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Reduced hip bone mineral density is associated with high levels of calciprotein particles in patients with Fabry disease

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Abstract

Summary Calciprotein particles (CPP) are nanoscale mineralo-protein aggregates that help stabilize excess mineral in the circulation. We examined the relationship between CPP and bone mineral density in Fabry disease patients. We found an inverse correlation with total hip and femoral neck density, but none with lumbar spine.

Purpose Calciprotein particles (CPP) are colloidal mineral-protein complexes made up primarily of the circulating glycoprotein fetuin-A, calcium, and phosphate. They form in extracellular fluid and facilitate the stabilization, transport, and clearance of excess minerals from the circulation. While most are monomers, they also exist in larger primary (CPP-I) and secondary (CPP-II) form, both of which are reported to be raised in pathological states. This study sought to investigate CPP levels in the serum of patients with Fabry disease, an X-linked systemic lysosomal storage disorder that is associated with generalized inflammation and low bone mineral density (BMD).

Methods We compared serum CPP-I and CPP-II levels in 59 patients with Fabry disease (37 female) with levels in an age-matched healthy adult cohort ($n=28$) and evaluated their association with BMD and biochemical data obtained from routine clinical review.

Results CPP-I and CPP-II levels were higher in male Fabry disease patients than female sufferers as well as their corresponding sex- and age-matched controls. CPP-II levels were inversely correlated with BMD at the total hip and femoral neck, but not the lumbar spine. Regression analyses revealed that these associations were independent of common determinants of BMD, but at the femoral neck, a significant association was only found in female patients.

Conclusion Low hip BMD was associated with high CPP-II in patients with Fabry disease, but further work is needed to investigate the relevance of sex-related differences and to establish whether CPP measurement may aid assessment of bone disease in this setting.

Keywords Bone mineral density · Calciprotein particles · DXA · Fabry disease · Fetuin-A · Lysosomal storage disorders

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Introduction

Fabry disease is a rare disorder caused by mutations in the X-linked gene (*GLA*) encoding the lysosomal enzyme α -galactosidase A. Poorly or non-functioning enzyme leads to a toxic build-up of uncleaved substrate, in particular the lipid globotriaosylceramide (Gb3) and its deacylated form globotriaosylsphingosine (lyso-Gb3).

The accumulation of unmetabolized glycolipids triggers immunogenic responses throughout the body, leading to a state of chronic inflammation characterized by high levels of circulating cytokines and chemokines [1, 2] and generalized activation of the innate and adaptive immune systems [3]. Over time, the condition leads to end-organ damage, most often involving the kidneys,

heart, and central nervous system, but also affecting many other organ systems including the skeleton. With respect to effects on bone quantity, prior studies have shown that individuals, in particular males, with Fabry disease have higher rates of osteoporosis or osteopenia compared to the general population [4–6], although the extent to which this is influenced by the disease compared to other factors such as medications (e.g., anticonvulsants commonly used for the management of neuropathic pain), low body mass index (BMI), inability to exercise, or low levels of vitamin D has not been definitively established [7]. Likewise, the mechanism by which bone density is reduced in Fabry patients has not been extensively explored. The current standard of care targeting Fabry disease includes enzyme replacement therapy (ERT) and oral chaperone therapy that helps misfolded enzyme reach maturity. ERT has been reported to improve bone mineral density (BMD) in Gaucher disease [8], a lysosomal storage disorder with much more prominent skeletal manifestations, but no analogous studies in Fabry disease are available.

Calciprotein particles (CPP) are nanoscale particles made up primarily of the circulating glycoprotein fetuin-A bound to ion clusters of calcium and phosphate [9]. They range from monomers consisting of a single unit of mineral-bound fetuin-A (CPM) to primary CPP (CPP-I) in which multiple units combine to form a spherical structure, to secondary CPP (CPP-II), which are larger, more elongated aggregates containing crystalline calcium phosphate [9, 10]. They form in supersaturated solutions of bone-forming minerals, thereby inhibiting crystallization and extraosseous calcification. Pathways involved in CPP production and accumulation are hypothesized to be linked to the processes of bone function and remodeling and may play a role in the paradoxical association of low BMD with higher vascular calcification, particularly in patients with chronic kidney disease (CKD) [11–13]. Furthermore, CPP levels correlate closely with bone turnover markers in bisphosphonate treated animals [14, 15], and humans with CKD [16, 17]. The existence of a “bone-vascular axis” has been suggested, implicating a complex interplay of the components regulating both systems [18], in which fetuin-A and CPP may function as mineral chaperones. High levels of circulating CPP have been reported in people with other chronic inflammatory disorders such as inflammatory bowel disease and inflammatory arthropathies [19], as well as in patients with end-stage kidney disease requiring dialysis therapy [20].

The bone phenotype and chronic inflammatory response in Fabry disease [21] implies a possible overlap with pathologies that have been associated with high CPP levels, and thus, we hypothesized that there may be dysregulation of CPP levels in individuals with the disease. The aim of this study was to examine the relationship between serum levels of CPP and BMD in a cohort of patients with Fabry disease.

Methods

Study design and cohort

This was a single-center observational study performed at the Royal Melbourne Hospital. All consecutive patients with a confirmed genetic diagnosis of Fabry disease were invited to participate. The final group consisted of 59 adults (22 male, mean age 45.0 ± 13.4). Enrolled patients underwent a fasting single venesection for serum CPP measurement during biannual clinical review. A separate cohort of 28 adults (15 male, mean age 44.7 ± 9.8 years) with no history of cardiovascular disease (exclusion criteria: previous myocardial infarction, stroke, heart failure, or receiving lipid lowering/antihypertensive therapy), type 2 diabetes mellitus, malignancy, recent infection, or trauma or with known renal disease were enrolled as controls over the same time period. Exclusion criteria included known pregnancy, age less than 16 years or greater than 90 years. Informed consent for both Fabry and control cohorts was part of the Fetuin-A Levels in Systemic disease and Kidney Impairment (FLEKSI) study [22] (HREC approval 2012.141). Additionally, Fabry disease patients underwent standard of care investigations including routine pathology and DXA scans to monitor BMD. This patient data forms part of the Fabry Outcome Survey (FOS), with research use approved by the Melbourne Health Human Research Ethics Committee (HREC approval 2001.144). All aspects of this study were performed in accordance with the Declaration of Helsinki. The Mainz Severity Score Index (FOS MSSSI) was used to evaluate disease severity, comprising four domains that include general, neurological, cardiovascular, and renal signs and symptoms of Fabry disease. Scores were weighted according to their contribution to the morbidity of the disease (maximal total score 64.5). To assess disease severity, the total score was then categorized as mild (<18), moderate (19–38), or severe (>38), as described previously [23]. End organ damage to the kidneys was defined as having chronic kidney disease (CKD) stage 3 or above (estimated GFR $<60\text{mL}/\text{min}/1.73\text{m}^2$) according to current Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guidelines for the Evaluation and Management of CKD, or previous renal transplantation. Transthoracic echocardiography was performed in the Department of Cardiology, Royal Melbourne Hospital, as previously described [24] and according to the American Society of Echocardiography recommendations [25] including measurement of interventricular septal thickness (IVSd). Standard assessment of diastolic function was performed, including pulsed-wave Doppler at the mitral valve tips, to determine mitral inflow pattern and to measure peak early (E) and late (A) wave

velocities. Doppler imaging of the mitral annulus was performed at the septal and lateral mitral valve annulus to allow direct measurement of E prime (E'), allowing assessment of average E/E'. Ratio of estimated LV filling pressure (E/E') was calculated giving an estimate LV filling pressure. The presence of left ventricular hypertrophy, particularly interventricular septal hypertrophy on transthoracic echocardiography, was considered diagnostic of Fabry cardiomyopathy.

Biochemical assessment

Fasting serum and urine were collected at a single time point during routine clinical care. Samples were collected to measure serum CPP by flow cytometry and the following parameters using standard laboratory methods: creatinine, albumin, calcium, phosphate, magnesium, bicarbonate, parathyroid hormone (PTH), thyroid stimulating hormone (TSH), total 25-hydroxy (OH) vitamin D, alkaline phosphatase (ALP), and urinary albumin/creatinine ratio (uACR). Serum calcium concentration was adjusted as follows if serum albumin was <40g/L: corrected serum calcium (mmol/L) = measured serum calcium (mmol/L) + 0.02 * (40 - serum albumin (g/L)). Serum T₅₀ (calcification propensity) was measured by turbidimetry at 400 nm and 37 °C ± 0.1 °C using a Synergy HTX Multi-Mode plate reader (BioTek Instruments, Winooski, VT) running Gen5 version 3 data analysis software, according to the method of Pasch et al. [26]. Each sample was run in quintuplicate, using the same serum sample that was used to measure CPP. The reference interval was determined locally in 170 healthy adults.

Dual X-ray absorptiometry

BMD measurements were obtained within the Royal Melbourne Hospital Bone Metabolism Unit at the lumbar spine, total hip, and mean of left and right femoral neck. BMD was measured in g/cm². T and Z scores were assessed using the National Health and Nutrition Examination Survey reference population [27]. WHO definitions of BMD categories were as follows: normal bone density (T score ≥ -1.0), osteopenia (T score between -1.0 and -2.5), and osteoporosis (T score ≤ -2.5) [28].

Measurement of CPP by flow cytometry

The levels of CPP-I and CPP-II were measured using flow cytometry as described in [29] with some modifications, as per [30]. Fasting blood samples were drawn using standard phlebotomy techniques and then allowed to separate on the bench before being centrifuged at 3000×g for 15 min. Serum was stored at -80 °C until analysis after a single thaw at 37 °C. Briefly, serum was diluted 1 in 4 with

double 0.22 µm-filtered Tris-buffered saline (TBS; made from 10x stock, Thermo Fisher Scientific, Waltham, MA, USA). It was then centrifuged for 30 min at 10,000×g at 4 °C, before vortexed supernatant was added to an equal volume of reaction buffer consisting of 0.5 µM OsteoSense 680EX (PerkinElmer, Waltham, MA, USA) and 0.12 µM phosphatidyl serine-binding lactadherin-FITC (BLAC-FITC; Haematologic Technologies Inc., Essex Junction, VT, USA) in TBS. The BLAC-FITC was included to label and allow exclusion of membrane-bound mineral-containing particles. After a 1-h incubation on a rotating mixer at 4 °C, the samples were read in duplicate on a BD FACSVerser flow cytometer (Becton Dickinson, San Jose, CA, USA) using high-sensitivity fluidics and detectors. Data were processed using FlowJo software version 10.7.1 (Ashland, OR, USA), giving reads in particles per mL. Mean values were used in subsequent analysis. Between-run analytical coefficients of variation were 7.0 and 11% for CPP-I and CPP-II, respectively.

Statistical analysis

Baseline characteristics of study participants were summarized as mean ± SD or median with 25th–75th percentile for continuous variables and percentages for categorical variables, dichotomised by sex. Continuous variables with skewed distributions were transformed by taking the natural logarithm of the raw values. Sex-related differences in each variable were evaluated using *t* tests with Welch's correction for unequal variance or Fisher's exact test, as appropriate. CPP levels in control and Fabry disease cohorts were compared by Brown-Forsythe and Welch one-way ANOVA and adjusted for multiple comparisons using Dunnett's T3 test. Pearson's correlation was used to examine the linear relationship between CPP levels with BMD scores at each skeletal site across the entire cohort and by sex. Linear regression was used to explore potential confounding between CPP-II and BMD indices, adjusting for sex, eGFR (either as a continuous function or categorically by CKD status), and drug therapy associated with CPP levels in univariate analysis. Kendall's tau (τ) correlation was used to look at associations between CPP-II and femoral neck BMD with the echocardiographic parameters E/E' and IV wall thickness due to their skewed distribution and high number of tied ranks. Further linear regression was then applied to assess confounders including CKD status, eGFR, and albuminuria with regard to the significant relationship between log-normalized PTH levels and IV wall thickness. All statistical analyses were performed in Stata/IC version 16.1 for Mac (StataCorp, College Station, TX, USA). All tests were two-sided and statistical significance was defined as *P*<0.05.

Results

Patient demographics, comorbidities, biochemistry, and echocardiographic parameters

Fifty-nine patients with a confirmed diagnosis of Fabry disease, of whom 37 were female and 22 were male, were enrolled in this study between September 2013 and January 2017. Baseline demographics and relevant serum and urine biochemistry are summarized in Table 1. Since the *GLA* gene is found on the X chromosome, males typically suffer from higher disease burden with end organ cardiac, renal, and central nervous system involvement, while in females, due to random X chromosome inactivation in early embryogenesis, symptoms range widely in severity [31]. For this reason, we looked at each of the parameters in the whole cohort, as well as by sex. Expectedly, disease severity scores were significantly higher in males than females. Age and body mass index (BMI) levels were similar between males and females, and while hypertension was more common among males, no other comorbidities distinguished the sexes. With respect to specific echocardiographic parameters related to diastolic dysfunction, IVSd, but not E/E', was higher in males compared to females. Five patients, two female and three male, had history of non-traumatic fractures. There were no differences in biochemical outcomes between sexes, apart from higher serum phosphate in females and higher PTH in males. Nineteen patients (11 male, 8 female) were biochemically deficient according to their 25-OH vitamin D levels.

Medication use

The patients in this study were taking a wide variety of medications, targeted both to the genetic defect (ERT, oral chaperone therapy (Migalastat), and substrate reduction therapy (Lucerastat)), and to the various symptoms of the disease (Table 2). Male patients were more likely than females to be on ERT and immunosuppression following renal transplant ($P<0.001$, $P=0.005$, respectively), reflecting greater disease severity. The use of mineralocorticoid receptor antagonist spironolactone was also higher in males than in females ($P=0.030$), although it is used at low dose, usually 12.5 mg/day.

Serum CPP levels

CPP-I were the more abundant species in serum (mean $88.6\pm 9.5\%$ of total CPP) and strongly correlated with CPP-II levels ($r=0.539$; $P<0.001$). Absolute levels of both CPP-I and CPP-II were higher in those with Fabry disease

compared to an age-matched adult control cohort (Figure 1), but with a similar predominance of CPP-I (mean $87.9\pm 8.7\%$ of total CPP), implying no disturbance in the tendency for particle transformation. Consistent with this, ex vivo calcification propensity (T_{50}) was within healthy reference intervals for the majority (75%) of the cohort.

With respect to the variables listed in Table 1, CPP levels only showed a significant association with patient sex, with the median levels of both CPP-I and CPP-II higher in male patients with Fabry disease compared to females. Interestingly, such sex-related differences in CPP were not apparent in controls, with higher levels of CPP-I and CPP-II in Fabry male and female patients compared to their corresponding sex-based control groups (Fig. 1).

Of note, neither CPP-I nor CPP-II levels were associated with FOS-MSSI disease severity scores, evidence of end-organ damage to the heart or kidney, and other conventional markers of mineral metabolism including PTH. With respect to specific echocardiographic parameters indicative of diastolic dysfunction, neither CPP-I nor CPP-II were associated with E/E' (CPP-I, $P=0.573$; CPP-II, $P=0.053$) or IVSd (CPP-I, $P=0.267$; CPP-II, $P=0.066$). Consistent with the strong predisposition in medication use by males (Table 2), both CPP-I and CPP-II levels were higher in patients prescribed ERT (CPP-I, $P=0.006$; CPP-II, $P=0.023$) and immunosuppression (CPP-I, $P<0.001$; CPP-II, $P=0.023$). CPP-II levels were also higher in those taking spironolactone ($P=0.008$).

BMD

DXA scan data was used from the closest scan to the date of serum sample collection (median [range] difference between serum collection and DXA scan = 319 [0, 2014] days). As before, we assessed patient data across the whole cohort and by sex. A pronounced reduction in BMD T and Z scores was observed for males at all sites, with osteopenia or osteoporosis present in 54.5% ($n=12$), 68% ($n=15$), and 82% ($n=18$) at the lumbar spine, total hip, and femoral neck, respectively. In contrast, only 28% ($n=10$), 32% ($n=11$), and 46% ($n=17$) of females had osteopenia or osteoporosis at the same sites (Table 3). Of the variables listed in Table 1, BMD at the 3 sites was strongly correlated with BMI (all $r>0.5$, $P<0.01$) and inversely associated with serum calcium concentrations (all $r>-0.3$, $P<0.05$). BMD at all 3 sites was unrelated to FOS-MSSI disease severity scores and there were no significant association with either of the echocardiographic parameters assessed. BMD at the femoral neck was higher in those on aspirin ($P=0.023$) and lower in those prescribed anticonvulsants ($P=0.035$), immunosuppression ($P=0.045$), or fish oil ($P=0.042$).

An inverse association between both CPP-I and CPP-II and BMD was evident in the entire population at the total

Table 1 Patient demographics, biochemical parameters, and clinical characteristics

	All patients (n=59)	Females (n=37)	Males (n=22)	P-value
Demographics				
Age (years)	45.0 ± 13.4	45.9 ± 15.4	43.3 ± 9.4	0.430
BMI (kg/m ²)	24.9 [21.9–28.0] (58)	25.1 [22.0–28.1] (36)	24.8 [21.8–27.1]	0.500
FOS-MSSI				
Mild	31 (52.5)	25 (67.6)	6 (27.3)	0.006
Moderate	21 (35.6)	10 (27.0)	11 (50.0)	
Severe	7 (11.9)	2 (5.4)	5 (22.7)	
Comorbidities				
Hypertension	12 (20.3)	4 (10.8)	8 (36.4)	0.041
Hyperlipidaemia	13 (22.0)	8 (21.6)	5 (22.7)	1.000
Diabetes mellitus	2 (3.4)	2 (5.4)	0 (0.0)	0.524
Smoker or ex-smoker	19 (32.2)	10 (27.0)	9 (41.0)	0.388
CKD (stage 3 or above)	6 (10.17)	2 (5.4)	4 (18.2)	0.183
Non-traumatic fracture	5 (8.5)	2 (5.4)	3 (13.6)	0.351
Echocardiographic parameters				
E/E' ^a	10 [8–13] (46)	8 [7–12.5] (28)	10.5 [9–13] (18)	0.197
IVSd, cm ^b	1.1 [0.8–1.4]	0.9 [0.8–1.2]	1.3 [1.1–1.5]	<0.001
Fabry cardiomyopathy	28 (47.5)	12 (32.4)	16 (72.7)	0.003
Biochemistry				
uACR, mg/mmol (<30 or <3) ^c	18.1 [4.4–159.0] (50)	10.6 [3.5–75.5] (32)	39.1 [9.3–159.8] (18)	0.130
eGFR, mL/min/1.73 m ² (≥60)	92.0 ± 25.2	90.1 ± 18.3	93.8 ± 34.2	0.650
T ₅₀ , min (250–450)	278.6 ± 49.2	273.2 ± 45.8	287.4 ± 54.4	0.295
Bicarbonate, mmol/L (22–32)	25.2 ± 2.8	25.1 ± 0.4	25.4 ± 0.6	0.740
Albumin, g/L (32–50)	39 ± 3	40 ± 3	39 ± 4	0.780
Adjusted calcium, mmol/L (2.10–2.60)	2.40 ± 0.11	2.41 ± 0.11	2.40 ± 0.11	0.710
Phosphate, mmol/L (0.75–1.50)	1.01 ± 0.18	1.07 ± 0.15	0.92 ± 0.19	0.003
Magnesium, mmol/L (0.70–1.10)	0.84 ± 0.06	0.84 ± 0.043	0.84 ± 0.08	0.793
ALP, IU/L (30–110)	73 [59–87]	72 [61–86]	73 [59–87]	0.810
25(OH) vitamin D, nmol/L (50–120)	67.9 ± 30.2	72.6 ± 27.2	59.9 ± 34.0	0.145
PTH, pmol/L (1.7–10.0)	5.9 [4.1–8.0] (53)	4.7 [3.9–7.5] (32)	7.2 [6.4–10.5] (21)	0.002
TSH, mU/L (0.3–4.2)	1.6 [1.2–2.1] (43)	1.6 [1.1–2.1] (25)	1.6 [1.2–2.1] (18)	0.231

Data are presented as mean ± SD, median [interquartile range], or number (%). Healthy reference interval is given in parentheses beneath each biochemical parameter. *N* is included in parentheses where it is less than the full cohort. *P*-value represents comparison between sexes

Abbreviations: *BMI* body mass index, *CKD* chronic kidney disease, *uACR* urine albumin-to-creatinine ratio, *ALP* alkaline phosphatase, *FOS-MSSI* Fabry Outcome Survey-Mainz Severity Score Index, *IVSd* interventricular septal wall thickness, *PTH* parathyroid hormone, *T₅₀* serum calcification propensity, *TSH* thyroid-stimulating hormone

^aE/E' <8 normal, 8–12 indeterminate, >12 elevated

^bIVSd >1.5 cm considered abnormal and indicative of increased risk of diastolic dysfunction

^cIf diabetic, clinically significant albuminuria when uACR >3 mg/mmol

Table 2 Medications taken by patients with Fabry disease at the time of blood collection for serum CPP levels

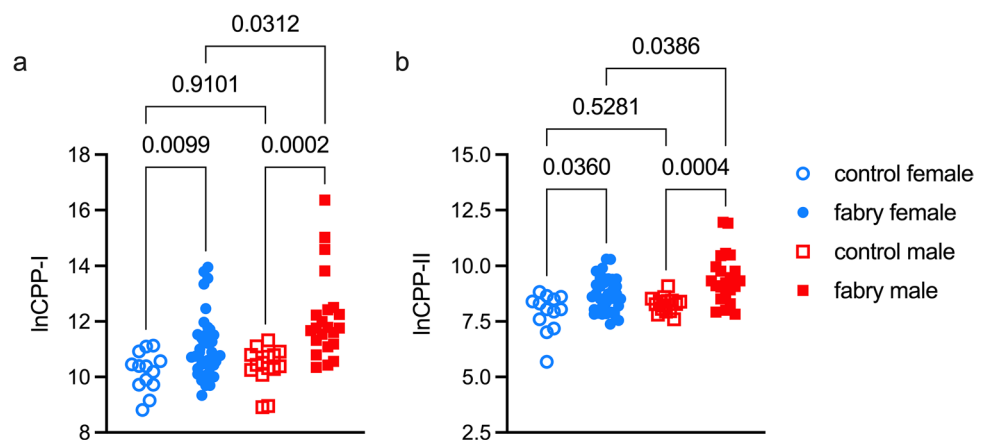
Medication	All patients (n=59)	Female (n=37)	Male (n=22)
ACE/ARB	28 (47.5)	14 (37.8)	14 (63.6)
Anticoagulant/antiplatelet	15 (25.4)	10 (27.0)	5 (22.7)
Anticonvulsant	17 (28.8)	8 (21.6)	9 (40.9)
Aspirin	39 (66.1)	24 (64.9)	15 (68.2)
BB	13 (22.0)	7 (18.9)	6 (27.3)
Calcium supplement	7 (11.9)	6 (16.2)	1 (4.6)
CCB	8 (13.6)	3 (8.1)	5 (22.7)
Cholecalciferol	15 (25.4)	11 (29.7)	4 (18.2)
Digoxin	4 (6.8)	3 (8.1)	1 (4.6)
ERT	19 (32.2)	5 (13.5)	14 (63.6)***
Fish oil	3 (5.1)	1 (2.7)	2 (9.1)
Immunosuppression ^a	5 (8.5)	0 (0.0)	5 (22.7)**
Lucerastat	2 (3.4)	2 (5.4)	0 (0.0)
Magnesium supplement	4 (6.8)	3 (8.1)	1 (4.6)
Migalastat	8 (13.6)	6 (16.2)	2 (9.1)
PPI	13 (22.0)	8 (21.6)	5 (22.7)
Spirolactone	26 (44.1)	12 (32.4)	14 (63.6)*
Statin	14 (23.7)	8 (21.6)	6 (27.3)

Data are given in number (%). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ comparing male and female usage

Abbreviations: ACE/ARB angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, BB beta blocker, CCB calcium channel blocker, ERT enzyme replacement therapy, PPI proton pump inhibitor

^aPost-renal transplant immunosuppressive therapy

Fig. 1 Fasting serum CPP-I (a) and CPP-II (b) levels in a cohort of healthy adult controls ($n=28$) and Fabry disease patients ($n=57$) according to sex. CPP levels (particles/mL) were transformed by taking natural logarithms and compared using Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons test. Adjusted P values for each pairwise comparison as indicated



hip and femoral neck, but not the lumbar spine. Only CPP-II levels were significantly correlated with raw density measurements and standardized coefficients for all indices were consistently higher than those for CPP-I, indicating a more robust association of CPP-II with BMD at these sites (Table 4).

Since males showed a strong tendency towards a lower BMD at the total hip and femoral neck, as well as significantly higher CPP-II levels compared to females, we considered whether sex might modify the relationship between CPP and BMD. While no effect modification was observed at the total hip, we found a strong and consistent interaction

between patient sex and CPP-II levels with BMD at the femoral neck, with CPP-II associated with raw density measurements, Z and T scores only in female Fabry disease patients (Fig. 2). CPP-I levels, on the other hand, were not associated with BMD indices at any site after adjustment for sex.

With respect to other potential confounders of the relationship between CPP-II and BMD, both spironolactone and immunosuppressant therapy were also associated with reduced T score at the femoral neck ($P=0.046$ and $P=0.034$, respectively), but not at other sites. While adjustment for immunosuppressant therapy had no effect on the relationship between CPP-II and BMD T scores at the femoral neck (data

Table 3 Bone mineral density (BMD) in patients with Fabry disease

BMD	All patients (<i>n</i> =59)	Females (<i>n</i> =37)	Males (<i>n</i> =22)	<i>P</i> -value
Lumbar spine (g/cm ²)	0.99 ± 0.14 (55)	1.01 ± 0.14 (0.74, 1.29)	0.96 ± 0.14 (0.74, 1.28)	0.231
Lumbar spine Z score	-0.18 ± 1.5 (59)	0.27 ± 1.46 (-2.88, 3.40)	-0.94 ± 1.27 (-2.90, 2.20)	0.002
Lumbar spine T score	-0.68 ± 1.3 (58)	-0.39 ± 1.28 (-3.05, 2.23)	-1.17 ± 1.25 (-3.18, 1.7)	0.026
Total hip (g/cm ²)	0.88 ± 0.14 (55)	0.90 ± 0.13 (0.66, 1.18)	0.85 ± 0.14 (0.56, 1.04)	0.177
Total hip Z score	-0.53 ± 1.23 (57)	-0.10 ± 1.14 (-2.60, 2.30)	-1.21 ± 1.07 (-3.30, 0.30)	<0.001
Total hip T score	-1.04 ± 1.22 (56)	-0.60 ± 1.12 (-2.64, 1.74)	-1.71 ± 1.06 (-3.91, -0.10)	<0.001
Femoral neck (g/cm ²)	0.76 ± 0.13 (55)	0.79 ± 0.13 (0.56, 1.11)	0.73 ± 0.12 (0.45, 0.95)	0.083
Femoral neck Z score	0.62 ± 1.37 (54)	-0.13 ± 1.31 (-3.12, 2.83)	-1.35 ± 1.14 (-3.72, 0.40)	<0.001
Femoral neck T score	-1.51 ± 1.39 (59)	-1.00 ± 1.31 (-3.30, 2.14)	-2.26 ± 1.15 (-4.79, -0.24)	<0.001

Data are given as mean ± SD (min, max) for BMD. BMD readings for all patients include number (*n*), where *n*<59 all missing scans are from female patients; *P*-values represent difference between sexes

Table 4 Association between bone mineral density and serum calciprotein particles in patients with Fabry disease

Skeletal site (<i>n</i>)	Serum CPP-I ^a		Serum CPP-II ^a	
	β ^b	<i>P</i> -value	β ^b	<i>P</i> -value
Lumbar spine g/cm ² (55)	-0.07	0.614	-0.18	0.191
Lumbar spine Z score (59)	-0.10	0.444	-0.13	0.304
Lumbar spine T score (58)	-0.08	0.552	-0.19	0.159
Total hip g/cm ² (55)	-0.22	0.101	-0.30	0.023
Total hip Z score (57)	-0.27	0.043	-0.29	0.028
Total hip T score (56)	-0.29	0.032	-0.37	0.005
Femoral neck g/cm ² (55)	-0.25	0.064	-0.32	0.017
Femoral neck Z score (55)	-0.30	0.028	-0.30	0.024
Femoral neck T score (54)	-0.30	0.028	-0.38	0.004

^aNatural log transformed

^bStandardized correlation coefficient

not shown), a significant interaction was again found according to both ERT and spironolactone use, with an inverse association only detected in those not on therapy and consistent with the strong preponderance of male patients taking these medications (Table 2).

Discussion

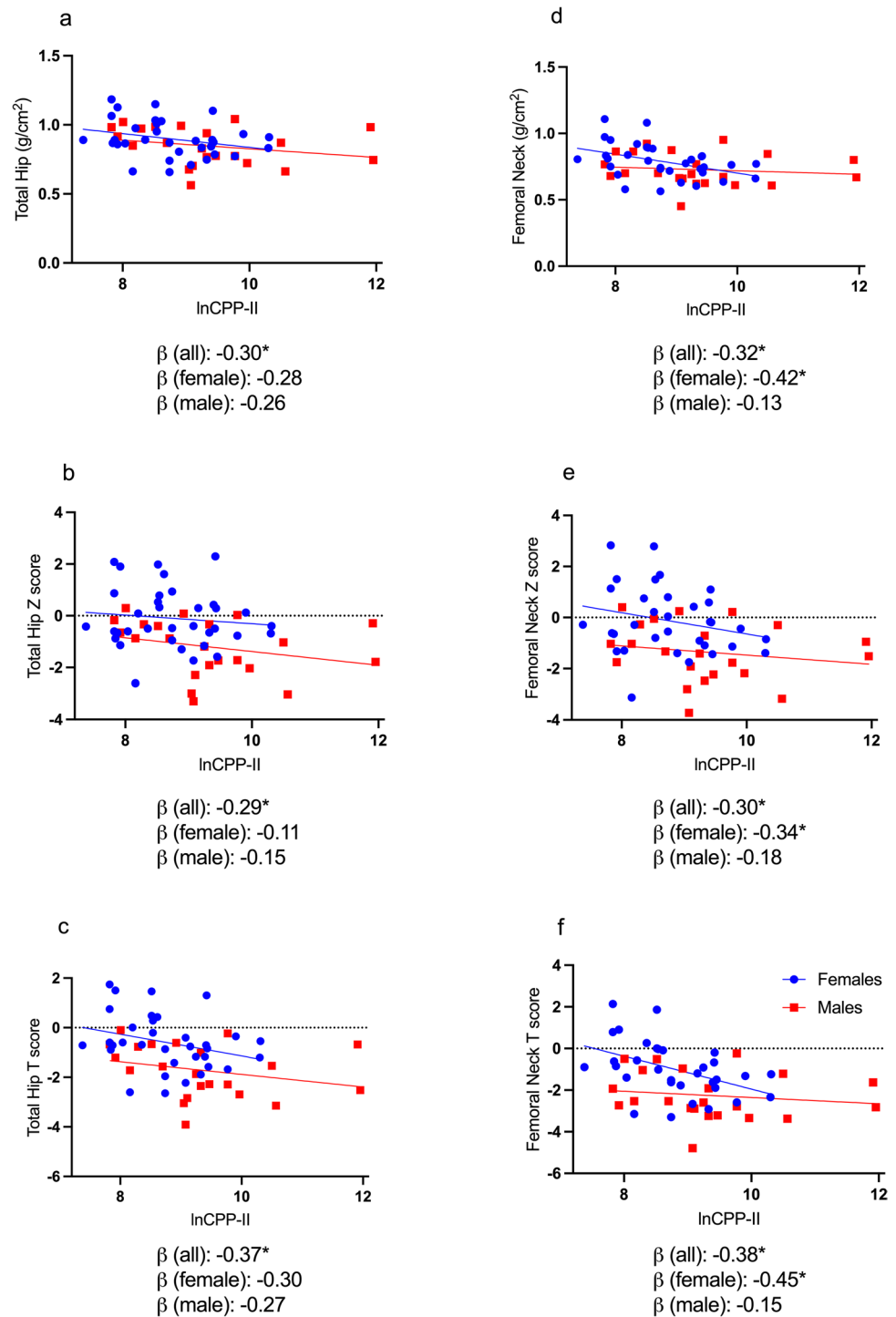
This is the first study to examine CPP levels in the serum of male and female individuals with Fabry disease, a systemic error in sphingolipid metabolism that often leads to generalized inflammation and reduced BMD. In doing so, we have found a significant relationship across the cohort associating high CPP-II levels with low BMD at the total hip and femoral neck. Our findings have therefore suggested a novel link between CPP and bone using bone mineral measurements. Previously, this relationship was inferred based on measurements of serum markers of formation and resorption.

The relationship between CPP and BMD could potentially be explained in one of two ways: either a low BMD points to low bone mineral turnover, which could lead to higher levels of mineral-bound particles in circulation, or higher levels of CPP could cause osteoblast dysfunction, leading to a reduction in BMD. Indeed, Akiyama et al. showed in mice that systemic CPP can access the bone compartment where osteoblasts reside [32], and CPP-II are known to inhibit osteoblast mineralization in vitro [11].

CPP-II are also associated with increased vascular calcification, although they are typically outnumbered 10:1 by CPP-I, which in turn are less plentiful than CPM, the main mediators of calcium and phosphate stability in serum [33]. CPP-II are thought to exist in appreciable numbers in relatively extreme pathological states and so their physiological function, if any, is uncertain [33]. Here, there was a strong association between levels of CPP-I and CPP-II, with CPP-I outnumbering CPP-II by an order of magnitude, in line with previous reports. CPP-I levels associated with reduced Z and T scores at the total hip and femoral neck, but not with raw BMD scores in g/cm² although *P* values approached significance. Generally, CPP-II levels were more closely associated with the femoral neck than the total hip, while neither form of CPP was increased along with lower BMD at the lumbar spine. This may be due to the greater proportion of trabecular versus cortical bone at that site, meaning that it has a higher rate of bone turnover [34], potentially compensating for an overload of CPP.

After adjusting for sex, there was only a strong interaction in female patients between CPP-II and BMD at the femoral neck and weak interactions in both sexes at the total hip (Fig. 2). Fabry disease is caused by a mutation on the *GLA* gene, which resides on the X chromosome, meaning that males typically suffer from worse disease than females, although heterozygotes present with a large variability in disease manifestations, and many women have severe phenotypes [35]. It was therefore interesting to note

Fig. 2 Scatter plots of BMD measurements (**a, d**) and scores (**b, c, e, f**) from total hip (**a–c**) and femoral neck (**d–f**) vs. ln(CPP-II), separated into females (blue) and males (red). Standardized correlation coefficients (β) are given below each plot, with linear relationships assessed by Pearson's correlation * $P < 0.05$



that while males had significantly higher CPP levels and lower BMD scores than females, the association between the two was stronger in females. We suspected that male sex may have been a proxy for disease severity in this setting; however, there was no clear association between FOS-MSSI severity scores and CPP levels or BMD indices at the total hip and femoral neck, indicating that we may be observing genuine sex-related differences in CPP,

and that sex could indeed modify the relationship between CPP and BMD in the femoral neck in patients with Fabry disease. These are novel and interesting suppositions that nevertheless need to be verified through further investigations, since the FOS-MSSI scoring system is subjective in nature and may contain inaccuracies, and the small group sizes in this study impose a lack of power to discern such differences.

Beyond subjective disease severity scores, we also looked at two objective echocardiographic indices of diastolic dysfunction: mitral E/E' and IVSd [24, 36, 37]. Although these parameters associated closely with each other and as expected, stratified with greater disease severity according to FOS-MSSI scores, neither were related to BMD at any of the 3 skeletal sites or CPP-II levels, overall, or when analyzed separately by sex. Taken together, this supports the assertion that the FOS-MSSI score does not capture bone manifestations of Fabry disease, but also implies that the processes leading to cardiovascular and skeletal end-organ damage may be mechanistically distinct.

Surprisingly, and in stark contrast to findings in CKD cohorts [17, 29], we found no association between serum CPP levels and any of the conventional markers of mineral metabolism. The reasons for this are unclear but may reflect differences in the origin of CPP in Fabry disease compared to CKD, where CPP may accumulate due to the manifest disturbances in systemic mineral handling. Interestingly, while PTH was not correlated with CPP or BMD in the present cohort, we observed that levels were modestly higher in male Fabry disease patients than female, even after adjusting for renal function. Further exploratory analyses uncovered a strong association with greater IV wall thickness ($r=0.426$, $P<0.001$). Contrary to expectations this association was minimally attenuated by adjustment for markers of renal function (eGFR and uACR), 25-OH vitamin D levels, and persisted after excluding those with CKD. Despite the small numbers, this relationship was apparent in both males and females, and it is notable that PTH levels even towards the upper end of the reference interval have been associated with risk of heart failure, especially in men [38]. Although the mechanistic links remain uncertain, further investigation of this association in a larger sample is clearly warranted.

While low BMD has previously been demonstrated to associate with low eGFR [39], in our study, eGFR was only inversely associated with Z scores at the total hip. Unexpectedly, there was no relationship between renal impairment (eGFR or uACR) and CPP-I or CPP-II levels, nor did adjustment for eGFR attenuate the relationship between BMD and CPP. This was not anticipated, since Fabry disease has prominent renal manifestations and CPP are known to be raised in CKD, particularly CPP-II, which may contribute to an inflammatory response involving tumor necrosis factor- α , C-reactive protein, and interleukin-6 [40]. The findings could be due to the small number of individuals ($n=6$) having CKD stage 3 or above, and five of these patients having excellent transplant GFR.

A previous study of Fabry disease patients from our group found that low BMD scores were associated with the use of anticonvulsant medication [6]. Here, the same trend was seen, although not at all sites; anticonvulsant drug use was associated with low density in g/cm^2 and low T scores at

the femoral neck, but there was no association with CPP levels. Medications that did associate with higher CPP included ERT, immunosuppression following renal transplant, and spironolactone. This may be a reflection of the reason for which these medications were prescribed—either for proteinuria control or renal transplant immunosuppression—so in turn relate to more advanced Fabry nephropathy. Interestingly, after adjustment for either ERT or spironolactone use, the association between low BMD and high CPP-II was only apparent in those not on therapy, consistent with the notion that both measures may simply be associated with a worse disease phenotype and male sex. A substantial proportion of both male and female patients were taking aspirin, and this was associated with increased BMD, in keeping with results from a recent meta-analysis that associated aspirin use with reduced fracture risk and potentially higher BMD [41]. The pharmacological chaperone Migalastat was associated with lower CPP-I levels, but again, small numbers of patients taking the medication preclude any assumption of causality.

In concordance with data from healthy populations [42], low BMD was clearly associated with low BMI at all sites and in all measures. Individuals with Fabry disease are often noted to have low BMI [43], a phenomenon that may be linked with gastrointestinal issues stemming from the disease [44, 45], although in our group, only 6 (10.2%) people had $\text{BMI} \leq 20$, while 6 (10.2%) had $\text{BMI} \geq 30$, meaning that around 80% of patients had BMI within a normal to overweight range. However, neither CPP-I nor CPP-II were associated with this important correlate of BMD.

This study has some limitations, including a small sample size, which limits the statistical power. The samples were collected over a period of several years and analyzed batch-wise for CPP levels, although this should not impact findings as storage was consistently at -80°C and samples were analyzed after a single thaw. Results were compared with retrospective BMD data collected at a date as closely matching the date of sample collection as possible; however, close matching was not possible for a proportion of participants. Patients undergo routine BMD every 5 years, and the median interval was over 300 days. Importantly, biochemistry data was taken from the same date as the serum collection, as these and CPP levels vary much more over time than do BMD readings. CPP levels are known to exhibit a post-prandial rise [46], but this is an unlikely confounder, since all samples were taken after an overnight fast. A third limitation is the lack of a control group with corresponding BMD measurements. As such, we are unable to speculate as to whether the associations observed between CPP levels and BMD are more broadly applicable, or if it is particular to Fabry disease. Given that both reduced BMD and increased CPP levels were seen here in males with Fabry disease, and that low BMD is more typically associated with menopausal changes in women,

it seems logical to assume that we are observing disease-specific effects, but more work is required to unravel this question, and to gain more clarity around the associations between CPP, BMD, and sex in health and disease. Further work should consider the relationship between CPP and bone turnover markers as well as other objective markers of cardiac fibrosis and inflammation that have proven useful in this setting [47–49]. Given the importance of nutrition to skeletal health, future studies should also capture data pertaining to dietary mineral intake and excretion.

In conclusion, we have uncovered a novel association between low BMD and high circulating CPP levels in a cohort of patients with Fabry disease, a systemic lysosomal storage disorder. It is the first time that CPP levels have been shown to have a direct relationship with quantitative bone mineral using data from DXA scans. Our work contributes to the knowledge surrounding the relatively newly described CPP and how their presence, particularly as CPP-II, can be indicative of a pathological state. While this is a small study, it is hypothesis-generating and should lead to future mechanistic studies to increase our understanding of the CPP-bone relationship in Fabry disease and pathophysiology more generally.

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Author contribution Study design: SB, SGH, ERS, IR; study conduct: SB, KMN, TDH, AST, SGH, ERS, IR. Data analysis: SB, ERS. Data interpretation: all authors. Drafting manuscript: SB. Revising manuscript content: all authors. Approving final version of manuscript: all authors. All authors take responsibility for the integrity of the data analysis.

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Declarations

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the *Melbourne Health Human Research Ethics Committee* (#2012.141 & #2001.144).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication All authors read and approved the final manuscript for publication.

Competing interests ERS owns stock in Calciscon AG, which commercializes the T₅₀ test and reports research funding from Amgen and Sanofi, outside the submitted work. SB reports no relevant conflicts of interest. KMN reports no relevant conflicts of interest. TDH reports no relevant conflicts of interest. AST reports no relevant conflicts of interest. SGH reports no relevant conflicts of interest. IR reports no relevant conflicts of interest.

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