant findings between Chapter 5 and previous works that had suggested epidemiological links between serum CPP and vascular endpoints, examination of further cohorts would be beneficial. Current methods of measuring CPM and CPP-I/II would preclude large, registry-based studies, such as that conducted in Chapter 2, meaning that the epidemiological study of these novel markers of mineral metabolism are currently limited to research cohorts. Nevertheless, further studies of CPP using hard, patient relevant outcomes would be useful to fully evaluate the prognostic significance of serum CPP in the CKD population.

While CPP have predominantly been studied in relation to cardiovascular endpoints, for the first time in Chapter 7, an association between CPM and progression of CKD itself was also described. This study was conceived based on three key developments that were published during the course of this thesis. Akiyama *et al* suggested that CPM may be the molecular signal that triggers osteocytic FGF23 release.²²⁵ Shiizaki *et al* proposed a mechanism that FGF23 mediated increases in the concentration of phosphate in the proximal tubular fluid may lead to calcium phosphate crystal formation which results in tubular damage.¹⁵¹ Finally, Koeppert *et al* showed that CPM are directly filtered at the glomerulus into the tubular fluid.²¹⁹ Consistent with these developments, in Chapter 7 it was found that higher levels of CPM were associated with progression of CKD, independent of a range of traditional risk factors. Taken together, it is plausible that the

development of impaired kidney function may lead to decreased CPM clearance, triggering increased FGF23 secretion and elevations in proximal tubular phosphate concentration, which ultimately results in the exacerbation of kidney damage via the mechanisms proposed by Shiizake *et al.* In addition to this possible schema, exploratory mediation analysis suggested that serum CPM may also be directly associated with eGFR slope, independent of its association via FGF23. Given the findings by Koeppert *et al* that CPM are rapidly filtered into the tubular fluid, it is fascinating to hypothesise whether CPM may more directly mediate tubular damage, although this requires further clinical confirmation and *in vitro* explanation. Notably, *in vitro*, CPM do not appear cytotoxic to tubular cells.²¹⁹ However, these experiments were conducted using standardised cell culture conditions, and it is unclear how CPM interact with the distinctive pH and biochemical composition of the urinary environment.

The findings in Chapter 7 provide additional insights into the known observation that elevated levels of serum phosphate and FGF23 are associated with progression of CKD.^{143, 147-151} The full clinical applications of this novel finding require further exploration. It is notable that in Chapter 5, CPP-I and CPP-II were not associated with change in eGFR. However, as already discussed, serum CPM were not analysed, and so it was not possible to verify the findings in Chapter 7 in this additional cohort. For this reason, a logical future direction would be to examine whether lanthanum carbonate has any effect on serum CPM in those with non-dialysis dependent CKD, and whether any reduction in those with high baseline CPM is associated with any change in FGF23 and progression of CKD.

8.8 Concluding remarks

Mineral metabolism abnormalities are intrinsic to CKD and undeniably contribute to adverse patient outcomes. Our ability to better detect and manage these disturbances is contingent on improving our understanding of normal mineral homeostasis, and how this becomes perturbed in CKD. Serum phosphate has long been at the centre of the classical understanding of CKD-MBD, and this thesis confirms that both high and low levels of serum phosphate remain independently associated with increased mortality in dialysis patients, irrespective of modality. In regards to CPP, as novel markers of mineral metabolism, this thesis provides direct evidence that the GIT is a major source of each phase of circulating CPP (CPM, CPP-I and CPP-II) in humans. This thesis also supports the notion that CPP play a role in normal mineral metabolism, and has implications for our understanding of whole body phosphate regulation in light of recent evidence that CPM stimulate FGF23 expression. Results in this thesis also strongly support that bone turnover is an additional determinant of circulating CPP. In terms of potential strategies to lower serum CPP, the studies presented here question the efficacy of the use of intestinal phosphate binders, and also of changes in commercially available HD membranes. A novel finding of this thesis is that fasting and post-prandial metabolism of CPM is disturbed in CKD, and related to this, it was found that elevated levels of CPM are associated with the progression of CKD itself. This thesis provides new insights into current and emerging markers of mineral metabolism and broadens our understanding of their physiological and interventional determinants. However, further study is needed to ultimately determine whether these findings can be translated into clinically applicable markers of risk and use as targets of therapeutic intervention.

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Appendices

ORIGINAL ARTICLE

NEPHROLOGY



WILEY

Serum phosphate and mortality in incident dialysis patients in Australia and New Zealand

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Abstract

Aim: Hyperphosphataemia is associated with increased adverse outcomes, including mortality. Re-examining this association using up-to-date data reflecting current and real-world practices, across different global regions and in both haemodialysis and peritoneal dialysis patients, is important.

Methods: We describe the association between serum phosphate and all-cause and cardiovascular mortality in incident dialysis patients between 2008 and 2018 using the Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry. Time-dependent Cox proportionate hazards models were used. Models were adjusted for available covariates and fitted for the overall cohort, and also each dialysis modality.

Results: 31 989 patients were followed over 97 122 person-years at risk (mean age at first dialysis 61 years, 38% female, 67% haemodialysis). We observed a U-shaped association between serum phosphate and all-cause mortality. In the fully adjusted model, categories of serum phosphate above and below 1.25–1.99 mmol/L were associated with progressively higher risk, reaching a hazard ratio of 2.13 (95% CI 1.93–2.36, $p < .001$) for serum phosphate ≥ 2.75 mmol/L, and 1.56 (95% CI 1.44–1.69, $p < .001$) for serum phosphate < 1.00 mmol/L. Low and high levels of serum phosphate were also associated with increased risk of cardiovascular mortality, however the association with high serum phosphate was more pronounced (“J-shaped relationship”). The associations were consistent across sub-analyses of patients receiving haemodialysis and peritoneal dialysis treatment.

Conclusion: In this large contemporary dialysis cohort, both high and low levels of serum phosphate were independently associated with increased risk of mortality. Future studies are required to determine whether treatment of abnormal serum phosphate levels improves mortality.

KEYWORDS

haemodialysis, hyperphosphataemia, hypophosphataemia, mortality, peritoneal dialysis, serum phosphate

SUMMARY AT A GLANCE

Among incident dialysis patients from a large study cohort, both high (1.99 mmol/L) and low (1.25 mmol/L) levels of serum phosphate were independently associated with increased risk of mortality.

1 | BACKGROUND

Patients with chronic kidney disease (CKD) experience significantly poorer outcomes compared to the general population, including greater all-cause and cardiovascular-related mortality.¹ Increased morbidity and mortality is partly attributable to the propensity for patients with CKD to be older and have an elevated burden of co-morbidities and cardiovascular risk factors. However, it is also clear that additional pathological processes specific to CKD play a role.² Decline in kidney function is paralleled by progressive abnormalities in mineral and bone metabolism. This multi-system process is collectively referred to as Chronic Kidney Disease–Mineral and Bone Disorder (CKD-MBD), and is almost universally present in advanced stages of CKD.³ CKD-MBD is associated with a range of deleterious patient outcomes, including accelerated cardiovascular disease and increased risk of fracture, and significantly contributes to the poor overall outcomes seen in CKD patients.

Abnormalities of phosphate metabolism are a key feature of CKD-MBD. In patients with kidney failure requiring dialysis, abnormalities in serum phosphate are highly prevalent and have been consistently linked to a range of adverse outcomes.⁴ In particular, hyperphosphataemia has been consistently associated with increased risk of all-cause and cardiovascular mortality.⁴ As a result, treatment of hyperphosphataemia has been a focus of international clinical guidelines for patients requiring dialysis.⁵ Conversely low levels of serum phosphate have also been reported to correlate with poor outcomes, in some, but not all studies, suggesting that serum phosphate may have a “U-shaped” association with mortality.^{6–12}

Evidence for the association of high and low serum phosphate with poor outcomes is predominantly based on observational studies and there is a lack of high quality trial evidence to show that treatment of abnormalities in phosphate improves patient-centred outcomes.¹³ Interventional trials are currently being conducted to clarify the utility of therapeutic manoeuvres in response to abnormal levels of serum phosphate, specifically the treatment of hyperphosphataemia in dialysis patients.^{14,15} Nevertheless, in the meantime, clinicians require up-to-date data to help guide their interpretation of biochemical markers encountered in everyday practice. Internationally, the demographic and comorbidity profile of the dialysis population continues to change,¹⁶ and given that dialysis and other aspects of clinical care also continue to evolve,¹⁷ it is conceivable that the relationship between phosphate and outcomes may not be stagnant over time. Therefore, there may be value in re-examining this association using up-to-date data that reflects current and real-world practices, including in different global regions. Furthermore, previous studies have predominantly focussed on patients being treated with haemodialysis (HD), while there is much less data relating to associations with serum phosphate in patients being treated with peritoneal dialysis (PD).

The objective of this study was to investigate the association between serum phosphate and all-cause and cardiovascular mortality, in a large and modern cohort of incident HD and PD patients from Australia and New Zealand.

2 | METHODS

2.1 | Study population

The Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry was utilized for this study. Full details of the structure and methods of ANZDATA have been published elsewhere.¹⁸ In brief, ANZDATA collects demographic and clinical information about all patients who are resident in Australia or New Zealand and are receiving long-term treatment for kidney failure with either dialysis or kidney transplantation. Patient data (including serum phosphate) is provided by the patient's treating unit through an annual survey. Additional data is also provided in “real-time”, for pre-specified events, such as change in dialysis modality or patient death. This analysis of the ANZDATA Registry was approved by the local ethics committee (Melbourne Health Human Research Ethics Committee reference number QA2020033).

2.2 | Patient inclusion criteria and follow up

Patients commencing dialysis between 2008 until 2018, who were at least 18 years of age at the time of first dialysis, and who had at least one measurement of serum phosphate were included. For each participant, follow up time was accrued from initiation of dialysis until date of death. Patients were censored at the time of receiving a kidney transplant, loss of follow up, or end of the study (31 December 2018), whichever occurred first. Given the objective of this analysis was to describe the long-term relationship between phosphate and mortality in dialysis patients, participants were excluded if they died or were censored within 30 days of first dialysis. Similarly, because the initial period after commencement of dialysis is associated with significant biochemical instability, biochemical measurements were excluded if they were taken within 30 days of first dialysis.

2.3 | Study exposure

The primary exposure variable was serum phosphate. ANZDATA instructs participating units to provide serum phosphate and other biochemical values which are measured midweek and, for HD patients, taken immediately pre-dialysis. Blood samples are analysed at local accredited laboratories, and units report values taken as close to the end of the annual survey period as are available.

2.4 | Study outcomes

The primary outcome of interest was all-cause mortality. Given phosphate has also been associated with cardiovascular disease and mortality, cardiovascular-related death was also analysed in a secondary analysis. The classification used by ANZDATA of categories of death, as well as a breakdown of cardiovascular-related death, is provided in Table S1.

2.5 | Study covariates

A range of demographic and clinical covariates that are collected by ANZDATA were included to adjust for potential confounding. The final fully adjusted model included the “fixed” covariates: age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer). The following “time-updated” covariates, which are updated with each annual survey were also included: dialysis modality (updated in real time), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

2.6 | Statistical analysis

Summary characteristics of the cohort were reported. Continuous variables with a normal distribution were expressed as mean and standard deviation (SD), and those with skewed distributions were expressed as median and interquartile range (IQR). Categorical data were presented as numbers and percentages.

Cox proportional hazards models were used to model the relationship between serum phosphate and mortality. Initially, the relationship between deciles of serum phosphate and all-cause mortality was analysed in a univariate model. Consistent with previous studies, the observed relationship between serum phosphate and mortality was non-linear. As a result, serum phosphate was categorized into 0.25 mmol/L intervals from <1.0 mmol/L to >2.75 mmol/L. These phosphate categories were empirically chosen to represent the distribution of phosphate observations. Serum phosphate between 1.50–1.74 mmol/L appeared to have the lowest hazard ratio (HR) in unadjusted analyses, and so was set as the reference category.

Unadjusted and multivariate adjusted models were fitted. As for serum phosphate, continuous covariates used in the adjusted models were categorized if they did not have a linear association with mortality. Patient sex and dialysis modality (HD or PD) were forced into the multivariate model, while other covariates were considered for inclusion if they were significant on univariate analysis ($p < .2$). Significant covariates were then entered into the main model using a manual forward stepwise procedure, where covariates were retained if they improved model goodness of fit, as assessed by Akaike information criterion (AIC). Current use of an erythropoiesis stimulating agent, and index of socioeconomic score were available as potential covariates, but were not selected in the final, fully adjusted, model. All other covariates that were selected for inclusion had p -values equal to or less than .001 on univariate analysis. All models were stratified by treatment centre. The proportional hazards assumption was checked by visual inspection of Kaplan Meier curves for categorical variables and by examining Schoenfeld residuals for continuous variables. For simplicity, only the unadjusted, age-adjusted and fully adjusted models are presented.

2.6.1 | Sub-analyses

To analyse the secondary outcome of cardiovascular-related death, each model was re-fitted for cardiovascular- and also non-cardiovascular-related mortality. To examine the effects of dialysis modality, models were also separately refitted after restricting data to patients receiving HD and PD. We also repeated the fully adjusted model for all-cause and also for cardiovascular mortality with an interaction term between serum phosphate and dialysis modality.

2.6.2 | Missing data and sensitivity analyses

Multiple imputation was used to assess for the effect of missing data for the fully adjusted model of all-cause mortality. For this analysis, 20 datasets were generated using the multivariate imputation by chained equations (MICE) approach, with the assumption that the data was missing at random.

For each model, serum phosphate and time-updated variables were included as time-dependent covariates. To account for potential bias introduced through the use of time-dependent covariates,¹⁹ the main, fully adjusted model was refitted for baseline and also for mean values of serum phosphate. For the baseline model, other time-updated variables were also entered using their baseline values. In the mean model, the mean of continuous time-updated variables was used, while categorical variables were left as time-dependent predictors.

We also fitted a model using baseline values of the comorbidities of lung disease, coronary artery disease, peripheral vascular disease and cerebrovascular disease, while leaving other time-updated variables as time dependent variables. Given that high serum phosphate has been associated with the development of cardiovascular disease, we included this model to account for the possibility of over-adjustment bias, due to adjusting for events that are potentially involved in the causal pathway.²⁰

Because of the potential to introduce bias through censoring patients who receive a kidney transplant in the Cox proportional hazard models,²¹ we also performed a competing risk analysis for all-cause mortality, where kidney transplant was a competing event. For this model, we used the Fine and Gray method,²² and adjustments were made for the same covariates as in the main, fully adjusted model.

p -Values <.05 were considered statistically significant. All data were analysed using Stata version 16.1 (StataCorp, College Station).

3 | RESULTS

There were a total of 32 227 adult incident dialysis patients who were registered with ANZDATA between 2008 and 2018 and had at least one determination of serum phosphate. Of these, 238 died or were censored within 30 days of first dialysis and were excluded, leaving 31 989 patients in the analysis. Baseline demographic and clinical details of these patients are displayed in Table 1. Mean age at first

TABLE 1 Participant demographics and characteristics at commencement of dialysis (by dialysis modality)

Characteristic	Overall (n = 31 989)	Baseline dialysis modality	
		Haemodialysis (n = 21 567)	Peritoneal dialysis (n = 10 422)
Age at first dialysis (years)	60.6 ± 14.9	61.3 ± 14.8	59.0 ± 15.0
No. female	12 203 (38.2)	8175 (37.9)	4028 (38.7)
<i>Self-reported ethnicity</i>			
European or other	21 615 (68.6)	14 790 (69.8)	6825 (66.3)
Australian Aboriginal or Torres Strait Islander	2854 (9.1)	2352 (11.1)	502 (4.9)
New Zealand Māori or Pacifica	3929 (12.5)	2500 (11.8)	1429 (13.9)
Asian	3092 (9.8)	1560 (7.4)	1532 (14.9)
<i>SEIFA (disadvantage score) quintile based on residential postcode</i>			
1st quintile	5135 (18.9)	3469 (18.4)	1666 (19.9)
2nd quintile	5899 (21.7)	4075 (21.7)	1824 (21.7)
3rd quintile	5042 (18.5)	3552 (18.9)	1490 (17.8)
4th quintile	5594 (20.6)	3902 (20.8)	1692 (20.2)
5th quintile	5532 (20.3)	3811 (20.3)	1721 (20.5)
<i>Primary aetiology of kidney disease</i>			
Glomerulonephritis	6210 (19.4)	3778 (17.5)	2432 (23.3)
Polycystic kidney disease or reflux	2402 (7.5)	1575 (7.3)	827 (7.9)
Diabetes	12 908 (40.4)	8935 (41.4)	3973 (38.1)
Other	8460 (26.5)	5900 (27.4)	2560 (24.6)
Unknown	2009 (6.3)	1379 (6.4)	630 (6.0)
First reviewed by nephrologist <3 months of first dialysis	6034 (19.1)	4515 (21.2)	1519 (14.7)
<i>Comorbid conditions at first dialysis</i>			
Chronic lung disease	5283 (16.6)	3814 (17.8)	1469 (14.1)
Coronary artery disease	12 072 (37.9)	8666 (40.4)	3406 (32.8)
Peripheral vascular disease	7365 (23.1)	5282 (24.6)	2083 (20.1)
Cerebrovascular disease	4248 (13.3)	3000 (14.0)	1248 (12.0)
Diabetes mellitus	16 569 (52.0)	11 591 (54.0)	4978 (47.9)
<i>BMI category at first dialysis</i>			
Underweight (<18.5 kg/m ²)	769 (2.5)	508 (2.4)	261 (2.6)
Normal (18.5–24.9 kg/m ²)	8684 (28.0)	5505 (26.4)	3179 (31.2)
Overweight (25–29.9 kg/m ²)	9728 (31.4)	6161 (29.6)	3567 (35.0)
Obese (>30 kg/m ²)	11 834 (38.2)	8655 (41.6)	3179 (31.2)
<i>Smoking status at first dialysis</i>			
Never	14 582 (46.3)	9663 (45.6)	4919 (47.7)
Former	12 853 (40.8)	8709 (41.1)	4144 (40.1)
Current	4079 (12.9)	2818 (13.3)	1261 (12.2)
History of cancer (excluding non-melanoma skin cancer)	3494 (10.9)	2637 (12.2)	857 (8.2)
Erythropoiesis stimulating agent use	25 106 (81.1)	17 878 (86.3)	7228 (70.7)
<i>Baseline serum biochemistry/haematology (taken >30 days after first dialysis)</i>			
Phosphate – mmol/L	1.64 [1.33–2.00]	1.63 [1.31–2.02]	1.65 [1.38–1.99]
Calcium – mmol/L	2.24 [2.11–2.36]	2.24 [2.12–2.36]	2.23 [2.10–2.35]
Haemoglobin – g/L	110 [100–120]	109 [98–119]	112 [102–122]
Ferritin – µg/L	303 [155–521]	323 [170–544]	260 [129–467.5]
Transferrin saturation – %	23 [17–30]	22 [16–30]	23 [18–30]

Note: Mean ± standard deviation, median [interquartile range] or number (%).

Abbreviations: BMI, body mass index; SEIFA, Socio-Economic Indexes for Areas 2011.²³

TABLE 2 Fully adjusted hazard ratios of all-cause mortality by categorical serum phosphate and dialysis modality

	Overall	Haemodialysis	Peritoneal dialysis
No. of deaths (all causes)	12 713	9784	2929
Person years at risk	97 122	72 824	24 298
Rate per 1000 person-years (95% CI)	130.9 (128.6–133.2)	134.3 (131.7–137.1)	120.5 (116.3–125.0)
<i>Adjusted HR for serum phosphate (95% CI)</i>			
<1.00 mmol/L	1.56*** (1.44–1.69)	1.50*** (1.37–1.63)	1.92*** (1.55–2.38)
1.00–1.24 mmol/L	1.17*** (1.08–1.26)	1.13** (1.05–1.23)	1.29** (1.07–1.54)
1.25–1.49 mmol/L	0.98 (0.92–1.06)	0.97 (0.90–1.05)	1.03 (0.86–1.22)
1.50–1.74 mmol/L	Reference	Reference	Reference
1.75–1.99 mmol/L	1.06 (0.98–1.14)	1.04 (0.96–1.14)	1.07 (0.90–1.28)
2.00–2.24 mmol/L	1.16*** (1.07–1.26)	1.16** (1.06–1.28)	1.16 (0.96–1.40)
2.25–2.49 mmol/L	1.24*** (1.12–1.38)	1.20** (1.07–1.35)	1.43** (1.15–1.78)
2.50–2.74 mmol/L	1.67*** (1.50–1.87)	1.59*** (1.40–1.81)	2.05*** (1.62–2.59)
≥2.75 mmol/L	2.13*** (1.93–2.36)	2.04*** (1.82–2.29)	2.42*** (1.91–3.06)

Note: Hazard ratio (HR) estimated by Cox proportionate hazards model. Models are stratified by treatment centre and adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

** $p < .01$; *** $p < .001$.

dialysis was 61 years and 38% were female. The most common primary aetiology of kidney disease was diabetes (40%) and the initial dialysis modality was HD for 67% of patients.

3.1 | Follow up and outcomes

During a median follow up time of 5.1 years (IQR 2.6–8.5), and a total of 97 122 person-years at risk, there were 12 713 deaths, while 6086 patients were censored at the time of receiving a kidney transplant, and 194 were censored due to loss of follow up. Overall, the crude mortality rate was 130.9 deaths per 1000 patient years for all-cause mortality and 41.5 deaths per 1000 patient years for cardiovascular mortality (Table 2).

3.2 | Association between serum phosphate and mortality

The unadjusted, age-adjusted and fully adjusted Cox-regression models are shown in Figure 1. The unadjusted model showed a U-shaped association between serum phosphate and all-cause mortality. This pattern persisted after adjusting for age and other covariates, albeit the magnitude of the association between low serum phosphate and mortality was partially attenuated, while the association between high serum phosphate and mortality was accentuated, in the fully adjusted model. These associations were also seen when models were analysed by dialysis modality (Figure 1). There was a significant

interaction between serum phosphate <1.00 mmol/L and PD (HR 1.37, 95% CI 1.10–1.71, $p = .01$), but other categories of phosphate did not have a significant interaction (Table S4).

The HR estimates for the fully adjusted models of all-cause mortality are displayed in Table 2. Overall, serum phosphate categories between 1.25 and 1.99 mmol/L conferred the lowest adjusted hazard of death, although serum phosphate of 1.75–1.99 mmol/L showed a trend towards increased mortality (HR 1.06, 95% CI 0.98–1.14, $p = .15$). There were incremental increases in HR observed with higher and lower serum phosphate categories outside of this range, reaching a HR of 2.13 (95% CI 1.93–2.36, $p < .001$) for phosphate ≥ 2.75 mmol/L, and a HR of 1.56 (95% CI 1.44–1.69, $p < .001$) for phosphate <1.00 mmol/L.

3.3 | Association between serum phosphate and cardiovascular mortality

The relationship between serum phosphate and mortality subtypes, in fully-adjusted models, is shown in Figure 2. Similar to all-cause mortality, the HR for cardiovascular mortality increased with high and low levels of serum phosphate (Table 3). However, there was a much more pronounced correlation between with high levels of serum phosphate and cardiovascular mortality than low serum phosphate, resulting in a “J-shaped” rather than a “U-shaped” curve. This also appeared to be present when the models were restricted to HD and PD groups, however the confidence intervals were noticeably larger, especially for the PD group. There were no significant interactions between serum phosphate and dialysis modality for cardiovascular mortality (Table S4).

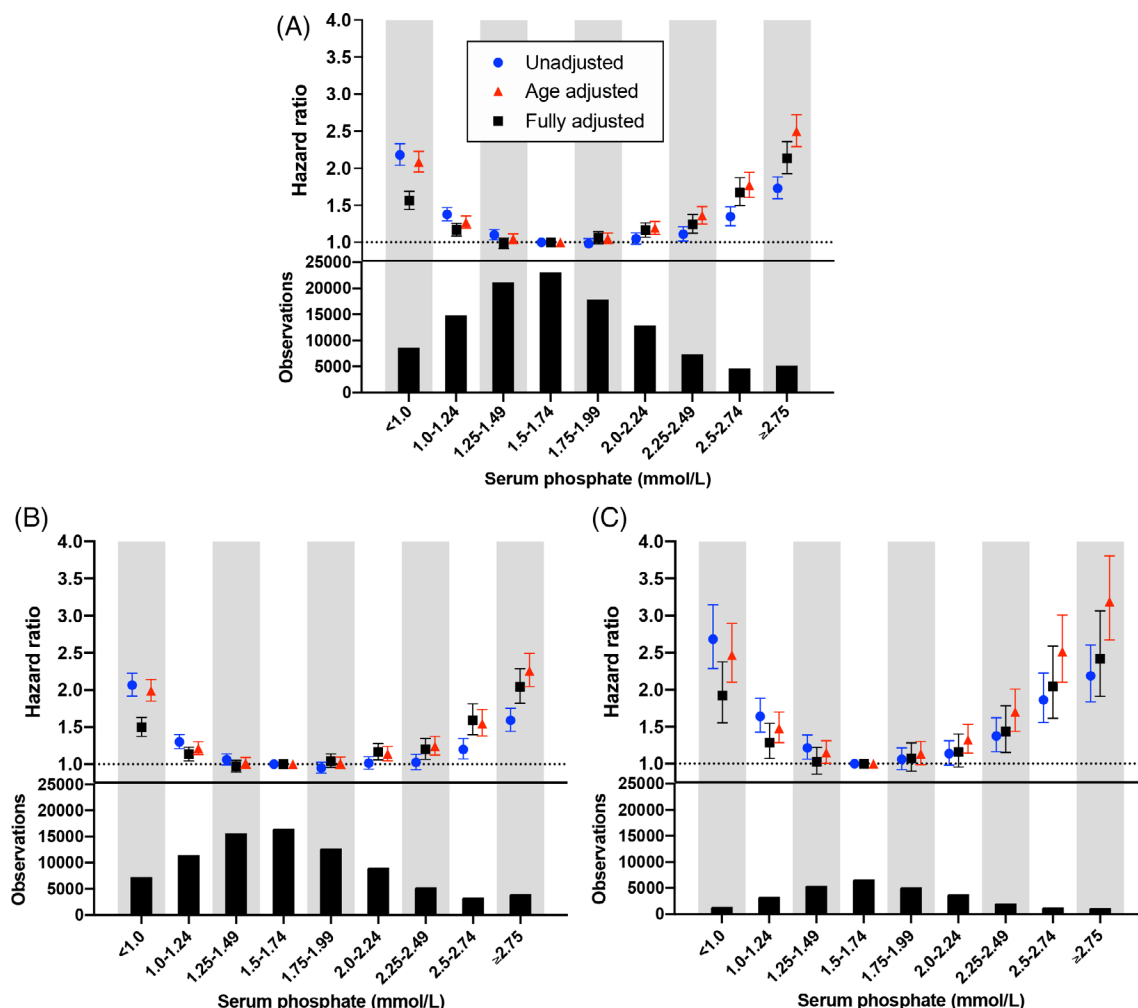


FIGURE 1 Unadjusted, age-adjusted and fully adjusted[†] models of association between serum phosphate and all-cause mortality in overall cohort (A), and in models restricted to patients on haemodialysis (B) and peritoneal dialysis (C). [†]Model adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation. "Observations" are the crude number of serum phosphate observations reported to ANZDATA during study period

3.4 | Sensitivity analyses

Overall rates of missing values for each variable were very low (Table S2), and imputation of missing values for the main fully adjusted model of all-cause mortality did not significantly change the pattern, or point estimates of hazard (not shown).

Models for baseline, and also for mean, values of serum phosphate and time-updated covariates are shown in Table S3. There were similar associations between serum phosphate and all-cause mortality across each model, but the time-dependent model ("main model") had the best overall fit as judged by AIC.

The model for baseline values of comorbidities (lung disease, coronary artery disease, peripheral vascular disease and cerebrovascular disease) had a marginally improved overall fit (as judged by AIC). However, there were only negligible differences in the point estimates of hazard, compared to the main model (Table S3).

Sub-hazard ratios for all-cause mortality from the fully-adjusted Fine and Gray model (with kidney transplant as a competing risk) are shown in Table S5. While cause-specific hazard ratios from the Cox proportional hazards model and sub-hazard ratios from the Fine and Gray model are not directly comparable,²⁴ in both models phosphate categories between 1.25 and 1.99 mmol/L were associated with the lowest risk, and each subsequent serum phosphate category above and below this range was associated with an incrementally higher risk.

4 | DISCUSSION

In this large, contemporary cohort of incident dialysis patients we reaffirm that both high and low levels of serum phosphate are independently associated with an increased hazard of all-cause mortality. While patients from Australia and New Zealand have been included in

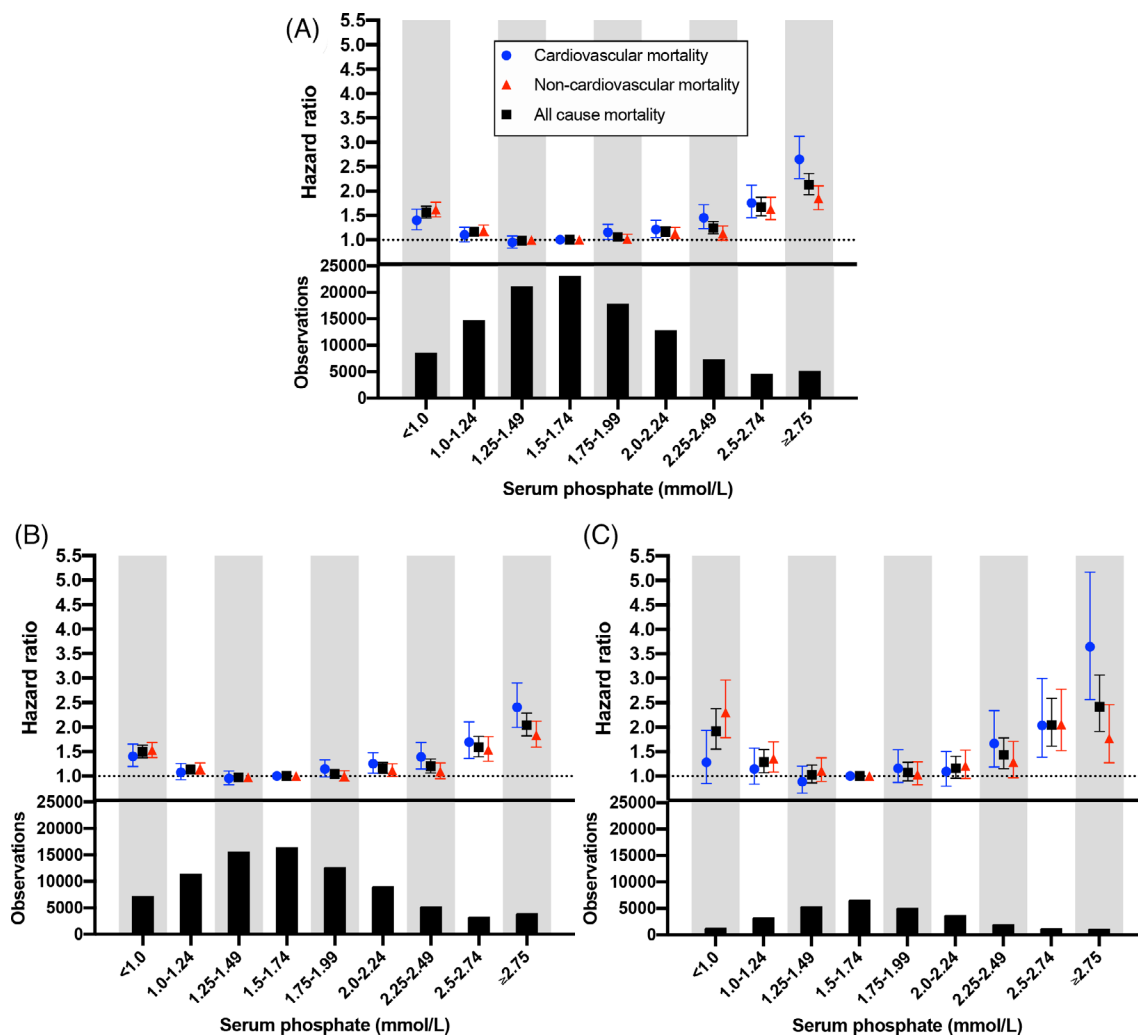


FIGURE 2 Fully adjusted[†] models of association between serum phosphate and cardiovascular, non-cardiovascular and all-cause mortality in overall cohort (A), and in models restricted to patients on haemodialysis (B) and peritoneal dialysis (C). [†]Model adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation. “Observations” are the crude number of serum phosphate observations reported to ANZDATA during study period

previous multi-national studies,²⁵ to our knowledge, this is the first time an analysis of serum phosphate and mortality has been reported from this region, and utilizing the ANZDATA Registry. Importantly, this work also contributes further outcome data for patients being treated with PD, who have previously been underrepresented in the literature.

After adjusting for significant covariates, high levels of serum phosphate had a positive association with all-cause mortality, with sequentially higher levels of serum phosphate conferring greater risk, in a dose-response fashion. In our analysis of mortality sub-type, we found that the association of high serum phosphate with cardiovascular mortality, was more pronounced than non-cardiovascular mortality. These observations have been consistently demonstrated, and support the notion that excess phosphate is directly (or indirectly) linked to cardiovascular disease

and mortality in the dialysis population.²⁶ Additionally, phosphate ostensibly induces vascular cell dysfunction²⁷ and calcification,²⁸ and drives elevated levels of parathyroid hormone and fibroblast growth factor-23, which themselves are associated with cardiovascular toxicity.²⁶ However, it remains debated whether phosphate is the direct mediator of these effects.^{13,29} Further, while current international clinical guidelines recommend lowering elevated serum phosphate towards the normal range, there is a notable lack of definite trial data to show that such therapeutic strategies improve patient-centred outcomes.⁵ Large interventional trials are currently underway, and will hopefully provide more clarity on this issue.^{14,15} Nevertheless, this study affirms that in the current era of dialysis care, elevated serum phosphate remains independently associated with increased risk of all-cause and cardiovascular death in both HD and PD patients.

TABLE 3 Fully adjusted hazard ratios of cardiovascular mortality by categorical serum phosphate and dialysis modality

	Overall	Haemodialysis	Peritoneal dialysis
No. of cardiovascular deaths	4030	3019	1011
Person-years at risk	97 122	72 824	24 298
Rate per 1000 person-years (95% CI)	41.5 (40.2–42.8)	41.5 (40.0–43.0)	41.6 (39.1–44.2)
<i>Adjusted HR for serum phosphate (95% CI)</i>			
<1.00 mmol/L	1.40*** (1.21–1.63)	1.40*** (1.19–1.65)	1.28 (0.85–1.94)
1.00–1.24 mmol/L	1.10 (0.96–1.26)	1.08 (0.93–1.26)	1.14 (0.83–1.57)
1.25–1.49 mmol/L	0.95 (0.83–1.08)	0.95 (0.82–1.10)	0.88 (0.65–1.20)
1.50–1.74 mmol/L	Reference	Reference	Reference
1.75–1.99 mmol/L	1.16* (1.01–1.32)	1.14 (0.98–1.33)	1.15 (0.87–1.54)
2.00–2.24 mmol/L	1.21** (1.05–1.40)	1.25** (1.06–1.48)	1.09 (0.79–1.50)
2.25–2.49 mmol/L	1.45*** (1.23–1.72)	1.39** (1.14–1.69)	1.67** (1.18–2.34)
2.50–2.74 mmol/L	1.76*** (1.45–2.12)	1.69*** (1.36–2.11)	2.04*** (1.38–3.00)
≥2.75 mmol/L	2.65*** (2.26–3.12)	2.41*** (1.99–2.90)	3.64*** (2.57–5.17)

Note: Hazard ratio (HR) estimated by Cox proportionate hazards model. Models are stratified by treatment centre and adjusted for age at commencement of dialysis, sex, ethnic origin, late referral to a nephrologist (commencement of dialysis within 90 days of referral), primary cause of kidney disease, smoking history, and history of cancer (excluding non-melanoma skin cancer), dialysis modality (for overall model), lung disease, coronary artery disease, peripheral vascular disease, cerebrovascular disease, diabetes mellitus, body mass index, uncorrected serum calcium, haemoglobin, serum ferritin and serum transferrin saturation.

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

* $p < .05$; ** $p < .01$; *** $p < .001$.

As has previously been described, we found that low levels of serum phosphate were also associated with increased risk of mortality.^{6–12} In contrast to the pattern observed between high levels of serum phosphate and subtypes of mortality, low levels of serum phosphate were more strongly associated with non-cardiovascular than cardiovascular death. Hypophosphataemia has been previously linked to a number of factors, including age,³⁰ infection,⁹ inflammation and malnutrition.^{10,31} These factors are all also important mortality risk factors in the dialysis population, and it is conceivable that serum phosphate may be a surrogate marker in these situations, rather than a direct mediator of pathology resulting in death. Whilst we were able to correct for body mass index in our analysis, ANZDATA does not collect other markers of inflammation or nutrition, such as serum albumin, limiting our ability to interrogate these effects further.

We found that serum phosphate <1.00 mmol/L was associated with higher risk of all-cause mortality in PD patients than HD patients. However, dialysis modality did not otherwise appear to have a widespread modifying effect, and associations between serum phosphate and all-cause and cardiovascular mortality were overall similar when models were restricted to subgroups of HD and PD patients. Previous studies have largely focussed on HD patients, which is not unexpected, given the relative predominance of HD as a treatment for kidney failure.³² Additionally, the regular contact with medical services and ease of obtaining regular blood tests, also make HD patients inherently suitable for large population-based studies. By contrast, studies examining phosphate that include patients receiving PD have been limited to smaller^{33–36} or older^{37–39} sets of data. There are a number of reasons why studies derived from HD cohorts may not be

directly applicable to PD patients. The different kinetics of phosphate removal with each modality mean that samples taken in routine clinical practice may not be comparable in terms of differences in total phosphate exposure. It has previously been observed that HD patients tend to have higher levels of serum phosphate than patients on PD.^{34,35} However, Evenepoel et al showed that despite this, PD patients may actually have less total phosphate clearance and higher overall exposure.⁴⁰ Evenepoel et al postulated that this discrepancy may be an artefact caused by the practice of measuring phosphate in HD patients immediately prior to dialysis, when serum levels are at their highest. With these considerations in mind it is reassuring to have further empirical evidence that serum phosphate levels in PD patients, as measured using routine and current collection methods, is a robust and independent marker of significant patient outcomes.

We entered serum phosphate as a time-dependent variable in our main models of all-cause and cardiovascular mortality. As with all epidemiological studies, it is important to consider whether this may have inadvertently introduced bias by misrepresenting biological effects.¹⁹ For example, it has been suggested that there may be a propensity for serum phosphate to progressively decrease in the immediate months preceding a significant event, such as hospitalization.¹⁰ In this scenario, a time-dependent Cox model would associate the most recent serum phosphate level with the event of interest, even if a patient previously had years of higher levels of serum phosphate - which ostensibly may have contributed to their eventual death through the accumulation of cardiovascular pathology. To assess for this effect, we refitted models using mean and baseline variables. These sensitivity analyses did not significantly change the associations between serum phosphate categories and mortality, suggesting that our

findings are robust. Other groups have recently suggested alternative modelling techniques, such as estimating the time that serum phosphate is above the reference range ("area under the curve").⁴¹ While this method showed strong association with cardiovascular mortality, by focussing on elevated serum phosphate this method may ignore the prognostic significance of low serum phosphate, which as shown in this study also imbues significant risk.

The strength of this study is the use of the ANZDATA Registry, which allows for analysis of up-to-date data from a large patient cohort, and with substantial numbers of both HD and PD patients. Consent for participation in ANZDATA is via an "opt-out" process, meaning that participation levels are high and risk of selection bias and loss to follow-up is minimal. These factors may improve the generalisability of our data to other populations of incident dialysis patients.

We acknowledge that our analysis has some limitations. Information about individual patients is restricted to the reporting fields collected as part of the ANZDATA Registry. This means that some potentially relevant covariates for this analysis, such as serum parathyroid hormone, serum albumin, as well as medications including phosphate binders, were not available. Some of these variables have previously been associated with mortality in dialysis patients,⁴ and while these associations have not been universal, it is possible that a lack of adjustment in this study may have resulted in an over-estimation of risk.

Another limitation of this study is that many of the clinical variables that were used, such as history of cardiovascular disease were categorical, and so it was not possible to further quantify the severity of these potential confounders. The nature of this registry analysis also means there may be variation in the collection and reporting methods of serum phosphate data. We do not have information about assays used by laboratories at different sites, and while ANZDATA provides instructions to standardize the timing of blood draws, it is possible that there is variation between, or even within sites. Further, we only have measures of serum phosphate on a yearly basis, which is less frequent than international clinical guideline recommendations.⁵ However, all of these limitations would be expected to bias our analysis towards the null, and arguably these conditions reflect contemporary, real world practice, which may improve the external applicability of our results. Finally, as an observational study, an inherent limitation is that causation between serum phosphate and mortality cannot be inferred, and the possibility of residual confounding must be considered.

5 | CONCLUSION

Our analysis shows that high and low levels of serum phosphate are associated with increased risk of mortality in patients with kidney failure being treated with dialysis. This association was independent of other covariates and of dialysis modality. In the current era of dialysis care, serum phosphate remains a relevant marker of risk of significant patient-centred outcomes.

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CONFLICT OF INTEREST

M.K.T., S.U., S.P.M., S.J.T. and N.M.L. declare no conflicts. N.D.T. reports honoraria, travel support, and research funding from Amgen, Shire and Sanofi.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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OPEN

Effect of nutritional calcium and phosphate loading on calciprotein particle kinetics in adults with normal and impaired kidney function

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Plasma approaches metastability with respect to its calcium and phosphate content, with only minor perturbations in ionic activity needed to sustain crystal growth once nucleated. Physiologically, calcium and phosphate are intermittently absorbed from the diet each day, yet plasma concentrations of these ions deviate minimally post-prandially. This implies the existence of a blood-borne mineral buffer system to sequester calcium phosphates and minimise the risk of deposition in the soft tissues. Calciprotein particles (CPP), endogenous mineral-protein colloids containing the plasma protein fetuin-A, may fulfill this function but definitive evidence linking dietary mineral loading with their formation is lacking. Here we demonstrate that CPP are formed as a normal physiological response to feeding in healthy adults and that this occurs despite minimal change in conventional serum mineral markers. Further, in individuals with Chronic Kidney Disease (CKD), in whom mineral handling is impaired, we show that both fasting and post-prandial levels of CPP precursors are markedly augmented and strongly inversely correlated with kidney function. This study highlights the important, but often neglected, contribution of colloidal biochemistry to mineral homeostasis and provides novel insight into the dysregulation of mineral metabolism in CKD.

Calcium and phosphate form highly insoluble salts in aqueous solution and while metabolism in all living organisms is dependent on these essential nutrients, their coexistence in biological fluids creates an inherent mineralisation risk and the need for tight regulation¹. Most extracellular fluids like plasma, are considered metastable or approaching metastability with respect to their calcium and phosphate ionic activities², readily sustaining crystal growth if nucleated. This tendency for calcium and phosphate to precipitate has been harnessed by vertebrates for its unique biomechanical properties during the evolution of the skeletal and dental tissues. However, at the same time, this mandated mechanisms of rapid mobilisation and efficient bulk transport of mineral precursors to meet the demands of growth and repair of these hard tissues, as well as strategies of restricting mineralisation to these sites and preventing unwanted calcification in soft tissues³. Indeed, even modest elevations in plasma calcium and phosphate concentrations are associated with increased risk of pathological calcification of the arteries and soft tissues⁴. Since excess calcium and phosphate are almost exclusively excreted by the kidney, this risk, along with its associated cardiovascular sequelae, is substantially higher in those with Chronic Kidney Disease (CKD), where disturbances in mineral metabolism become evident with relatively small decrements in glomerular filtration rate (GFR)^{5,6}. Given the abundance of calcium and phosphate in the diet to which mammals are intermittently exposed, one might suspect the risk of ectopic mineralisation to be highest following a meal due to the anticipated surge in mineral ions absorbed from the intestine. Yet, contrary to this expectation, plasma calcium and phosphate concentrations change minimally in the post-prandial period and while increased urinary excretion helps to maintain homeostasis⁷, this response is not instantaneous and lags by some hours after ingestion of millimolar amounts of calcium and phosphate⁸. As a solution to this physiological challenge,

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a blood-borne system for buffering and transporting mineral has long been suspected⁹, and recent attention has turned to colloidal mineral-protein complexes that could fulfill this function and safely chaperone the intermittent flux of mineral through extracellular fluid to sites of utilisation or disposal¹⁰.

In plasma, the most potent and abundant protein mineralisation regulator is the liver-derived glycoprotein fetuin-A¹¹, which interacts with nascent colloidal mineral-protein complexes to form calciprotein particles (CPP)^{12,13}. Analogous to the manner in which apolipoproteins solubilise their lipid cargo for transport, fetuin-A stabilises poorly soluble calcium phosphates, preventing crystal growth and precipitation, while facilitating their uptake in tissues for utilisation or clearance^{14–16}. An inability to make or sufficiently stabilise CPP, as seen in fetuin-A knockout mice, results in one of the most severe phenotypes of ectopic calcification known¹⁷, where mineral-containing complexes precipitate directly in the lumen of the microvasculature leading to occlusion, ischaemia, necrosis and fibrosis¹⁸. CPP are generated through a series of ordered steps, initially with the binding of spontaneously formed clusters of calcium and phosphate ions to fetuin-A, forming calciprotein monomers (CPM)¹⁹, which then serve as the building blocks for larger polymeric structures; first, coalescing to form spherical primary CPP (CPP-I) containing amorphous calcium phosphate, before transforming into larger and denser ellipsoidal secondary CPP (CPP-II) containing crystalline hydroxyapatite¹².

Beyond the purported physiological role of CPP formation in sequestering and dispersing excess mineral, elevated levels are also observed in states of impaired mineral metabolism, including in CKD²⁰. In this setting, higher serum levels of CPM and CPP have been linked to an increased risk of cardiovascular events^{21–25} and mortality²². Pre-clinical studies suggest that CPP may directly induce vascular smooth muscle cell calcification^{26,27}, activation of cellular inflammatory and cytotoxic pathways^{28,29}, as well as vascular luminal and endothelial lesions³⁰. Taken together, it has been proposed that prolonged exposure to chronically elevated levels of serum CPP may help explain the links between excess dietary mineral and poor patient outcomes in CKD^{14,31}. Intriguingly, recent data also suggests that circulating CPM are filtered at the glomerulus²⁹, implying that kidney impairment may affect CPP metabolism via multiple mechanisms.

Recent methodological advancements permit direct quantification of CPM³², CPP-I and CPP-II³³, with these assays now applied across a number of observational and interventional clinical studies^{24,25,34–39}. Another novel complementary method for assessing this system is the T50 test, a functional assessment of the capacity of serum to resist ex vivo formation of CPP-II, when challenged with supersaturating amounts of calcium and phosphate⁴⁰. A lower T50 has been consistently associated with increased risk of vascular pathology and mortality in individuals with normal kidney function⁴¹, as well as in various CKD cohorts, including both non-dialysis^{22,42,43}, and also dialysis-dependent CKD⁴⁴. In addition to the kinetics of CPP-II formation, the size (hydrodynamic radius) of the CPP-II molecules generated in the T50 assay can also be measured^{40,45}, and may provide additional prognostic information about vascular risk^{46,47}.

The notion of a dietary origin of CPP is strongly supported by the observation that high-phosphate feeding is associated with increased ambient levels of CPM⁴⁸ and CPP³³ in animal studies, and the reduction in serum CPM³⁴ and CPP³⁷ in haemodialysis patients treated with intestinal phosphate binders. However, definitive observation of the effect of nutritional mineral intake on acute CPP kinetics in humans is lacking. Furthermore, the impact of CKD on post-prandial CPP metabolism has yet to be documented. To address these key evidence gaps, we conducted a controlled study of the effect of standardised food intake on serum CPM, CPP-I, CPP-II, T50 and CPP-II size, in fasted adults with normal or impaired kidney function.

Methodology

Study population. We studied 14 individuals with CKD and 16 age- and gender-matched healthy controls. Each participant had to be at least 18 years of age to be eligible. Participants were excluded if they: (i) had a history of mineral or bone disease, other than related to CKD; (ii) were being treated with an intestinal phosphate binder or calcitriol; or (iii) had a gastrointestinal disorder, history of lactose intolerance or were unwilling to consume the study meal. For the CKD group, we recruited seven individuals with an estimated glomerular filtration rate (eGFR) between 30 and 60 mL/min/1.73 m² and seven with an eGFR < 30 mL/min/1.73 m², excluding participants who required dialysis or with a previous kidney transplant. Healthy controls had no history of chronic medical conditions and had normal kidney function (eGFR > 60 mL/min/1.73 m²). The study was conducted in accordance with the Declaration of Helsinki.

All individuals provided written informed consent, and the study was approved by the local ethics committee (Melbourne Health Human Research Ethics Committee MH2018.363).

Procedure. Each participant was studied after an overnight fast and sample collection commenced between 7.30 and 9.30 am. An intravenous cannula was inserted at the start of the study period. Before each blood sample was collected, an initial 5 mL draw from the cannula was discarded. Two initial fasting blood samples were taken 30 min apart to account for baseline variation. The mean values of these two timepoints were used as “time 0”. Immediately after collection of the second fasting sample, participants consumed a standardised meal (Sanitarium Up&Go liquid breakfast; 250 mL, vanilla flavour) containing 815 kJ energy, 300 mg calcium and 188 mg phosphate (Table 1). Participants were instructed to consume the entirety of the drink within 5 min. Serial blood samples were collected at five post-prandial timepoints (30, 60, 120, 180 and 240 min) from the commencement of the meal. During the study period participants were allowed to drink water but were not allowed to consume any other food or drink.

Outcome measures. Blood was collected for repeated measurement of novel markers of mineral metabolism (CPM, CPP-I, CPP-II, T50 and CPP-II size) at each timepoint. Fetuin-A was measured at each timepoint, given its role as the principal mineral-binding protein present in CPM and CPP. We also measured serum phos-

Parameter	Contents of meal ⁷¹	Recommended dietary intake ^a
Energy (kJ)	815	–
Protein (g)	8.3	13%
Fat (g)	4.3	–
Carbohydrate (g)	28.4	–
Sodium (mg)	158	17–34%
Potassium (mg)	450	45%
Calcium (mg)	300	30%
Phosphate (mg)	188	19%

Table 1. Nutritional information for standardised meal (Sanitarium Up&Go liquid breakfast; 250 mL, vanilla flavour). ^aPercentage of recommended dietary intake based on Australian adult male⁷² (where generalised recommendations are available).

phate, total calcium, magnesium, albumin and bicarbonate at each timepoint, and serum intact parathyroid hormone (PTH) and intact fibroblast growth factor-23 (iFGF23) at three timepoints (0, 120 and 240 min). Serum citrate was measured at 0, 30 and 60 min. Serum urea, creatinine and 1,25 dihydroxyvitamin D were measured once at fasting baseline (0 min). Blood samples for novel markers of mineral metabolism, PTH, iFGF23, 1,25 dihydroxyvitamin D and serum citrate, were allowed to clot for 30 min before centrifugation, and then serum aliquots were stored at -80°C until batch-analysis. All other biochemical measurements were performed at the time of sample collection using standard laboratory methods.

Gel-filtration and flow cytometric assays for CPM and CPP. We employed two complementary assays to quantitate different fractions of the circulating CPP pool⁴⁹. Both assays use the fluorescently-labelled bisphosphonate derivative, OsteoSense 680EX (Perkin Elmer), which binds specifically to solid-phase calcium phosphate and preferentially to crystalline phases (e.g. hydroxyapatite). The ‘gel-filtration’ method of Miura et al.³² was used to measure small (<50 nm diameter), low-density mineral-laden fetuin-A colloids. Briefly, frozen serum samples were thawed for 24 h at 25°C to induce aggregation of CPM and phase transition to crystalline calcium phosphate. Samples were then centrifuged for 30,000g for 2 h at 4°C to remove larger CPP-I and CPP-II, leaving less dense crystal-laden fetuin-A monomer and multimers for staining with OsteoSense (0.5 μM) in HEPES-buffered DMEM (pH 8.0). Unbound dye was subsequently removed by gel filtration (Micro Bio-Spin Columns with Bio-Gel P-30, Bio-Rad) and the resultant fluorescence measured using an infrared scanner (Odyssey CLx, LI-COR; EX 685 nm, EM 700 nm). Miura and colleagues³² referred to the mineral detected as low-density (L)-CPP, however, here we refer to them as CPM to reflect the origin of this mineral fraction in vivo. In our hands, the mean interassay analytical coefficient of variation (CV_A) for the CPM assay was 4.9%.

For flow cytometric analysis, aliquots of frozen serum were processed using the standardised procedures described previously⁵⁰. CPP-I and CPP-II were measured as previously described using a BD FACSVerser flow cytometer setup to resolve particles < 200 nm from background and operating with fluorescence triggering on OsteoSense-positive events^{33,37}. In this assay, CPP are distinguished from membrane-delimited mineral-containing extracellular vesicles using phosphatidyl serine-binding lactadherin-FITC (Haematologic Technologies Inc., Essex Junction, VT). CPP-I and CPP-II were distinguished by differences in side scatter (SSC) intensity (related to particle size) and OsteoSense fluorescence intensity. Interassay CV_A for CPP-I and CPP-II were < 15% and < 10%, respectively.

Other mineral markers. Serum T50 was measured by Calciscion AG, Biel, Switzerland, as previously described using a Nephelostar nephelometer (BMG Labtech, Ortenberg, Germany)⁴⁰. The mean interassay CV_A for T50 was 3.4%. CPP-II hydrodynamic radius was measured by dynamic light scattering using a DynaPro Plate Reader II (Wyatt Technology, Santa Barbara, CA, USA) as described by Chen et al.⁴⁷ The interassay CV_A for CPP-II size was 4%. Commercial immunoassays were used to measure iFGF23 (Kainos Laboratories, Tokyo, Japan), 1,25 dihydroxyvitamin D (Immunodiagnostic Systems, Boldon, UK), and fetuin-A (R&D Systems, Minneapolis, USA) according to the manufacturer’s instructions. Mean interassay CV_A were 3.8%, 5.5%, and 3.2%, respectively. Serum citrate was measured using a colorimetric assay (Sigma-Aldrich, Darmstadt, Germany) with a mean interassay CV_A of 3.5%.

Statistical analysis. Using GLIMMPSE, a validated linear mixed model power and sample size calculator⁵¹, we estimated that 10 participants would provide > 90% power to detect a doubling of CPP in the post-prandial period in with a type I error rate of 0.05. The study was not powered to detect a difference between groups.

Demographic and fasting biochemical data were compared between groups using unpaired t-test or Kruskal–Wallis test for normal and skewed continuous variables respectively, and chi-squared test for categorical variables.

Our aim was to describe the within and between-group post-prandial response for each repeated parameter. In order to do this, we fitted linear mixed-effects models (LMM) for each parameter, using a restricted maximum likelihood approach and with an unstructured covariance matrix⁵². For each LMM we modelled group, categorical time and group-by-time interaction as fixed effects and a random intercept was included for each participant

	Healthy control (n = 16)	CKD (n = 14)	p-value
Participant characteristics			
Age (years)	44.1 ± 13.5	44.3 ± 16.0	0.917
Female sex, n (%)	9 (56.3)	8 (57.1)	0.961
CKD stage, n (%)			
Stage 3a or stage 3b	–	7 (50.0)	
Stage 4 or stage 5 (non-dialysis)	–	7 (50.0)	
Primary cause of kidney disease, n (%)			
Diabetic nephropathy	–	2 (14.3)	
Glomerulonephritis	–	6 (42.9)	
Reflux nephropathy	–	2 (14.3)	
Polycystic kidney disease	–	4 (28.6)	
Fasting biochemical parameters			
Creatinine (μmol/L)	71.6 ± 9.9	239 ± 110.3	0.001
eGFR ^a (mL/min/1.73 m ²)	99.8 ± 10.2	29.2 ± 14.5	<0.001
Urea (mmol/L)	4.8 ± 1.4	15.2 ± 7.4	<0.001
Calcium (mmol/L)	2.29 ± 0.08	2.25 ± 0.10	0.262
Phosphate (mmol/L)	0.96 ± 0.14	1.17 ± 0.24	0.006
Magnesium (mmol/L)	0.84 ± 0.06	0.80 ± 0.08	0.132
Albumin (g/L)	40 ± 2.0	36 ± 2.9	<0.001
Bicarbonate (mmol/L)	27 ± 2.6	22 ± 3.0	<0.001
PTH (pmol/L)	5.2 (4.6–6.75)	16.9 (7.1–31.0)	0.001
1,25-dihydroxyvitamin D (pmol/L)	137 (103–173)	42 (31–56)	<0.001
Intact FGF23 (pg/mL)	48.8 (32.9–68.8)	132.5 (75.1–238.9)	0.002

Table 2. Participant demographics and clinical characteristics. P-value is for between group difference, examined using unpaired t-test or Kruskal–Wallis test for normal and skewed continuous variables respectively, and chi-squared test for categorical variables. Mean ± SD, number (percentage) or median (interquartile range). CKD chronic kidney disease, eGFR estimated glomerular filtration rate, FGF23 fibroblast growth factor-23, PTH intact parathyroid hormone. ^aeGFR—calculated using CKD—Epidemiology Collaboration equation.

to account for correlation of repeated measures. The control group and time ‘0’ were used as the reference values for group and time, respectively. Coefficient estimates for group-by-time interaction terms were used to test for differences in post-prandial response between the CKD and control group. After fitting each LMM, we also performed post hoc pairwise comparisons to test for differences in mean values between groups at each time-point, and to test for deviation from the fasting baseline within each group. For these pairwise comparisons we used Bonferroni correction method to adjust for multiple comparisons. CPM, CPP-I, CPP-II, CPP-II size, PTH and iFGF23 were natural log transformed before fitting LMMs to ensure normal distribution of residuals. For ease of interpretation, coefficient estimates for interaction terms from these models were then exponentiated to derive estimates of percentage change. For the LMM of CPM, group by time interaction terms suggested a significant difference in post-prandial response between groups. To further explore the effect of kidney function on post-prandial levels of CPM we also calculated the area under the curve (AUC) for CPM using the cubic spline method (time 0 to 240 min), and examined the relationship between eGFR, AUC and maximum concentration of CPM (using Spearman rank correlation) as well as between CKD and time of maximum CPM concentration (using chi squared test).

Several samples had undetectable levels of CPP-I (2 samples in the control group, and 6 in the CKD group) or CPP-II (5 in the control group and 10 in the CKD group). For the main analyses, the lower limit of quantification for the assay (133 particles/mL) was used for these left-censored values. To assess for potential bias from this approach, we performed a sensitivity analysis where LMMs for CPP-I and CPP-II were refitted after imputing left-censored values using multi-level Tobit regression, where time and group were entered as independent variables⁵³.

Two-tailed p values < 0.05 were considered significant. All data were analysed using Stata MP version 17.0 (StataCorp, College Station, USA) and figures were produced using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, USA).

Results

Participant demographics and clinical characteristics. The demographics and participant characteristics for each group are displayed in Table 2. Overall, the groups were well matched for age and sex. The mean eGFR within the CKD group was 29.2 mL/min/1.73 m². Consistent with group allocation, participants within the CKD group had higher fasting levels of serum creatinine, urea, phosphate, PTH and iFGF23, and lower fasting eGFR, serum albumin, bicarbonate and 1,25-dihydroxyvitamin D. Fasting serum total calcium and magnesium were comparable between groups. Among the 14 CKD participants, one was hyperphosphataemic

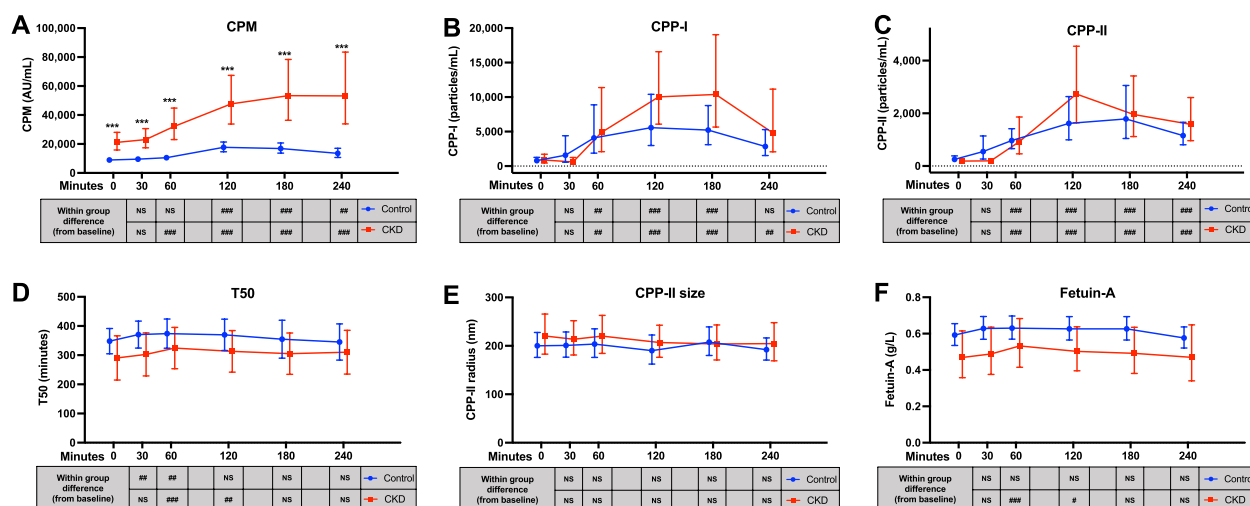


Figure 1. Post-prandial response of novel markers of mineral metabolism after standardised meal in participants with normal and impaired kidney function. Repeated measures of novel markers of mineral metabolism depicted for control and CKD group when fasting (“0”) and then post consumption of a standardised meal. Data are presented as mean and 95% confidence interval or geometric mean and 95% confidence interval. Data points have been offset for clarity. Between-group and within-group pairwise comparisons shown for each timepoint, with Bonferroni correction for multiple comparisons. **(A)** Calciprotein monomers (CPM); **(B)** primary calciprotein particles (CPP-I); **(C)** secondary calciprotein particles (CPP-II); **(D)** T50; **(E)** secondary calciprotein particle size (CPP-II size); **(F)** Fetuin-A. *** $p < 0.001$ for CKD group versus healthy controls. NS: $p \geq 0.05$ versus group baseline; ##: $p < 0.01$ versus group baseline; ###: $p < 0.001$ versus group baseline.

(> 1.50 mmol/L) and 10 (71%) were hyperparathyroid (> 10.0 pmol/L), while all participants in the control group had values within the respective population-based reference intervals for these two parameters. Medication use for the CKD group is shown in Supplementary Table S1.

Novel markers of mineral metabolism. Levels of novel markers of mineral metabolism at fasting and after the standardised meal for each group are depicted in Fig. 1. Summary data and group-by-time coefficient estimates are provided in Supplementary Table S2.

CPM. Serum CPM was significantly higher in CKD participants than in controls when fasting, and at each post-prandial timepoint (Fig. 1A). A significant increase in serum CPM was observed post meal within each group. This increase began at 120 min post meal in the control group and 60 min post meal in the CKD group. Levels of CPM remained above fasting levels in both groups at the end of the study period (240 min).

Group-by-time interaction coefficients showed significant between-group differences for CPM at multiple timepoints, indicating that the post-prandial increase in CPM was proportionately larger in the CKD group than in controls (Supplementary Table S2). The peak difference between groups was seen at 240 min, where the increase from fasting levels was 73.3% higher in the CKD group than in controls (95% CI 30.8, 129.6, $p < 0.001$). To further explore the apparent effect of kidney function in post-prandial profile, CPM AUC, maximum concentration, and time of maximum concentration were also examined (Supplementary Fig. S1). There was strong, inverse correlation between eGFR and CPM AUC ($r = -0.806$, $p < 0.001$) and maximum CPM concentration ($r = -0.779$, $p < 0.001$). In addition, the time of maximum concentration for CPM occurred significantly later in the CKD group compared to controls.

CPP-I and CPP-II. Significant post-prandial excursions were observed within both groups for CPP-I and for CPP-II (Fig. 1B,C). For CPP-I, serum levels were significantly increased from fasting baseline in both groups at 60 min. At the end of the observation period, CPP-I remained significantly higher than fasting values in the CKD group but were comparable to baseline in the control group. For CPP-II, significant post-prandial increases were evident in both groups at 60 min, and levels remained above fasting values at the end of the study period.

There was no significant difference between group means in pairwise comparisons at any timepoint for CPP-I or CPP-II (Fig. 1B,C). However, there was a significant group-by-time interaction for CPP-II at 120 min (+130.1% for the CKD group [95% CI 4.1, 408.6], $p = 0.039$) (Supplementary Table S2). Refitting LMMs for CPP-I and CPP-II after re-imputing left censored values using Tobit regression did not materially alter coefficient estimates, or post hoc between group or within group pairwise comparisons (Supplementary Table S3 and S4). In contrast to CPM, we found no association between eGFR and the AUC, maximal concentration or time to maximum concentration for serum CPP-I or CPP-II (Supplementary Fig. S2).

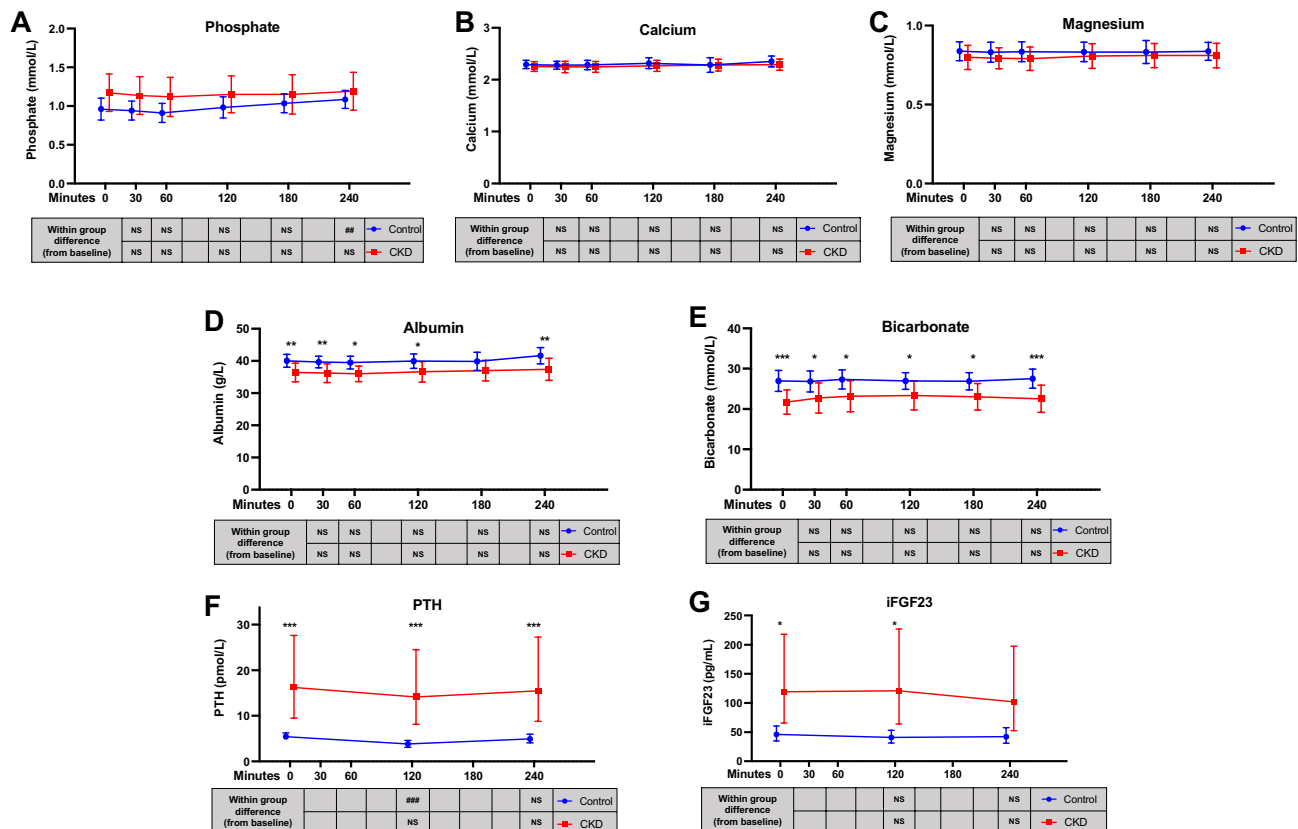


Figure 2. Post-prandial response of conventional markers of mineral metabolism after standardised meal in participants with normal and impaired kidney function. Repeated measures depicted for control and CKD group when fasting (“0”) and then post consumption of a standardised meal. Data are presented as mean and 95% confidence interval or geometric mean and 95% confidence interval. Data points have been offset for clarity. Between-group and within-group pairwise comparisons shown for each timepoint, with Bonferroni correction for multiple comparisons. (A) Phosphate; (B) calcium; (C) magnesium; (D) albumin; (E) bicarbonate; (F) intact parathyroid hormone (PTH); (G) intact fibroblast growth factor-23 (iFGF23). * $p < 0.05$ for CKD group versus healthy controls; ** $p < 0.01$ for CKD group versus healthy controls; *** $p < 0.001$ for CKD group versus healthy controls. NS: $p \geq 0.05$ versus group baseline; #: $p < 0.01$ versus group baseline; ###: $p < 0.001$ versus group baseline.

T50, CPP-II size, fetuin-A and citrate. In both control and CKD groups, early but transient within-group increases in T50 from fasting levels were observed (Fig. 1D). This was evident at 30 and 60 min in the control group and at 60 and 120 min in the CKD group. There were no between-group pairwise differences, or significant group-by-time interactions for T50 at any timepoint. Within each group, CPP-II size appeared stable over the post-prandial period, with no significant excursions from baseline values in either group (Fig. 1E). There were no significant pairwise differences between groups at any timepoint, however at 180 min there was a modest, but significant, group-by-time interaction (-10.9% for CKD group [95% CI $-19.5, -1.3$]; $p = 0.027$). For serum fetuin-A, there were no significant pairwise group differences, or group by time interactions (Fig. 1F). However, in the CKD group, there was a transient post-prandial increase in fetuin-A, which was coincident with the increase in T50. In further ad hoc exploratory analysis of all participants, a strong correlation between deviation in T50 and fetuin-A from baseline was evident, irrespective of renal function ($r = 0.839$, $p < 0.001$; Supplementary Fig. S2). Since the study meal was noted to contain citrate, a known inhibitor of CPP formation and modulator of T50⁵⁴, we measured serum citrate concentrations in available stored serum at baseline, 30, and 60 min. Although a small increase in serum citrate was observed in the CKD group at 30 min (Supplementary Table S3), this did not coincide with the observed changes in T50 in this group, nor was there any overall correlation between change in T50 and serum citrate (Supplementary Fig. S3).

Conventional biochemical markers. Fasting and post-prandial levels of conventional biochemical markers are depicted in Fig. 2, and summary data and group-by-time coefficient estimates are also shown in Supplementary Table S4 and Supplementary Table S5.

There was a trend for serum phosphate to increase from fasting levels post meal in the control group, which reached significance at 240 min (Fig. 2A). By comparison, no within group post-prandial change was observed in the CKD group. This subtle difference in post-prandial response between groups was reflected in significant group-by-time interactions at 180 min (-0.09 mmol/L for CKD group [95% CI $-0.17, -0.02$], $p = 0.018$) and at

240 min (-0.10 mmol/L [95% CI $-0.19, -0.02$], $p=0.019$). For serum calcium and magnesium, there were no significant between-group or within-group differences, nor significant group-by-time interactions (Fig. 2B,C, Supplementary Table S4). Serum albumin was lower in the CKD group compared to controls at all but one timepoint and remained unchanged from baseline levels in both groups post-prandially (Fig. 2D). Serum bicarbonate was higher in control than in CKD participants at all timepoints (Fig. 2E). There was no significant deviation from baseline in either group, however there was a significant group-by-time interaction at 120 min ($+1.64$ mmol/L for CKD group [95% CI $0.25, 3.04$], $p=0.021$). This coincided with the T50 peak in the CKD group, and in ad hoc analysis, there was correlation between the deviation in T50 and bicarbonate from baseline (Supplementary Fig. S4), albeit this was weak compared to the apparent interdependence of T50 and fetuin-A (Supplementary Fig. S2).

With respect to the major hormonal mineral regulators, serum PTH was significantly higher in the CKD group than in controls at all measured timepoints (Fig. 2F). There was a transient drop in PTH from fasting to 120 min in the control group, which was not seen in the CKD group. This corresponded to a significant group-by-time interaction at 120 min ($+25.2\%$ for CKD group [95% $5.5, 48.5$], $p=0.01$). Serum iFGF23 was significantly higher in the CKD than control group at baseline and 120 min, but not at 240 min (Fig. 2G). No significant post-prandial within group changes were observed.

Discussion

To the best of our knowledge, this is the first study to report a significant post-prandial effect of nutritional intake on serum levels of CPM, CPP-I and CPP-II in humans. These effects were common to individuals with normal and impaired kidney function; however, the post-prandial excursion of serum CPM was much more pronounced in CKD participants. We also found an early and transient post-prandial effect on T50, which was present regardless of kidney function, and accompanied by a concomitant increase in serum fetuin-A.

Our findings are consistent with the notion that intestinal absorption of a dietary mineral load can directly lead to formation of circulating CPM, CPP-I and CPP-II. This has previously been proposed^{10,15,16}, but based largely on animal data^{33,48}. In contrast, evidence in humans has been indirect, coming from studies of intestinal phosphate binders in haemodialysis dependent CKD patients^{34,37,55}. A small study by Yamada et al. suggested diurnal variation in serum CPP with post-prandial peaks, however this study used an older assay technique that was unable to separately quantify CPM and CPP sub-species, and participants were all hospitalised for management of unstable diabetes⁵⁶. In contrast, all participants in this study were clinically stable, and as far as we are aware, for the first time we have demonstrated that post-prandial excursions are seen not only in individuals with CKD, but also in healthy adults, substantiating a role of CPM and CPP formation in the normal physiological response to the ingestion of food.

Surges of serum CPM and CPP were seen even after relatively modest, and physiologically relevant, nutritional mineral loads (Table 1). In contrast, there was minimal post-prandial variation in the more conventional markers of mineral metabolism. Previous studies have similarly shown limited post-prandial deviation in phosphate^{7,57}, except when subjects are challenged with large pharmacological loads^{58,59}. In health, total body phosphate is regulated, such that net intestinal absorption is matched by urinary excretion⁸. However, this response is not instantaneous, and a lag of several hours may be seen before augmentation of urinary phosphate excretion occurs, even when the phosphate load is given intravenously⁸. Instead, animal models have demonstrated that other, non-renal, mechanisms serve to maintain serum ionic concentrations more acutely, via distribution to bone and other tissues^{59–61}. It is plausible that formation of CPM and CPP may be an important additional temporary depot of phosphate (and calcium), that is able to acutely buffer local mineral loads, such as from the gastrointestinal system¹⁴. The physiochemical properties of CPM and CPP mean that potentially large quantities of otherwise insoluble mineral can exist in the circulation without risk of precipitation, which ostensibly facilitates mineral to be safely transported in bulk to sites of use or clearance.

We observed a transient increase in serum fetuin-A in the CKD group. In contrast, fetuin-A appeared stable in controls, however, when pairwise comparisons were repeated without correction for multiple comparisons, there were significant increases at 30 and 60 min in controls (Supplementary Table S8), indicating that we may have been underpowered to detect an underlying effect. Beyond being a negative acute phase reactant, with levels strongly suppressed in response to acute and chronic inflammation⁶², little is known about other mechanisms that directly regulate the hepatic synthesis and secretion of fetuin-A, and there is a paucity of previous data about diurnal, or acute post-prandial variation in any species. Given the observed rise in serum fetuin-A after feeding, especially in the CKD group, and the requisite role of fetuin-A in CPM and CPP formation, it is intriguing to consider whether feeding may be “sensed” via a yet unknown mechanism, leading to hepatic release of fetuin-A to coincide with an influx of mineral from the intestines. Indeed, Uedono et al. recently suggested that CPP itself, may be a trigger for fetuin-A expression in cultured hepatocytes⁶³. Thus, mechanisms controlling fetuin-A release and their response to mineral loading warrant further investigation.

T50 is a functional assessment of the serum's ability to resist ex vivo CPP-II formation, representing a composite of various potentiating (including calcium and phosphate) and inhibiting (including fetuin-A, albumin, magnesium, citrate and bicarbonate) calcification factors⁴⁰. We observed an early but transient increase in T50. This was seen in both groups, but in the CKD group, the peak was coincident with increases in serum fetuin-A. Further, among all participants, change in T50 from baseline was very closely correlated with change in serum fetuin-A (Supplementary Fig. S2), suggesting that fetuin-A may be the main factor underlying the observed changes in T50. Indeed, we did not find corresponding early post-prandial changes in other known modulators of T50 (serum phosphate, calcium, bicarbonate, magnesium, or albumin), although this does not exclude more subtle changes in one, or a combination, of these factors. For instance, a positive group-by-time interaction was present for serum bicarbonate in the CKD group at 120 min, which overlapped with the increase in T50. As

for fetuin-A, there was also correlation between change in bicarbonate and T50 from baseline (Supplementary Fig. S4), albeit this was relatively weak compared to the correlation with the former ($r = 0.385$ vs. $r = 0.839$). Nevertheless, the post-prandial “alkaline tide” is a recognised phenomenon, and it is plausible that multiple factors contributed to the observed post-prandial changes in T50⁶⁴. The study meal also contained a surprising amount of citrate (~16 mM), a known potent inhibitor of CPP formation, however, although there was a small increase in serum citrate in the CKD group, this change did not coincide with the observed changes in T50, nor was there any overall correlation between change in T50 and serum citrate across both groups. Other previously described modulators of T50, such as pyrophosphate and zinc, were not measured in this study. Although it is possible that these, or other yet unknown, factors may also have contributed to the observed post-prandial increase in T50, we note that quite substantial changes in concentration of these small inorganic molecules are generally required to significantly impact T50⁶⁵. In contrast to the post-prandial effects on T50, the hydrodynamic radius of CPP-II remained ostensibly unchanged in both groups.

While post-prandial excursions of CPM were clearly observed in both groups, CPM levels were consistently higher in the CKD group at each of the timepoints, and group-by-time interaction terms indicated that the magnitude of the post-prandial response was significantly larger in CKD participants than in controls. Accordingly, in exploratory analysis, there was strong correlation between eGFR and CPM AUC and maximum concentration. Peak levels of CPM also tended to occur later in the CKD group. Theoretically, these differences could indicate an increased production of CPM following nutritional intake and/or an impaired capacity to clear CPM in those with CKD. The latter is supported by recent data by Koeppert et al., who used live two-photon microscopy to show that circulating CPM are predominantly cleared by glomerular filtration in mice²⁹. A reasonable prediction therefore, may be that the capacity to clear CPM becomes impaired as GFR declines. However, alterations in other aspects of mineral homeostasis, including bone turnover⁶⁶, and delayed renal excretion of acute phosphate loads^{7,59} are also commonly seen in CKD and may well have contributed to elevations in post-prandial levels. Recent evidence has questioned the long-held notion that CKD affects net intestinal phosphate absorption^{57,67,68}. We did however note that six of the 14 individuals in the CKD group were taking cholecalciferol. Considering the potential for stimulatory effects of vitamin D on intestinal mineral absorption, we performed an ad hoc exploratory analysis to test whether use of nutritional vitamin D impacted the acute response of CPM, CPP-I and CPP-II to feeding (Supplementary Tables S9–S11). Apart from a single positive group-by-time interaction for CPP-I at 30 min in those taking cholecalciferol, all other interaction terms and post hoc pairwise comparisons for CPM, CPP-I and CPP-II were not significant, suggesting that cholecalciferol use was unlikely to have had a substantial effect on post-prandial CPM/PP kinetics. While the small sample size and potential for confounding may preclude definitive conclusions, the lack of effect may also reflect the predominance of phosphate absorption via the paracellular pathway when mineral is abundant, which is not actively regulated by the vitamin D axis⁶⁹.

In contrast to CPM, we did not observe a strong effect of kidney function on either CPP-I or CPP-II. Unlike CPM, circulating CPP is primarily cleared from the circulation by non-renal mechanisms. Animal and in vitro models have suggested rapid clearance of CPP-I predominantly by liver sinusoidal endothelial cells²⁸, and of CPP-II by resident macrophages of the liver and spleen⁷⁰. It is plausible that while participants in our CKD group displayed evidence of altered CPM metabolism, that these discrete CPP clearance pathways were sufficient to maintain normal post-prandial CPP profiles. We had anticipated that the CKD group would have discerningly higher levels of CPP compared to controls based on previous studies, albeit these studies enrolled patients with more advanced CKD who were dialysis dependent³³, or used the older and indirect method of CPP measurement^{20,23}. It is possible that individuals with more advanced CKD than those studied here may exhibit greater differences in fasting and post-prandial CPP levels. The lack of separation for CPP between our CKD and control group may also be due to our limited participant numbers, which also limited our ability to formally test for an effect of CKD stage (Supplementary Fig. S6). Indeed, our study was powered based on examining post-prandial responses rather than between group differences. Of note, CPP-I appeared to return to fasting levels earlier in controls than CKD participants, and there was a positive group-by-time interaction for CPP-II in CKD participants at 120 min. Both findings potentially signal that we may have observed more pronounced between-group effects in a larger cohort. Higher analytical imprecision for flow cytometry-based CPP measurements may have also contributed to the null findings.

In addition to providing new insights into the physiology of CPM and CPP metabolism, our study also has direct relevance for optimising the use of these novel markers of mineral metabolism in future studies. While each of these novel assays have shown promise in early clinical work, very few studies have reported⁴³ or controlled for fasting/absorptive status^{46,56}. In this study a number of individuals had undetectable levels of CPP-I or CPP-II in the fasting and early post-prandial period, and this profound effect of fasting on serum CPP is in itself a previously unreported and notable finding. In contrast, fasting CPM levels were measurable and significantly higher in the CKD group than in controls, which suggests that sustained elevations in CPM may not necessarily manifest in elevated CPP levels in those with non-dialysis-dependent CKD.

Previous epidemiological studies have suggested links between elevated levels of CPM and CPP with a range of surrogate markers of vascular disease^{21,23,24}, as well as with cardiovascular events²⁵ and all-cause mortality²². Further, lab-based studies have provided plausible mechanisms by which CPP may themselves mediate these pathological vascular outcomes^{26–28}. However, while in vitro studies have suggested that many of these toxicities are induced in a dose-dependent manner, if CPP do have a normal physiological role in health then it is unclear at what point these particles may become injurious. Knowledge of the effect of nutritional intake on CPP kinetics as revealed here may prove crucial to further understanding this process. It is possible that a threshold concentration for the onset of pathological effects of CPM and CPP may (at least initially) only be reached in the post-prandial state in vivo. If so, dynamic testing of the post-prandial response may provide additional opportunities to evaluate earlier manifestations of dysregulated mineral metabolism, as well as the risk of associated

vascular disease. Another important possibility is that the composition and thus the intrinsic toxicity of CPP-I and CPP-II differs among health and CKD⁴⁵.

Limitations. We acknowledge that this study has several limitations, including limited patient numbers, as already discussed. Notably, we also used a standardised meal given after an overnight fast, and so cannot comment on the effect of varying meal composition or the effect of subsequent meals given throughout the rest of the day. We chose the meal based on its commercial availability, allowing for standardisation between participants, and because it represented physiologically relevant nutritional loads (Table 1). It is however possible that if participants were challenged with larger mineral loads, that further separation between those with normal and impaired kidney function may have been apparent. Similarly, given we only observed individuals for four hours post-meal, it is conceivable that the cumulative effect of subsequent meals may have also distinguished groups further.

We did not recover faeces or urine and so cannot comment on total mineral absorbed or excreted during the study. We assume that changes in each measure seen after feeding are directly related to nutritional intake. This assumption is supported indirectly by detailed imaging studies showing flux of calcium and phosphate ions following food intake⁵⁷, as well as direct evidence from animal studies showing that acute oral gavage of mice with a buffered phosphate solution results in serum spikes in CPM/CPP⁴⁸. Nevertheless, we did not observe participants over extended fasting conditions, and so cannot conclusively account for non-dietary related diurnal fluctuations in any of the studied parameters. We did however have two fasting samples, the average of which was used as “time 0”, and variability between these fasting samples was trivial compared to the magnitude of changes seen in the post-prandial period (Supplementary Fig. S5).

We used the Bonferroni method to account for the multiple post hoc pairwise comparisons between and within groups for each parameter. This is undoubtedly a conservative approach, and to our knowledge not one adopted by no other similar feedings studies of post-prandial mineral metabolism. We chose this approach given the large number of timepoints and comparisons that were made and reasoned that it was preferable to focus on the most robust and significant signals. However, as a result we may have missed smaller, but potentially relevant effects. We did not correct for multiple testing across different mineral parameters given the likelihood of interdependent physiologically linked changes.

Conclusion

Our study has revealed for the first time that nutritional mineral intake leads to the formation of CPM and CPP in blood as a normal physiological response to feeding. These findings corroborate the hypothesis that CPM/CPP formation helps to buffer post-prandial mineral loads, functioning as a temporary circulating store of bulk calcium phosphates ultimately destined for utilisation/storage (e.g., mineral precursor for bone mineralisation) or elimination. We also observed higher fasting levels of serum CPM and a larger post-prandial response in those with impaired kidney function, suggesting that CPM metabolism is manifestly altered in CKD. Analysis of post-prandial CPM/CPP handling may provide new insights into the mechanisms linking excessive dietary calcium and phosphate intake to increased risks cardiovascular disease in patients with impaired mineral excretion. More broadly, these novel findings underscore the important, but often neglected, contribution of colloidal biochemistry to mineral homeostasis.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Author contributions

Conceptualisation: M.K.T., M.M.X.C., N.D.T., E.R.S. Funding acquisition: M.M.X.C. Collection and processing of biological samples and clinical data: M.K.T., S.J.T. Laboratory analysis: M.K.T., A.P., E.R.S. Statistical analysis: M.K.T., E.R.S. Original draft: M.K.T. Review and editing of manuscript: All authors. Supervision: S.J.T., N.D.T., E.R.S.

Competing interests

M.K.T. and M.M.X.C. report no competing interests. N.D.T. reports research funding and honoraria from Amgen, Sanofi and Takeda. S.J.T. reports honoraria from AstraZeneca. A.P. is an inventor of the T50 test and a stockholder of Calciscon AG (Biel, Switzerland), which commercialises the test used in this study. E.R.S. is a stockholder of Calciscon AG (Biel, Switzerland), which commercialises the T50 test used in this study. He also reports research funding from Sanofi and Baxter.

Additional information

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Reduction of Calciprotein Particles in Adults Receiving Infliximab for Chronic Inflammatory Disease

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ABSTRACT

Patients with chronic inflammatory diseases (CID) experience accelerated loss of bone mineral density, which is often accompanied by increased vascular calcification. These disturbances can be attenuated by therapies for inflammation, such as the tumor necrosis factor inhibitor infliximab. Calciprotein particles (CPP) are circulating colloidal aggregates of calcium and phosphate together with the mineral-binding protein fetuin-A, which have emerged as potential mediators of vascular calcification. The precise origins of serum CPP are unclear, but bone turnover may be an important source. In this longitudinal observational study, we studied patients with CID undergoing treatment with infliximab to assess the temporal relationship between bone turnover and circulating CPP. Ten patients with active CID receiving infliximab induction therapy and an additional 3 patients with quiescent CID on maintenance infliximab therapy were studied for 8 weeks with repeated measures of bone turnover markers as well as CPP (calciprotein monomers [CPM], primary CPP [CPP-I], and secondary CPP [CPP-II]). Therapeutic response was appraised using validated disease activity scores. At baseline, those with active CID had elevated markers of bone resorption and suppressed bone formation markers as well as higher CPM and CPP-I compared with those with quiescent CID. In responders, there was an early but transient reduction in resorption markers by week 1, but a more sustained increase in bone formation markers compared with non-responders at week 8. This was accompanied by reductions in CPM ($\beta = -6.5 \times 10^3$ AU [95% CI $-11.1, -1.8$], $p = 0.006$) and CPP-I ($\beta = -23.4 \times 10^4$ particles/mL [95% CI $-34.8, -11.9$], $p < 0.001$). In contrast, no significant changes in any markers were observed in non-responders or those receiving maintenance therapy. Thus, CPP have a dynamic association with changes in bone turnover in response to infliximab therapy, adding to accumulating evidence of the role of bone as a determinant of systemic levels. © 2021 The Authors. *JBMR Plus* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research.

KEY WORDS: BIOCHEMICAL MARKERS OF BONE TURNOVER; DISORDERS OF CALCIUM/PHOSPHATE METABOLISM; OSTEOIMMUNOLOGY; PTH/VIT D/FGF23

Introduction

A classic consequence of the aging process is a progressive loss of bone mineral content.⁽¹⁾ The most prominent complication of this is a significant burden of fracture-related morbidity and mortality.⁽²⁾ However, loss of bone mineralization is often also accompanied by a parallel, and paradoxical, increase in mineralization of blood vessels with an associated increased risk of cardiovascular disease (“the calcification paradox”).⁽³⁾ A propensity for accelerated bone and vascular disease is also found in several pathological conditions, including chronic kidney disease (CKD),⁽⁴⁾ as well as in chronic inflammatory diseases

(CID) such as inflammatory bowel disease and inflammatory arthritis.^(5,6)

There is mounting evidence that bone loss and vascular calcification are directly linked through a series of interconnected pathways. Both processes share a number of common underlying risk factors, such as hypertension, diabetes, physical inactivity, smoking, excess alcohol consumption, as well as age and inflammation itself.⁽⁷⁾ In addition, newer insights into overlapping molecular pathways have strengthened the biological plausibility of a “bone-vascular axis.”^(8,9)

An emerging pathway that has been implicated in vascular calcification is the formation of fetuin-A-containing calciprotein

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particles (CPP). Fetuin-A is a hepatically derived plasma protein, which is a powerful inhibitor of ectopic mineralization.^(10,11) In biological solutions, fetuin-A is able to bind clusters of calcium and phosphate ions, forming calciprotein monomers (CPM). CPM may then self-aggregate into 30- to 100-nm amorphous primary calciprotein particles (CPP-I), which may then transform into larger, denser 100- to 250-nm secondary calciprotein particles (CPP-II).⁽¹²⁾ This ordered formation of mineral-protein colloids serves an important physiological role in facilitating bulk transport of mineral in circulation while preventing precipitation and ectopic deposition.⁽¹³⁾ However, in certain pathological conditions, including CKD and CID, elevated levels of circulating CPP are observed⁽¹⁴⁾ and have been positively associated with adverse events, including vascular stiffness,⁽¹⁵⁾ vascular calcification,⁽¹⁶⁾ and mortality.⁽¹⁷⁾ In vitro studies have shown that CPP can induce vascular smooth muscle cell calcification and expression of inflammatory mediators,^(18,19) while inhibiting osteoblast mineralization.⁽²⁰⁾ Animal models have shown that circulating CPP can also induce vascular luminal⁽²¹⁾ and endothelial⁽²²⁾ lesions. Taken together, it is proposed that elevated levels of CPP may not be just a marker of disordered mineral metabolism, but may themselves be mediators of vascular and bone pathology.

The precise origins of CPP in circulation and the drivers of elevated levels in pathological conditions are yet to be fully described. Previous animal⁽²³⁾ and human^(24–26) studies have suggested that there may be some relationship with dietary intake. However, there is also a line of evidence to suggest that bone may be an important determinant of serum CPP. In early animal models, it was shown that CPP levels could be altered by manipulating bone turnover in rats with normal^(27,28) and impaired⁽²⁹⁾ kidney function. There are limited data exploring the link between bone and CPP in human studies. In patients with CKD, CPP levels have been observed to correlate with serum parathyroid hormone (PTH)⁽³⁰⁾ and markers of bone turnover.^(15,31) Beyond CKD, whether bone metabolism may also be linked to elevations in serum CPP in the setting of CID has not previously been explored in clinical studies.

Outcomes of patients with various forms of CID have been dramatically improved with the introduction of anti-inflammatory and immunomodulatory biologic therapies such as tumor necrosis factor (TNF)-alpha inhibitors, including infliximab. Exact indications are specific to individual conditions, but these agents are

generally used in patients with moderate to high disease activity, often after failure to respond to conventional therapies. Their use in CID has been associated with improved bone mineral density and cardiovascular outcomes.^(32–35)

Bone loss in CID is driven by both suppressed bone formation and enhanced bone resorption, and TNF-alpha inhibitor therapy has been shown to normalize markers of bone turnover.^(36,37) Given the hypothetical links between CPP and bone turnover, we hypothesized that treatment of active CID with infliximab would lower circulating CPP levels and be temporally related to changes in markers of bone turnover.

Materials and Methods

In this hypothesis-generating, exploratory study, we recruited a cohort of patients with CID, who were planned to receive treatment with the TNF-alpha inhibitor infliximab for active disease. This study was undertaken as part of the Fetuin-A Levels in Systemic disease and Kidney Impairment (FLEKSI) study.⁽¹⁴⁾

We recruited a cohort of 10 patients with current active CID who were planned to receive induction therapy with infliximab. Participants had either inflammatory bowel disease (ulcerative colitis or Crohn's disease) or inflammatory arthritis (psoriatic arthritis or ankylosing spondylitis) and had disease-specific indications for infliximab use (outlined in Table 1). For comparison, we also recruited an additional group of three patients with quiescent CID who were receiving maintenance therapy with infliximab (two with Crohn's disease and one with ulcerative colitis). Participants were recruited via the outpatient clinics at The Royal Melbourne Hospital. All participants were at least 18 years of age and gave written informed consent to participate in this study. Key exclusion criteria were participants who were unable to give informed consent or who were pregnant or breastfeeding. This study was approved by the local ethics committee (Melbourne Health Human Research Ethics Committee reference number MH 2012.141) and conducted in accordance with the Declaration of Helsinki.

Participant groupings and infliximab dosing

Patients with active disease received intravenous infliximab (Remicade, Janssen Immunology, Horsham, PA, USA) 5 mg/kg

Table 1. Baseline Demographics

	Overall (n = 13)	Induction therapy		Maintenance therapy
		Non- responder (n = 4)	Responder (n = 6)	Quiescent (n = 3)
Age (years), mean (SD)	40.9 (11.8)	46.5 (15.4)	37.2 (11.9)	41 (4)
Sex—female, n (%)	7 (53.8)	4 (100)	2 (33.3)	1 (33.3)
Inflammatory arthritis, n (%)	3 (23.1)	1 (25)	2 (33.3)	—
Diagnosis and original indication for infliximab, n (%)				
Moderate to severe (Mayo score ≥ 6) active ulcerative colitis despite conventional therapy	4 (30.8)	1 (25)	2 (33.3)	1 (33.3)
Moderate to severe Crohn's disease (Crohn's Disease Activity Index score ≥ 220) despite conventional therapy	6 (46.2)	2 (50)	2 (33.3)	2 (66.7)
Active ankylosing spondylitis not responding to nonsteroidal anti-inflammatory drugs	1 (7.7)	—	1 (16.7)	—
Severe active psoriatic arthritis not responding to conventional therapy	2 (15.4)	1 (25)	1 (16.7)	—

at 0, 2, and 6 weeks according to local dosing guidelines for induction therapy and in line with current international recommendations.⁽³⁸⁾ The quiescent group received maintenance treatment with intravenous infliximab, at a dose of 5 mg/kg on an 8-week schedule. Patients with active disease who received induction therapy were classified into “non-responders” and “responders” based on their clinical and biochemical response to treatment. Criteria used for response was a decrease in Mayo score of ≥ 3 or a score of 0 for participants with ulcerative colitis; a decrease in Harvey-Bradshaw Index ≥ 4 for participants with Crohn’s disease; a decrease in Disease Activity Score-28 ≥ 1.2 for participants with psoriatic arthritis; or a decrease in Ankylosing Spondylitis Disease Activity Score ≥ 1.1 for participants with ankylosing spondylitis.^(39–43) Assessment against these criteria were made by the participant’s clinicians, who were otherwise not involved in the conduct of this study or analysis of results. These clinicians were also solely responsible for decisions regarding eligibility for and use of infliximab; however, each participant met local clinical eligibility criteria that are required for government funding of infliximab therapy⁽⁴⁴⁾ and are consistent with international prescribing guidelines.⁽⁴⁵⁾

Variables

We performed serial measurements of the bone formation (osteoblast activity) markers bone-specific alkaline phosphatase (bsALP) and procollagen type 1 N-terminal propeptide (P1NP), bone resorption (osteoclast activity) markers tartrate-resistant

acid phosphatase 5b (TRAcP 5b) and C-terminal telopeptide (CTx), CPP parameters (CPM, CPP-I, CPP-II), as well as intact and C-terminal FGF23 (iFGF23 and cFGF23, respectively).

In the participants with active disease who were receiving induction therapy, we measured bone turnover markers (BTM), CPP, and FGF23 1 week before treatment as well as on the day of treatment and then on week 1, week 2, week 6, and week 8 after the initial dose of infliximab. To account for variation, the mean of values taken 1 week before and on the day of initial treatment was used as baseline (“week 0”) for our analysis. Participants receiving maintenance infliximab for quiescent disease had BTM, CPP, and FGF23 measured 1 week before and on the day of infliximab, as well as week 8 post dose. As for participants in the active group, for the quiescent group, we combined the values for the week before and the day of infliximab to calculate week 0. All participants also had blood collected at week 0 and week 8 for standard biochemistry as well as the cytokines interleukin (IL)-6, -8, -10, -12, -17A, and -23, and fetuin-A.

Venous blood samples were collected using standard phlebotomy procedures into serum, lithium heparin and K₂-EDTA tubes. Serum samples were allowed to clot at room temperature for 1h and then centrifuged at 2500g for 10 min. Plasma specimens were processed immediately following collection. Aliquots were stored at -80°C until batched analysis following a single thaw. Other biochemistries were performed by our institution in an accredited laboratory. Commercial immunoassay kits were used to measure serum IL (BioLegend, San Diego, CA, USA), serum fetuin-A (R&D Systems, Minneapolis, MN, USA), BTM

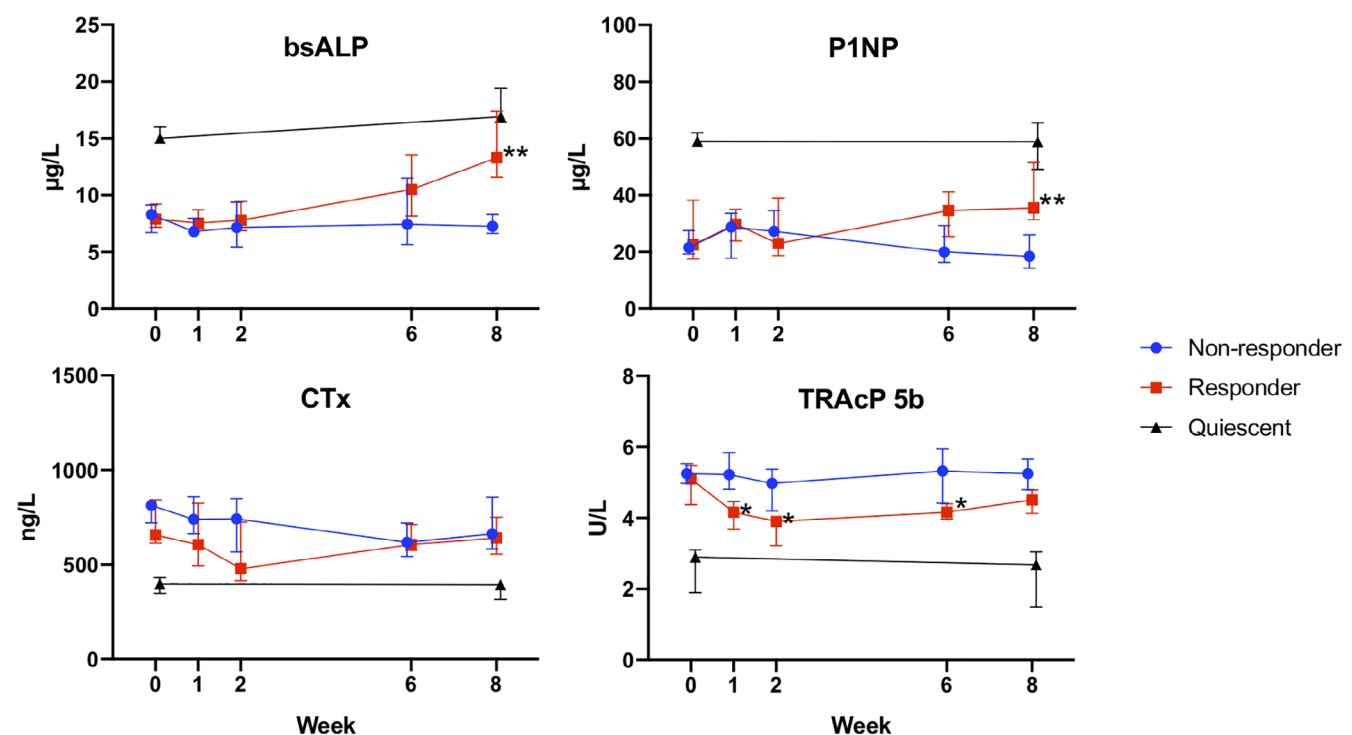


Fig 1. Change in bone turnover markers with infliximab treatment. Comparison between “non-responder” and “responder” groups of bone formation (osteoblast) markers bsALP and P1NP as well as bone resorption (osteoclast) markers CTx and TRAcP 5b, during induction therapy with infliximab for active disease. Quiescent group also displayed for reference. Data presented as median with error bars showing interquartile range. Data points offset for clarity. * $p < 0.05$ for responder versus non-responder, ** $p < 0.01$ for responder versus non-responder. bsALP = bone-specific alkaline phosphatase; P1NP = procollagen type 1 N-terminal propeptide; TRAcP 5b = tartrate-resistant acid phosphatase 5b; CTx = C-terminal telopeptide.

(Immunodiagnostic Systems, East Boldon, UK), and serum iFGF23 (Kainos Laboratories, Tokyo, Japan) and plasma cFGF23 (Immunotopics Inc, San Clemente, CA, USA) according to the manufacturer's instructions. Plasma CPM was measured by gel-filtration method as previously described.⁽⁴⁶⁾ Serum CPP-I and CPP-II were measured using a fluorescent probe-based assay running on a BD FACSVerser flow cytometer as previously described.^(23,25)

Statistical analysis

Demographics and standard biochemical and cytokine data are presented using descriptive statistics. Mean and standard deviation or median and interquartile range are presented for continuous variables as appropriate, and number and percentage are presented for categorical variables.

Because there were no published data pertaining to the effect of infliximab on CPP or clinically important differences in levels, sample size calculations were based on our observation of a patient with Takayasu's arteritis,⁽⁴⁷⁾ in whom infliximab treatment (5 mg/kg at 0 and 4 weeks) resulted in reduction in total CPP of 31% ($25 \times 10^4/\text{mL}$) from baseline to 8 weeks. We used GLIMMPSE, a validated linear mixed model power and sample size calculator,⁽⁴⁸⁾ to conduct a priori sample size calculations. Assuming a mean baseline CPP-I of $65 \times 10^4/\text{mL}$ and standard

deviation of $8 \times 10^4/\text{mL}$ at each of the five time points, we estimated that a total of seven patients would be required to provide >90% power in detecting a linear decline in CPP-I levels of 30% over 8 weeks at a 1% significance level (Geisser–Greenhouse corrected). Data for individual CPP components were not available to estimate appropriate sample sizes.

For patients with active disease receiving induction therapy with infliximab, we examined longitudinal between-group differences for the non-responder and responder groups for each measure of BTM, CPP, and FGF23. To do this while accounting for repeated measures, we fitted linear mixed-effects models using a restricted maximum likelihood approach with an unstructured covariance structure. In each model response group, categorical time and time-response group interaction were modeled as fixed effects, and we included a random intercept for each participant to allow for individual differences and correlation between repeated measures. In all these models, week 0 and non-responder group were used as the reference levels for time and group, respectively. For the model of bsALP, we naturally log transformed values before fitting the model to ensure normal distribution of residuals. Estimates for bsALP were then back transformed for ease of interpretation. There was an apparent between-group difference in the responder group compared with the non-responder group in multiple BTM, CPP,

Table 2. Linear Mixed-Effect Models for Bone Turnover Markers in Patients With Active CID Receiving Infliximab Induction Therapy

	bsALP ^a μg/L	P1NP μg/L	CTx ng/L	TRAcP 5b U/L
Group median (IQR)				
Non-responder				
Baseline	8.3 (7.2, 9.0)	21.5 (19.5, 26.0)	813 (750, 824)	5.3 (5.1, 5.5)
Week 1	6.8 (6.6, 7.7)	28.8 (19.9, 33.6)	739 (686, 821)	5.2 (4.9, 5.7)
Week 2	7.2 (6.0, 8.7)	27.3 (26.4, 32.3)	742 (592, 847)	5.0 (4.5, 5.3)
Week 6	7.5 (5.9, 10.5)	20.0 (16.4, 27.4)	618 (567, 687)	5.3 (4.5, 6.0)
Week 8	7.3 (6.7, 8.2)	18.5 (15.6, 23.6)	664 (589, 814)	5.3 (4.9, 5.6)
Responder				
Baseline	7.9 (7.2, 8.3)	22.5 (20.0, 38.0)	657 (618, 839)	5.1 (4.4, 5.4)
Week 1	7.6 (7.2, 8.4)	29.8 (27.6, 34.5)	606 (522, 816)	4.2 (3.8, 4.4)
Week 2	7.8 (7.1, 9.3)	23.0 (20.4, 38.9)	480 (419, 680)	3.9 (3.4, 4.0)
Week 6	10.5 (8.5, 12.6)	34.7 (29.4, 40.5)	607 (584, 672)	4.2 (4.0, 4.4)
Week 8	13.3 (11.9, 17.3)	35.6 (33.8, 50.5)	640 (577, 738)	4.5 (4.2, 4.6)
Between-group coefficient estimate ^b				
Week 1	0.5 (−1.8, 3.7)	−0.8 (−9.6, 7.9)	−36.8 (−231.9, 158.4)	−0.9* (−1.7, −0.2)
Week 2	0.6 (−1.7, 3.8)	−5.5 (−14.3, 3.2)	−84.1 (−279.4, 111.2)	−1.0* (−1.7, −0.2)
Week 6	2.4 (−0.4, 6.4)	8.2 (−0.6, 16.9)	97.6 (−97.9, 293.1)	−0.8* (−1.6, −0.1)
Week 8	6.4** (2.5, 11.9)	16.8** (8.1, 25.6)	27.7 (−168.1, 223.5)	−0.5 (−1.2, 0.3)
Coefficient estimate versus baseline for responder group only ^c				
Week 1	−0.5 (−1.9, 1.2)	3.2 (−1.5, 7.8)	−70.4 (−209.1, 68.3)	−0.9** (−1.4, −0.4)
Week 2	−0.3 (−1.8, 1.4)	1.1 (−3.6, 5.7)	−152.2* (−291.1, −13.4)	−1.3** (−1.8, −0.8)
Week 6	2.2* (0.4, 4.6)	7.3** (2.6, 12.0)	−62.8 (−201.8, 76.3)	−0.8** (−1.3, −0.3)
Week 8	5.5** (3.0, 8.5)	13.7** (9.0, 18.4)	−58.4 (−197.9, 81.1)	−0.5 (−1.0, 0.0)

CID = chronic inflammatory diseases; bsALP = bone-specific alkaline phosphatase; P1NP = procollagen type 1 N-terminal propeptide; CTx = C-terminal telopeptide; TRAcP 5b = tartrate-resistant acid phosphatase 5b; IQR = interquartile range.

Table shows group median (IQR) or mixed-effects model coefficient (95% confidence interval).

Week 0 and non-responder group used as reference groups for each model.

^absALP was naturally log transformed before fitting regression models to ensure normal distribution of residuals. Estimates have been back transformed for ease of interpretation.

^bCoefficient estimate is for responder group compared with non-responder group.

^cCoefficient estimate is for categorical time compared with baseline for models fitted to responder group only.

* $p < 0.05$.

** $p < 0.01$.

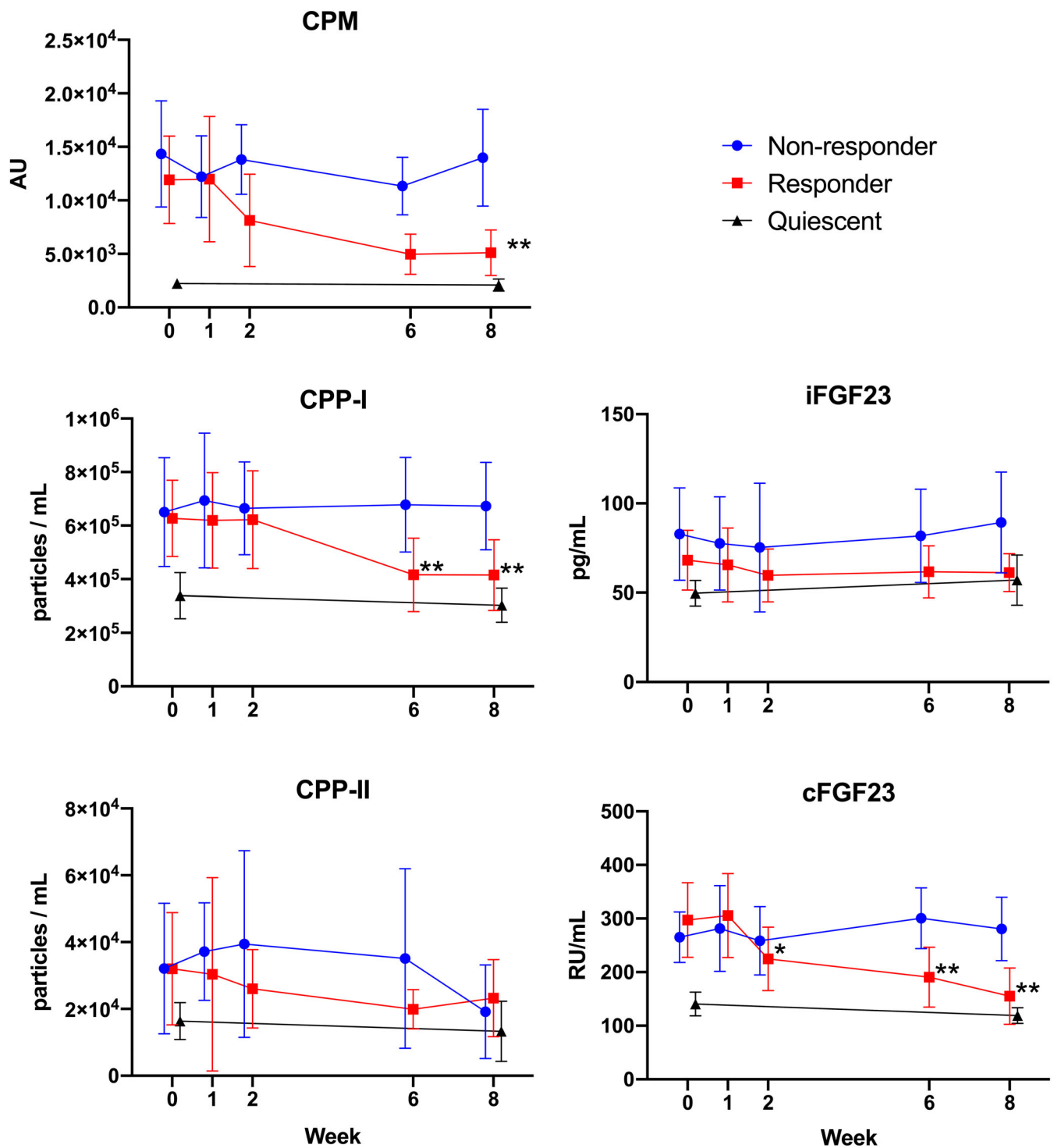


Fig 2. Change in calciprotein particles and fibroblast growth factor-23 with infliximab treatment. Comparison between “non-responder” and “responder” groups of CPM, CPP-I, CPP-II, iFGF23, and cFGF23 during induction therapy with infliximab for active disease. Quiescent group also displayed for reference. Normally distributed data (CPM, CPP-I, and cFGF23) presented as mean with error bars showing standard deviation. Skewed data (CPP-II and iFGF23) presented as median with error bars showing interquartile range. Data points offset for clarity. * $p < 0.05$ for responder versus non-responder, ** $p < 0.01$ for responder versus non-responder. CPM = calciprotein monomer; CPP = calciprotein particle; FGF23 = fibroblast growth factor-23.

and FGF23 variables. As a result, in further analyses, we fitted linear mixed effects models, restricted to data from the responder group, and using categorical time as the fixed effect, in order

to confirm whether between-group differences represented changes from baseline. Because this was an exploratory, hypothesis-generating study, no correction for multiple testing

Table 3. Linear Mixed-Effect Models for Calciprotein Particles and Fibroblast Growth Factor-23 in Patients With Active CID Receiving Infliximab Induction Therapy

	CPM ×10 ³ AU	CPP-I ×10 ⁴ /mL	CPP-II ×10 ³ /mL	iFGF23 pg/mL	cFGF23 RU/mL
Group mean (SD) or median (IQR)					
Non-responder					
Baseline	14.3 (5.0)	65.1 (20.4)	33.3 (15.8, 48.4)	85.0 (64.0, 101.6)	265.2 (47.2)
Week 1	12.2 (3.8)	69.4 (25.1)	36.7 (24.8, 49.5)	79.2 (58.0, 97.0)	281.4 (80.2)
Week 2	13.8 (3.3)	66.4 (17.3)	39.7 (16.5, 62.3)	69.1 (49.9, 100.7)	258.5 (63.7)
Week 6	11.3 (2.7)	67.8 (17.7)	30.5 (14.0, 56.2)	83.3 (64.9, 98.5)	300.3 (56.5)
Week 8	14.0 (4.5)	67.3 (16.3)	13.7 (10.9, 27.4)	90.7 (69.1, 109.5)	280.4 (58.8)
Responder					
Baseline	11.9 (4.1)	62.7 (14.2)	30.3 (15.2, 43.3)	70.7 (59.1, 77.2)	297.4 (69.7)
Week 1	12.0 (5.9)	61.9 (17.8)	16.2 (9.2, 52.0)	63.7 (51.0, 88.4)	305.8 (78.6)
Week 2	8.1 (4.3)	62.2 (18.2)	29.2 (15.0, 34.8)	54.3 (52.6, 74.7)	224.6 (59.2)
Week 6	5.0 (1.9)	41.5 (13.7)	17.4 (15.4, 26.3)	61.6 (48.2, 71.4)	190.5 (56.1)
Week 8	5.1 (2.1)	41.5 (13.1)	24.7 (15.9, 30.3)	61.4 (57.9, 65.4)	155.2 (52.4)
Between-group coefficient estimate ^a					
Week 1	2.2 (−2.5, 6.8)	−5.1 (−15.5, 5.3)	−6.7 (−38.4, 24.9)	2.7 (−13.5, 18.9)	−7.9 (−70.9, 55.2)
Week 2	−3.3 (−7.9, 1.4)	−1.9 (−12.5, 8.8)	−13.3 (−45.2, 18.5)	−1.2 (−18.0, 15.5)	−66.1* (−130.5, −1.7)
Week 6	−4.0 (−8.6, 0.7)	−23.8** (−34.7, −12.8)	−15.2 (−47.3, 17.0)	−5.3 (−23.0, 12.4)	−142.0** (−208.6, −75.4)
Week 8	−6.5** (−11.1, −1.8)	−23.4** (−34.8, −11.9)	4.2 (−28.5, 36.8)	−13.6 (−32.6, 5.3)	−157.4** (−227.0, −87.8)
Coefficient estimate versus baseline for responder group only ^b					
Week 1	0.1 (−3.0, 3.1)	−0.8 (−8.0, 6.5)	−1.7 (−18.0, 14.6)	−2.6 (−13.0, 7.8)	8.4 (−36.1, 52.8)
Week 2	−3.8* (−7.0, −0.6)	−0.5 (−7.8, 6.8)	−6.0 (−22.8, 10.8)	−8.8 (−20.1, 2.6)	−72.9** (−118.6, −27.1)
Week 6	−7.0** (−10.5, −3.4)	−21.1** (−28.4, −13.7)	−12.2 (−29.8, 5.5)	−6.5 (−19.2, 6.3)	−106.9** (−154.8, −59.1)
Week 8	−6.8** (−10.7, −2.9)	−21.2** (−28.6, −13.8)	−8.8 (−27.6, 10.0)	−7.2 (−21.7, 7.4)	−142.2** (−192.8, −91.6)

CID = chronic inflammatory diseases; CPM = calciprotein monomer; CPP = calciprotein particle; cFGF23 = c-terminal fibroblast growth factor 23; iFGF23 = intact fibroblast growth factor-23; SD = standard deviation; IQR = interquartile range.

Table shows group mean (SD), median (IQR), or mixed-effects model coefficient (95% confidence interval).

Week 0 and non-responder group used as reference groups for each model.

^aCoefficient estimate is for responder group compared with non-responder group.

^bCoefficient estimate is for categorical time compared with baseline for models fitted to responder group only.

* $p < 0.05$.

** $p < 0.01$.

was made. Two-tailed p values <0.05 were considered significant. All data were analyzed using Stata version 16.1 (StataCorp, College Station, TX, USA) and figures were produced using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA, USA).

Results

Of the 10 participants with active CID, 4 were adjudged by their treating clinician using disease-specific criteria to be “non-responders” and 6 to be “responders” after completing the induction regimen. The demographics of these participants, as well as the additional 3 patients with quiescent disease are presented in Table 1. The overall mean age of the cohort was 51 years and 54% were female. Three patients had inflammatory arthritis (2 in the responder group and 1 in the quiescent group), while the other 10 patients had inflammatory bowel disease.

Conventional biochemical measures as well as inflammatory markers at week 0 and week 8 are presented in Supplemental Table S1. All participants had normal kidney function at both time points. Groups appeared to have comparable levels of serum calcium, phosphate, albumin, bicarbonate, magnesium, total alkaline phosphatase, and PTH, while participants in the

non-responder group appeared to have lower levels of 25-hydroxyvitamin D at both week 0 and week 8.

Expectedly, there were differences in the inflammatory marker C-reactive protein (CRP) and interleukin panel between subgroups at baseline and over the 8-week study period (Supplemental Table S1 and Supplemental Fig. S1). Participants with active disease tended to have higher levels of CRP and pro-inflammatory cytokines at week 0 compared with those with quiescent disease. In the responder group, there were reductions in CRP, IL-6, IL-8, and IL-17A from baseline to week 8, while there was an increase in the regulatory cytokine IL-10. In contrast, levels of these markers appeared unchanged in non-responders and those with quiescent disease. Consistent with fetuin-A being a negative acute-phase reactant, patients with active disease appeared to have lower levels of serum fetuin-A than those with quiescent disease at baseline; however, levels appeared to rise in the responder group by week 8 (Supplemental Table S1).

Bone turnover markers

Repeated measures of BTM are displayed in Fig. 1 and Table 2. Coefficient estimates for linear mixed effects models fitted to the non-responder and responder groups for each BTM are also presented in Table 2. At baseline, patients in the quiescent group

appeared to have higher levels of the bone formation (osteoblast) markers bsALP and P1NP and lower levels of the bone resorption (osteoclast) markers CTx and TRAcP 5b than those with active disease.

In the responder group, a significant increase was observed at week 8 for both bsALP ($\beta = 6.4 \mu\text{g/L}$ [95% confidence interval (CI) 2.5, 11.9], $p < 0.001$) and P1NP ($\beta = 16.8 \mu\text{g/L}$ [95% CI 8.1, 25.6], $p < 0.001$), whereas TRAcP 5b showed a transient reduction in levels at week 1 ($\beta = -0.93 \text{ U/L}$ [95% CI -1.66, -0.19], $p = 0.014$), week 2 ($\beta = -0.96 \text{ U/L}$ [95% CI -1.70, -0.22], $p = 0.011$), and week 6 ($\beta = -0.81 \text{ U/L}$ [95% CI -1.55, -0.06], $p = 0.011$), but not at week 8 ($\beta = -0.48 \text{ U/L}$ [95% CI -1.23, 0.27], $p = 0.207$). In contrast, no change in CTx levels was detected at any time point in either group.

Calcioprotein particles

Participants in the quiescent group appeared to have lower levels of CPM and CPP-I than the groups with active disease (Fig. 2 and Table 3). Coefficient estimates for linear mixed effects models fitted to the non-responder and responder groups for CPP and FGF23 are presented in Table 3. In the responder group, there was a significant reduction in CPM at week 8 ($\beta = -6.5 \times 10^3 \text{ AU}$ [95% CI -11.1, -1.8], $p = 0.006$), while for CPP-I, a significant reduction was evident at week 6 ($\beta = -23.8 \times 10^4 \text{ particles/mL}$ [95% CI -34.7, -12.8], $p < 0.001$) and was sustained at week 8 ($\beta = -23.4 \times 10^4 \text{ particles/mL}$ [95% CI -34.8, -11.9], $p < 0.001$).

For CPP-II, there was no clear difference between those with active and quiescent disease at baseline or serially, and no between-group difference was observed at week 8 ($\beta = 4.2 \times 10^3 \text{ particles/mL}$ [95% CI -2.9, 3.7], $p = 0.803$).

Fibroblast growth factor-23

The quiescent group appeared to have lower levels of cFGF23 than the non-responder and responder groups at baseline (Fig. 2 and Table 3). Longitudinally, a significant reduction in cFGF23 was observed for the responder group, which was evident by week 2 ($\beta = -66.1 \text{ RU/mL}$ [95% CI -130.5, -1.7], $p = 0.044$) and persisted at week 6 ($\beta = -142.0 \text{ RU/mL}$ [95% CI -208.6, -75.4], $p < 0.001$) and at week 8 ($\beta = -157.4 \text{ RU/mL}$ [95% CI -227.0, -87.8], $p < 0.001$). In contrast, no significant difference in iFGF23 between the non-responder and responder groups were observed at any time point.

Linear mixed-effect models restricted to responder group

Given the between-group differences found in several of the above variables, linear mixed effect models were fitted to data restricted to the responder group to confirm whether between-group differences also represented change from baseline values (Tables 2 and 3). These analyses confirmed that there was a significant increase at week 8 for bsALP ($\beta = 5.5 \mu\text{g/L}$ [95% CI 3.0, 8.5], $p < 0.001$) and P1NP ($\beta = 13.7 \mu\text{g/L}$ [95% CI 9.0, 18.4], $p < 0.001$) compared with baseline. There were also significant decreases for CPM ($\beta = -6.8 \times 10^3 \text{ particles/mL}$ [95% CI -10.7, -2.9], $p = 0.001$), CPP-I ($\beta = -21.2 \times 10^4 \text{ particles/mL}$ [95% CI -28.6, -13.8], $p < 0.001$), and cFGF23 ($\beta = -142.2 \text{ RU/mL}$ [95% CI -192.8, -91.6], $p < 0.001$) at week 8 compared with baseline.

Discussion

In this pilot, hypothesis-generating observational study, our data suggest that modulation of inflammation in patients with CID is associated with dynamic changes in bone turnover profile and that this is paralleled by changes in CPP and FGF23 metabolism. To our knowledge, this is the first study to report that interventions with anti-inflammatory and immunomodulatory anti-TNF biologics like infliximab might be useful in modifying CPP levels. At baseline, we found that patients with active inflammation had evidence of suppressed bone formation (osteoblast activity) and high bone resorption (osteoclast activity), which was accompanied by high levels of CPM, CPP-I, and cFGF23. Serially, patients who showed response to infliximab demonstrated improvements in bone formation markers, which were accompanied by reductions in CPM, CPP-I, and cFGF23. Notably, all these longitudinal changes appeared to trend significantly toward values observed in the quiescent group.

Our data add to the accumulating evidence that alterations in bone metabolism may be a significant determinant of CPP and its precursors (CPM) in the circulation. While at baseline, elevated levels of CPM and CPP-I were observed alongside both suppressed bone formation and high bone resorption, it is notable that only effects on bsALP and P1NP appeared sustained at week 8 in responders, whereas changes in the osteoclast marker TRAcP 5b occurred much earlier, were transient, and not mirrored by changes in CTx. Indeed, the increase in bone formation markers appeared to more closely parallel reductions in CPM and CPP-I. Given these observations, we speculate that bone formation may be the dominant driver underlying serum levels of CPP in CID. This contrasts with observations in dialysis cohorts, where CPP positively correlate with the degree of hyperparathyroidism, a condition where high turnover bone disease and enhanced osteoclastic activity is a prominent feature.⁽³⁰⁾ Moreover, in a post hoc analysis of a randomized trial of hemodialysis patients comparing standard to high dialysate magnesium, Bressendorff and colleagues found that while CPP were weakly negatively correlated with bsALP, the association with TRAcP 5b was nearly twice as strong.⁽³¹⁾ Taken together, this suggests both formation and resorption components of remodeling may contribute to changes in circulating CPP and it is the net balance or coupling of these processes that determines effects on the serum CPP pool. Further work is needed to validate these findings against gold standard assessment of bone turnover by histomorphometric analysis of bone biopsies.

Compatible with these observations in humans, early animal models by Price and colleagues found that use of etidronate at supratherapeutic doses known to acutely inhibit bone formation was associated with the appearance of CPP in circulation.⁽²⁷⁾ In subsequent work, they found that this effect was ameliorated when the animals were first pretreated with various inhibitors of bone resorption.⁽²⁸⁾ A notable point of difference between these experiments and a valuable clinical data is that the supratherapeutic doses of etidronate also led to significant fluctuations in serum calcium and phosphate and potentially resulting in intravascular CPP formation. Nevertheless, one plausible explanation of this experimental data, as well as previous clinical studies in CKD and now in CID, is that bone acts as both a source and a sink of CPP or its precursors.⁽²³⁾ Therefore, it may be that in situations where rates of bone formation and bone resorption are geared toward accelerated net bone loss, this leads to elevated serum CPP, especially when mineral excretion is impaired as in CKD.

The hypothesis eluded to here is still lacking a complete mechanistic explanation of how alterations in bone turnover would eventually result in elevations of CPP in circulation. In lab-based experiments, under physiological conditions, the process from assembly of CPM, which coalesce to form CPP-I and ultimately CPP-II, takes hours.⁽⁴⁹⁾ By comparison, injected artificial CPP particles are rapidly cleared from circulation within minutes,⁽¹⁹⁾ and so doubts have previously been raised whether CPP could be assembled in significant numbers within the serum itself.⁽⁴⁹⁾ Additionally, a “bone-centric” paradigm of CPP formation also does not exclude the gastrointestinal tract from being an important contributor. Previous studies in animals⁽²³⁾ and dialysis patients^(25,26) have shown that serum CPP can be manipulated through the gastrointestinal (GI) tract, and it is possible that an interrelationship between the gastrointestinal tract, bone, and CPP may exist.⁽¹²⁾ Indeed, given that the majority of patients in this pilot study had GI disease, it is plausible that improvement in GI tract barrier function in response to infliximab may have imparted effects on the absorption of mineral.⁽⁵⁰⁾

The clinical interest in CPP stems mostly from its apparent role as a mediator of vascular toxicity. However, almost all of the human data linking CPP to clinically relevant events come from patients with CKD. While CPP have also been found to be elevated in CID, larger studies incorporating vascular, patient relevant outcomes are needed to clarify the significance of CPP in this setting. While we found a reduction of CPM and CPP-I with treatment of the underlying inflammatory condition, there was less clear delineation for CPP-II between those with active and quiescent disease at baseline and no clear longitudinal change between response groups. This is potentially of significance, given that several preclinical studies have suggested that CPP-II is the main mediator of cellular toxicity, albeit more recent preclinical,⁽¹⁹⁾ and clinical data have suggested that smaller CPP species are also associated with important outcomes.^(16,17) Our failure to detect a change in CPP-II may reflect our small sample size; however, it is notable that some,^(25,30) but not all,⁽³¹⁾ previous clinical studies using the same flow cytometric assay also found that only CPP-I was responsive to intervention. Given the potential link between CPP and bone, if CPP is also confirmed to be associated with vascular outcomes in CID, then this may suggest a role of CPP in the “bone-vascular axis.” Importantly, given the reductions of CPM and CPP-I with treatment, it is possible that CPP could act as not only a marker but also a modifiable risk factor for bone and/or vascular outcomes, through therapeutic interventions targeting drivers of high bone turnover, including systemic inflammation. Clearly, further work is needed to clarify the mechanistic role, and ultimately, the clinical utility of CPP measured in these processes.

Fetuin-A, an indispensable component of CPP as well as its precursors, has notable links to inflammation, as well as bone and mineral metabolism. Fetuin-A is considered a negative acute-phase reactant,⁽⁵¹⁾ and consistent with this, we observed lower serum levels in those with active disease, which numerically increased in responders after induction therapy with infliximab. Although primarily secreted by hepatocytes in adults, fetuin-A extravasates from extracellular fluid to mineralized tissues because of its high affinity for hydroxyapatite and in bone represents one of the most abundant of the non-collagenous proteins.⁽⁵²⁾ Fetuin-A has also previously been associated with significant clinical outcomes, with serum levels showing inverse associations with vascular calcification,⁽⁵³⁾ aortic stiffness,⁽⁵⁴⁾ and mortality.⁽⁵⁵⁾ Nevertheless, these associations have not been uniform,⁽⁵⁶⁾ and one explanation for this variability is that the

mineral-bound fetuin-A (ie, CPP) may be the more relevant metric of pathology than total serum levels.⁽⁵⁷⁾

The peptide hormone FGF23 is another factor with evidence of biological toxicity and with intriguing links to inflammation and bone.⁽⁵⁸⁾ FGF23 is itself bone-derived and primarily functions as an endocrine regulator of phosphate excretion and vitamin D metabolism in the kidney.⁽⁵⁹⁾ In CKD populations, it has consistently been positively associated with cardiovascular events and death, although whether this is through direct causation is unclear.⁽⁶⁰⁾ While the exact mechanism(s) regulating FGF23 in health and disease remain under investigation, levels have previously been shown to positively correlate with inflammation, including in clinical studies of patients with rheumatoid arthritis and inflammatory bowel disease.^(61,62) FGF23 has also been observed to correlate with CPP,^(23,24) and recently, CPP have been proposed to be a direct mediator of FGF23 expression in bone cells.⁽⁶³⁾

Inactivation of FGF23 by posttranslational cleavage is believed to be an important step in the regulation of FGF23 bioactivity,⁽⁶⁴⁾ and this can be captured *ex vivo* by measurement of FGF23 in blood using intact and C-terminal assays in tandem. Whereas intact FGF23 assays exclusively measure the full-length protein, the C-terminal assay detects both the full-length hormone and cleaved C-terminal fragments. In this study, the pattern of changes in cFGF23 largely followed that of CPM and CPP-I, with patients with active CID demonstrating higher levels than the quiescent group at baseline, and those responding to infliximab therapy showing progressive reductions in levels over 8 weeks. By comparison, we did not find evidence of differences in intact FGF23 at baseline or longitudinally. Interestingly, pro-inflammatory stimuli have been found to differentially effect intact and C-terminal FGF23 concentrations in osteocyte cultures and mouse models.^(65,66) Thus it is possible that inflammation, and potentially CPP, might drive an increase FGF23 transcription in CID, but that this is accompanied by an appropriate and compensatory upregulation of cleavage to maintain overall bioactivity and null effects on phosphaturia and vitamin D. This could explain the elevation in cFGF23 in patients with active inflammation at baseline which “normalized” in responders, while also accounting for the lack of signal in iFGF23.

Our study has a number of limitations. We studied a small and heterogeneous group of patients. Inflammatory bowel disease and inflammatory arthritis each encompass diverse disease entities with distinct pathogenesis and clinical manifestations. Nonetheless, these conditions share a number of common features, including an apparent environmental stimulus, often in individuals with genetic susceptibility, triggering an exaggerated and sustained inflammatory response. Indeed, most CIDs also share a propensity for accelerated systemic bone loss and vascular calcification. Importantly, another shared feature of these disease entities is the efficacy of treatment with TNF-alpha inhibitors, such as infliximab, supporting the idea of shared pathological pathways.

Given the small sample size, we did not undertake formal comparison testing of between-group demographics, nor did we attempt to make adjustments for any demographic variable in our models. For similar reasons, we also considered it inappropriate to formally test for correlations between variables or to include the quiescent group in our linear mixed models, as this would have significantly added to the number of comparisons applied to our limited number of participants. Hence, formal hypothesis testing was limited to a small number of predefined outcomes relating to CPP and bone markers parameters. This

was always intended as a hypothesis-generating study, and larger trials are needed to test the robustness of the apparent associations that we describe. Nevertheless, considering our small heterogenous cohort, the delineation between patients with active disease and quiescent disease in several of the study variables at baseline was remarkable. Further, the tendency of multiple variables in the responder group to trend toward values found in the quiescent group was also impressive in their uniformity. Exceptions to this were the bone resorption markers CTx and TRAcP 5b. However, considering the small numbers and relatively short observational period, it is possible that TRAcP 5b and/or CTx may “normalize” in larger or longer-term studies, as has previously been suggested.^(36,37)

Given the observational nature of this study, our findings are associative, and we can only speculate on potential underlying mechanistic links. Our notion of CPP being directly linked to bone turnover requires confirmation in future carefully designed studies. In particular, we speculate that inflammation drives dynamic changes in bone turnover, which sequentially results in elevations in CPP. These assumptions are largely based on the well-described effects of inflammation on bone turnover, driven by the cross-talk between multiple cytokine and bone cell signaling pathways.⁽⁶⁷⁾ Given that our observations are temporarily linked to the administration of infliximab, it seems reasonable to assume that our observations of CPP are “downstream” to the effects of inflammation modulation and bone turnover. However, it is notable that in lab studies, CPP itself has been found to induce cellular inflammatory responses and also to inhibit osteoblast mineralization.^(18–20) As such, if interactions between inflammation, bone turnover, and CPP are confirmed, they may not necessarily be unidirectional. Similarly, both inflammation⁽⁶⁵⁾ and CPP⁽⁶³⁾ have previously been proposed as modulators of FGF23 expression, and our data do not allow us to clarify the hierarchy of these effects.

In conclusion, our data suggest that infliximab induction therapy is associated with an increase in bone formation markers and concomitant reductions in CPM and CPP-I in patients with active CID. This raises the possibility that bone turnover may be a significant determinant of serum CPP levels and that in situations of net bone loss commonly found in CID and CKD may lead to expansion of the circulating CPP pool. Given the growing evidence implicating CPP as a mediator of vascular disease, this may also suggest that elevations in CPP form part of the disturbed bone-vascular axis in CID.

Disclosures

MKT reports no disclosures. ERS holds stock in Calciscon and has received grant support from Amgen, Baxter, and Sanofi. NDT reports research funding and honoraria from Amgen, Sanofi, and Shire Pharmaceuticals. HFA-K reports no disclosures. SGH reports research funding from Amgen, Baxter, and Sanofi.

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Peer review

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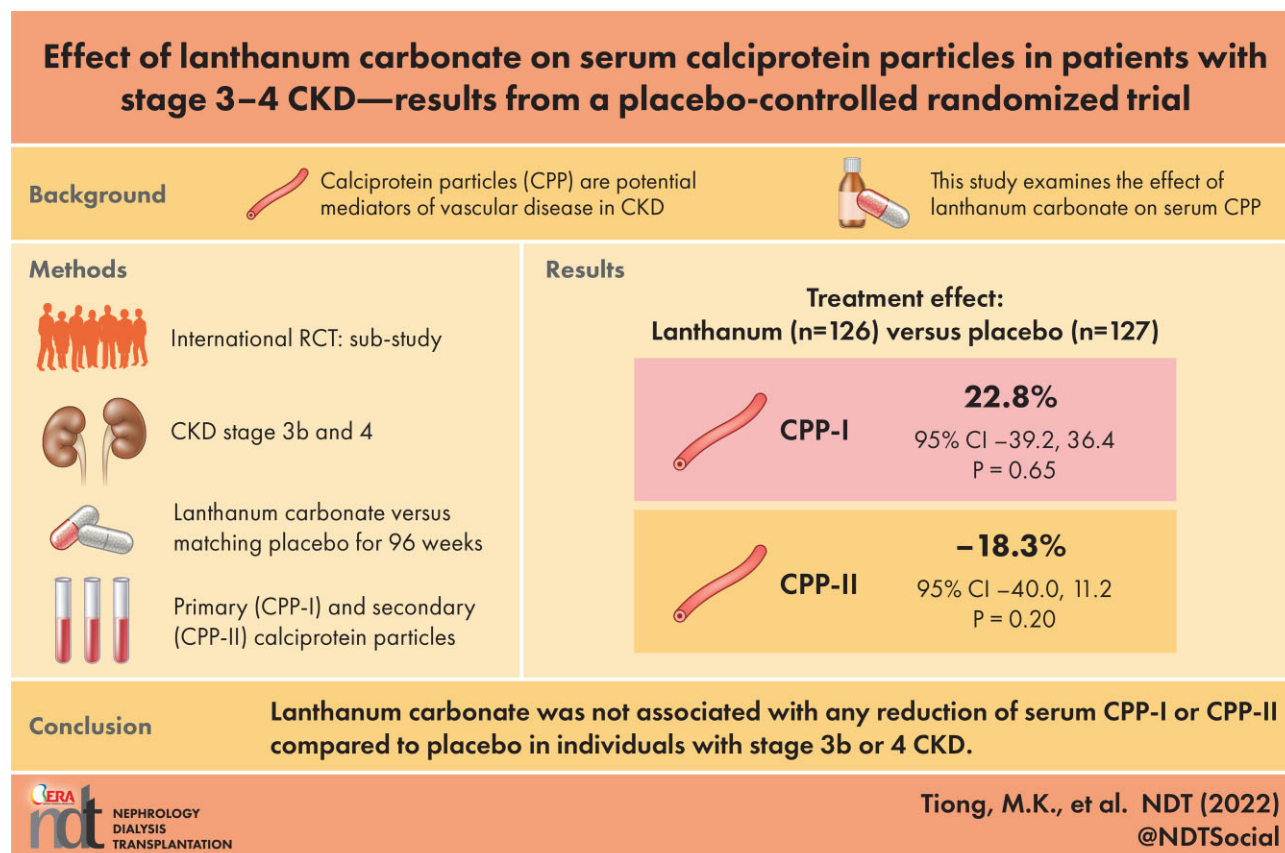
Effect of lanthanum carbonate on serum calciprotein particles in patients with stage 3–4 CKD—results from a placebo-controlled randomized trial

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GRAPHICAL ABSTRACT



KEY LEARNING POINTS

What is already known about this subject?

- Calcioprotein particles (CPP) are colloidal aggregates of calcium phosphate and the mineral-binding protein fetuin-A. CPP have emerged as potential mediators of cardiovascular disease in chronic kidney disease (CKD).
- Previous studies have suggested that non-calcium-containing phosphate binders may reduce serum CPP in patients with dialysis-dependent CKD. It is unclear whether similar interventions are effective in patients with earlier stages of CKD, who tend to also have less advanced cardiovascular disease and may benefit from early intervention.

What this study adds?

- Use of the non-calcium-containing phosphate binder lanthanum carbonate was not associated with any change in serum CPP in patients with non-dialysis-dependent CKD.
- We were unable to confirm previous associations between serum CPP and surrogate markers of cardiovascular disease in this large, multinational cohort of individuals with CKD stage 3b/4.

What impact this may have on practice or policy?

- Intestinal phosphate binders may not be an effective strategy to lower serum CPP in patients with non-dialysis-dependent CKD.

ABSTRACT

Background. Calcioprotein particles (CPP) are colloidal aggregates of calcium phosphate and the mineral-binding protein fetuin-A, and are potential mediators of cardiovascular disease in chronic kidney disease (CKD). Emerging evidence suggests non-calcium-containing phosphate binders may reduce serum CPP in patients with kidney failure who require dialysis; however, it is unclear whether similar interventions are effective in patients with earlier stages of CKD.

Methods. The IMPact of Phosphate Reduction On Vascular End-points in CKD (IMPROVE-CKD) was a multi-centre, placebo-controlled, randomized trial of lanthanum carbonate on cardiovascular markers in 278 participants with stage 3b/4 CKD. In this pre-specified exploratory analysis, primary (CPP-I) and secondary CPP (CPP-II) were measured in a sub-cohort of participants over 96 weeks. Treatment groups were compared using linear mixed-effects models and the relationship between serum CPP and pulse wave velocity (PWV) and abdominal aortic calcification (AAC) was examined.

Results. A total of 253 participants had CPP data for baseline and at least one follow-up timepoint and were included in this analysis. The mean age was 62.4 ± 12.6 years, 32.0% were female and the mean estimated glomerular filtration rate (eGFR) was 26.6 ± 8.3 mL/min/1.73 m². Baseline median serum CPP-I was 14.9×10^4 particles/mL [interquartile range (IQR) 4.6–49.3] and median CPP-II was 3.3×10^3 particles/mL (IQR 1.4–5.4). There was no significant difference between treatment groups at 96 weeks in CPP-I [22.8% (95% confidence interval –39.2, 36.4), $P = 0.65$] or CPP-II [–18.3% (95% confidence interval –40.0, 11.2), $P = 0.20$] compared with a placebo. Serum CPP were not correlated with baseline PWV or AAC, or with the progression of either marker.

Conclusions. Lanthanum carbonate was not associated with a reduction of CPP at 96 weeks when compared with a placebo in a CKD cohort.

Keywords: cardiovascular disease, calcioprotein particles, chronic kidney disease, mineral metabolism, phosphate binders

INTRODUCTION

Patients with chronic kidney disease (CKD) are at increased risk of cardiovascular disease and mortality. This risk is highest in patients with kidney failure who require dialysis, however, vascular pathology often begins much earlier in the course of CKD [1]. Patients with CKD have a high burden of traditional risk factors for cardiovascular disease, however, non-traditional risk factors, such as abnormalities in mineral metabolism, may also be important. Abnormalities in mineral metabolism are intrinsic to CKD and disturbances in serum phosphate, parathyroid hormone (PTH), as well as vitamin D metabolism, are almost universally present by the time patients reach kidney failure. However, abnormalities in mineral homeostasis are detectable much earlier in CKD [2] and it is conceivable that early intervention may prevent or reduce the progressive burden of cardiovascular disease as CKD advances.

Calcioprotein particles (CPP) are nanoscale mineral protein colloids that are emerging as novel markers of mineral and cardiovascular pathology in CKD [3, 4]. In biological fluids, nascent clusters of calcium and phosphate ions are bound by the liver-derived glycoprotein fetuin-A, forming calcioprotein monomers (CPM). These mineral–protein clusters may then self-aggregate to form primary calcioprotein particles (CPP-I) before transforming into larger and denser secondary calcioprotein particles (CPP-II). In health, the formation of CPM and CPP appear to have a physiological role in facilitating the bulk transport of mineral ions without extrasosseous precipitation. However, elevated levels of serum CPM and CPP may be a marker of ‘mineral stress’ in CKD and have been shown to positively correlate with a range of intermediate cardiovascular endpoints such as aortic stiffness [5] and coronary artery

calcification [6, 7], as well as with patient outcomes such as cardiovascular events [8] and mortality [9]. Laboratory studies have also suggested that CPP directly mediate a range of toxic effects such as induction of vascular smooth muscle cell calcification and expression of inflammatory mediators, as well as causing vascular luminal and endothelial lesions [10–12]. Consequently, elevated levels of CPP have been suggested not only to be a correlate of vascular pathology in CKD, but may also be directly involved as a mediator of these processes [4].

Reduction of gastrointestinal phosphate load has been suggested as a potential strategy to reduce elevated levels of serum CPP in CKD. While the exact determinants of CPP in circulation are yet to be fully elucidated, animal models have shown that dietary phosphate loading leads to increased CPP in the serum of CKD model rats [13]. Additionally, in patients with kidney failure requiring dialysis, it has been shown that non-calcium-based intestinal phosphate binders are associated with lower levels of serum CPM and CPP than calcium-based binders [14, 15]. In these studies, however, it was not clear if reductions in CPM and CPP were predominantly related to a reduction in intestinal phosphate absorption *per se*, or the avoidance of exogenous calcium loading associated with calcium-based binders. Given that previous studies have been in patients requiring dialysis, it is also unclear whether phosphate binders can be used to lower serum CPP in patients with earlier stages of CKD, who notionally tend to also have less advanced cardiovascular disease and so may ultimately benefit more from intervention.

The Impact of Phosphate Reduction On Vascular End-points in CKD (IMPROVE-CKD) trial [16] was a placebo-controlled, randomized trial assessing the effect of the non-calcium-based phosphate binder lanthanum carbonate on cardiovascular markers over 96 weeks in 278 participants with stage 3b/4 CKD. In this pre-specified exploratory analysis, we examined the effect of lanthanum carbonate on serum CPP in a non-dialysis-dependent CKD population. We included all participants who had CPP data at baseline and at least one follow-up measure.

MATERIALS AND METHODS

Full details of the study protocol [17], participant characteristics [18] and the primary trial results [16] from IMPROVE-CKD have been reported. In brief, IMPROVE-CKD was an international study, conducted across 17 sites in Australia, New Zealand and Malaysia and coordinated by the Australasian Kidney Trials Network (AKTN). Ethical approval was obtained by each local institutional ethics committee and the trial was prospectively registered (ACTRN12610000650099) and conducted in accordance with the declaration of Helsinki.

All participants provided written informed consent, were at least 18 years of age, had stage 3b or 4 CKD [estimated glomerular filtration rate (eGFR) 15–44 mL/min/1.73 m²] and had at least one recording of serum phosphate ≥ 1.00 mmol/L in the 6 months prior to enrolment. Potential participants were excluded if they had a serum phosphate ≤ 0.8 mmol/L at screening, or if they had a kidney transplant, a medical condition that may affect calcium and phosphate metabolism

(apart from CKD), a gastrointestinal disorder, were pregnant or breast-feeding, or had been hospitalized or had a cardiovascular event within 1 month of screening.

Participants were randomized to receive either 500 mg lanthanum carbonate or a matching placebo three times a day with meals for 96 weeks. Study medication was up-titrated by local investigators if serum phosphate remained persistently >1.60 mmol/L, to a maximum total dose of six tablets per day. Other phosphate binders were ceased for 2 weeks prior to the commencement of the study medication; however, other phosphate binders could be prescribed during the study for persistent hyperphosphataemia if the study medication had already been maximally titrated.

Outcome measures

For this sub-study, blood samples for CPP were collected at baseline and after 24, 48, 72 and 96 weeks. Blood was collected via standard venepuncture techniques and allowed to clot for 30 min before centrifugation. Serum aliquots were stored at -80°C until blinded batch-analysis at a central laboratory. Serum CPP-I and CPP-II were measured using a fluorescent probe-based assay running on a BD FACSVerser flow cytometer as previously described [13, 14].

Surrogate cardiovascular markers

As previously described [16–18], vascular stiffness was assessed using pulse wave velocity (PWV), which was measured as the mean of two carotid-femoral measurements at each time-point using a SphygmoCor device (AtCor, PWV Inc., Sydney, Australia), while abdominal aortic calcification (AAC) was determined by Agatston score from computed tomography at baseline and 96 weeks. Agatston score was calculated from the density and total size of calcification in the abdominal aortic wall over a length of 10 cm below the upper end plate of the L2 lumbar vertebral body. As well as PWV and AAC, the binary variables of ‘PWV >10 m/s’ as well as ‘presence of AAC’ were also included as variables at baseline and 96 weeks. PWV ≥ 10 m/s was used as it is considered as the threshold where risk of cardiovascular events is significantly increased [19]. Presence of AAC was defined as Agatston score greater than zero.

Statistical analysis

The main outcome of interest was the effect of lanthanum carbonate on CPP-I and CPP-II at 96 weeks. Between-group differences for both CPP-I and CPP-II were analysed by fitting linear mixed effects models with repeated measurements (MMRM). For each model, treatment group, categorical time, group–time interaction and baseline values were fitted as fixed effects and an unstructured covariance structure was used to model within-patient correlation. Missing baseline values (six participants for CPP-I and seven participants for CPP-II) were replaced by mean imputation. Values for both CPP-I and CPP-II were natural log-transformed before fitting each model to ensure normal distribution of residuals. To aid

Table 1. Participant demographics and characteristics at baseline

	Overall (<i>n</i> = 253)	Placebo (<i>n</i> = 127)	Lanthanum (<i>n</i> = 126)
Age (yrs)	62.4 ± 12.6	61.8 ± 13.1	63.1 ± 12.1
Female sex, <i>n</i> (%)	81 (32.0)	41 (32.3)	40 (31.7)
Country of study site, <i>n</i> (%)			
Australia	182 (71.9)	92 (72.4)	90 (71.4)
New Zealand	30 (11.9)	15 (11.8)	15 (11.9)
Malaysia	41 (16.2)	20 (15.7)	21 (16.7)
Ethnicity, <i>n</i> (%)			
White/Caucasoid	157 (62.1)	85 (66.9)	72 (57.1)
Australian Aboriginal or Torres Strait Islander	1 (0.4)	0	1 (0.8)
New Zealand Māori or Pasifika	6 (2.4)	1 (0.8)	5 (4.0)
Asian	62 (24.5)	29 (22.8)	33 (26.2)
Other	27 (10.7)	12 (9.4)	15 (11.9)
Body mass index (kg/m ²)	29.8 ± 6.0	30.1 ± 6.2	29.6 ± 5.8
Waist circumference (cm)	102.4 ± 15.8	103.0 ± 16.0	101.9 ± 15.7
CKD stage, <i>n</i> (%)			
Stage 3b	83 (32.8)	42 (33.1)	41 (32.5)
Stage 4	170 (67.2)	85 (66.9)	85 (67.5)
Primary cause of kidney disease, <i>n</i> (%)			
Diabetic nephropathy	78 (30.8)	41 (32.3)	37 (29.4)
Hypertension/vascular	42 (16.6)	19 (15.0)	23 (18.3)
Glomerulonephritis	36 (14.2)	21 (16.5)	15 (11.9)
Reflux nephropathy	9 (3.6)	5 (3.9)	4 (3.2)
Polycystic kidney disease	12 (4.7)	4 (3.1)	8 (6.3)
Other	38 (15.0)	17 (13.4)	21 (16.7)
Unknown	38 (15.0)	20 (15.7)	18 (14.3)
Diabetes mellitus, <i>n</i> (%)	113 (44.7)	54 (42.5)	59 (46.8)
Hypertension, <i>n</i> (%)	228 (90.1)	114 (89.8)	114 (90.5)
Dyslipidaemia, <i>n</i> (%)	198 (78.3)	100 (78.7)	98 (77.8)
Cardiovascular disease, <i>n</i> (%)	75 (29.6)	35 (27.6)	40 (31.7)
Peripheral vascular disease, <i>n</i> (%)	21 (8.3)	10 (7.9)	11 (8.7)
Cerebrovascular disease, <i>n</i> (%)	16 (6.3)	9 (7.1)	7 (5.6)
Smoking status, <i>n</i> (%)			
Never	123 (48.6)	65 (51.2)	58 (46.0)
Former	115 (45.5)	57 (44.9)	58 (46.0)
Current	15 (5.9)	5 (3.9)	10 (7.9)

Mean ± SD or number (percentage).

interpretation, the regression coefficients and 95% confidence limits were exponentiated to obtain estimates of the percentage change. Models for CPP-I and CPP-II were repeated after (i) adjusting for medication compliance, (ii) restricting the analysis to participants with at least 80% compliance, (iii) adjusting for CKD stage, age and diabetes. For these analyses, compliance was defined as the number of study follow-up visits for which the participant was taking the randomized treatment.

In further exploratory analyses, we used Pearson correlation to examine the relationship between baseline serum CPP and demographic and biochemical variables. We also examined correlations between baseline CPP and both baseline and change in surrogate cardiovascular markers, as well as change in eGFR.

No adjustments for multiple comparisons were made. All statistical hypothesis testing was two-sided with $P < 0.05$ considered statistically significant. Analyses were performed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA).

RESULTS

Participant characteristics and medication use

A total of 278 participants were enrolled in IMPROVE-CKD. Of these, 253 had CPP data for baseline and at least one follow-up timepoint and were included in this analysis. Participant demographic, biochemical and clinical characteristics at baseline were well-matched between groups (Tables 1 and 2 and Supplementary data, Table S1). Overall, the mean age of the cohort was 62.4 ± 12.6 years, 32.0% were female, 67.2% had stage 4 CKD and 91% were normophosphataemic (serum phosphate ≤ 1.50 mmol/L). At baseline, median serum CPP-I was 14.9×10^4 particles/mL [interquartile range (IQR) 4.6–49.3] and median CPP-II was 3.3×10^3 particles/mL (IQR 1.4–5.4).

There was no significant group difference in the median number of study medication tablets consumed per day (2.69 for placebo; 2.55 for lanthanum) and the proportion of participants using a non-study phosphate binder during the study was low in each group (8.7% for placebo; 7.9% for

Table 2. Biochemical and clinical parameters at baseline

	Overall (<i>n</i> = 253)	Placebo (<i>n</i> = 127)	Lanthanum (<i>n</i> = 126)
Haemoglobin (g/L)	124.1 ± 16.4	123.2 ± 16.4	125.1 ± 16.4
Albumin (g/L)	39.0 ± 4.4	38.7 ± 4.2	39.2 ± 4.6
Bicarbonate (mmol/L)	23.4 ± 3.4	23.5 ± 3.2	23.3 ± 3.7
Urea (mmol/L)	15.9 ± 5.1	15.6 ± 4.8	16.2 ± 5.4
Creatinine (µmol/L)	221.2 ± 59.9	218.9 ± 58.6	223.5 ± 61.4
eGFR (mL/min/1.73 m ²)	26.6 ± 8.3	27.0 ± 8.3	26.2 ± 8.2
Uric acid (mmol/L)	0.5 ± 0.11	0.5 ± 0.1	0.5 ± 0.1
Glucose (mmol/L)	6.3 ± 3.0	6.3 ± 2.8	6.4 ± 3.2
Calcium (mmol/L)	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
Phosphate (mmol/L)	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
ALP (U/L)	88.9 ± 31.3	92.4 ± 30.3	85.4 ± 32.0
Bone specific ALP (µg/L)	14.2 (10.4–18.1)	14.8 (10.9–18.2)	13.3 (9.8–17.9)
PTH (pmol/L)	12.1 (7.2–17.8)	11.8 (7.9–16.4)	12.9 (6.4–19.4)
25-hydroxyvitamin D (nmol/L)	70.3 ± 31.2	68.8 ± 30.0	71.8 ± 32.4
1,25-dihydroxyvitamin D (pmol/L)	77.8 ± 26.8	75.5 ± 26.7	80.3 ± 26.9
C-terminal FGF23 (RU/mL)	222.0 (155.7–334.1)	225.8 (161.0–328.2)	220.7 (151.7–353.1)
Intact FGF23 (pg/mL)	133.0 (89.1–202.0)	136.1 (89.1–202.0)	124.8 (88.9–208.5)
Urinary phosphate excretion (mmol/24 h)	24.1 ± 11.6	25.8 ± 12.9	22.4 ± 9.9
Cholesterol (mmol/L)	4.5 ± 1.1	4.5 ± 1.1	4.6 ± 1.2
Triglyceride (mmol/L)	2.1 ± 1.3	2.0 ± 1.1	2.2 ± 1.5
C-reactive protein (mg/L)	4.8 ± 6.4	4.6 ± 6.3	5.0 ± 6.5
Protein excretion (mg/24 h)	805 (209–1765)	920 (310–1880)	570 (120–1730)
Urine ACR (mg/mmol)	73 (20–138)	86 (23–147)	53 (16–137)
Pulse-wave velocity (m/s)	10.7 ± 3.6	10.8 ± 3.6	10.7 ± 3.5
Pulse wave velocity ≥ 10 m/s	115 (47.7%)	55 (45.5%)	60 (50.0%)
Aortic calcification	174 (80.6%)	81 (76.4%)	93 (84.5%)
Agatston score (HU)	1288 (58–5178)	710 (8–3373)	1975 (257–5744)

Mean ± SD, number (percentage) or median (interquartile range).

eGFR calculated using CKD–Epidemiology Collaboration equation.

ACR, albumin-to-creatinine ratio; ALP, alkaline phosphatase; FGF23, fibroblast growth factor-23; PTH, parathyroid hormone.

lanthanum). The use of cholecalciferol (22.5% overall) and calcitriol (9.1% overall) was similar in each group.

Primary outcome

Summary data for CPP-I and CPP-II at each time point are displayed in Figure 1. After 96 weeks, lanthanum carbonate was not associated with a significant difference in CPP-I [22.8% [95% confidence interval (CI) −39.2, 36.4], *P* = 0.65] or CPP-II [−18.3% (95% CI −40.0, 11.2), *P* = 0.20] compared with placebo (Table 3). Adjustment for medication compliance, participants with at least 80% compliance and for CKD stage, age and diabetes, did not significantly alter the results of these analyses. There was notable variation in the observed distribution of values of CPP-I and CPP-II between sites (Supplementary data, Figure S1), however further adjustment for the study site yielded similar estimates of treatment effect (not shown).

Correlation between CPP and other variables

There were no correlations between serum CPP-I or CPP-II and markers of vascular stiffness (PWV and PWV > 10 m/s) or vascular calcification (AAC Agatston score and binary presence of AAC) at baseline, nor was there any correlation between baseline levels of CPP-I or CPP-II and change in PWV, Agatston score, or eGFR at 96 weeks (Supplementary data, Table S2). There were few correlations between patient

baseline demographics and conventional biochemistry with serum CPP (Supplementary data, Table S3).

DISCUSSION

In this planned exploratory analysis of a large, international, randomized clinical trial, we found that the use of lanthanum carbonate, a non-calcium containing phosphate binder, in patients with stage 3b and 4 CKD was not associated with any significant change in serum CPP-I or CPP-II over 96 weeks. This finding was not altered by adjusting the analysis for medication compliance, or other potential confounders, including CKD stage and diabetes. Our findings suggest that intestinal phosphate binders may not be an effective strategy to lower serum CPP in patients with non-dialysis-dependent CKD.

The lack of effect of lanthanum carbonate on serum CPP is perhaps not unexpected given that in the IMPROVE-CKD trial, lanthanum carbonate was also not associated with a reduction in serum phosphate when compared with placebo [16]. While serum phosphate is arguably an insensitive marker of total phosphate balance, other measures of phosphate metabolism, including 24-h urinary phosphate excretion, serum PTH and serum fibroblast growth factor-23, were also unaltered by lanthanum carbonate in IMPROVE-CKD [16]. In the absence of other measurable effects of lanthanum carbonate on mineral metabolism in this cohort, the lack of

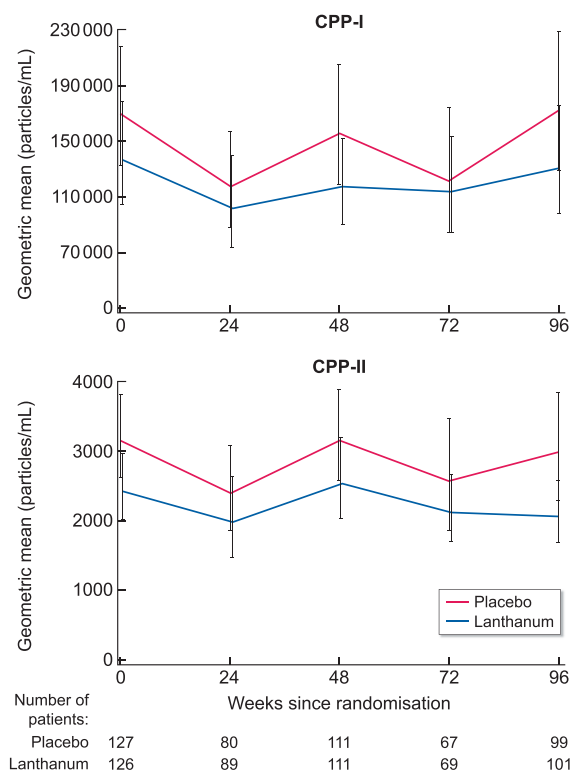


FIGURE 1: Serum primary (CPP-I) and secondary (CPP-II) calciprotein particles over 96 weeks by treatment group (geometric mean and 95% confidence interval).

a treatment effect on CPP would appear consistent with these other null observations.

Our results do, however, contrast with previous smaller and shorter trials in haemodialysis patients, which reported that alterations of phosphate binders were associated with changes in serum CPP, as well as its precursor serum CPM, despite an absence of change in serum phosphate. In an open-label randomized control trial of 31 haemodialysis patients, Smith *et al.* found that sevelamer carbonate was associated with significant reductions in CPP-I compared to calcium carbonate at 24 weeks [14]. In a separate study measuring CPM by gel-filtration method [20], Nakamura *et al.* demonstrated that changing patients from calcium carbonate to lanthanum carbonate resulted in lower levels of CPM in a 16-week crossover study of 24 haemodialysis patients [15].

Notably, though, both previous studies were in the setting of kidney failure requiring dialysis, and participants in these

studies had numerically higher baseline values of conventional markers of mineral metabolism, including serum phosphate, than in our cohort. Additionally, recent evidence has suggested that CPM appears to be predominantly cleared by the kidneys [21]. A reasonable prediction may be that as CKD progresses, CPM and as a result CPP, progressively accumulate. It is plausible that in more severe stages of CKD, as the homeostatic role of the kidneys is progressively lost, that gastrointestinal mineral load may become a more prominent determinant of serum CPP and perhaps also more responsive to therapeutic interventions with phosphate binders.

Another potential explanation for the differing results between IMPROVE-CKD and previous studies by Smith *et al.* and Nakamura *et al.* is that the latter two studies used calcium carbonate as the comparator, rather than a placebo. Given that calcium is a significant component of CPP, it is conceivable that in these studies the exogenous calcium load from calcium carbonate [22] may have contributed to elevated levels of CPP, making it challenging to isolate the effects of intestinal phosphate binding in these studies. Given the divergent results of previous studies and our study, carefully designed future trials are required to separate and characterize the individual effect of phosphate-binding from calcium load, as well as to clarify whether the severity of CKD modifies any effect of phosphate binders on serum CPP.

It is notable that we found a lack of correlation between serum CPP and two surrogate cardiovascular markers. Previous clinical studies suggested a positive correlation between serum CPP (and its precursor CPM) and vascular endpoints in CKD, such as vascular stiffness [5] coronary artery calcification [6, 7] the risk of cardiovascular events [8] and mortality [9]. Additionally, *in vitro* data suggest that CPP directly mediate a range of cellular responses that typify pathological processes in CKD, including expression of inflammatory mediators and calcification [10, 23, 24]. In this regard, it was unexpected that we found no correlation between CPP and markers of arterial stiffness or aortic vascular calcification at baseline, nor was the baseline level of CPP associated with progression of either of these surrogate cardiovascular measures at 96 weeks. Interestingly though, in the overall study cohort, conventional markers of mineral metabolism, including serum phosphate, calcium and PTH, were also not predictive of these measures [18]. We acknowledge that the majority of participants in this study already had evidence of advanced vascular stiffness and calcification at baseline, given a mean carotid-femoral PWV of the cohort greater than 10 m/s and 81% of participants

Table 3. Calciprotein particles (CPP) at baseline and after 96 weeks, with between-group differences

	Baseline		Week 96		Treatment effect versus placebo at Week 96	
	N	Median (IQR)	N	Median (IQR)	% change (95% CI)	P-value
CPP-I ($\times 10^4$ particles/mL)						
Placebo	127	16.3 (6.5–59.7)	99	17.6 (6.0–54.8)	–	–
Lanthanum		12.4 (3.7–48.5)		15.2 (4.6–35.8)	22.8 (–39.2, 36.4)	0.648
CPP-II ($\times 10^3$ particles/mL)						
Placebo	126	3.8 (1.5–5.8)	99	3.5 (1.3–5.8)	–	–
Lanthanum		3.0 (1.3–5.1)		2.6 (1.0–4.0)	–18.3 (–40.0, 11.2)	0.198

Change in calciprotein particles (CPP) compared with baseline, estimated from mixed effects models with repeated measurements. Models were fitted to natural log transformed data in order to ensure normal distribution of residuals; thereafter the regression coefficients have been exponentiated into percentage change to aid interpretation.

with AAC on CT. This relative lack of heterogeneity may have reduced our ability to properly assess the association between serum CPP and arterial stiffening and calcification than in a more diverse population.

There was also a general lack of correlation between serum CPP and other demographic and biochemical markers. We utilized a next-generation flow-cytometric assay, which allows discrimination of CPP from other mineral containing components in blood, as well as separately quantifying CPP-I and CPP-II subtypes [13]. This method uses a rapid freeze protocol and avoids prolonged incubation, minimizing *ex vivo* generation of mineral precipitation artefact, and readouts have been shown to be stable after a freeze–thaw cycle [14]. In addition, *post hoc* analyses did not show any correlation between the storage time of samples and levels of either CPP-I or CPP-II (not shown). We adopted the same collection protocols with centralized batch analysis that has reliably been used in several recent single-centre [14, 25, 26] and multicentre [27] studies. We did observe unexpected variation in the distribution of CPP values between sites. However, there was no suggestion of a centre effect when our main MMRMs were refitted with adjustment for the study site and results were consistent when analyses were restricted to data from the largest study site, which contributed 48 participants (19%) to the overall study cohort (not shown). These additional analyses provide some reassurance that variation in sample collection and processing between sites is unlikely to be the explanation for our null findings, although we acknowledge that this is not possible to definitively exclude in retrospect. Nevertheless, future studies utilizing this assay may help to clarify the relationship of serum CPP with other markers of mineral metabolism, as well as vascular risk, in patients with non-dialysis-dependent CKD.

Strengths and limitations

IMPROVE-CKD was a multi-national randomized controlled trial that, in comparison with other studies assessing serum CPP using the flow-cytometric assay, had large participant numbers and long follow-up. In comparison with previous trials assessing the effect of phosphate binders on serum CPM and CPP, our study was the only trial to use placebo as the comparator and so we were able to exclude potential confounding from exogenous calcium loading.

We acknowledge that this sub-study had several limitations. IMPROVE-CKD was designed to test the effect of lanthanum carbonate on surrogate cardiovascular markers rather than serum CPP and so our analysis is exploratory by nature. Another permutation of the design is that trial medication was titrated against serum phosphate, rather than serum CPP, and so we are unable to answer whether an alternative strategy to titrate lanthanum carbonate directly to serum CPP would have been more effective. However, given that the median number of lanthanum tablets taken by participants was 3 per day, even if higher doses of lanthanum achieved reductions in serum CPP, it would be questionable whether such a therapeutic regimen would be feasible in routine clinical practice.

It is also important to note that blood draws were not standardized to time of day or in relation to meals. It is unclear

whether serum CPP, as measured using the flow cytometric method, is liable to diurnal or post-prandial variation. Data about last food intake and time of sample collection were not routinely collected in this study, and so it was not possible to examine for these possible effects; however, a previous study estimating serum CPP by the earlier fetuin-A ELISA-based method in a small group of diabetic patients suggested that post-prandial spikes may be seen [28]. Finally, we utilized surrogate cardiovascular markers of vascular stiffness and calcification, as assessed by PWV and AAC and it is not clear if serum CPP levels ascertained using this second-generation assay may still hold value in assessing the risk of hard patient outcomes in this population, such as cardiovascular events and death.

CONCLUSION

Our study found that the use of the non-calcium-containing phosphate binder lanthanum carbonate was not associated with any reduction of serum CPP-I or CPP-II in participants with stage 3b or 4 CKD over 96 weeks when compared with placebo. Further, we were unable to confirm the purported relationship between CPP and the surrogate cardiovascular markers of PWV and AAC.

SUPPLEMENTARY DATA

Supplementary data are available at *ndt* online.

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AUTHORS' CONTRIBUTIONS

All authors were involved in study design and concept. M.K.T., E.R.S., E.M.P. and N.D.T. analysed the data. M.K.T. drafted the manuscript; all authors reviewed and edited the manuscript and approved the final version.

CONFLICT OF INTEREST STATEMENT

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ORIGINAL ARTICLE

Adequacy

Effect of a medium cut-off dialyzer on protein-bound uremic toxins and mineral metabolism markers in patients on hemodialysis

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Abstract

Introduction: Hemodialysis (HD) with medium cut-off (MCO) dialyzers may expand molecular clearance, predominantly larger middle molecules (molecular weight 25–60 kDa). However, the impact of MCO dialyzers on long-term clearance of various other components of the uremic milieu is unknown. The tRial Evaluating Mid cut-Off Value membrane clearance of Albumin and Light chains in HemoDialysis patients (REMOVAL-HD) provided an

opportunity to assess the effect of MCO dialyzers on protein-bound uremic toxins and novel markers of mineral metabolism.

Methods: This exploratory sub-study of REMOVAL-HD evaluated changes in protein-bound solutes (total and free indoxyl sulfate [IS] and p-cresyl sulfate [PCS]) and mineral metabolism markers (intact fibroblast growth factor-23 [iFGF23], fetuin-A and endogenous calciprotein particles [CPP-1 and CPP-2]). Mid-week, pre-HD serum samples were collected at baseline and after 12 and 24 weeks of MCO use in stable adult patients. Change from baseline to Week 12 and 24 was estimated using linear mixed effects models.

Findings: Eighty-nine participants were studied (mean age 67 ± 15 years, 38% female, 51% diabetic, median urine output 200 ml/24 h). Serum iFGF23 was reduced at Week 12 compared to baseline (-26.8% [95%CI $-39.7, -11.1$], $p = 0.001$), which was sustained at Week 24 (-21.7% [95%CI $-35.7, -4.5$], $p = 0.012$). There was no significant change in serum IS, PCS, fetuin-A, CPP-1, or CPP-2.

Discussion: The use of a MCO dialyzer over 24 weeks was associated with a sustained reduction in FGF23, while other measured components of the uremic milieu were not significantly altered. Further studies are required to determine whether FGF23 reduction is associated with improved patient outcomes.

KEYWORDS

calciprotein particles, fetuin-A, fibroblast growth factor 23, medium cut-off dialyzer, protein-bound uremic toxins

BACKGROUND

A hallmark of chronic kidney disease (CKD) is the increased concentration of inorganic and organic molecules in circulating plasma.¹ This is initially related to progressive retention of molecules usually excreted in the urine. Secondary abnormalities in homeostatic controls, such as mineral metabolism, also evolve and become significant in the milieu of CKD.^{2,3}

Globally, hemodialysis (HD) remains the principal therapy for kidney failure.⁴ The implicit goal of HD is to correct circulating levels of excess molecules, primarily through dialysis clearance. High flux (HF) dialyzers have become standard in HD and allow rapid clearance of small (<500 Da) water soluble molecules and some of the so-called *small* middle molecules (0.5–25 kDa). Medium cut-off (MCO) dialyzers are a novel class of dialysis membrane with larger pore sizes than HF membranes. The putative advantage of the MCO dialyzer is the permissive passage of an expanded range of molecules to include *large* middle molecules (25–60 kDa), while retaining essential molecules with higher molecular weights, such as albumin (66 kDa).^{5,6} Dialysis may affect the milieu of

circulating molecules directly through clearance or indirectly by addressing the drivers of secondary metabolic abnormalities.⁷ There are limited data on how MCO membranes affect specific subgroups of biologically significant molecules, such as protein-bound uremic toxins and novel markers of mineral metabolism.

Protein-bound uremic toxins, such as indoxyl sulfate (IS) and p-cresyl sulfate (PCS), are a group of molecules that are progressively retained in CKD and thought to mediate deleterious effects.^{3,8} Despite most molecules in this class being of small size (<500 kDa), they are poorly cleared by current dialysis technologies, owing to their protein binding. In advanced stages of CKD, elevated levels of total and free IS and PCS have both been linked to a range of pathological effects, including endothelial damage and cardiovascular disease.^{8–10}

Fibroblast growth factor-23 (FGF23) is a bone-derived phosphate and vitamin-D-regulating hormone that rises early in CKD, with the highest circulating levels seen in patients on dialysis.^{11,12} FGF23 has been independently and positively associated with a range of adverse events, including all-cause mortality in patients with and without CKD.^{12–14} While its role as a direct cellular toxin is

still debated, FGF23 remains of interest as a potential therapeutic target in dialysis patients.^{5,6}

Fetuin-A is a hepatically derived protein, which has a key role in the prevention of ectopic mineralization.¹⁵ Fetuin-A buffers the constant flux of calcium and phosphate into the circulation by forming colloidal protein mineral complexes.¹⁶ Clusters of calcium and phosphate ions bind to fetuin-A, forming calciprotein monomers, which may self-aggregate into 30–100 nm diameter primary calciprotein particles (CPP-1) before transitioning into larger and denser secondary calciprotein particles (CPP-2) with a diameter between 100 and 250 nm.¹⁷ This system frequently becomes dysregulated in CKD. Low serum levels of fetuin-A and elevated levels of CPP have been associated with an increased risk of cardiovascular disease and mortality in dialysis patients.^{18–21} Fetuin-A has a molecular weight of approximately 58 kDa, and so may ostensibly be lost during HD with an MCO dialyzer, although this has not previously been studied. Given the complex and heterogeneous composition of endogenous CPP,²² it is also unclear whether HF and MCO dialyzers may differentially affect levels of CPP in the circulation.

The tRial Evaluating Mid cut-Off Value membrane clearance of Albumin and Light chains in Hemodialysis patients (REMOVAL-HD) was a multicenter device study, primarily examining the safety of MCO dialyzers over 6 months in comparison to baseline use of HF dialyzers.²³ Here, we report the results of a preplanned exploratory analysis of REMOVAL-HD, evaluating the effects of an MCO membrane on the protein-bound uremic toxins IS and PCS, as well as the novel mineral metabolism markers FGF23, fetuin-A, CPP-1, and CPP-2.

MATERIALS AND METHODS

The full protocol and the main results for REMOVAL-HD have been previously published.^{23,24} In brief, REMOVAL-HD was a nonrandomized, single-arm device study involving nine sites across Australia and New Zealand. Consenting adult patients who had been treated with HD for at least 12 weeks and had a functioning arteriovenous fistula or graft were eligible. All participants were either anuric or oliguric (<500 ml/24 h). Key exclusion criteria were anticipated change in dialysis modality, dialysis site or kidney transplant within the study period; presence of chronic infection or inflammatory condition or currently receiving immunosuppressants; life expectancy < 12 months; and serum albumin < 30 g/L within 4 weeks of screening. Ethical approval was obtained from Institutional Ethics Committees for participating sites and REMOVAL-HD was conducted in accordance with the Declaration of Helsinki.

Participants received in-center HD three times per week throughout the study. A standardized HF dialyzer (Revaclear R400; Baxter Healthcare, Sydney, Australia) was used for a 4-week wash-in period, followed by 24 weeks with an MCO dialyzer (Theranova 400; Baxter Healthcare). Dialysis prescription remained the responsibility of the patient's usual care provider throughout the study; however, sites were instructed to maintain the same dialysis prescription for the study duration where possible and to maintain a target blood flow rate > 300 ml/min and a dialysate flow rate of 500 ml/min. Participants received usual care for other CKD and non-CKD related issues.

Outcome measures

Blood samples were collected at baseline after wash-in but before first MCO dialyzer use, and then after 12 and 24 weeks of dialysis with the MCO dialyzer. All blood samples were collected predialysis prior to the mid-week session. Blood for IS, PCS, FGF23, fetuin-A, and CPP was centrifuged and serum aliquots were stored at –80°C until batch analysis. Other biochemistry was performed locally at an accredited laboratory.

Total and free concentrations of the protein-bound uremic toxins IS and PCS were analyzed by ultra-performance liquid chromatography using a fluorescence detection method (Waters Corporation, Milford, MA).²⁵ We measured intact-FGF23 (iFGF23) and fetuin-A using commercial ELISA kits (Kainos Laboratories, Tokyo, Japan and R&D Systems, MN, respectively) according to the manufacturers' instructions. Serum CPP-1 and CPP-2 were measured using a fluorescent probe-based assay running on a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA) as previously described,²⁶ with a minor modification where phosphatidyl serine-binding lactadherin-FITC (Haematologic Technologies Inc., Essex Junction, VT) was substituted for PKH67 reagent.²⁷

Statistical analysis

Baseline characteristics and descriptive data are presented as mean ($\pm$ standard deviation [SD]) or, when skewed, median (interquartile range [IQR]) for continuous variables, and as number and percentage for categorical variables. Shapiro–Wilk test and distribution plots were used to assess normality, and right-skewed data were natural log transformed prior to analysis.

The main outcomes of this secondary analysis were change in predialysis serum levels of total- and free- IS

and PCS, iFGF23, fetuin-A, CPP-1 and CPP-2 after 12 and 24 weeks of dialysis with an MCO dialyzer compared to baseline. For each molecule of interest, a linear mixed effects model was fitted using a restricted maximum likelihood approach. To test for longitudinal change with MCO dialyzer use, time was modeled as a fixed effect factor variable and random intercepts were used for each participant to allow for individual variation and repeated measures. Dunnett's test was used when analyzing each of the measures to correct for the multiple comparisons that occurred when comparing each follow-up time point to the common control baseline. For these models, iFGF23, CPP-1 and CPP-2 were natural log-transformed, to ensure that the residuals were normally distributed. To aid interpretation, the regression coefficients for iFGF23, CPP-1 and CPP-2 were exponentiated to obtain estimates of percentage change. All available data at each time point were used for the main analysis, but models were also refitted using only participants with complete data to assess for sensitivity to missing data.

In further exploratory analyses, correlation between longitudinal change (from baseline to 24 weeks) in mineral metabolism markers and other variables were examined using Pearson correlation for normally distributed and Spearman's rank correlations for skewed data. For these analyses, only participants with data at baseline and 24 weeks were included and no correction for multiple testing was made. All hypothesis tests were assessed at 5% level of significance. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) and R (version 4.0.2). The linear mixed models were fitted in R using the "lmerTest" package.²⁸

RESULTS

The full CONSORT participant flow diagram for REMOVAL-HD has been published elsewhere.²³ Eighty-nine participants completed 4-week wash-in with the standardized HF dialyzer and commenced treatment with the MCO dialyzer. Participant demographics are listed in Table 1. The mean age was 67 ± 15 years, 38% were female and 51% were diabetic. Forty-five percent of participants were anuric, and among oliguric participants, the median urine output was 200 ml/day with an IQR of 130–300 ml/day.

All available samples from each of the 89 participants were included in the analysis. Baseline values for protein-bound uremic toxins, FGF23, fetuin-A, and CPP are displayed in Table 2, and summary data at each time-point are shown in Figure 1. For each analyte, previously reported serum concentrations in apparently healthy

TABLE 1 Participant demographics and baseline characteristics

Participant characteristic (n = 89)	
Sex	
Female	34 (38)
Age (yrs)	67 $\pm$ 15
Ethnicity	
Caucasoid	66 (74)
Aboriginal or Torres Strait Islander	1 (1)
New Zealand Māori	8 (9)
Pacific Islander	4 (4.5)
Asian	6 (7)
Other	4 (4.5)
Body mass index (kg/m ²)	28.8 $\pm$ 5.8
Primary cause of renal disease	
Diabetic nephropathy	33 (37)
Hypertension/vascular	13 (15)
Glomerulonephritis	10 (11)
Reflux nephropathy	3 (3)
Polycystic kidney disease	6 (7)
Other	19 (21)
Unknown	5 (6)
Urine output	
Anuric	40 (45)
Oliguric (<500 ml/day)	49 (55)
Residual urine volume (n = 49) (ml/24 h)	200 [130–300]
Diabetes mellitus	45 (51)
Ischemic heart disease	21 (24)
Smoking (current or former)	46 (52)

Note: Mean $\pm$ standard deviation, median [interquartile range] or number (%).

adults, and measured using the same assays as in this study, are also provided in Table 2 for reference.

Protein-bound uremic toxins

There were no significant changes in total or free levels of IS or PCS after 12 or 24 weeks of MCO use compared to baseline (Table 3).

Intact FGF23

There was a significant reduction in predialysis iFGF23 between baseline and 12 weeks (-26.8% [95% CI $-39.7, -11.1$], adjusted $p = 0.001$) which was

TABLE 2 Baseline serum concentrations of study analyte

Analyte	Baseline mean or median	Previously reported values in healthy adults for assay ^a
Indoxyl sulfate (n = 86)		
Total (μmol/L)	138 [96–185]	0.7–6.3 ²⁵
Free (μmol/L)	11 [7–16]	0.0–0.2 ²⁵
p-cresyl sulfate (n = 86)		
Total (μmol/L)	209 [121–314]	0.0–38.4 ²⁵
Free (μmol/L)	13 [5–22]	0.1–2.4 ²⁸
Intact FGF23 (n = 87) (pg/ml)	3985 [1414–18,362]	42.0 ± 9.9 ²⁹
Fetuin-A (n = 86) (mg/L)	511 ± 148	303–671 ³⁰
CPP-1 (n = 87) – ×10 ⁵ particles/ml	8.2 [3.7–13.9]	2.2–17.3 ²⁶
CPP-2 (n = 87) – ×10 ⁴ particles/ml	4.1 [1.9–6.6]	1.7–28.2 ²⁶

Note: Mean ± standard deviation, median [interquartile range], or range minimum-maximum.
Abbreviations: CPP, calciprotein particles; FGF23, fibroblast growth factor-23.
^aValues are serum concentrations previously reported using the same assay for each analyte in apparently healthy adults.

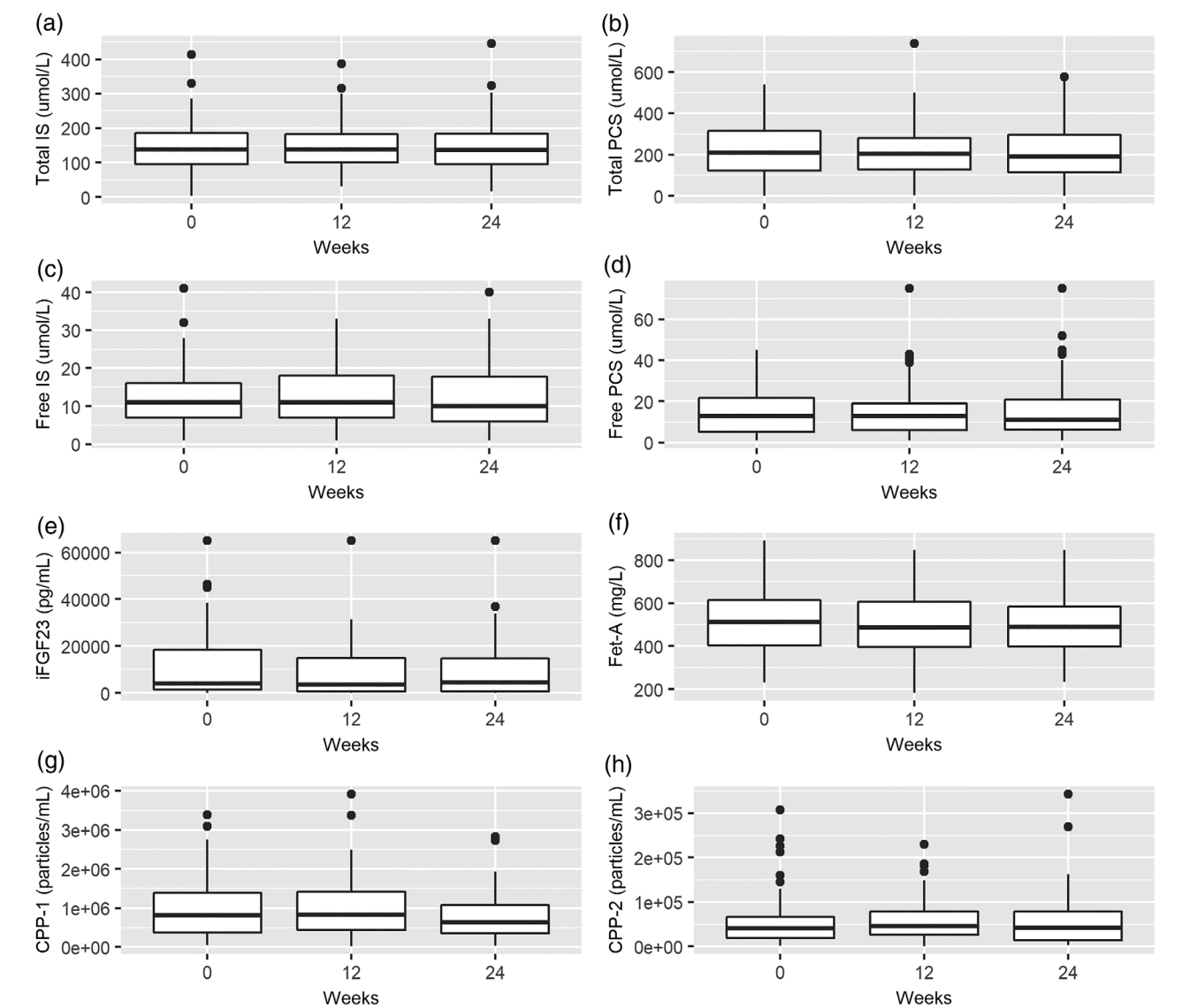


FIGURE 1 Box plot of study molecules before first MCO use and after 12 and 24 weeks. Box shows median and interquartile range. CPP, calciprotein particles; Fet-A, fetuin-A; iFGF23, intact fibroblast growth factor-23; IS, indoxyl sulfate; PCS, p-cresyl sulfate

TABLE 3 Change in protein-bound uremic toxins and mineral metabolism markers

Baseline			Week 12			Week 24				
Analyte	n	Mean or median	n	Mean or median	Change from baseline (95% CI) ^a	Change at week 12 <i>p</i> -value ^b	n	Mean or median	Change from baseline (95% CI) ^a	Change at week 24 <i>p</i> -value ^b
Protein bound uremic toxins										
Total IS (μmol/L)	86	138 [96–185]	82	138 [101–183]	–2.6 (–18.4, 13.3)	0.91	78	136 [96–185]	2.3 (–13.8, 18.4)	0.93
Free IS (μmol/L)	86	11 [7–16]	82	11 [7–18]	0.3 (–1.4, 2.0)	0.92	78	10 [6–18]	0.2 (–1.6, 1.9)	0.97
Total PCS (μmol/L)	86	209 [121–314]	82	203 [128–280]	–11.5 (–39.5, 16.4)	0.56	78	191 [114–297]	–13.1 (–41.5, 15.3)	0.49
Free PCS (μmol/L)	86	13 [5–22]	82	13 [6–19]	–0.4 (–2.9, 2.1)	0.92	78	11 [6–21]	–0.4 (–2.9, 2.1)	0.92
Mineral metabolism markers										
iFGF23 (pg/ml)	87	3985 [1414 – 18,362]	83	3594 [688–14,888]	–26.8% (–39.7, –11.1)	0.001	79	4526 [844–14,657]	–21.7% (–35.7, –4.5)	0.012
Fetuin-A (mg/L)	86	511 ± 148	80	500 ± 155	–11.0 (–47.8, 25.8)	0.73	77	496 ± 142	–7.2 (–44.5, 30.2)	0.88
CPP-1 (×10 ⁵ particles/ml)	87	8.2 [3.8–13.9]	83	8.2 [4.5–14.2]	9.1% (–14.0, 38.3)	0.63	78	6.4 [3.6–10.8]	–16.0% (–34.0, 7.1)	0.20
CPP-2 (×10 ⁴ particles/ml)	87	4.1 [1.9–6.6]	83	4.6 [2.6–7.9]	11.4% (–17.6–50.7)	0.64	79	4.2 [1.3–7.8]	–6.4% (–31.1, 27.2)	0.85

Note: Mean ± standard deviation or median [interquartile range].

Abbreviations: CI, confidence interval; CPP, calciprotein particles; iFGF23, intact fibroblast growth factor-23; IS, indoxyl sulfate; PCS, p-cresyl sulfate.

Bold values are statistically significant (*p*-value < 0.05).

^aChange in analytes compared to baseline, estimated from linear mixed model. For iFGF23, CPP-1, and CPP-2, linear models were fitted to natural log transformed data in order to ensure normal distribution of residuals; thereafter the regression coefficients have been exponentiated into percentage change to aid interpretation.

^bAdjusted *p*-value using Dunnett's post estimation test.

TABLE 4 Correlation between longitudinal change (baseline to 24 weeks) in novel mineral markers and selected variables

	Change in iFGF23		Change in fetuin-A		Change in CPP-1		Change in CPP-2	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Change in fetuin-A	−0.08	0.481	—	—	—	—	—	—
Change in CPP-1	0.37	0.001	−0.07	0.563	—	—	—	—
Change in CPP-2	0.33	0.004	−0.24	0.038	0.38	0.001	—	—
Change in albumin	0.01	0.913	0.10	0.387	0.08	0.491	−0.13	0.261
Change in urea reduction ratio	−0.17	0.182	0.07	0.603	−0.03	0.807	−0.09	0.470
Change in hemoglobin	−0.11	0.356	0.04	0.767	−0.17	0.141	0.10	0.381
Change in hsCRP	0.08	0.471	0.07	0.582	0.01	0.929	0.00	0.973

Note: Urea reduction ratio = (predialysis urea − postdialysis urea)/predialysis urea × 100%.
Abbreviations: CPP, calciprotein particles; hsCRP, high sensitivity C-reactive protein; iFGF23, intact fibroblast growth factor-23.
Bold values are considered statistically significant (*p*-value < 0.05).

sustained at 24 weeks (−21.7% [95% CI −35.7, −4.5] adjusted *p* = 0.012) (Table 3).

Linear mixed models were refitted with complete cases (*n* = 76) to examine the effect of missing data. This sensitivity analysis restricted to complete cases did not change the main finding of reductions in serum iFGF23 levels at 12 and 24 weeks of MCO use (−27.4% [−41.0, −10.8] adjusted *p* = 0.001 and −21.8% [−36.4, −3.9] adjusted *p* = 0.016, respectively).

Fetuin-A and calciprotein particles

There were no significant changes in predialysis serum fetuin-A, CPP-1 or CPP-2 concentrations between baseline and Week 12 or baseline and Week 24 (Table 3).

Correlations of mineral metabolism markers with selected variables

The correlations between change in novel mineral metabolism markers and other variables were examined for participants who had data available at both baseline and 24 weeks (Table 4). There was no longitudinal correlation between any of the novel mineral metabolism markers and predialysis serum albumin, urea reduction ratio, hemoglobin or high-sensitivity C-reactive protein (hsCRP).

There were moderate positive correlations between iFGF23 and both CPP-1 (*r* = 0.37, *p* = 0.001) and CPP-2 (*r* = 0.33, *p* = 0.004). Fetuin-A negatively correlated with CPP-2 (*r* = −0.24, *p* = 0.038) but not CPP-1 (*r* = −0.07, *p* = 0.563). There was also a moderate correlation between CPP-1 and CPP-2 (*r* = 0.38, *p* = 0.001).

DISCUSSION

We report that the use of a MCO dialyzer over 24 weeks was associated with a sustained reduction in serum concentrations intact FGF23 in patients on HD, while other measured components of the uremic milieu were not significantly altered.

Despite advances in dialysis technology and techniques, patients requiring dialysis for kidney failure continue to experience significant excess morbidity and mortality compared to those without CKD.⁴ Part of this increased risk may be attributable to retained molecules that are incompletely cleared by current therapies and may directly mediate toxicity or indirectly drive disordered metabolic processes, such as abnormalities in mineral metabolism. MCO dialyzers offer the putative advantage over current HF dialyzers of expanding the range of molecules cleared during HD. Clinical efficacy studies of MCO dialyzers have mainly examined a selection of molecules, which are either thought to be clinically significant, or are chosen to give a representative spread of molecule weights.^{23,31–35} These studies are used to inform predictions about how other molecules with similar physiochemical properties may be affected by a MCO dialyzer. However, given the complex, and often inter-related, milieu of abnormalities in CKD, it is important to test these predictions, particularly for molecules that may have significant biological and therapeutic implications.

We report that continuous MCO dialyzer use was associated with a sustained reduction in predialysis serum iFGF23 concentration. Intact FGF23 is a middle molecule, with an approximate molecular weight of 32 kDa, and would be expected to be cleared more efficiently through dialysis with a MCO than HF membrane. We did not directly measure FGF23 in spent dialysate

and so were not able to confirm that clearance was the mechanism behind this observed reduction. There are limited previous data on circulating FGF23 comparing relative effects of HD with a MCO and a standard HF dialyzer. Previous studies by Belmoaz et al. and by Kim et al. have suggested that a MCO dialyzer is associated with a higher reduction ratio of FGF23 compared to a HF dialyzer, implying removal through MCO use.^{36,37} Of note though, neither study demonstrated a reduction in predialysis levels. In addition, the trend toward increased reduction ratio in the study by Kim et al did not reach statistical significance; however, this was a small study (six patients) and used lower blood flow rates (250 ml/min), both of which may have contributed to the equivocal result. This study also used a C-terminal FGF23 assay, in contrast to the intact assay in our study. MCO membranes are designed to improve clearance of larger middle molecules (>25 kDa). Given that the C-terminal assay measures both intact FGF23 and the smaller C-terminal fragment (12 kDa), use of this assay may be less discriminatory between these membrane types, although in patients receiving dialysis virtually all circulating FGF23 is thought to be intact.^{38,39}

Hypothetically, the observed reduction in FGF23 with MCO dialyzer use could be due to mechanisms apart from direct clearance. For instance, phosphate balance is known to correlate with FGF23 production,⁴⁰ and we did not measure serum phosphate or phosphate clearance in this cohort. However, it is worth noting that previous studies have suggested there is minimal, if any, difference between MCO and HF dialyzer clearance of small solutes, including phosphate.³³ We observed a correlation between FGF23 and CPP, which has previously been demonstrated.^{20,26,41} It has also recently been shown that CPP induces FGF23 expression in osteoblasts.⁴² However, given there was no significant longitudinal change in CPP-1 or CPP-2 in this study, it is less likely that a link between FGF23 and CPP explains the reduction in FGF23.

FGF23 has consistently and positively been linked to adverse outcomes^{12–14} and in vitro studies have demonstrated biologically plausible mechanisms of cardiovascular toxicity.^{43,44} Taken together, this adds weight to the assertion that elevated levels of FGF23 in CKD may directly mediate adverse outcomes and makes FGF23 a potential therapeutic target in patients on dialysis. However, previous studies have shown mixed benefits of interventions associated with FGF23 reduction,^{45,46} and further studies are required to determine whether the reductions with MCO dialyzer use in this study will result in improved patient outcomes. Indeed, the percentage reductions in intact FGF23 observed here are only

modestly greater than the biological variation of this hormone in healthy adults.³⁹

We found that MCO dialyzer use was not associated with a change in predialysis levels of the protein-bound uremic toxins IS or PCS. Both IS and PCS are predominantly albumin-bound,⁴⁷ and previous models of extracorporeal removal have suggested that their clearances are directly related to the free fractions in circulation.⁴⁸ It is conceivable that if dialysis membrane types vary in albumin loss, then this could indirectly affect clearance of these compounds. Albumin loss could have the dual effects of removing IS and PCS bound to lost albumin and also reducing the circulating pool of albumin thereby increasing the free fraction available for diffusion across a dialysis membrane. Given that we did not observe a decrease in albumin in this study,²³ it is not unexpected that IS and PCS levels were also unchanged.

Pore size is a key determinant of the range of molecules that can be cleared by a dialysis membrane. MCO dialyzers are specifically manufactured to have a distribution of pore sizes that allow passage of larger middle molecules (25–60 kDa) while restricting loss of albumin (66 kDa).^{5,49} Fetuin-A (58 kDa) has a beneficial role in the prevention of ectopic mineralization, and given that it has a molecular weight within the upper limit of the cut-off range of a MCO dialyzer, it is reassuring that predialysis levels were unchanged across the study period. The large diameter of CPP (50–100 nm for CPP-1 and 100–250 nm for CPP-2), mean it was unlikely that there would be direct clearance by an MCO membrane (effective pore size 3.0–3.5 nm),⁴⁹ and so it is unsurprising that levels were unchanged. However, given the complex composition²² and metabolic pathways⁵⁰ of CPP, it is useful to have empiric evidence to confirm this prediction.

As discussed above, one limitation of this study is that we did not directly measure clearance of the study molecules in spent dialysate, and so can only speculate on mechanisms of change (or lack thereof). However, change in serum levels better reflects time-averaged exposure to the molecule of interest, as it accounts for the effects of intercompartmental sequestration and interdialytic generation.¹ This arguably makes predialysis concentrations a more biologically relevant metric than the kinetics of dialysis clearance in isolation. It is also important to note that while this was a preplanned sub-study, it is in essence an exploratory analysis, and so caution is required when interpreting results. Specifically, in regards to the main finding of a significant reduction in intact FGF23, while we did adjust for multiple hypothesis testing at Week 12 and Week 24, we did not adjust for multiple hypothesis tests across the various molecules

and so we cannot exclude that this result was due to chance. Finally, this was a study of surrogate markers, and while each has a body of evidence supporting biological relevance, only larger long-term outcome studies can properly examine the potential clinical benefits of HD with a MCO dialyzer.

CONCLUSION

This study found that HD with a MCO dialyzer was associated with a sustained reduction of intact FGF23 in serum compared to a HF membrane. There were no changes in other markers of mineral metabolism, including no evidence of loss of the beneficial protein fetuin-A. MCO dialyzer use did not appear to have a significant impact on serum concentrations of protein-bound uremic toxins. Whether the reduction in intact FGF23 is associated with improved patient outcomes requires further assessment in future trials.

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CONFLICT OF INTEREST

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Serum Calciprotein Monomers and Chronic Kidney Disease Progression

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Keywords

Calciprotein monomers · Calciprotein particles · Chronic kidney disease · Mineral metabolism disorders

Abstract

Introduction: Elevated levels of fibroblast growth factor-23 (FGF23) in chronic kidney disease (CKD) are associated with progression of CKD. FGF23 inhibits proximal tubular phosphate reabsorption, raising phosphate concentrations in the tubular fluid of functioning nephrons, predisposing to spontaneous precipitation of calcium phosphate crystals and resultant tubular injury. Calciprotein monomers (CPM) form spontaneously in biological fluids when clusters of calcium phosphate ions are bound by the liver-derived glycoprotein fetuin-A. Serum CPM are elevated in CKD and are postulated to trigger FGF23 secretion. CPM are also readily filtered at the glomerulus into the tubular fluid, suggesting that higher CPM levels could be associated with progression of CKD via FGF23-mediated increased phosphate load but also through direct effects in the proximal tubule. **Methods:** ACADEMIC was a prospective observational study of 200 stable outpatients with CKD stages 3 and 4. Participants were followed until commencement of dialysis or death. In this study, we examined a sub-cohort of 189 participants who had baseline serum available for measurement of CPM. Cox proportionate

hazard regression models were used to examine the association between CPM and a composite kidney disease outcome (commencement of dialysis or reduction in estimated glomerular filtration rate [eGFR] >30%). Linear regression models were used to examine the association between CPM and annualized eGFR slope. **Results:** Relative to the lowest tertile, the highest tertile of CPM was associated with increased risk of the composite kidney disease outcome in univariate models and after sequential adjustment for conventional risk factors for progression of CKD (adjusted hazard ratio 4.22; 95% confidence interval [CI] 1.91, 9.33, $p < 0.001$). Natural log-transformed CPM was also inversely associated with eGFR slope in univariate and multivariate adjusted models (adjusted β -coefficient -1.66 , 95% CI: -3.10 , -0.22 , $p = 0.024$). In exploratory mediation analysis, the association between serum CPM and eGFR slope was partially mediated by iFGF23; however, the majority of the association was direct and independent of the iFGF23 pathway. **Conclusion:** Elevated levels of CPM are associated with the progression of CKD. This association was partially mediated via FGF23, consistent with recent evidence that FGF23 predisposes to spontaneous precipitation of calcium phosphate crystals leading to tubular injury. However, serum CPM also appeared to have a direct association with eGFR slope, raising the possibility that CPM may also be associated with progression of CKD through additional pathways.

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Introduction

Mineral metabolism disorders are a classical feature of chronic kidney disease (CKD). Elevated serum levels of phosphate and, its principal regulatory hormone, fibroblast growth factor-23 (FGF23) are almost ubiquitous by the time individuals reach kidney failure [1, 2] and are consistently associated with an increased risk of adverse patient outcomes, including cardiovascular events and mortality [3–5]. Emerging evidence has suggested that these associations may in part be mediated via increased exposure to calciprotein particles (CPP) [6], with higher levels also linked to an increased risk of cardiovascular disease [7–11] and mortality [8].

CPP are endogenous mineral-protein colloids that are mainly composed of solid-phase calcium phosphates and the liver-derived mineral-binding glycoprotein fetuin-A (see www.karger.com/doi/10.1159/000526609 for all online suppl. material). In vitro studies have revealed that CPP are formed through a series of ordered steps. This begins with the spontaneous formation of clusters of calcium phosphate ions, which readily bind to fetuin-A [12]. These mineral-laden fetuin-A molecules are referred to as calciprotein monomers (CPM), which can consolidate into rounded primary CPP (CPP-I), before eventually transforming into larger, denser secondary CPP (CPP-II), containing mineral as hydroxyapatite [13]. The formation of CPM and CPP appears to have a role in stabilizing bulk quantities of mineral in extracellular fluid for transport to sites of use or disposal. Circulating CPM and CPP levels rise acutely following a meal, while serum phosphate (sPi) and calcium remain relatively stable, suggesting that a principal physiological function of these mineral-protein colloids is to buffer acute mineral loads derived from the diet [14]. Since CPM may also stimulate phosphaturia via induction of osteocytic FGF23 expression [15], CPM provide a plausible link between dietary mineral and endocrine mineral regulation in the kidney.

Apart from cardiovascular outcomes, there is emerging interest in the role that disordered mineral metabolism may contribute to the progression of CKD itself. In epidemiological studies, elevated levels of phosphate [16–18] and FGF23 [4, 19, 20] have been associated with increased risk of kidney function decline. It has been suggested in small clinical studies [21, 22] and in CKD animal models [23–25] that the association between phosphate exposure and progression of CKD may be specifically related to a proportionate increase in filtered phosphate load in the kidney, relative to the reduced number of functional nephrons. Recently, Shiizaki et al.

[20] proposed that these observations may be explained by the spontaneous precipitation of calcium phosphate crystals in the tubular fluid, which may then induce cell dysfunction and interstitial inflammation, culminating in tubular damage. The propensity to form tubular crystals appeared to be related to the concentration of phosphate in the proximal tubular fluid, higher levels of which were found to predict progression of CKD in mice and humans. Although Shiizaki et al. [20] suggested that calcium phosphate crystals form in situ in the tubular fluid, another possibility is that this mineral derives partly from circulating CPM (8.4 nm), which unlike much larger CPP-I (30–100 nm) and CPP-II (100–250 nm) are reported to be readily filtered at the glomerulus [6, 26]. Consistent with this, post-prandial rises in CPM are higher in CKD patients compared to age-matched controls without CKD, with the magnitude of the rise inversely correlated with estimated glomerular filtration rate (eGFR) [14].

To further examine the potential relationship between CPM and the progression of CKD, we analyzed data from the ACADEMIC study [8], a prospective observational study of 200 stable outpatients with CKD stages 3 and 4 who were followed prospectively until time of death or commencement of kidney replacement therapy. We hypothesized that higher serum levels of CPM would be associated with increased risk of a progression to dialysis and faster decline in eGFR.

Materials and Methods

Study Population and Follow-Up

Full methodology of the ACADEMIC cohort has previously been published [8]. In brief, participants were recruited from nephrology outpatient clinics at Brighton and Sussex University Hospitals NHS Trust between March 2006 and September 2009. To be eligible, participants had to be between the age of 40 and 90 years and have CKD stages 3 and 4 at the time of recruitment. The study was approved by the West Sussex Research Ethics Committee [REC Ref.# 05/Q1911/89], and patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Follow-up time was accrued from the time of recruitment until the commencement of kidney failure, death, or the end of the study (1 November 2012), whichever occurred first. Participant status was gathered prospectively via biannual study visits and hospital electronic medical records.

Exposure Assessment

The primary exposure analyzed in this study was serum CPM. Blood samples were collected at baseline study visit using standard venepuncture techniques. Samples were centrifuged after being allowed to clot, and serum samples were stored at -70°C until batched analysis. Serum CPM was measured by gel filtration meth-

od as previously described [27], with minor modifications [14]. This assay uses a fluorescently labeled bisphosphate derivative (OsteoSense 680EX, Perkin Elmer) to detect solid-phase calcium phosphate crystals formed from CPM and other small (<50 nm) low-density fetuin-A-mineral aggregates that remain in serum after sedimentation by high-speed centrifugation (30,000 g, 2 h). For simplicity, we refer to them here as CPM. The mean inter-assay analytical coefficient of variation (CV) for the CPM assay was 4.9%.

Covariate Assessment

Assessment of demographics and baseline clinical characteristics were recorded at study entry. Blood and urine samples were collected at study entry for standard biochemical measurements, which were performed at the time of collection by the hospital's accredited pathology service. eGFR was calculated using the CKD-Epidemiology Collaboration equation.

Serum intact-FGF23 (iFGF23) was measured using a commercial ELISA from KAINOS Laboratories (Tokyo, Japan), with an inter-assay CV of <5%. Serum and urine fetuin-A was measured using ELISA kits from BioVendor (Brno, Czech Republic), and inter-assay CVs were <3.5% and <6.5%, respectively. Urine neutrophil gelatinase-associated lipocalin (uNGAL) was measured using the particle-enhanced turbidimetric immunoassay as previously described with an inter-assay CV of <3.5% [28].

Urinary fetuin-A and uNGAL were each normalized to the urine creatinine concentration. Estimated proximal tubular phosphate (ePTFp) was calculated based on measured sPi and urinary fractional excretion of phosphate (FEp) using the equation ($ePTFp = sPi \times FEp \times 3.33$) proposed by Shiizaki et al. [20], which assumes that 70% of the filtered fluid fraction is reabsorbed along the proximal tubule [29].

Study Outcomes

We examined progression of kidney disease using a composite kidney disease outcome and by analyzing eGFR slope. The composite kidney disease outcome was defined as commencement of kidney replacement therapy or drop in eGFR >30% from study entry (as suggested by Coresh et al. [30]). eGFR slope was defined as average change in eGFR per year [31], using all available measures of eGFR taken after enrolment in the study, including from biannual study visits and from hospital electronic medical records (excluding those recorded during an acute illness).

Statistical Analysis

Baseline characteristics of the cohort are summarized across CPM tertile, using mean and standard deviation for normally distributed, or median and interquartile range (IQR) for skewed data, respectively. Categorical data are presented as number and percentages. *p* value for trend in baseline characteristics across CPM tertiles was estimated using linear regression, with CPM tertile as an ordinal variable.

Cox proportionate hazard models were used to examine the relationship between the serum CPM and the composite kidney disease outcome. Serum CPM was categorized into tertiles, given that visual inspection of Martingale residuals suggested that the association between serum CPM and outcome was nonlinear. An unadjusted and three hierarchical multivariable adjusted models (Models 1–3) were created. The multivariate models were sequentially adjusted for participant demographics and for conventional

risk factors for CKD progression which were defined a priori, based on known clinical associations. For Model 1, we adjusted for participant age and sex. For Model 2, we adjusted for the covariates in Model 1 plus eGFR and urinary albumin-to-creatinine ratio (uACR). For Model 3, we adjusted for the covariates in Model 2 plus cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medication use (use of an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, beta blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker). Interaction terms between CPM tertile and covariates were assessed, and Schoenfeld residuals were used to assess the proportionate hazard assumption.

Univariate and multivariate linear regression models were used to examine the relationship between serum CPM and eGFR slope. The multivariate regression models were sequentially adjusted for the same covariates as the Cox models. Serum CPM, as well as other skewed covariates, were natural log-transformed before fitting these models, and we used robust confidence interval (CI) estimates to account for heteroskedasticity of residuals. For the multivariate models, collinearity was assessed using the variance inflation factor.

Additional Analyses

In the unadjusted and multivariate adjusted Cox models, participants were censored at time of death or end of study follow-up (whichever occurred first). Because death precludes progression of CKD, we also performed a competing risk analysis for the composite kidney disease outcome using the Fine and Gray method [32]. For this analysis, death was the competing event and the model was adjusted for the same covariates as used in Model 3.

In our main Cox and linear regression models, we did not adjust for conventional markers of mineral metabolism (serum calcium, phosphate, and parathyroid hormone), given that each is potentially involved in the hypothesized pathway. We performed a sensitivity analysis where these variables were included in addition to the covariates in Model 3.

In our main analyses, we found that serum CPM was associated with increased risk of the composite kidney disease outcome and also greater eGFR decline. Given the hypothesis that CPM may be related to kidney outcomes via the pathway proposed by Shiizaki et al., we also performed a mediation analysis to investigate whether the association between CPM and eGFR slope was explained by FGF23 and ePTFp. To do this, we constructed a simplified directed acyclic graph (online suppl. Fig. 2) with serum CPM as the independent variable, eGFR slope as the dependent variable, and iFGF23 and ePTFp as the mediators. We modeled for the potential confounding effect of baseline eGFR on serum CPM, iFGF23, ePTFp, and eGFR slope as well as the potential indirect effect of baseline eGFR on eGFR slope via uACR. For the mediation analysis, we naturally log-transformed skewed variables before estimating linear coefficients, and 95% CIs were bias corrected using bootstrapping with 2,000 resamples. Where variables were significantly associated with eGFR slope via direct and indirect pathways, we also calculated the proportion of the association that was directly mediated.

Two-tailed *p* values <0.05 were considered significant. All data were analyzed using Stata/MP version 17.0 (StataCorp, College Station, USA). Graphics were produced using the same software.

Table 1. Baseline participant clinical and biochemical characteristics

Participant characteristic	Overall (n = 189)	Tertile 1 (n = 63)	Tertile 2 (n = 63)	Tertile 3 (n = 63)	p value for trend
Serum CPM – $\times 10^3$ AU/mL	26.6 [15.8–51.1]	12.6 [8.9–15.8]	26.6 [22.6–31.5]	66.7 [51.1–92.7]	–
Age, years	69.1 $\pm$ 11.3	69.9 $\pm$ 10.9	69.8 $\pm$ 11.3	67.5 $\pm$ 11.7	0.228
Female sex	52 (27.5)	14 (22.2)	21 (33.3)	17 (27.0)	0.552
Baseline comorbidities					
Cardiovascular disease	81 (57.1)	28 (44.4)	28 (44.4)	25 (39.7)	0.591
Diabetes mellitus	45 (23.8)	12 (19.1)	16 (25.4)	17 (27.0)	0.298
Smoking history – pack years among smokers	25 [7–40] (n = 115)	33 [8–40] (n = 41)	20 [7–40] (n = 42)	26 [7–43] (n = 32)	0.297
Systolic blood pressure, mm Hg	152 $\pm$ 21.5	150 $\pm$ 20.4	154 $\pm$ 20.4	151 $\pm$ 23.8	0.782
Diastolic blood pressure, mm Hg	81 $\pm$ 11.3	82 $\pm$ 11.5	82 $\pm$ 11.1	81 $\pm$ 11.3	0.777
Body mass index, kg/m ²	29.4 $\pm$ 5.6	29.7 $\pm$ 5.1	29.4 $\pm$ 6.4	29.2 $\pm$ 5.4	0.587
Baseline biochemical parameters					
eGFR ¹ , mL/min/1.73 m ²	32.7 $\pm$ 10.8	35.6 $\pm$ 10.4	34.9 $\pm$ 9.8	27.6 $\pm$ 10.5	<0.001
Hemoglobin, g/L	127 $\pm$ 16.8	133 $\pm$ 17.2	128 $\pm$ 16.6	120 $\pm$ 14.4	<0.001
Serum albumin, g/L	43 $\pm$ 3.2	43 $\pm$ 3.2	43 $\pm$ 2.8	42 $\pm$ 3.6	0.105
uACR, mg/mmol	5.3 [1.7–30.5]	3.1 [1.0–16.1]	5.1 [1.3–34.9]	12.5 [3.0–48.2]	0.001
Urinary protein-to-creatinine ratio, mg/mmol	27.2 [13.2–64.7]	21.9 [11.4–42.0]	18.9 [11.3–47.1]	40.7 [22.1–101.7]	<0.001
Calcium (adjusted) ² , mmol/L	2.29 $\pm$ 0.12	2.30 $\pm$ 0.11	2.30 $\pm$ 0.11	2.28 $\pm$ 0.13	0.327
Phosphate, mmol/L	1.08 $\pm$ 0.20	0.93 $\pm$ 0.14	1.09 $\pm$ 0.14	1.22 $\pm$ 0.20	<0.001
Parathyroid hormone, ng/L	76 [50–118]	70 [49–105]	67.5 [50–95]	103 [57–161]	0.002
iFGF23, pg/mL	53.6 [35.4–83.9]	40.9 [25.7–63.0]	46.4 [34.3–70.6]	71.7 [53.9–113.3]	<0.001
uFet-A/creat ratio, μ g/mmol	106.4 [43.7–271.2]	66.2 [23.0–154.8]	98.7 [28.8–342.5]	168.4 [70.7–376.9]	0.004
Urinary fractional excretion of calcium, %	1.7 [1.2–2.2]	1.6 [1.2–2.0]	1.6 [1.1–2.2]	1.8 [1.3–2.7]	0.015
Urinary FE _p , %	29.8 [21.6–40.3]	25.9 [19.6–34.7]	26.6 [20.7–33.8]	39.9 [25.8–53.0]	<0.001
ePTFP ³ , mmol/L	1.01 [0.74–1.45]	0.81 [0.56–1.04]	1.00 [0.80–1.25]	1.77 [0.99–2.22]	<0.001
C-reactive protein, mg/L	2.27 [0.94–5.63]	1.73 [0.76–3.56]	2.68 [1.01–8.01]	2.52 [1.05–5.91]	0.043
Serum fetuin-A, mg/L	209 $\pm$ 63.0	226 $\pm$ 67.6	200 $\pm$ 55.8	200 $\pm$ 62.4	0.018
uNGAL/creatinine ratio, μ g/mmol	1.7 [0.6–4.3]	1.3 [0.6–2.6]	1.6 [0.4–2.3]	4.4 [1.1–14.6]	<0.001
Baseline use of medications related to mineral metabolism					
Cholecalciferol	7 (3.7)	3 (4.8)	4 (6.4)	0 (0.0)	0.159
Calcitriol or alfacalcidol	15 (7.9)	6 (9.5)	1 (1.6)	8 (12.7)	0.512
Bisphosphonate	13 (6.9)	4 (6.4)	5 (7.9)	4 (6.4)	1,000
Calcium supplement or calcium-based phosphate binder	14 (7.4)	5 (7.9)	3 (4.8)	6 (9.5)	0.735
Noncalcium-based phosphate binder	3 (1.6)	1 (1.6)	1 (1.6)	1 (1.6)	1,000
Baseline use of other medications					
Angiotensin-converting enzyme inhibitor or angiotensin receptor blocker	124 (65.6)	45 (71.4)	34 (54.0)	45 (71.4)	1,000
Beta blocker	62 (32.8)	25 (39.7)	18 (28.6)	19 (30.2)	0.257
Calcium channel blocker	88 (46.6)	26 (41.3)	29 (46.0)	33 (52.4)	0.213
Diuretic	102 (54.0)	31 (49.2)	32 (50.8)	39 (61.9)	0.154
Alpha blocker	60 (31.8)	21 (33.3)	16 (25.4)	23 (36.5)	0.704
Statin	110 (58.2)	42 (66.7)	34 (54.0)	34 (54.0)	0.150
Erythropoiesis stimulating agent	11 (5.8)	3 (4.8)	3 (4.8)	5 (7.9)	0.449

Mean $\pm$ SD, median [IQR], or number (%). *p* value for linear trend determined by linear regression with tertile as an ordinal variable. CPM, calciprotein monomers; CPP-I, primary calciprotein particles; CPP-II, secondary calciprotein particles; eGFR, estimated glomerular filtration rate; ePTFP, estimated proximal tubular phosphate concentration; uNGAL, urine neutrophil gelatinase-associated lipocalin; uFet-A/creat ratio, urinary fetuin-A-to-creatinine ratio; SD, standard deviation. ¹ eGFR calculated according to the CKD-Epidemiology Collaboration equation. ² Serum calcium adjusted for serum albumin concentration according to the following equation: measured calcium (mmol/L) + 0.02 (40–albumin [g/L]) ³ ePTFP calculated based on measured sPi and urinary FE_p using the equation proposed by Shiizaki et al. [20].

Fig. 1. Cumulative hazard function for composite kidney disease outcome and serum CPM tertile (commencement of kidney replacement therapy or drop in eGFR >30%). **a** The cumulative hazard function for the composite kidney disease outcome (commencement of kidney replacement therapy or drop in eGFR >30%). **b, c** The individual component hazard functions of the composite outcome.

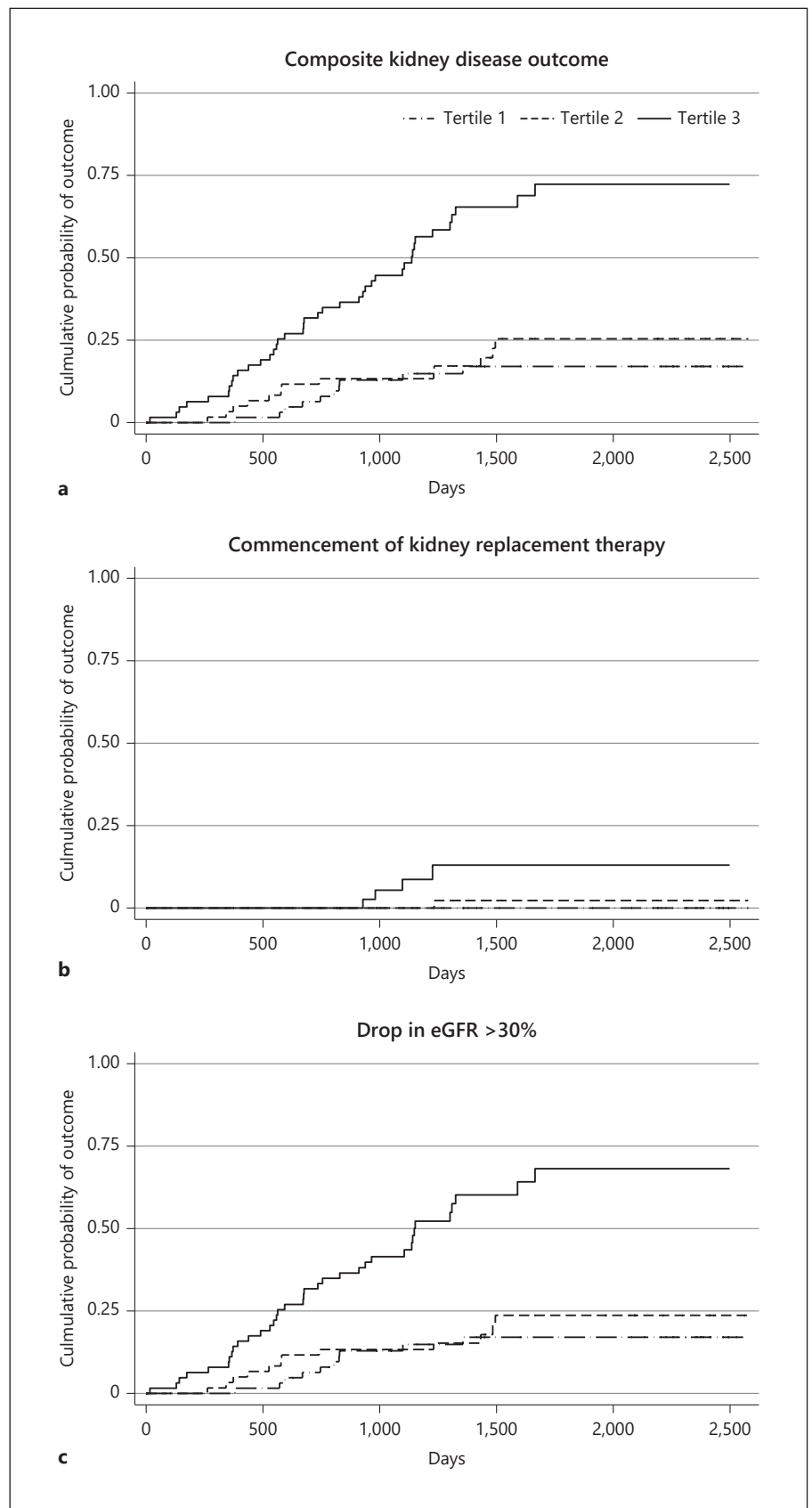


Table 2. Association between CPM tertile and composite kidney disease outcome (commencement of kidney replacement therapy or drop in eGFR >30%)

	Tertile 1		Tertile 2		Tertile 3	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Serum CPM	(3.5–19.3 × 10 ³ AU/mL)		(19.4–36.2 × 10 ³ AU/mL)		(37.8–183.4 × 10 ³ AU/mL)	
Unadjusted	Reference	–	1.37 (0.60–3.13)	0.454	5.88 (2.93–11.82)	<0.001
Model 1	Reference	–	1.41 (0.62–3.22)	0.418	6.32 (3.13–12.75)	<0.001
Model 2	Reference	–	1.23 (0.53–2.86)	0.638	4.21 (2.02–8.79)	<0.001
Model 3	Reference	–	1.05 (0.42–2.64)	0.922	4.22 (1.91–9.33)	<0.001

Hazard ratio (HR) estimated by Cox proportionate hazard model. Model 1 adjusted for participant age and sex. Model 2 adjusted for the covariates in Model 1 plus eGFR and uACR. Model 3 adjusted for the covariates in Model 2 plus cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, beta blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker). CPM, calpionin monomers; HR, hazard ratio; 95% CI, 95% confidence interval.

Results

Baseline Demographic and Clinical Characteristics

One hundred and eighty-nine of 200 participants in the ACADEMIC study had stored baseline serum samples available for measurement of CPM and were included in this analysis. No participants in this sub-study were lost to follow-up.

Baseline demographic details and clinical characteristics are displayed in Table 1. Of the 189 individuals, 186 self-identified as Caucasian, 2 as Middle Eastern, and 1 as Black African. The mean age of the study cohort was 69.1 ± 11.3 years, 27.5% were female, and 23.8% had a history of diabetes. At study entry, the mean eGFR was 32.7 ± 10.8 mL/min/1.73 m² and median uACR was 5.3 mg/mmol [IQR 1.7–30.5]. The distribution of primary cause of CKD for the cohort is listed in online supplementary Table 1.

Across CPM tertiles, there was no trend in age, sex, blood pressure, baseline comorbidities, or medication use. However, ascending tertiles of serum CPM were associated with lower eGFR at baseline (*p* value for trend <0.001). Consistent with this, participants with higher serum levels of CPM also had lower hemoglobin, higher urinary albuminuria, and proteinuria, and higher sPi, parathyroid hormone, iFGF23, C-reactive protein levels, and ePTFp. Total serum fetuin-A concentrations were lower in those with higher serum CPM, whereas urine fetuin-A levels increased across the tertiles. uNGAL, a marker of kidney injury, was also higher in those with higher serum CPM, and itself associated with higher

ePTFp (*r* = 0.198, *p* = 0.007), iFGF23 (*r* = 0.418, *p* < 0.001), fetuinuria (*r* = 0.332, *p* < 0.001).

Association between CPM and Composite Kidney Disease Outcome

During a median follow-up of 3.6 years (IQR 2.5–5.9), and a total of 730.3 person-years, there were 63 kidney disease events, while 21 participants died, and the remaining 105 participants were censored at the conclusion of study follow-up. The overall event rate was 8.6 kidney disease events per 100 person-years. Of the 63 recorded composite kidney disease events, the majority (92%) were due to drop in eGFR >30%.

The unadjusted cumulative hazard function for the composite kidney disease outcome by tertile of serum CPM is depicted in Figure 1. The cumulative incidence curves for the lower two tertiles of serum CPM converged; however, there was clear delineation of the highest tertile. Unadjusted and adjusted Cox proportionate hazard models examining the association between CPM and the composite kidney disease outcome are displayed in Table 2. The highest tertile of serum CPM was associated with increased risk of the composite kidney disease outcome in the unadjusted model, and this association persisted in all three multivariate adjusted models, with only a modest attenuation in the hazard ratio.

Association between CPM and eGFR Slope

During the study period, there was a median of 21 (IQR 14–33) eGFR recordings available for review per participant. The mean eGFR slope of the cohort was –0.8

Table 3. Association between CPM and eGFR slope

	β coefficient (95% CI)	<i>p</i> value
Ln CPM		
Unadjusted	−2.10 (−3.45, −0.74)	0.003
Model 1	−2.08 (−3.44, −0.72)	0.003
Model 2	−1.75 (−3.27, −0.23)	0.024
Model 3	−1.66 (−3.10, −0.22)	0.024

eGFR slope defined as average change in eGFR per year [30]. β coefficient and 95% CI estimated by linear regression models using robust CI estimates to account for heteroskedasticity of residuals. CPM as well as other skewed covariates were natural log-transformed. Model 1 adjusted for participant age and sex. Model 2 adjusted for the covariates in Model 1 plus eGFR and uACR. Model 3 adjusted for the covariates in Model 2 plus cardiovascular risk factors (history of cardiovascular disease, baseline systolic blood pressure, history of diabetes mellitus, smoking history) and medications (use of an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, beta blocker, statin, thiazide diuretic, loop diuretic, alpha blocker, or calcium channel blocker). CPM, calciprotein monomers; eGFR, estimated glomerular filtration rate; 95% CI, 95% confidence interval.

± 4.9 mL/min/1.73 m²/year. Unadjusted and multivariate adjusted linear regression models examining the association between CPM and eGFR slope are shown in Table 3. Consistent with Cox models for composite kidney disease outcome, higher serum CPM was associated with greater eGFR decline per year, and this association remained significant after sequential adjustment for demographics and risk factors for CKD progression, again with minimal attenuation of the coefficient.

Additional Analyses

Adjusted fine and gray models using death as a competing risk are displayed in online supplementary Table 2. Consistent with the main Cox proportionate hazard models, the highest tertile of CPM was associated with increased risk of kidney disease events. Further adjustment of our main Cox and linear regression models for conventional markers of mineral metabolism (calcium, phosphate, and PTH) did not substantially change the association between serum CPM and the composite kidney disease outcome or eGFR slope (online suppl. Tables 3, 4).

The directed acyclic graph and pathway coefficients from mediation analysis are shown in online supplementary Figure 2. The proportion of direct to total association is also displayed in online supplementary Table 5 for serum CPM, which was found to have both significant di-

rect and indirect associations with eGFR slope. In mediation analysis, the effect of CPM on eGFR slope appeared to be partially mediated by iFGF23; however, there was also a significant direct association between CPM and eGFR slope (75.6% of the total association). Notably, the association between iFGF23 and eGFR slope did not appear to be significantly mediated by ePTFp in this exploratory analysis.

Discussion

In this analysis of 189 individuals with stage 3 and 4 CKD, we found that serum CPM, a novel marker of mineral metabolism, were associated with disease progression. Specifically, higher levels of serum CPM were significantly associated with both increased risk of kidney disease events and eGFR decline, independent of a range of traditional risk factors for progression of CKD.

Disordered mineral metabolism is near ubiquitous in the advanced stages of CKD [1, 2] and has long been associated with worse patient cardiovascular and mortality outcomes [33]. The (patho)physiology of CPM and CPP metabolism has recently emerged as a novel pathway in the understanding of these processes and has been suggested to be a possible mediator of cardiovascular outcomes in CKD [6, 34]. While clinical studies to date have focused on the relationship between CPM/PPP and cardiovascular complications, this is the first to suggest that measurement of CPM may also have utility in evaluating the risk of progression of CKD itself.

Previous epidemiological studies have suggested that the presence of disordered mineral metabolism in CKD may be independently associated with loss of kidney function [4, 16–20]. Like our study, these previous studies have largely been observational and so do not definitively show causation. However, Shiizaki et al. [20] have recently suggested that the formation of calcium phosphate crystal particles in the proximal tubule lumen may initiate cytotoxic responses that result in tubular cell loss. In their study, it was proposed that this process is triggered by an initial reduction of functional nephrons, leading to a compensatory rise in FGF23 to maintain total urinary phosphate excretion. This ostensibly adaptive change results in increased per nephron phosphate excretion, which is reflected in an increased concentration of proximal tubular fluid phosphate, causing a predisposition for calcium phosphate crystal formation. Our findings are largely compatible with this proposed mechanism which is further supported by several other observations: first

that serum levels of CPM and CPP have been found to increase with reduced eGFR [27, 35], second that CKD patients experience greater elevations in CPM following a meal compared to non-CKD controls [14], and third that CPM have been elucidated as the molecular signal that leads to increased FGF23 expression in osteocytes in response to phosphate loading [15]. Taken together, it is plausible that as kidney function declines, elevated CPM may trigger increased FGF23 expression, which leads to vicious cycle of increased proximal tubular fluid phosphate and progressive tubular loss due to the formation of calcium phosphate crystals.

In exploratory mediation analysis, a significant proportion of the association between serum CPM and eGFR slope was explained by its association with iFGF23, notably though the association between iFGF23 and eGFR slope did not appear to be explained by ePTFp. There are several potential explanations for this. First, it is possible that iFGF23 may be associated with CKD progression via alternative mechanisms than that proposed by Shiizake et al., such as direct activation of injury primed renal fibroblasts, as has previously been suggested [36, 37]. Second, while ePTFp did correlate with kidney disease outcomes in mice and human cohorts studied by Shiizake et al. [20], it is important to remember that this is an indirect estimation of true end proximal tubular concentration of phosphate (and precipitation potential), and it is unclear whether this estimation method is prone to diurnal or post-prandial variation. Related to this, given our limited patient numbers and the availability of only single blood and urine samples, it is possible that we may have been underpowered to demonstrate an effect of ePTFp on eGFR slope in our analysis. Third, it is likely that the propensity to form calcium phosphate crystals in the tubular fluid is dependent on multiple additional local factors that we did not account for in this analysis, such as pH, as well as concentrations of calcium, magnesium, and zinc, among others. We note that Shiizake et al. [20] proposed a 2-segmented linear association between ePTFp and injury markers, with an inflexion point above 0.75 mmol/L. Given that the majority (75%) of participants in our study had an ePTFp above this threshold, we used a single linear function. Repeating our analyses with restriction to individuals with an ePTFp equal to or greater than 0.75 mmol/L did not substantially change the association between ePTFp and eGFR slope in linear regression or mediation analysis (not shown).

It was notable that in mediation analysis the majority of the association between serum CPM and eGFR slope appeared to be direct and independent of iFGF23. Shi-

izaki et al. did not examine for the presence of fetuin-A in the calcium phosphate crystal particles they observed; however in a separate study, Koeppert et al. [26] recently showed that CPM are rapidly filtered at the glomerulus using fluorescently labeled fetuin-A in a live mouse model. While in this latter study it was suggested that filtered fetuin-A is mostly reabsorbed by proximal tubular cells, it is tempting to speculate whether this resorptive capacity may become overwhelmed in situations of chronically elevated serum CPM, such as in CKD, which would presumably lead to correspondingly increased glomerular filtration of CPM by the remnant functioning nephrons. Consistent with this possibility, we found an association between higher CPM and higher urinary fetuin-A excretion. Thus, one hypothetical explanation for the direct association between serum CPM and eGFR slope may be that under permissive conditions (i.e., high phosphate in the proximal tubular fluid, low inhibition), filtered CPM could exacerbate the formation of calcium phosphate crystals in the tubular fluid, serving as a source of additional mineral ions, or as nuclei for in situ precipitation. In this context, however, it is worth acknowledging that fetuin-A may well be protective, as shown in recent models of ischemic and fibrotic kidney injury [38]. So, while excessive levels of CPM might ultimately potentiate injury, fetuin-A-bound mineral is certainly less toxic than naked calcium phosphate crystals [39], congruent with its functions as a mineral chaperone. Indeed, Koeppert et al. [26] also reported that CPM by itself did not induce any cytotoxicity in their tubular cell cultures. Ultimately, future studies are required to better quantify the composition of proximal tubular fluid and to elucidate how various components, including ions (such as calcium, phosphate, magnesium, and zinc), proteins (such as albumin and fetuin-A), physiochemical properties (such as pH), and filtered CPM, may interact to potentiate kidney damage. Functional measures of calcification propensity in urine, akin to the T50 in serum, may be highly informative in this regard.

Limitations

In this study, we did have complete participant follow-up with minimal missing data, albeit our study cohort was small. We only had single baseline measurements available for serum CPM. However, the availability of only single measurement would be expected to lead to underestimation, rather than overestimation, of the true association between serum CPM and CKD outcomes. Our study is observational, and so our findings are associative by nature. As outlined, recent mechanistic studies have

provided biologically plausible explanations through which elevated CPM in CKD could contribute to kidney function decline. Nevertheless, we acknowledge that future studies are required to confirm these associations, and additionally, that only controlled interventional studies will ultimately be able to determine whether targeting mineral metabolism is a useful intervention to slow progression of CKD.

In conclusion, CPP are novel markers of disordered mineral metabolism in CKD. While elevated levels of CPM and CPP have been associated with adverse cardiovascular outcomes in individuals with CKD, our study suggests that elevated levels of CPM may also be associated with increased risk of progression of CKD itself. This adds to accumulating evidence that disordered mineral metabolism in CKD may directly contribute to increased tubular damage and loss of kidney function.

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Statement of Ethics

The study was approved by the West Sussex Research Ethics Committee [REC Ref.# 05/Q1911/89], and patients gave written informed consent to participate. The study was conducted in accordance with the Declaration of Helsinki.

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Conflict of Interest Statement

E.R.S. is a stockholder of Calciscon AG (Biel, Switzerland). He also reports research funding from Sanofi and Baxter, outside the submitted work. M.K.T. reports consultation fees from Vifor Pharma outside the submitted work. M.L.F. reports no conflicts of interest. S.G.H. reports research funding, travel support, and consultation fees from Amgen, AstraZeneca, Baxter, Otsuka, and Sanofi, outside the submitted work.

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Author Contributions

Stephen Holt, Martin Ford, and Edward Smith were involved in the design and conduct of the ACADEMIC Study. Edward Smith and Mark Tiong conceived and designed this sub-study. Mark Tiong analyzed the data and drafted the manuscript under the supervision of Edward Smith. All authors contributed to the interpretation of the results and critical review of the manuscript.

Data Availability Statement

The data used in this study are not publicly available due to privacy or ethical restrictions. Queries about the data should be directed to the corresponding author.

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