Novel Aspects in the Pathogenesis of Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD)

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DECLARATION

This is to certify that:

i. This thesis comprises only my original work towards the PhD except where indicated in the preface

ii. Due acknowledgment has been made in the text to all other materials used, and

iii. The thesis is fewer than 100,000 words in length, exclusive of tables bibliographies and appendices

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Irene Ruderman

PREFACE

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

Chapter 2. The Australian Calciphylaxis Registry was established in 2013, by a Steering Committee of Nephrologists including Professor Grahame Elder, Professor Carmel Hawley, Associate Professor Nigel Toussaint and Associate Professor Genie Pedagogos. Associated Professor Rathika Krishnasamy was subsequently appointed to this committee. These clinicians were essential for the development and ongoing maintenance of the registry. Trial nurses, co-ordinators and treating clinicians from participating Nephrology centres uploaded data electronically onto the registry webpage. This data was used to evaluate the five-year registry outcomes described in Chapter 2. Results of this study have been published in *Nephrology Dialysis Transplantation* in 2019.

For this chapter, I was involved in conception and design of the study, analysed the data and wrote the manuscript. The extent of my contribution was 80%. The following co-authors contributed to the work:

Name	**Nature of contribution**
Nigel Toussaint:	Conception, study design, commented on and revised manuscript
Carmel Hawley	Commented on and revised manuscript
Rathika Krishnasamy	Commented on and revised manuscript
Eugenia Pedagogos	Commented on and revised manuscript
Nicole Lioufas	Patient data collection and revised manuscript
Grahame Elder	Commented on and revised manuscript

Chapter 3. Funding from Melbourne Health (Victor Hurley Grant in Aid) supported the basic science research presented in Chapter 3. Intraoperative skin samples were kindly collected by the Nephrology Surgical Team at The Royal Melbourne Hospital. This work has been accepted for publication following peer review by *BMC Nephrology* in 2020.

For this chapter, I was involved in conception, design of the study, performed all basic science work, analysed the data, and wrote the manuscript. The extent of my contribution was 80%. The following co-authors contributed to the work:

Name	**Nature of contribution**
Tim Hewitson	Contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work
Ed Smith	Contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work.
Steve Holt	Contributed to critical appraisal of paper and drafting of the work
Belinda Wigg	Contributed data interpretation
Nigel Toussaint	Contributed to the conception, design, critical appraisal of paper and drafting of the work.

Chapter 4. This study was funded by an investigator-initiated research grant from Amgen Australia. I would like to acknowledge the help of Ms Connie Karschimkus in collecting study samples over the trial period. Results of this study have been published in *BMC Nephrology* in 2018.

For this chapter, I was involved in data and statistical analysis, performed all basic science work, analysed the data, and wrote the manuscript. The extent of my contribution was 75%. The following co-authors contributed to the work:

Name	**Nature of contribution**
Ed Smith	Contributed to data and statistical analysis, data interpretation, critical appraisal of the paper and drafting of the work
Nigel Toussaint	Contributed to conception and study design, critical appraisal of the paper and drafting of the work
Tim Hewitson	Contributed to critical appraisal of the paper and drafting of the work
Steve Holt	Contributed to conception and study design, data interpretation, critical appraisal of the paper and drafting of the work

Chapter 5. For this chapter, I was involved in conception and design of the study, data collection, analysis and wrote the manuscript. The extent of my contribution was 80%. Results of this study have been published in the *Internal Medical Journal* in 2018.

The following co-authors contributed to the work:

Name	**Nature of contribution**
Steve Holt	Commented on and revised manuscript
Geoffrey Kirkland	Patient data collection and revised manuscript
Sophie Maslen	Patient data collection and revised manuscript
Carmel Hawley	Patient data collection and commented on and revised manuscript
Veronica Oliver	Patient data collection and revised manuscript
Rathika Krishnasamy	Patient data collection and commented on and revised manuscript

Nicholas Gray	Patient data collection and revised manuscript
Girish Talaulikar	Patient data collection and revised manuscript
Craig Nelson	Patient data collection and revised manuscript
Yogeshwar Rajaram	Patient data collection and revised manuscript
Hilton Gock	Patient data collection and revised manuscript
Eric Au	Patient data collection and revised manuscript
Grahame Elder	Commented on and revised manuscript
Rahul Mainra	Patient data collection and revised manuscript
Nigel Toussaint	Conception, study design, commented on and revised manuscript

Chapter 6. Imaging for the study in Chapter 6 was performed by the Department of Radiology at The Royal Melbourne Hospital. This work has been accepted for publication following peer review by *Bone Reports* in 2020.

In the case of Chapter 6, I was involved in the conception and design of the study, patient recruitment, data analysis and wrote the manuscript. The extent of my contribution was 75%. The following co-authors contributed to the work:

Name	**Nature of contribution**
Chamith Rajapakse	Contributed to image interpretation and image data analysis, commented on and revised manuscript
Angelica Opperman	Contributed to image interpretation and image data analysis
Patricia Robertson	Commented on and revised manuscript
Rosemary Masterson	Commented on and revised manuscript
Mark Tiong	Commented on and revised manuscript
Nigel Toussaint	Contributed to conception and study design, critical appraisal of the paper and drafting of the work

Additionally, I would to acknowledge postgraduate funding from the Australian Government Research Training Program Scholarship.

The undersigned herby certify that the above declaration correctly reflects the nature and extent and the candidate's and co-authors' contribution to this thesis.

Candidates' Signature	Date
Main Supervisor's Signature	Date

ACKNOWLEDGEMENTS

The work presented in this thesis was primarily carried out in the Department of Nephrology at the Royal Melbourne Hospital, with the help and encouragement of many of the staff members and patients, for which I am deeply grateful. There are several individuals who have played a significant role that deserve a specific mention.

Firstly, I would like to thank my supervisors, Associate Professors Nigel Toussaint, Tim Hewitson and Edward Smith for their time, mentorship, words of wisdom and dedication throughout my PhD journey. Thank you for allowing me the opportunity to undertake the PhD with you and providing me with an excellent foundation of both clinical research and basic science skills, which have encouraged me to grow and develop both as a clinician and researcher.

I would like to thank my co-supervisor, Professor Steve Holt for his intellectual expertise and support throughout my studies.

I am grateful to Ms Belinda Wigg for her advice, assistance, encouragement and patience with my laboratory endeavours.

I would like to acknowledge Dr Moira Finley from the Department of Pathology at The Royal Melbourne Hospital for her intellectual expertise with histology interpretation in Chapter 3.

Thank you to Dr Jean Tan for wonderful mentorship, friendship and many coffees throughout this journey.

Last, but not least, thank you to my amazing family, in particular my husband Nicholas for his unwavering support, love and patience throughout this journey.

TABLE OF CONTENTS

SUMMARY

Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD) is a clinical entity, broadly defined by (i) disturbances in mineral metabolism, (ii) abnormal bone remodeling and (iii) accelerated vascular calcification. CKD-MBD is ubiquitous as kidney function deteriorates and contributes significantly to increased fracture risk and excess cardiovascular morbidity and mortality. Due to the complex and multifactorial nature of this condition, there remains many unanswered questions on how to best investigate and manage all aspects of CKD-MBD.

The general aim of this thesis was to investigate the pathophysiology, clinical outcomes and novel assessment strategies of the three domains of CKD-MBD. More specifically, this thesis aimed to 1) evaluate the outcomes of calciphylaxis in Australian patients, 2) develop a greater understanding of changes in skin and subcutaneous tissue in patients with kidney disease, 3) demonstrate the consequences of severe secondary hyperparathyroidism (SHPT) in the absence of calcimimetic treatment, 4) understand the temporal relationship between calciprotein particles and biochemical markers of CKD-MBD and 5) evaluate bone microarchitecture in patients with severe SHPT using a novel imaging modality.

Although calciphylaxis is a rare disease, it can be considered a form of accelerated vascular calcification. It predominantly affects patients with chronic kidney disease (CKD) and is associated with significant morbidity and mortality due to progressive cutaneous calcification, necrotic ulceration and infection. Clinical registries have been established to better understand risk factors, optimal treatments and disease outcomes of calciphylaxis. To better understand outcomes of Australian patients with calciphylaxis, **Chapter 2** investigated the five-year outcomes from the internet-based Australian Calciphylaxis Registry. Data was recorded on patient characteristics, biochemical parameters, treatments and disease outcomes. The Australian Calciphylaxis Registry highlights risk factors for calciphylaxis including diabetes, obesity

and vitamin K antagonist use, whilst significantly elevated parathyroid hormone (PTH) levels were not a consistent finding at the time of diagnosis or in the preceding 12 months in this cohort. Unfortunately, resolution of calciphylaxis remains uncommon despite multimodal therapy and mortality from calciphylaxis in the first year following diagnosis is high.

Vascular calcification is well described in large- and medium-sized vessels in patients with CKD, especially in those with end-stage kidney disease (ESKD) on dialysis. Medial calcification is particularly prevalent in this population and contributes to arterial stiffness and increased cardiovascular mortality and morbidity. Apart from in the setting of calciphylaxis, few studies have assessed skin and subcutaneous calcification and associations with abnormalities of bone and mineral metabolism in patients with CKD. In **Chapter 3**, patients with varying stages of CKD undergoing elective surgery underwent intraoperative incisional skin biopsy to evaluate for the histological presence of vascular calcification and upregulation of pro-calcific gene transcripts. This study reports the novel finding of dermal and subcutaneous small vessel calcification in multiple anatomical locations in 38% of patients with advanced CKD or ESKD undergoing elective surgery but free from calciphylaxis. Expression of pro-calcific gene transcripts, specifically *TNAP* or *RUNX2*, was not increased in samples from patients with CKD or those with histological evidence of vascular calcification.

SHPT in patients with CKD is associated with cardiovascular and bone pathology. Measures to achieve target PTH values and control biochemical abnormalities associated with SHPT require complex therapies, and severe SHPT often requires parathyroidectomy or the calcimimetic cinacalcet. In Australia, cinacalcet prescription, in dialysis patients was reimbursed by the government from 2009 to 2015. However, after this time funding was withdrawn following publication of the EVOLVE study, which resulted in most patients on cinacalcet ceasing therapy. Changes to reimbursement of cinacalcet in Australia provided an opportunity to assess effects of medication cessation on biochemical and clinical outcomes in dialysis patients, including changes to novel biomarkers such as calciprotein particles (CPP). CPP are

nanoparticles of mineral and protein in the circulation associated with increased vascular calcification in patients with CKD. **Chapter 4** focused on changes to novel biochemical markers following cinacalcet withdrawal in dialysis patients with SHPT from a single centre with CPP levels assessed at four different time points over a 12-month period. Outcomes were compared with an age- and gender-matched cohort of cinacalcet-naïve dialysis patients. Cinacalcet withdrawal in the real-world setting demonstrated increases in PTH, serum calcium and the novel prognostic marker CPP over a 12-month period. Following on from work in Chapter 4, **Chapter 5** involved a multi-centre retrospective study of dialysis patients who ceased cinacalcet after August 2015 at 11 Australian institutions. Clinical outcomes and changes in biochemical parameters were assessed over a 12- and 24-month period from drug cessation. Significant increases in serum PTH, calcium and alkaline phosphatase occurred over a 12-month period following withdrawal of cinacalcet, without an associated increase in cardiovascular mortality and morbidity, fractures or calciphylaxis.

SHPT in patients with CKD leads to complex bone disease, affecting both trabecular and cortical bone with resulting increased fracture risk. Optimal assessment of bone in patients with CKD is yet to be determined. High-resolution magnetic resonance imaging (MRI) can provide three-dimensional assessment of bone microarchitecture, as well as determination of mechanical strength with finite element analysis (FEA). **Chapter 6** evaluated bone microarchitecture in patients with severe SHPT undergoing parathyroidectomy. Correlation of MRI findings of bone microarchitecture were made with biochemical markers. MRI in this patient population demonstrated evidence of significant changes in bone microarchitecture with trabecular deterioration, low trabecular and cortical bone volume, and reduced mechanical competence of bone with overall poor bone mechanical strength.

In summary, this thesis presents insights into the vascular, biochemical and bone domains of CKD-MBD with both clinical and basic science research focusing on

pathophysiology, novel biochemical markers and imaging techniques. The novel findings obtained in this thesis have broadened the understanding of all three domains of CKD-MBD, particularly with regards to CPP as a potential novel biomarker to evaluate biochemical derangement and vascular risk, and MRI as a novel imaging modality to gain a better understanding of bone microarchitecture. Further clinical and basic science studies are required to validate findings and to explore concepts established in this thesis, however, data presented here establishes important concepts for future research in this field.

ABBREVIATIONS

1,25(OH)D	1,25-dihydroxyvitamin D
25[OH]D	25 hydroxy vitamin D
ACR	Australian Calciphylaxis Registry
ALP	alkaline phosphatase
ANZSN	Australian and New Zealand Society of Nephrology
ANZDATA	Australia and New Zealand Dialysis and Transplant Registry
AVF	arteriovenous fistula
BMI	body mass index
BMD	bone mineral density
BMP2	bone morphogenic protein 2
BVF	bone volume fraction
BV/TV	bone volume/ total volume
CAC	coronary artery calcification
cAMP	cyclic adenosine monosulphate
CaSR	calcium sensing receptor
cDNA	complementary deoxyribonucleic acid
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease mineral and bone disorder
CPP	calciprotein particles
CPP II	primary calciprotein particles
CPP II	secondary calciprotein particles
CTh	cortical thickness
CRP	C-reactive protein
CT	computer tomography
CUA	calcific uraemic arteriolopathy
CVD	cardiovascular disease

DNA	deoxyribonucleic acid
DXA	dual-energy X-ray absorptiometry
eGFR	estimated glomerular filtration rate
E/I	erosion index
ESKD	end stage kidney disease
FEA	finite element analysis
FGF23	fibroblast growth factor 23
FFP	fresh frozen plasma
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GN	glomerulonephritis
H&E	haematoxylin and eosin
HD	haemodialysis
HDF	haemodiafiltration
HLD	high density lipoproteins
HR-pQCT	high resolution peripheral quantitative computer tomography
IEL	internal elastic lamina
IHD	ischaemic heart disease
IL-6	interleukin 6
iPTH	intact parathyroid hormone
KDIGO	Kidney Disease: Improving Global Outcomes
MBP	mineral binding proteins
MGP	matrix Gla protein

MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
PD	peritoneal dialysis
PEX	plasma exchange
PKD	polycystic kidney disease
PTH	parathyroid hormone
PTH1R	parathyroid hormone 1 receptor
PTH2R	parathyroid hormone 2 receptor
PTHrP	parathyroid hormone related peptide
PVD	peripheral vascular disease
PWV	pulse wave velocity
RCT	randomized controlled trial
RF	radiofrequency
RNA	ribonucleic acid
RT- PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
S/C	surface to curve ratio
SHPT	secondary hyperparathyroidism
STS	sodium thiosulphate
TbTh	trabecular thickness
TbN	trabecular number
TbS	trabecular separation
TBS	tris buffered saline
TLR4	toll-like receptor 4
TNAP	tissue non-specific alkaline phosphatase
TNFα	tumor necrosis factor alpha

USRDS	United States Renal Data System
VC	vascular calcification
VKA	vitamin K antagonist
VSMC	vascular smooth muscle cells

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Chapter 2

LIST OF FIGURES

Chapter 1

LIST OF PUBLICATIONS

Manuscripts resulting directly from the work encompassed in this thesis

Ruderman, I., Holt, S. G., Kirkland, G. S., Maslen, S., Hawley, C. M., Oliver, V., Krishnasamy, R., Gray, N. A., Talaulikar, G. S., Nelson, C.L., Rajaram, Y., Gock, H., Au, E., Elder, G. J., Mainra, R., Toussaint, N. D. (2018). Outcomes of cinacalcet withdrawal in Australian dialysis patients. *Internal Medicine Journal*. doi:10.1111/imj.14036

Ruderman, I., Smith, E. R., Toussaint, N. D., Hewitson, T. D., & Holt, S. G. (2018). Longitudinal changes in bone and mineral metabolism after cessation of cinacalcet in dialysis patients with secondary hyperparathyroidism. *BMC Nephrology, 19*(1), 113. doi:10.1186/s12882-018-0910-9

Ruderman, I., Toussaint, N. D., Hawley, C. M., Krishnasamy, R., Pedagogos, E., Lioufas, N., & Elder, G. J. (2019). The Australian Calciphylaxis Registry: reporting clinical features and outcomes of patients with calciphylaxis. *Nephrology, Dialysis, Transplantation*. doi:10.1093/ndt/gfz256

Ruderman, I., Hewitson, T.D., Smith, E. R., Holt, S. G., Wigg, B., Toussaint, N. D. (2020). Vascular calcification in skin and subcutaneous tissue in patients with chronic and end stage kidney disease *BMC Nephrology, accepted for publication*

Ruderman, I., Rajapakse, C. S., Opperman, A., Robertson, P. L., Masterson, R., Tiong, M., K., Toussaint, N., D. (2020). Bone microarchitecture in patients undergoing parathyroidectomy for management of severe secondary hyperparathyroidsim. *Bone Reports, accepted for publication*

Manuscripts related to work contained in this thesis

Ruderman I, Holt, S. G., Hewitson, T. D., Smith, E. R., & Toussaint, N. D. (2018). Current and potential therapeutic strategies for the management of vascular calcification in patients with chronic kidney disease including those on dialysis. *Semin Dial, 31*(5), 487-499. doi:10.1111/sdi.12710

Toussaint, N. D., & **Ruderman, I.** (2017). What Is the Role of Vitamin D Supplementation Vascular Health in CKD? *Clinical Journal of the American Society of Nephrology*. doi:10.2215/cjn.07170717

LIST OF CONFERENCE PRESENTATIONS

The following conference abstracts have been presented by Irene Ruderman during her PhD candidature directly relating to work in this thesis:

Ruderman I, Holt S, Hewitson T, Smith E, Krishnasamy R, Gray N, Hawley C, Oliver V, Talaulikar G, Kirkland G, Maslen S, Toussaint T. Changes in bone and mineral metabolism markers after cessation of Cinacalcet in dialysis patients with secondary hyperparathyroidism. **Mini-oral presentation** at 2017 Australian and New Zealand Society of Nephrology Conference, Darwin, Australia

Ruderman I, Smith E, Toussaint N, Hewitson T, Holt S. Longitudinal changes in bone and mineral metabolism after cessation of Cinacalcet in dialysis patients with secondary hyperparathyroidism. **Mini-oral presentation** at 2018 Australian and New Zealand Society of Nephrology Conference, Sydney, Australia and **Cleveland Young Investigator closing plenary presentation** at 2017 Melbourne Health Research Week, Melbourne, Australia

Ruderman I, Hewitson T, Smith E, Wigg B, Holt S, Toussaint T. Dermal and subcutaneous vascular and other extraosseous calcification in patients with chronic kidney disease without presence of calcific uraemic arteriolopathy. **Mini oral presentation** at 2019 Melbourne Health Research Week, Melbourne, Australia

LIST OF AWARDS

Australian Government Research Training Program Scholarship 2017-2020

Victor Hurley Medical Research Grant in Aid 2017

Cleveland Young Investigator Award, Melbourne Health Research Week 2017

1. GENERAL INTRODUCTION

1.1 Physiological mineral handling

The kidneys play an important role in maintaining healthy bone mass and structure. This is achieved through homeostasis of calcium and phosphate with gastrointestinal absorption balanced by renal excretion. Within the body, the majority, approximately 85%, of calcium and phosphate is stored in bone and is tightly connected to the process of bone mineralisation. Of the remaining 15%, approximately 14.5% is in the soft tissue and 0.5% is present in the extracellular and intracellular spaces, with both ions playing a prominent role in regulation of cellular function (Rizzoli & Bonjour, 2006).

Parathyroid hormone (PTH) is the main hormone involved in calcium homeostasis. Hypocalcemia acts as the main driver for pulsatile PTH secretion, whilst a high ionized calcium acts to inhibit PTH synthesis, driven by interactions with the cell surface calcium sensing receptor (CaSR) (Felsenfeld, Rodriguez, & Aguilera-Tejero, 2007; Silver, Naveh-Many, Mayer, Schmelzer, & Popovtzer, 1986). PTH is a single-chain 84 amino acid hormone produced by chief cells in the parathyroid glands. PTH exerts its effects through the interaction of its first 34 amino acids with the type 1 PTH/PTH-related peptide (PTHrP) receptor (PTH1R), which is highly expressed in bone and kidney, but also in lower levels in the vasculature. Peripheral metabolism of the hormone fragments occurs in the liver and kidneys (Hruska, Korkor, Martin, & Slatopolsky, 1981). Fragments containing carboxyl (C)- or amino (N)-terminal portions of the molecule are present in the circulation at varying half-lives, with C-terminal (possibly inactive or antagonistic) fragments existing 5-10 times longer in the circulation than PTH (1-84), which has a half-life of 2-4 minutes. As a result, circulating PTH is a heterogeneous mixture of full-length hormone and fragments (Evenepoel, Bover, & Urena Torres, 2016). Another PTH receptor, the PTH2R, is mainly expressed in the

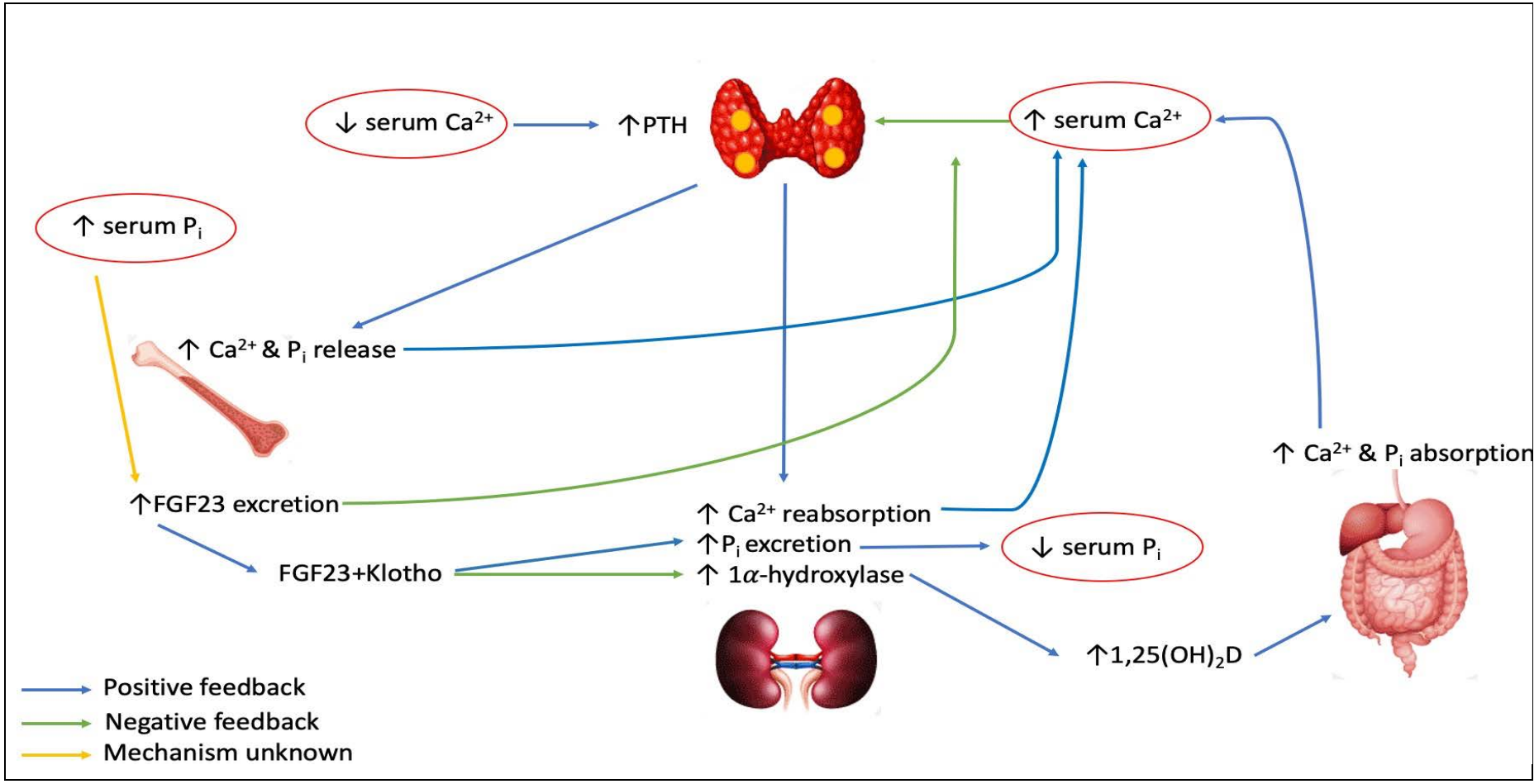
central nervous system, thyroid, gastrointestinal tract, pancreatic islet cells and the cardiovascular system. Like PTH1R, the PTH2R responds to PTH, with generation of cAMP and increase in tricellular calcium (Rodriguez & Lorenzo, 2009).

PTH exerts its main action in the kidney and bone. In the kidney, PTH stimulates calcium reabsorption in the distal convoluted tubule by activating specific ion channels, whilst promoting urinary phosphate excretion by regulating sodium-phosphate cotransporters in the proximal convoluted tubule (Goldfarb, 1996). PTH also indirectly enhances small intestinal calcium and phosphate absorption by stimulating renal production of 1,25 dihydroxyvitamin D (1,25$[OH]_2$D) (Lee & Partridge, 2009). In the bone, PTH plays an important role in bone remodeling. The acute response to a low ionized calcium (minute-hours) is calcium release from a rapid skeletal exchange pool in bone as well as osteocyte mediated osteolysis. A slower response, which takes several days to weeks, is driven by bone cells involved in the remodeling process (Evenepoel et al., 2016). Bone resorption predominates in response to continuous exposure to high circulating PTH levels, whereas intermittent PTH administration leads to a net increase in bone mass. PTHR1 is also expressed in various tissues, including pancreas, bone marrow and vasculature. PTH exerts acute vasodilatory actions through PTHR1 activation in vascular smooth muscle cells and reduces vascular oxidative stress and pro-calcific and profibrotic signals that drive atherosclerotic disease (Cheng et al., 2010).

Calcitriol or 1,25$[OH]_2$D is another major regulator of mineral metabolism, having a stimulatory effect on calcium and phosphate metabolism (Khundmiri, Murray, & Lederer, 2016). Renal synthesis of calcitriol occurs when 1-α hydroxylase, a key enzyme produced in the renal tubular cells converts 25-hydroxyvitamin D (25[OH]D) to 1,25$(OH)_2$D. This leads to enhanced intestinal absorption of calcium and phosphate. Thereby, PTH and calcitriol form a tightly controlled feedback cycle, with PTH being a major stimulator of calcitriol synthesis in the kidney, whilst calcitriol exerts negative feedback on PTH secretion.

Phosphate homeostasis is not completely understood, and in particular no 'phosphate sensor' has been convincingly demonstrated in mammals. Over the past decade much work has gone into studying the protein fibroblast growth factor 23 (FGF23) and its co-receptor Klotho, both of which play an in important role in phosphate metabolism. FGF23 is primarily secreted by osteocytes (Quarles, 2008) and has several endocrine effects on mineral metabolism. Many of the physiological effects of FGF23 are dependent on the presence of α-klotho, which is highly expressed in the kidney and the parathyroid glands. The interaction of α-klotho with the FGF receptor, creates a unique binding pocket for FGF23, facilitating the actions of this protein(Kurosu et al., 2006; Richter & Faul, 2018; Urakawa et al., 2006). The main functions of FGF23 are to decrease the serum levels of 1,25$[OH]_2$D through inhibition of 1-α hydroxylase (Hasegawa et al., 2010; Shimada et al., 2004) and enhance phosphaturia by inhibiting phosphate proximal tubular resorption by reducing luminal sodium phosphate cotransporters NaPi2a and NaPi2b (Berndt & Kumar, 2007; Liu & Quarles, 2007). FGF23, along with calcitriol also forms part of the negative feedback loop to inhibit secretion of PTH (Ben-Dov et al., 2007).

Figure 1.1 Diagrammatic representation of physiological mineral handling illustrating the interaction between the kidney, parathyroid gland, intestine and bone



The main reservoir for calcium and phosphate in the body is the skeleton. The skeleton is composed of two types of bone: cortical and trabecular. Cortical bone is heavily calcified and fulfills a mainly structural and protective role. Trabecular bone is less heavily calcified and has a greater surface area which allows it to be metabolically active. Overall, the adult skeleton is about 80% cortical bone and 20% trabecular bone. The proportion of trabecular and cortical bone varies by the skeletal site (Walsh, 2015). The role of calcium and phosphate in bone is to form hydroxyapatite crystal deposited in specific regions of intra- and interfibrillar collagen fibril networks. In the extracellular fluid, precipitation of calcium and phosphate is prevented in part due to presence of proteins which prevent accretion of mineral at the nanoparticle level. One such nanoparticle complex is the fetuin calciprotein particle (CPP). These particles are increasingly recognized as important intermediates of mineral trafficking and may play a key homeostatic role in buffering against pro-calcific changes in mineral metabolism (Holt & Smith; Jahnen-Dechent, Schäfer, Ketteler, & McKee, 2008).

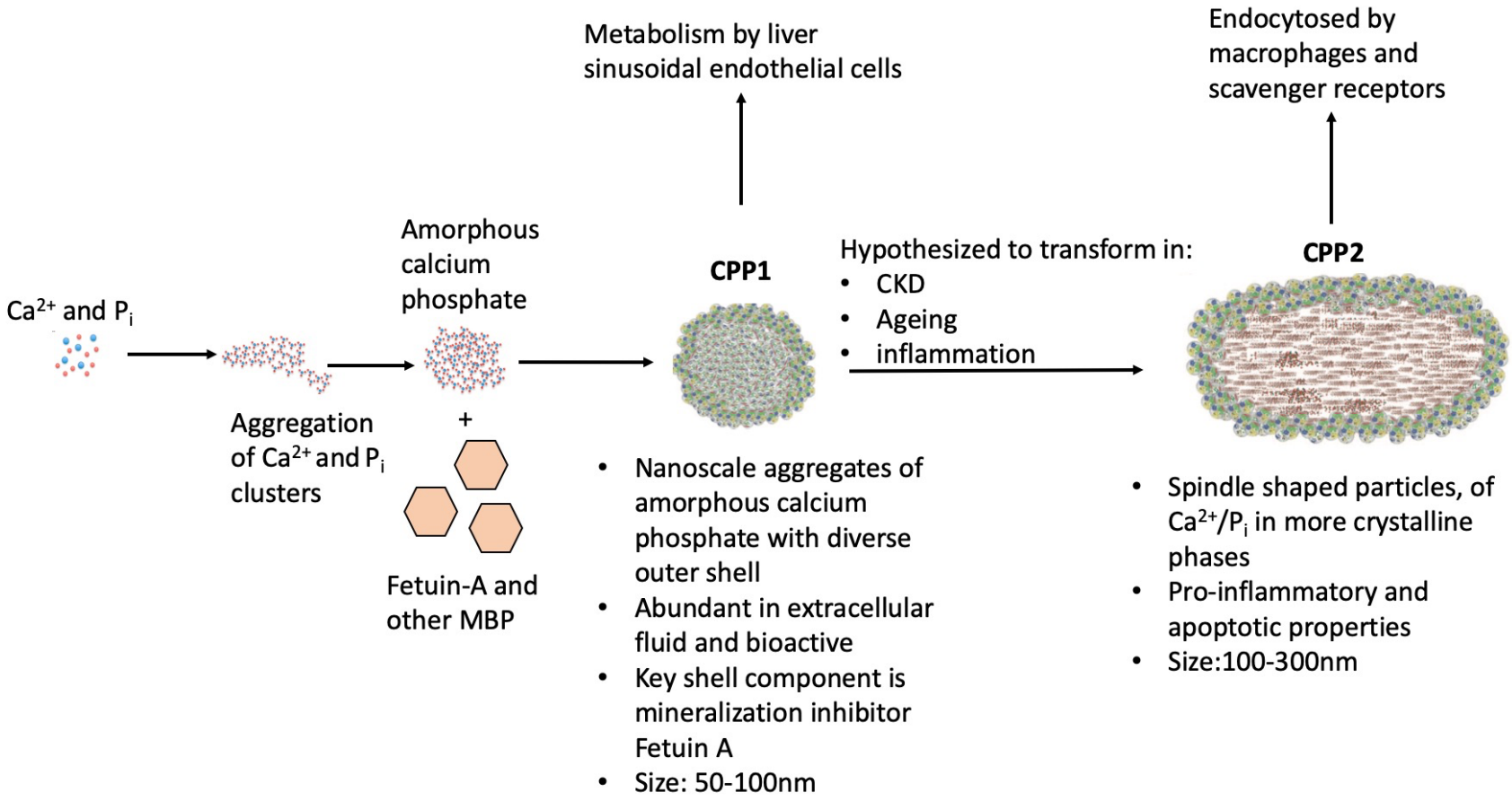
Although the structural aspects of CPP have been well described the function of these nano-molecules in humans is currently being investigated, *in vitro* studies have demonstrated that they are important in halting the progression of crystal growth, whilst facilitating clearance and metabolism of insoluble calcium phosphate (Andreas Pasch, Jahnen-Dechent, & Smith, 2018). In *in vitro* models*,* protein-bound calcium phosphate ion clusters associate to form spherical structures, 50-100 nm in diameter (Wald et al., 2011) containing mineral as amorphous calcium phosphate (known as primary CPP or CPP1). CPP1 can subsequently undergo “ripening” to larger and more spindle shaped particles 100-300 nm in diameter (known as secondary CPP or CPP2) containing more crystalline phases like hydroxyapatite (Andreas Pasch et al., 2018). The circulating pool of CPP is pleomorphic with mineral held in various states of crystallization.

The outer “shell” of endogenous CPP contains a diverse range of proteins (such as fetuin-A and other mineral binding proteins (MBP), apolipoproteins, fatty acid binding

proteins), bioactive macromolecules (including lipids, nucleic acids, magnesium and zinc), DNA fragments, small RNA and microbe-derived components (Smith, Hewitson, Hanssen, & Holt, 2018). The composition reflecting the biological fluid in which they are made. One key protein, fetuin-A, is a major regulator of mineralization. It inhibits the growth of small calcium phosphate crystal nuclei through a molecular shielding mechanism mediated by its amino-terminal cystatin-like domain (Berchtold et al., 2016).

Animal *in vivo* studies have demonstrated that CPP1 are cleared by the liver sinusoidal endothelial cells, whereas CPP2 are endocytosed by macrophages in the liver and spleen via scavenger receptor A (Herrmann et al.; Koppert et al., 2018). Removal of CPP2 is not without potential toxicity, *in vitro* studies have shown that CPP2 clearance can induce toll-like receptor 4 (TLR4)-dependent TNFα and inflammasome-dependent IL-β secretion in macrophages. This evidence supports the role of prolonged CPP presence in inflammation and calcification (Koppert et al., 2018). Levels of CPP are significantly higher in patient cohorts known to develop premature ageing and arterial calcification, such as patients with CKD (especially those on dialysis) (Smith et al., 2017) and those with inflammatory diseases (Smith et al., 2013), compared to healthy controls.

Figure 1.2 Proposed maturation and development of calciprotein particles in serum



1.2 Mineral handling in CKD

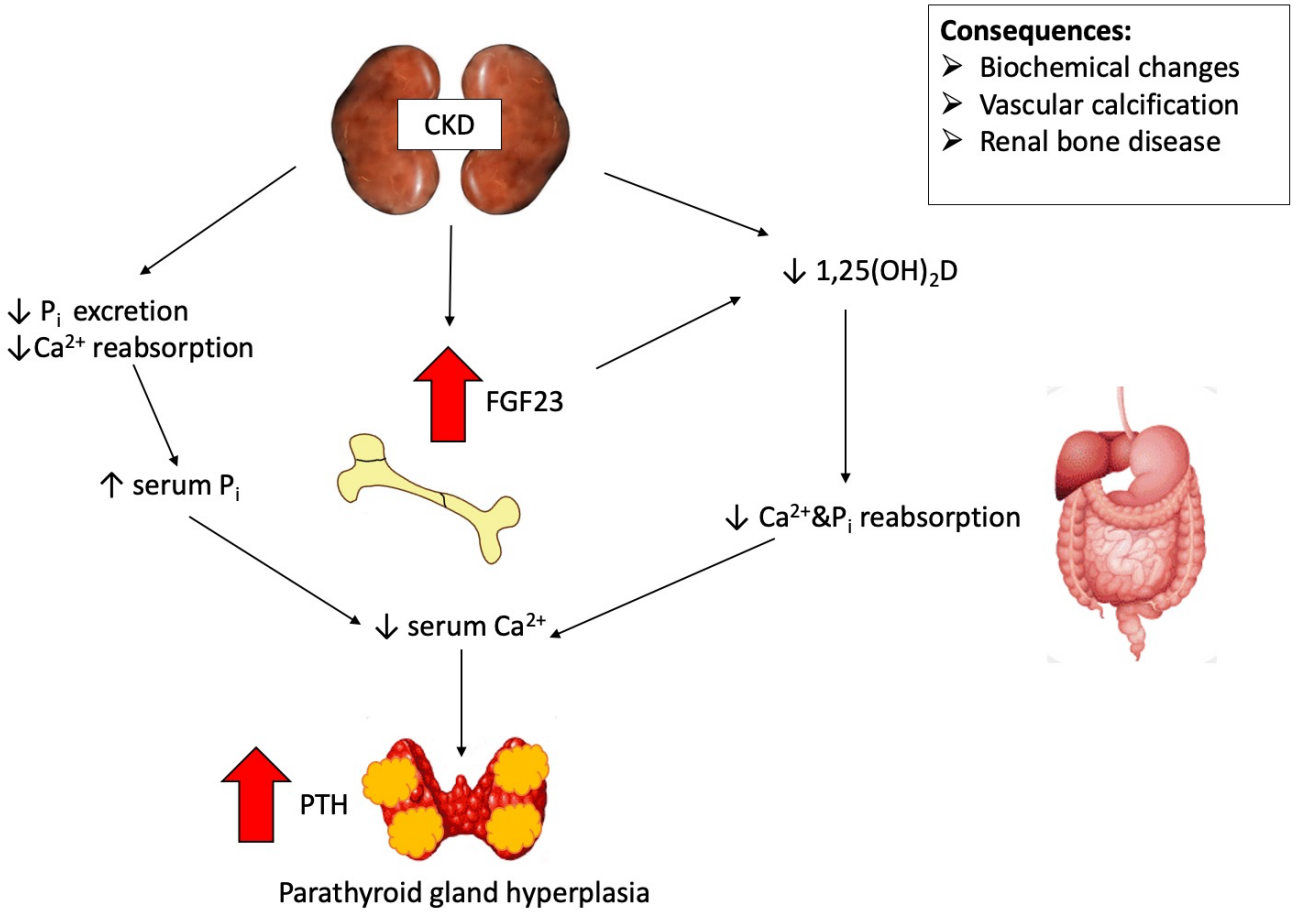
In chronic kidney disease (CKD), as kidney function declines, there is continuing disruption to normal mineral metabolism. The earliest endocrine abnormality currently detectable is a progressive increase in FGF23 levels, which occurs concurrently as glomerular filtration rate declines (Gutierrez et al., 2005; Larsson, Nisbeth, Ljunggren, Juppner, & Jonsson, 2003). An increase in this phosphaturic hormone leads to a downregulation of 1-α hydroxylase expression in proximal renal tubular cells, with resulting reduced production of $1,25[OH]_2D$ as well as direct suppression of PTH. Although initially elevated FGF23 levels directly suppress PTH gene expression, its inhibitory effect becomes limited due to decreased co-receptor klotho expression in the parathyroid glands of patients with advanced CKD (Komaba & Fukagawa, 2010).

In response to a down-regulation of $1,25[OH]_2D$, low ionized calcium and elevated serum phosphate levels, upregulation of parathyroid chief cell proliferation occurs to promote PTH synthesis (Slatopolsky et al., 1996). As this vicious cycle continues, rising PTH levels increase 1-α hydroxylase expression in the kidney and mobilize ionized calcium from bone. Persistent hyperparathyroidism results in development of parathyroid hyperplasia (Wang, Palnitkar, & Parfitt, 1997), known as secondary hyperparathyroidism (SHPT). This is initially present as polyclonal parathyroid cell proliferation (diffuse hyperplasia) and later progresses to monoclonal expansion of adenomatous-like tissue (nodular hyperplasia) (Goto, Komaba, & Fukagawa, 2008). As cells become increasingly adenomatous they lose responsiveness to vitamin D and CaSR activation.

Decline in kidney function also contributes to disturbed PTH metabolism, with an accumulation and marked prolongation of the half-life of C-terminal PTH fragments. Mounting evidence indicates that C-terminal PTH fragments, by binding to the PTHR1

exert biological effects that are a distinct opposite of (1-84)PTH (Wesseling-Perry et al., 2010). Another factor contributing to impaired action of PTH in CKD is PTH hyporesponsiveness, also referred to as PTH resistance. Factors associated with PTH resistance in CKD include phosphate loading, calcitriol deficiency, oxidative stress, magnesium deficiency, accumulation of PTH fragments and uraemic toxins (Evenepoel et al., 2016), as well as a decrease in PTH1R expression and function.

Figure 1.3 Diagrammatic representation of the pathogenesis of disturbed mineral handling in CKD

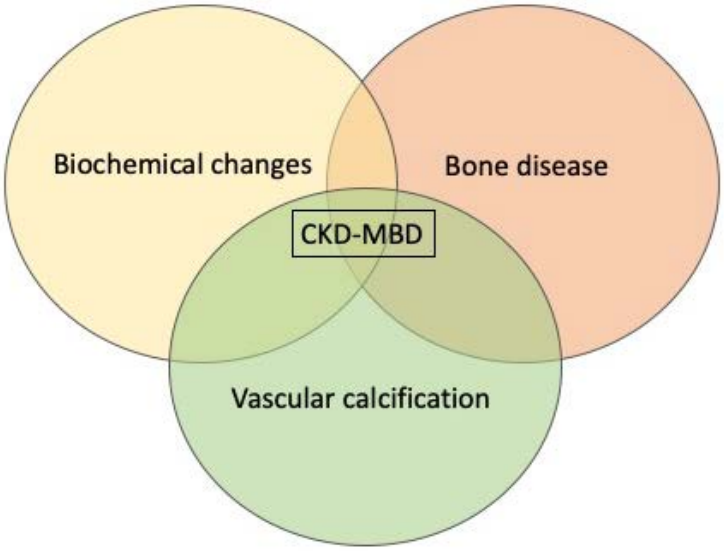


1.3 Complications of disturbed mineral handling in CKD

The combination of interrelated biochemical, bone and vascular abnormalities arising as a result of disordered bone and mineral metabolism in CKD encompass the broader clinical syndrome known as Chronic Kidney Disease Mineral and Bone Disorder (CKD-MBD) (KDIGO, 2009). This term was coined in 2005 by the international clinical guideline organization Kidney Disease: Improving Global Outcomes (KDIGO) after it became evident that the traditional definition of bone changes in renal disease (at the time known as renal osteodystrophy) did not accurately encompass the diverse clinical spectrum of abnormalities found in CKD. It is now well recognized that CKD-MBD significantly contributes to the increased mortality and morbidity in the CKD population (G. Block & Port, 2003). Improvement in clinical outcomes with newer therapeutic strategies, however, hasn't eventuated, partially due to lack of understanding of the pathological drivers of this disease as well as adequate diagnostic tools to assess different domains of this syndrome.

Figure 1.4 The three domains of disturbed mineral handling in CKD-MBD

Adaption from (KDIGO, 2009)



1.4 Biochemical complications of CKD-MBD

Traditionally, the key biochemical derangements that encompass this domain of CKD-MBD, specifically elevated PTH, reduced vitamin D levels, reduced total calcium and elevated serum phosphate, were thought to begin from stage 3 CKD, with the rate and severity of these abnormalities highly variable among patients. With greater understanding of the pathophysiology of FGF23, it is now known that FGF23 levels are significantly elevated by stage 2 and early stage 3 CKD (Larsson et al., 2003) and it is this excess of FGF23 which contributes to insufficient 1-α hydroxylase activity and the cascading biochemical consequences outlined above.

More novel biochemical markers such as CPP levels are also elevated in CKD (Hamano et al., 2010; Smith et al., 2013; Smith et al., 2014; Smith et al., 2012) and are positively associated with serum phosphate, inflammatory and bone turnover markers (Smith et al., 2012), coronary artery calcification (Bundy et al., 2019; Hamano et al., 2010), aortic

stiffness (Smith et al., 2012) and mortality (Smith et al., 2014). Very high levels of CPP have been reported in patients on dialysis with calciphylaxis, reflecting the widespread calcification and poor prognosis of this condition (Smith et al., 2013). However, unlike PTH and FGF23 which rise exponentially with progressive CKD, a temporal relationship of CPP and kidney function decline is yet to be established. In patients on dialysis with SHPT, use of parathyroidectomy or calcimimetics resulted in a reduction in CPP (Hamano et al., 2010), suggesting that abnormal bone metabolism is associated with mineral stress, leading to an imbalance in production and removal of CPP. Current human studies support an association between CPP and inflammation although causality in this cohort is yet to be proven with robust clinical studies or animal models.

CPP have also been temporally implicated in the development of VC *in vivo*, appearing in the circulation of adenine-fed animals before presence of detectable aortic mineral deposition (Matsui et al., 2009). *In vitro* studies have demonstrated that mineral nanocrystals drive mineralization (Sage, Lu, Tintut, & Demer, 2011) and CPP2 are able to induce oxidative stress, inflammation and mineralization in VSMC (Aghagolzadeh et al.; Aghagolzadeh et al., 2017; M. M. X. Cai, Smith, Tan, Hewitson, & Holt, 2017). Detection of elevated levels of CPP in the circulation in pathology may serve as a novel biomarker of mineral stress and cardiovascular risk (Aweeka et al., 1999). There remain many gaps in knowledge of the role this novel biomarker plays not only in normal mineral handling but in the pathogenesis of calcification and inflammation in CKD and end-stage kidney disease (ESKD) which require further exploration.

1.5 Bone complications of CKD-MBD

The bone-disease component of CKD-MBD has far-reaching clinical ramifications. It contributes to increased risk of fractures, bone pain and deformities in growing children with CKD and ESKD. The incidence of hip fractures in patients on dialysis is 4.4 times higher than in the general population independent of age and gender (Alem et al., 2000),

and mortality in patients on dialysis following a hip fracture is 2.7 times higher than in the fracture-free dialysis patients and 2.4 times higher than in non-CKD patients with a hip fracture (Coco & Rush, 2000). Similar outcomes have been consistently shown in multiple studies using large observational data from the US Renal Data System (Danese et al., 2006; Kaneko, Foley, Gilbertson, & Collins, 2007; Mittalhenkle, Gillen, & Stehman-Breen, 2004).

CKD-MBD encompasses a variety of disturbances of cortical and trabecular bone. Typically, bone changes in SHPT are most commonly related to high bone turnover disease, known as osteitis fibrosa, with excessive rates of bone resorption by osteoclasts in response to high levels of PTH. Resulting in decreased bone mass and lamellar structure and bone marrow fibrosis (Sherrard et al., 1993). This has been demonstrated in an animal model, where continuous infusion of supraphysiological levels of PTH in 5/6 nephrectomized rats led to high-turnover disease and had an osteoporotic effect on the bone (Neves et al., 2007). Dialysis patients with PTH levels > 900pg/ml (equivalent to 198pmol/L) versus PTH 150-300pg/ml (33-66pmol/L), had a significantly increased risk of new fractures (relative risk (RR)=1.72 [95% confidence interval (CI)1.02-2.90], $P<0.05$ (Jadoul et al., 2006).

Based on histomorphometric studies, other forms of bone disease can also be present in patients with CKD-MBD including osteomalacia due to deficiency of $1,25[OH]_2D$ or adynamic bone disease. Adynamic bone disease is characterized by reduced bone formation and resorption without a corresponding increase in osteoid. This may occur as a result of skeletal resistance to PTH in CKD, which occurs due to a decrease in osteoblast PTH receptor expression, accumulation of the inactive 7-84 PTH fragments, and accumulation of osteoprotegerin (Iwasaki et al., 2006). The majority of histomorphometric studies are limited to the animal setting and human studies typically provide a single time point assessment of bone pathology. Presently, the effect of SHPT on bone microarchitecture has not been clearly delineated.

As CKD progresses, bone abnormalities can evolve and overlap (like post-menopausal or age-related osteoporosis) which contributes to the challenges of accurate diagnosis. Unlike osteoporosis where bone fragility is due to low bone mineral content, CKD-MBD can lead to an abnormal bone quality even in the setting of a normal or high bone mineral content. The gold standard for diagnosis of bone abnormalities in CKD-MBD is a bone biopsy, and is recommended by KDIGO for patients in whom the aetiology of bone disease is not certain (Ketteler et al., 2017). The current system for evaluating bone biopsy changes in CKD is based on determining bone turnover, bone mineralization and bone volume. Although a bone biopsy provides the best assessment of bone histology, this investigation has gone out of vogue due its invasive nature, difficulty in sample processing and analysis and challenges in monitoring longitudinal changes.

In clinical practice, bone turnover markers are routinely used to identify and monitor bone abnormalities for common bone pathologies such as osteoporosis. Unfortunately, standard serological markers of bone turnover do not reliably distinguish between different renal bone pathologies and commonly used biomarkers can have assay limitations and inconsistent results in CKD (Coco & Rush, 2000; Danese et al., 2006; Sprague et al., 2016). This is particularly the case in adynamic bone disease (Sprague et al., 2016). Therefore, radiological techniques have become important for measuring bone mineral density (BMD). Currently, dual-energy X-ray absorptiometry (DXA) and (in-specialized centres) quantitative computer tomography (CT) are used for patients with CKD and ESKD. There are three main issues with DXA. Firstly, DXA scans are unable to differentiate between cortical and trabecular bone. This is a particular issue when looking at the vertebral body which is 42% trabecular bone (Nottestad, Baumel, Kimmel, Recker, & Heaney, 1987), and also becomes important in CKD as bone remodeling is different in trabecular bone compared to cortical bone. Secondly, DXA scans can overestimate vertebral BMD due to presence of aortic calcification or extensive osteophyte formation. Finally, bone density is not a good predictor of fracture rate in patients with progressive CKD (West et al., 2015). Quantitative CT overcomes some of these issues as it can separately measure cortical and trabecular bone, however it exposes patients to ionizing radiation and requires specialized CT scanners, which are

not widely available. Routine assessment and monitoring of bone disease in CKD-MBD still has many short falls, which in turn prohibits adequate assessment of treatment strategies in this population. A promising new technique is micro- MRI scanning, a radiation-free and novel method, which requires further investigation in the CKD and ESKD population to assess bone microarchitecture.

1.6 Vascular complications of CKD-MBD

Vascular calcification (VC) is highly prevalent in patients with CKD and makes up the third domain of CKD-MBD. Observational studies report the prevalence of VC in up to 25% of patients with CKD stage 3 and over 70% in patients on dialysis (Sigrist, Taal, Bungay, & McIntyre, 2007). Once present, VC progresses rapidly, irrespective of age (Foley, Parfrey, & Sarnak, 1998; Shroff et al., 2008). Two types of VC are identified in patients with CKD (Goodman, 2002) – intimal calcification, associated with atherosclerotic plaques, which depending on the plaque composition can erode and rupture, and medial calcification of large elastic arteries and arterioles, resulting in increased arterial stiffness and cardiac afterload (Abedin, Tintut, & Demer, 2004; London, Marchais, & Guerin, 2004; Virmani, Burke, Kolodgie, & Farb, 2002). These subtypes do not occur in isolation and both pathologies can be present in the same vascular bed. In large epidemiological studies, VC is associated with, but not necessarily causative of, increased risk of cardiovascular mortality (London et al., 2003) and morbidity (Lehto, Niskanen, Suhonen, Ronnemaa, & Laakso, 1996). There is also a well described inverse association between VC and reduced BMD, with increased fracture risk (Braun et al., 1996).

Medial calcification has been identified in multiple anatomical locations in patients with ESKD including small arteries and arterioles in malignant breast tissue (W. C. O'Neill & Adams, 2014), as well as subcutaneous tissue of lower limb amputations (Ellis & O'Neill, 2018) In both locations, VC has been identified in vessels in the presence of other risk factors for calcification, including active malignancy or peripheral vascular

disease. The presence of VC of small vessels in the skin and subcutaneous tissue has only been described in patients with calciphylaxis (Mochel, Arakaki, Wang, Kroshinsky, & Hoang, 2013), although recent studies suggest that this may not be a specific finding (Ellis & O'Neill, 2018). The skin is the largest organ of the body and is comprised of three layers: the epidermis, dermis and deeper subcutaneous tissue. The role and composition of each layer is unique; all three layers receive blood supply from a vast network of arterioles and capillaries, with the largest calibre vessels located in the deep subcutaneous layer made up of fat and connective tissue. Presently there is little information about the prevalence of calcification in the skin and subcutaneous tissue in patients with CKD and ESKD outside of the calciphylaxis setting and whether it holds any clinical significance.

VC in patients with CKD is complex and pathogenesis is not fully understood. This process likely results from multiple interlinked pathways involving aberrant bone metabolism, inflammatory changes and dysregulation of endogenous calcification inhibitors and promoters (Reynolds et al., 2004; Speer & Giachelli, 2004). Both biochemical (passive) and cellular (active) processes may contribute to ectopic deposition in vascular smooth muscle cells. These processes can also impact on one another and it is uncertain whether one is a true biological process or a secondary reactive phenomenon. Phenotypic alteration to osteoblastic phenotype has become an accepted mechanism and is considered by some to be the most important pathological process, although this is far from the case in all experimental settings. Other changes include elastic degradation and calcinosis, and passive deposition of calcium. The initial trigger for calcification remains contentious and likely depends on the type of calcification (intimal versus medial) and location of the involved vessel, as different vessels are more prone to different calcification pathologies. In patients with CKD, and ESKD, a significant contribution to VC is the altered expression of inhibitors of calcification in association with deranged calcium and phosphate metabolism disrupting the homeostatic balance.

Numerous experimental studies have demonstrated the process of osteogenic transformation of vascular smooth muscle cells (VSMCs) into an osteoblast-like phenotype (Giachelli, 2004) with the ability to produce collagenous extracellular matrix on which mineral is deposited (Ciceri et al., 2012; Shanahan, Crouthamel, Kapustin, & Giachelli, 2011). Differentiation of VSMCs is characterized by expression of genes that are normally restricted to bone tissue, such as bone morphogenic protein 2 (BMP-2), osterix, runt-related transcription factor 2 (RUNX2) and tissue non-specific alkaline phosphatase (TNAP) (Jono et al., 2000). Expression of these osteo-inducing genes is accompanied by downregulation of VSMC lineage markers, such as transgelin and calponin (Steitz et al., 2001). Another problem arising in patients with CKD is the development of a pro-inflammatory milieu (increased C-reactive protein (CRP) and interleukin-6 (IL-6), and reduction in fetuin-A) which contributes to endothelial dysfunction and likely aides in the development of intimal calcification in this population.

At a tissue level, important VC inhibitors include endogenous pyrophosphate and carboxylated matrix Gla protein (MGP). Pyrophosphate acts via binding to mineralization surfaces to prevent crystal growth. Extracellular levels of pyrophosphate are tightly regulated by ectoenzyme tissue nonspecific alkaline phosphatase (TNAP). As TNAP is vital for healthy skeletal development, a fine balance must exist in order to maintain calcification within specific tissue confines. MGP is another key protein expressed by chondrocytes and VSMCs. It requires vitamin K as an essential cofactor for enzymatic carboxylation and function. MGP inhibits calcification by binding to and inactivating pro-calcific BMP2 (Sweatt, Sane, Hutson, & Wallin, 2003; Y. T. Zhang & Tang, 2014). There remain many unanswered questions regarding the pathogenesis of this process, however as more is discovered about VC, we are likely to uncover interlinked mechanisms and multiple pathways between various calcification inhibitors, which work together to prevent this process in healthy adults.

1.6.1 Calciphylaxis-an extreme example of calcification

Given the burden of VC in CKD and ESKD, clinical situations of extreme calcification can potentially provide useful information with regards to disease pathophysiology and progression. Calciphylaxis, also known as calcific uremic arteriolopathy (CUA), is regarded as a template for accelerated VC (Kramann et al., 2013), and is a useful condition in which to evaluate the effects of systemic calcification. Calciphylaxis is a rare disease, with a reported annual incidence of 4 per 10,0000 dialysis patients(Brandenburg, Kramann, et al., 2017). It predominately affects patients with CKD, especially those with ESKD on dialysis, although it can also occur in patients with normal kidney function and following kidney transplantation (Nigwekar et al., 2015). This condition was first described in 1898, however the term 'calciphylaxis' was coined in 1961 by Hans Selye after laboratory experiments induced generalized subcutaneous soft tissue calcification in rats using a two-step process of sensitization and application of a challenging agent (such as local trauma) (Selye, Gentile, & Jean, 1961; Selye, Gentile, & Prioreschi, 1961; Selye, Grasso, & Dieudonne, 1961). In humans, the pathogenesis of calciphylaxis is not considered to be a hypersensitivity reaction, rather accelerated vascular and extraosseous calcification with evidence of osteogenic transformation of VSMC, together with vessel thrombosis and fibrointimal hyperplasia (Kramann et al., 2013). This results in vascular endothelial dysfunction and panniculitis leading to skin break down and impaired wound healing (Brandenburg, Sinha, Specht, & Ketteler, 2014; Kramann et al., 2013). Although the exact pathogenesis and initial driver for this process remain unknown, VC remains an important histopathological component of this disease.

Calciphylaxis presents with painful subcutaneous lesions that can develop into necrotic ulcers. Due to the progressive and unrelenting nature of cutaneous calcification, calciphylaxis is associated with significant morbidity and mortality due to extreme pain, non-healing wounds and lengthy hospitalizations. One-year mortality is reported as high as 45-80%, mainly due to complications of wound sepsis, adverse effects of

treatment, and treatment withdrawal due to uncontrollable disease burden (Fine & Zacharias, 2002; Nigwekar et al., 2015; Weenig, Sewell, Davis, McCarthy, & Pittelkow, 2007).

Currently, there are no agreed diagnostic criteria for calciphylaxis. Due to the rarity of this condition, diagnosis and treatment is based largely on case series or observational studies that are predominantly retrospective, with no randomized control trials to date. Diagnosis is most commonly based on clinical impression, supporting features and suggestive risk factors, whilst treatment varies depending on clinician and unit preferences, it typically involves wound management together with intensifying dialysis therapy and reducing calcium intake.

In conclusion, there remain significant gaps in our understanding of VC in CKD and ESKD. The focus of current investigations has been to identify the ramifications of VC in central vessels in patients CKD and ESKD. What happens in the small vessels of skin is relatively unknown. Particularly outside the calciphylaxis setting, we don't know whether small vessel calcification in the skin is a feature of CKD-MBD.

1.7 Overview of management of CKD-MBD

There is significant overlap in the current management of the complications of CKD-MBD and cardiovascular disease (CVD) in patients with CKD. Much of the evidence for management comes from observational data, without large randomized control trials to back up treatment choices. Nevertheless, KDIGO guidelines provide a useful framework for clinicians to consider approach to treatment (Ketteler et al., 2017).

Medical management of biochemical abnormalities of hyperparathyroidism and hyperphosphataemia are aimed at controlling phosphate by means of restricting

intake, optimizing dialysis and administering phosphate binders, as well as modulating calcium balance and suppressing PTH release with activated vitamin D and calcimimetics. Additional medical management of vascular and cardiovascular complications includes addressing traditional CVD risk factors. Although treatment for CVD in CKD and ESKD is aimed at multiple different physiological targets, efficacy is significantly reduced compared to the general population (Tomlinson et al., 2011).

There is currently no targeted treatment for bone complications of CKD-MBD other than the treatment of osteoporosis identified in patients with Stage 1-3 CKD (Ketteler et al., 2017). As previously outlined, identification of the type of renal bone disease present in patients remains challenging and longitudinal follow up of bone abnormalities is near impossible with current imaging modalities. Therefore, management of renal bone disease significantly overlaps with management of biochemical abnormalities and centres around control of SHPT.

1.8 Current medical management of CKD-MBD

Given the significant overlap in medical treatment for CKD-MBD, the simplest way to categorize therapies is to differentiate them into those that address traditional CVD risk factors, therapies aimed at lowering phosphate and therapies aimed at managing SHPT. When medical management of SHPT fails, patients proceed to surgical parathyroidectomy, which is outlined below.

All patients with CKD are at an increased risk of CVD, therefore addressing and modifying CVD risk factors in this population are imperative. Compared to the general population, patients with ESKD have a higher prevalence of many traditional CVD risk factors, including hypertension, diabetes and hypertriglyceridemia (Longenecker et al., 2002). Tackling traditional CVD risk factors plays an important management role in this patient group. Unfortunately, few studies have looked specifically at risk factor

modification in this patient group and most evidence is derived from *post hoc* analysis or meta-analysis. Blood pressure management plays a significant role in progression and complications of renal disease. Although there is no single optimal target for all CKD patients, KDIGO guidelines recommend targeting a blood pressure of ≤ 140mmHg systolic and ≤90mmHg diastolic for patients without albuminuria and ≤130mmHg systolic and ≤90mmHg diastolic for patients with albuminuria (Wheeler & Becker, 2013; SPRINT Research Group et al., 2015). Evidence regarding blood pressure targets becomes less concrete once patients commence dialysis, however two meta-analyses (Agarwal & Sinha, 2009; Heerspink et al., 2009) found benefit in antihypertensive agents compared to placebo for cardiovascular protection in dialysis patients. Whether benefit of antihypertensive management is through direct effect of reducing VC or other benefits of blood pressure lowering such as reduced myocardial fibrosis and left ventricular hypertrophy is uncertain.

The lipid profile of patients with CKD and ESKD can vary significantly from the general population, typically with higher levels of triglycerides and low HDL. Low HDL can contribute to complex inflammatory and oxidative stress milieu and likely exacerbate uraemic endothelial dysfunction (Keane, Tomassini, & Neff, 2013). Use of statins in CKD, as in the general population, is consistently found to reduce cardiovascular mortality, with a recent meta-analysis reporting statins associated with a relative risk (RR) of 0.72 (95% CI; 0.66-0.79) for major cardiovascular events (Palmer et al., 2014). Once patients transition to dialysis, statin therapy has not translated to improved cardiovascular outcomes based on three large randomized controlled trials (RCT) that have targeted specifically dialysis patients (SHARP (Baigent et al., 2011), AURORA (Fellstrom et al., 2009) and 4D (Wanner et al., 2004) trials), showing no significant impact on major cardiovascular events or all-cause mortality. There are no studies to support the use of fenofibrates in ESKD patients. Management of other traditional CVD risk factors, including diabetes, obesity and smoking cessation is in line with recommendations for the general population.

The second component of medical management centres around lowering serum phosphate. This is achieved through a combination of low phosphate diet, providing adequate dialysis and use of phosphate binders. Observational studies have shown significant and independent associations between elevated serum phosphate levels and all-cause mortality and CVD in people with normal kidney function and in patients with CKD. This relationship has been consistently reported across the spectrum of CKD from mild renal impairment to patients with ESKD on dialysis (G. A. Block, Hulbert-Shearon, Levin, & Port, 1998; Kestenbaum et al., 2005). Despite these associations, mechanisms by which elevated serum phosphate may contribute to CVD and death remain unclear and could, in part, potentially involve direct endothelial dysfunction as well as increased development of VC.

Phosphate binders have become pivotal in the standard care of dialysis patients; however, there are conflicting views on their role and the ideal serum phosphate target, with the most appropriate phosphate binder also not agreed upon universally. In a recent meta-analysis of 71 RCTs assessing phosphate binder use in dialysis patients, there was no difference in cardiovascular mortality between the two different classes of binders (calcium and non-calcium based)(RR 2.54, 95% CI 0.67-9.62) (Sekercioglu et al., 2016). Although studies relating to this issue are conflicting, the most consistent finding is that limiting exogenous calcium burden is considered beneficial as highlighted by recommendations in the current KDIGO CKD-MBD guidelines to restrict the use of calcium-based binders in all patients with CKD (Ketteler et al., 2017).

Addressing SHPT and rising PTH levels is the final component of medical management in CKD-MBD. This involves correcting vitamin D deficiency and lowering PTH levels with calcimimetics. Vitamin D deficiency in observational studies has been associated with VC, arterial stiffness and cardiovascular mortality in patients with CKD (Barreto et al., 2009), (Pilz, Iodice, Zittermann, Grant, & Gandini, 2011) as well as in the general population (Zittermann et al., 2012). In patients with CKD and those on dialysis, the

efficacy of both nutritional and activated vitamin D on vascular health has been investigated however, beneficial effects are uncertain. Presently, there is inconsistent evidence and conflicting literature regarding the form and dose of vitamin D supplementation. Of three recent RCTs (Kendrick et al., 2017; Kumar et al., 2017; Levin et al.) examining the role of vitamin D supplementation on markers of vascular stiffness in non-dialysis CKD patients, only two (Kumar et al., 2017; Levin et al.) reported improvement in surrogate markers of vascular stiffness (pulse wave velocity [PWV] and flow mediated dilation) with nutritional supplementation but not active vitamin D. The KDIGO CKD-MBD guidelines recommended nutritional vitamin D for treatment of vitamin D deficiency but not routine use of calcitriol or vitamin D receptor analogs in pre-dialysis patients, given the risk of hypercalcaemia and likely associated pro-calcific effects, unless prescribed for 'severe and progressive SHPT' (Ketteler et al., 2017).

Calcimimetics are used for the treatment of SHPT in patients with ESKD. They act to allosterically modulate the CaSR, which is expressed in a variety of tissues, including the parathyroid gland and VSMCs. In the parathyroid gland, this results in down regulation of PTH secretion. The two current preparations include oral cinacalcet and an intravenous formulation etelcalcitide. Multiple studies have reported the clinical efficacy of calcimimetics in reducing PTH levels (G. A. Block et al., 2004; Ketteler et al., 2012; Lindberg et al., 2005; Lindberg et al., 2003; Quarles et al., 2003; Raggi et al., 2011). Cinacalcet has also been associated with reduced progression of abdominal aortic calcification over a 12-month period (Nakayama et al., 2014) and, in *post hoc* analysis, Cunningham *et al* (Cunningham, Danese, Olson, Klassen, & Chertow, 2005) combined data on clinical outcomes from four RCTs and showed that treatment with cinacalcet resulted in a significant reduction in the risk of cardiovascular hospitalization (HR 0.61, 95% CI 0.43-0.86). Further benefits of calcimimetics in reducing VC were seen in the ADVANCE (A Randomized Study to Evaluate the Effects of Cinacalcet Plus Low Dose Vitamin D on Vascular Calcification in Subjects with CKD) study of 360 haemodialysis patients with SHPT (Raggi et al., 2011) treated with either cinacalcet plus low dose vitamin D or flexible doses of vitamin D alone. There was a 24% increase

in coronary artery calcification (CAC) for the cinacalcet plus vitamin D group compared to 31% for the vitamin D group alone (non-significant stratified median treatment difference was -10.3%; 95% CI -22.6-to 0.8%, p=0.07). There was also a stratified median treatment difference in aortic valve calcification of -44.7% (95% CI -85.8% to -6.1%, p=0.01). The major limitation of this trial was the short study duration (12 months), which was unlikely to be sufficient for the detection of substantial changes in VC.

Despite the potential benefits on VC, cardiovascular benefits of calcimimetics were not conclusive in the Effect of Cinacalcet on Cardiovascular disease in Patients Undergoing Dialysis (EVOLVE) trial (Investigators, 2012), which to date remains the largest RCT conducted in a dialysis population (3883 participants). The EVOLVE trial failed to show a difference in primary composite end point (death, myocardial infarction, hospitalization for unstable angina, heart failure or peripheral vascular event) in the cinacalcet compared to placebo group (hazard ratio [HR] 0.93, 95% CI 0.85-1.02, p=0.11). Results of this trial however should be interpreted with caution because of numerous limitations including a one-year age difference between study arms (55 and 54 years in the cinacalcet and placebo group respectively), and high study treatment cross over (significant drop-in and drop-out rate) due to commercially available cinacalcet. Although the primary analysis of the EVOLVE trial was negative, *post hoc* analysis demonstrated reduced incidence of calciphylaxis (unadjusted relative hazard, 0.31; 95% CI, 0.13 to 0.79; p=0.014) (Floege, Kubo, Floege, Chertow, & Parfrey, 2015), a trend towards reduced fracture rate (adjusted relative hazard 0.83 (95%CI, 0.72-0.98) (Moe et al., 2015) and decreased risk of death and cardiovascular events in patients over the age of 65 years (adjusted relative hazard 0.7, 95% CI 0.6 to 0.81) (Parfrey et al., 2015).

In Australia, cinacalcet was a commonly prescribed medication for the management of biochemical changes associated with moderate to severe SHPT in patients on dialysis from its introduction to the market in 2007. Following the publication of the EVOLVE

study (Investigators, 2012) the pharmaceutical benefits advisory committee in Australia withdrew reimbursement in August 2015. Cinacalcet is now available only on private prescription and at a significant cost to patients.

1.9 Current surgical management of CKD-MBD

Despite medical management, patients with severe and progressive SHPT may require surgical management with a parathyroidectomy. Although parathyroidectomies improve biochemical parameters and clinical symptoms related to severe SHPT, they are associated with an increased 30-day mortality and risk of re-hospitalization with hypocalcaemia (Kestenbaum et al., 2004). Surgical management also may not be suitable for older patients with complex co-morbidities, which encompasses a large proportion of dialysis patients in the Western world. In some cases, the effect of parathyroidectomy is also transient, with recurrence in up to 14% (Agha, Loss, Schlitt, & Scherer, 2012).

There are currently no RCTs that define an optimal PTH level for patients with CKD or clinical endpoints of hospitalization, fracture or mortality. According to the 2017 KDIGO CKD-MBD guidelines, treatment of SHPT should be based on progressive and persistently elevated PTH levels rather than single elevated values (Ketteler et al., 2017). As a result of these published guidelines, parathyroidectomy remains a valid treatment option especially in cases when PTH-lowering therapies fail.

Surgery is typically advocated in the following circumstances (Lau, Obi, & Kalantar-Zadeh, 2018):

1. Persistent hypercalcaemia (corrected calcium >2.5mmol/L) and hyperphosphataemia (serum phosphate >1.8mmol/L) despite patient compliance to diet and optimized vitamin D analog/calcimimetic doses

2. Elevated risk or presence of calciphylaxis
3. Erythropoietin-resistant anaemia, when other modifiable factors, such as iron deficiency or gastrointestinal bleeding have been excluded

Compared with medical management, parathyroidectomy is associated with decreased all-cause mortality (RR 0.74 [95%CI, 0.66-0.83]) in ESKD patients with SHPT but not in kidney transplant patients (Apetrii et al., 2017). Patients undergoing surgery had decreased cardiovascular mortality (RR 0.59; 95% CI, 0.46-0.76) in 6 observational studies that included almost 10,000 patients (Apetrii et al., 2017; Conzo et al., 2013; Costa-Hong et al., 2007; Iwamoto et al., 2012; Komaba et al., 2015; H. C. Lin et al., 2014). Unfortunately, most of the trials looking at surgical outcomes were performed in the pre-calcimimetic era, with no direct comparison between the two treatment modalities in a RCT. Other surgical benefits include improvements in erythropoietin-resistant anaemia in patients with marked hyperparathyroidism (Chow, Chan, Ho, & Lam, 2007; Jemcov, Petakov, Bogdanovic, Djukanovic, & Lezaic, 2008) and improvements in nutritional status and humoral and cellular immunity (Tzanno-Martins, Futata, Jorgetti, & Duarte, 2000; Yasunaga et al., 1999) as well as an increase in left ventricular function 1-2 weeks following surgery (Drueke et al., 1980).

Despite improvement in cardiovascular mortality, surgical management has failed to demonstrate a protective effect on VC. A recent prospective observational study of 19 haemodialysis patients with severe SHPT reported that parathyroidectomies resulted in increased CAC ($p=0.02$) and were associated with a shift from high to low bone turnover disease on bone biopsy over a 12-month period (90% of patients evolved to either very low or low bone turnover) (Hernandes et al., 2017). The presence of low bone turnover disease has previously been associated with increased risk of VC (London, Marty, et al., 2004).

Few studies have evaluated the short-term bone changes occurring following parathyroidectomy due to the practical difficulty of performing a bone biopsy before

and after surgery. The studies that have looked at this issue have demonstrated that parathyroidectomy suppresses bone resorption as well as cause a transient marked increase in bone formation and an increase in normal lamellar osteoid seams (Yajima et al., 2003). These findings indicate an uncoupling of bone formation and bone resorption, which explains the rapid uptake of calcium and phosphate by the skeleton following parathyroidectomy (known as hungry bone syndrome). In accordance with increased bone formation and mineralization following parathyroidectomy, observational studies have consistently reported an increase in BMD following surgery (Abdelhadi & Nordenstrom, 1998; Abugassa, Nordenstrom, Eriksson, Mollerstrom, & Alveryd, 1990; Chou, Chen, Lee, Chen, & Sheen-Chen, 2001; Fang et al., 2018; Yamanouchi et al., 2013; Yano et al., 2003). Only one study using US Renal Data System data has assessed the risk of fracture following parathyroidectomy by comparing long term fracture rates among haemodialysis patients who underwent parathyroidectomy with a matched control group. The investigators demonstrated that surgery was associated with a significantly lower risk for hip fracture (HR 0.68; 95% CI, 0.54-0.86) and combined fracture (HR 0.69; 95% CI, 0.57-0.83)(Rudser, de Boer, Dooley, Young, & Kestenbaum, 2007).

1.10 Current Australian clinical practice

Clinical consequences CKD-MBD remain a challenging problem for the Australian nephrology community. Despite rapid scientific expansion in the field of mineral metabolism, our armamentarium for this condition remains small. Following the withdrawal of cinacalcet in dialysis patients with SHPT, we have seen a rise in what is considered acceptable PTH levels, and consequently more patients are being referred and undergoing parathyroid surgery. We are yet to see the consequences of this clinical approach, particularly the effect it has on CVD mortality and morbidity, fracture and calciphylaxis rate.

1.11 Thesis Aims

The aims of this thesis are to explore the pathophysiology, clinical outcomes and novel assessment strategies of the three domains of CKD-MBD.

The aims specifically focused on addressing the pathophysiology and clinical outcomes in CKD-MBD are:

- To evaluate outcomes of calciphylaxis in Australian patients
- To develop a great understanding of changes in skin and subcutaneous tissue in patients with kidney disease
- To demonstrate the consequences of severe SHPT in the absence of calcimimetic treatment

The aims specifically focused on evaluating novel assessment strategies and biomarkers in CKD-MBD are:

- To understand the temporal relationship between CPP and biochemical markers of CKD-MBD
- To evaluate bone microarchitecture in patients with severe SHPT using a novel imaging modality

2. LESSONS AND CLINICAL OUTCOMES FROM THE AUSTRALIAN CALCIPHYLAXIS REGISTRY

2.1 Introduction

Traditionally, calciphylaxis has been considered a manifestation of severely deranged mineral metabolism, and originally PTH and vitamin D were used as sensitizing agents in rats to evoke this process (Selye, 1962). Since the first published descriptions of calciphylaxis the early 1960s, we now appreciate that dysregulated mineral metabolism is not the sole risk factor or cause for calciphylaxis. Nevertheless, calciphylaxis is still considered an accelerated template of VC with devastating clinical consequences. Given the burden of VC in CKD and ESKD, a study of the natural history of such extreme calcification can potentially provide useful insight into the pathophysiology and progression of CKD-MBD.

Calciphylaxis remains a feared condition associated with significant morbidity and mortality, due to extreme pain, non-healing wounds and lengthy hospitalizations. One-year mortality is reported as high as 45-80%, mainly due to complications of wound sepsis, adverse effects of treatment, and treatment withdrawal due to uncontrollable disease burden (Fine & Zacharias, 2002; Nigwekar et al., 2015; Weenig et al., 2007). Diagnosis and management of calciphylaxis remain challenging due to the rare nature of this condition. There are currently no completed, randomized controlled trials to guide treatment, with evidence largely based on case series or observational studies that are predominantly retrospective. Calciphylaxis registries have been established in several countries to provide real-world information regarding risk factors, diagnosis and management of this disease.

The German Calciphylaxis Registry (http://www.calciphylaxie.de) was the first online registry, and has reported a calciphylaxis incidence of 4 per 10,000 patients on dialysis

in Germany (Brandenburg, Kramann, et al., 2017). This registry also highlighted the high use of vitamin-K antagonists (VKA) in patients with calciphylaxis and suggested that low rather than high PTH values may be key risk factors for calciphylaxis, with 45% of dialysis patients having PTH values below the recommended target range. This finding was in contrast to previous case reports showing an association with high PTH (Mohammed, Sekar, Bubtana, Mitra, & Hutchison, 2007; Velasco, MacGregor, Innes, & MacKay, 2006), and results from the EVOLVE trial, which demonstrated that the calcimimetic cinacalcet decreased PTH levels and was associated with a reduced incidence of calciphylaxis (Floege et al., 2015).

Registries for calciphylaxis may provide important insights and information to increase our knowledge about the epidemiology and clinical aspects of this condition and may help to optimize therapeutic management. The German Calciphylaxis Registry (Brandenburg, Kramann, et al., 2017) was limited to collecting data at a single time point, and did not collect data on longer term outcomes, including mortality. The Australian Calciphylaxis Registry (ACR) became active in 2013, with the aim to develop a large database of cases of calciphylaxis in Australia, which could provide a resource for research into causes and evidence-based management. The registry was also designed to evaluate the safety and efficacy of therapies, and assess overall patient care and outcomes with prospective, longitudinal data collection.

2.2 Aims

The aim of this chapter is to provide a detailed and comprehensive summary of the first 5 years of data collection from the ACR; looking specifically at the presentation of calciphylaxis, patient risk factors, biochemical parameters associated with CKD-MBD at the time of diagnosis, patient treatment and outcomes. Additionally, following withdrawal of government re-imbursement for cinacalcet in 2015, I was interested to identify whether this would contribute to an increased incidence of calciphylaxis.

2.3 Methods

2.3.1 Registry design and development

The ACR is accessible at http://www.calciphylaxis.org.au, and was established in association with investigators involved in the development of the German Calciphylaxis Registry in 2013. The registry is non-interventional, prospective and observational, and participants provided informed consent for deidentified data collection and storage. The registry received ethical approval from the Western Sydney Local Health District Human Research Ethics Committee in New South Wales, Australia, with reciprocal ethics and governance arranged at each additional involved site including The Royal Melbourne Hospital (RMH), Parkville, Australia. Registry activities are supported by the Australian and New Zealand Society of Nephrology (ANZSN), and by an unrestricted grant from Amgen Australia (2013 to 2017). Case recruitment commenced in 2014 at Westmead Hospital.

2.3.2 Registry data collection

The registry includes a comprehensive online database similar to the German Calciphylaxis Registry with questions covering >70 parameters and items regarding patient-related and laboratory data, clinical background and presentation as well as therapeutic strategies and outcomes of treatment (Appendix A). The online registry questionnaire provides drop down boxes for item selection and includes free text options for questions surrounding dialysis prescription, medications, and treatment outcomes. Photos of skin lesions can also be uploaded online to support the diagnosis.

Patients were followed longitudinally to determine disease and patient outcomes. Missing or ambiguous registry data for this project were clarified with either the

treating physician or research nurse at the participating centre. In the event that implausible data could not be clarified, such data or the entire dataset was excluded from analysis.

The registry also recorded biochemical and haematological parameters of serum creatinine, urea, potassium, bicarbonate, calcium, albumin, total protein, phosphate, magnesium, uric acid, ALP, PTH, C-reactive protein, haemoglobin, white cell count, and platelets. For each parameter, standard procedures were applied according to the local participating laboratory. There remains some variability between different intact PTH platforms available (Eddington et al., 2014) at each site, however this variability across units in different states was unavoidable. For serum albumin levels < 40g/L, the adjusted serum calcium was calculated as calcium (mmol/L) + 0.02 (40 - serum albumin).

2.3.3 Statistical analysis

Descriptive statistics were performed for the entire cohort as well as for subgroups of patients stratified by gender. All data are summarised and results reported as mean (+/- standard deviation [SD]) or median (inter-quartile range [IQR]) for continuous data, and number (percentage, %) for categorical variables.

Paired t-tests and Wilcoxon signed-rank tests were used for between group comparisons. Categorical variables were analyzed using the Chi-square test. Two-tailed P-values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPSS, Chicago, IL), graphics were created with GraphPad Prism 8 for Macintosh (La Jolla, CA, USA).

2.4 Results

2.4.1 Participant characteristics

From May 2014 to May 2019, seven participating Australian nephrology units provided data to the registry and recorded 50 calciphylaxis cases. Three patients were excluded from the final analysis due to missing data (n=2) or an alternative diagnosis to calciphylaxis being established (n=1), leaving 47 patients diagnosed with calciphylaxis. The mean patient age was 66 ± 11 years, with 51% female. The majority of patients were Caucasian (93%) and mean body mass index (BMI) was 35 ± 9kg/m^2. Median notification rate was 8 cases per year in complete years (minimum 5, maximum 12) across the seven centres. Baseline patient demographics are outlined in Table 1.

Table 2.1 Patient demographics, divided by gender

Patient demographics	Total (n=47)	Female (n=24)	Male (n=23)
Age (years)	66 ± 11	64 ± 11	68 ± 12
Gender, female	24 (51%)		
Race, Caucasian	43 (91%)	22 (92%)	21 (91%)
BMI (kg/m2)	35 (9)	36 (10)	33 (5)
CKD/ESKD/dialysis modality			
- Normal kidney function	1	0	1
- CKD, non-dialysis	5	3	2
- Kidney transplant recipient	2	0	2
- PD	10	5	5
- HD and HDF	29	15	13
Time on dialysis, years	4.8 [1.7-7.4]	4.9 [1.8-7]	4.0 [1.6-8.3]
Aetiology of CKD			
- Diabetic nephropathy	28 (60%)	16	12
- Renovascular disease	5 (11%)	2	3
- Glomerulonephritis	7 (15%)	1	4
- Polycystic kidney disease	1 (2%)	0	1
- Reflux nephropathy	4 (8%)	3	1
- Other	2 (4%)	1	1
Hospitalized within 3 months of diagnosis	28 (60%)	13	15

Results presented as mean± SD, median [IQR]

Calciphylaxis cases predominantly involved patients with ESKD (83%), with haemodialysis (HD) and haemodiafiltration (HDF) the most common dialysis modality (61%). Diabetic nephropathy was the most common cause of CKD (60%) although 76% of all patients had diabetes. Calciphylaxis was seen in five patients with CKD not on dialysis and in two patients with a functioning kidney transplant. One patient was

reported to have calciphylaxis with normal kidney function. Sixty percent of patients had been hospitalized within three months prior to the diagnosis of calciphylaxis.

Patients with calciphylaxis had a high co-morbidity burden including cardiovascular disease, arrhythmias, and metabolic syndrome (Table 2). Compared to incident dialysis patients in Australia, as recorded by the Australian and New Zealand Dialysis and Transplant Registry (ANZDATA) in the 2017 report (Registry, 2017a), calciphylaxis patients in the registry were more likely to be female, have a history of diabetes as well as vascular and coronary artery disease (Table 3). VKA were used by 16 patients (34%) prior to diagnosis (female to male ratio 14:2) and other commonly administered medications at diagnosis included active vitamin D (calcitriol) and calcium-based phosphate binders (Table 4). Biochemical parameters at diagnosis are listed in Table 5. Median PTH was 32 pmol/L [14-50 pmol/L] and was within the target range recommended by the KDIGO clinical guidelines (Ketteler et al., 2017). PTH levels in the 12 months prior to and following diagnosis of calciphylaxis were evaluated. There was no significant change in PTH within 12 months prior to diagnosis ($p=0.18$), although there was a statistically significant increase in PTH over the 12 months following calciphylaxis ($p=0.04$) (Figure 1).

Table 2.2 Patient co-morbidities

Co-morbidities	N=47
Ischaemic heart disease	25 (54%)
Arrhythmia	21(45%)
Valvular disease	9 (20%)
Hypertension	37 (80%)
Diabetes	35 (76%)
Hyperlipidaemia	22 (48%)
Peripheral vascular disease	25 (54%)
Peripheral neuropathy	14 (30%)
Chronic obstructive airways disease	5 (11%)
Previous fracture	5 (11%)
Gastrointestinal/peptic ulcer disease	7 (15%)
Malignancy	10 (22%)
Current or ex-smoker	17 (37%)
Previous calciphylaxis	4 (9%)

Results presented as number (%)

Table 2.3 Comparison of demographic details between patients with calciphylaxis and incident dialysis patients from the ANZDATA registry (2017 report)

Demographics	ACR (n=47)	ANZDATA (n=3056)
HD (satellite)	60%	51%
PD	21%	19%
Gender (female)	50%	38%
Race (Caucasian)	91%	63%
Diabetes	76%	50%
Coronary artery disease	54%	29%
Peripheral vascular disease	54%	14%
Chronic lung disease	11%	2%

Results presented as number (%)

Table 2.4 Medication prescribed at the time of calciphylaxis diagnosis

Medication	N=47
Cinacalcet	4 (9%)
VKA (warfarin)	16 (34%)
Active vitamin D (calcitriol)	19 (41%)
Phosphate binder	
- Calcium-based binder	20 (43%)
- Sevelamer	11 (24%)
- Lanthanum	7 (15%)
- Aluminium-based binder	3 (7%)
- Magnesium-based binder	5 (11%)
Erythropoiesis-stimulating agents	29 (63%)
Intravenous iron	29 (63%)

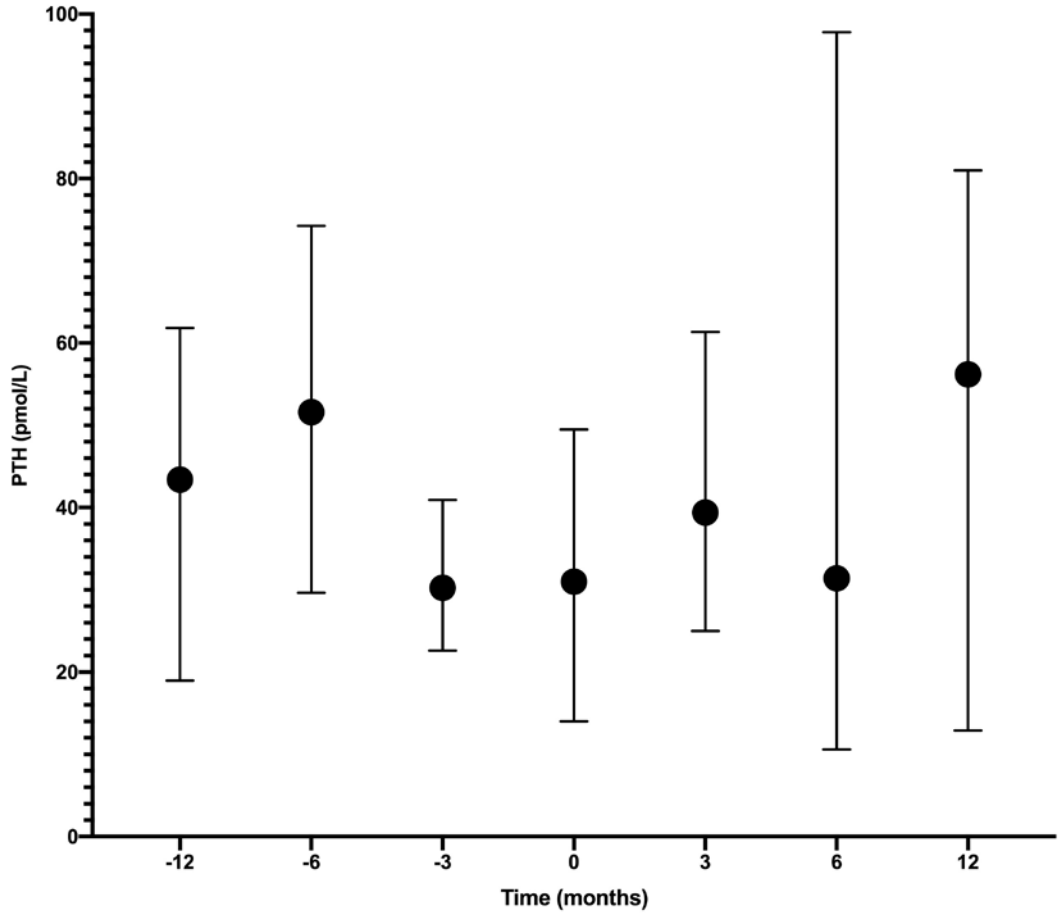
Results presented as number (%)

Table 2.5 Blood and serum laboratory parameters at the of calciphylaxis diagnosis

Laboratory parameter	N=47	Reference range
Albumin (g/L)	26 ± 6	35 – 55
Adjusted calcium (mmol/L)	2.56 ± 0.22	2.20 – 2.70
Phosphate (mmol/L)	1.7 ± 0.8	1.12 – 1.45
Magnesium (mmol/L)	0.86 ± 0.15	0.60 – 1.10
PTH (pg/L)	32 [14-50]	10 – 65
ALP (IU)	147 [113-238]	44 – 147
Haemoglobin (g/L)	95 ± 15	103 – 180 (males)
		120 – 160 (females)
CRP (mg/L)	36 [12-97]	<5
Bicarbonate (mmol/L)	23 ± 4	23 – 30

Results presented as mean± SD, median [IQR]

Figure 2.1 PTH levels 12 months prior to and following diagnosis of calciphylaxis



(Time=0); error bars refer to median with interquartile range

2.4.2 Diagnosis and site of calciphylaxis

Calciphylaxis was diagnosed by clinical impression in 31 cases, by skin biopsy in 21 cases, and by both in 11 patients (Table 6). Twelve patients were documented to have a suspected contributing event that may have precipitated calciphylaxis, including trauma (such as at injection sites) (n=10) and dialysis non-compliance (n=2). The lower limb was the most common site of calciphylaxis (n=31) and nine patients had more than one affected area. Females had a predilection to calciphylaxis affecting the abdomen and breasts, which compared to feet, lower limbs and thighs as more common sites for males.

Table 2.6 Characteristics of calciphylaxis lesions, divided by gender

Clinical features	Total (n=47)	Female (n=24)	Male (n=23)
Contributing event to calciphylaxis			
Trauma to site	8	1	7
(Injection to site)	2	2	0
Non-compliance with dialysis	2	1	1
No precipitating event	11	6	5
Not recorded	23	12	11
Site of calciphylaxis			
Lower extremities	30	13	18
Feet	9	4	5
Hands	1	0	1
Thighs	3	0	3
Abdomen	8	7	1
Buttocks	2	1	1
Breast	3	3	0
Penis	1	0	1
>1 site involved	9	3	6
Diagnosis of calciphylaxis			
Clinical impression	31	15	16
Skin biopsy	21	8	13
Radiograph	4	2	2
Nuclear medicine	1	0	1
>1 modality used for diagnosis	11	3	8
Outcome			
Resolution of lesion	10	9	1
Time to resolution (months)	8 [5-24]	6.7 [4.8-15]	32
Deceased	25	12	13

Results are number of cases, or median [IQR]

2.4.3 Treatment and outcome of calciphylaxis

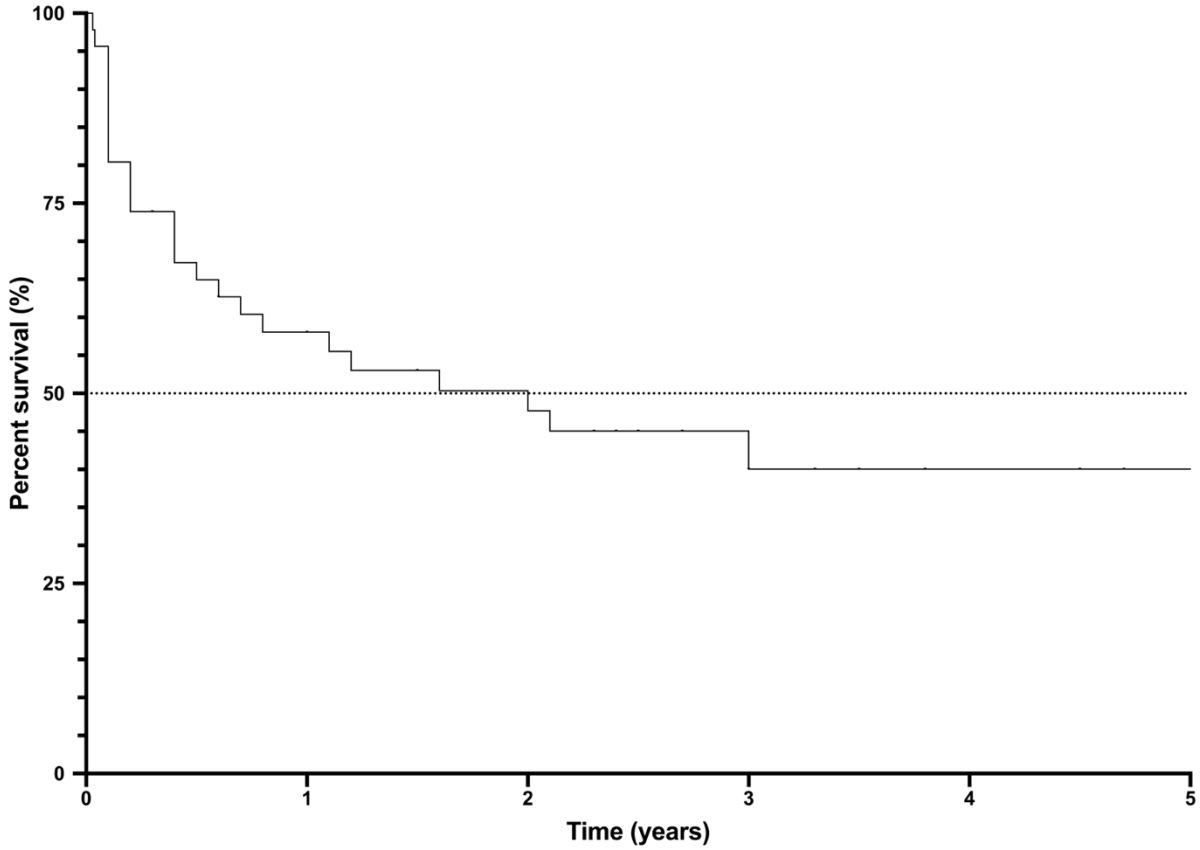
Treatment of calciphylaxis was multimodal and multidisciplinary (Table 2.7). In all cases, analgesia and wound care were provided, and antibiotics were prescribed in 29 cases (62%). Specific treatments for calciphylaxis are outlined in Table 2.7, and in all but two cases, more than one treatment option was administered. The most common treatment options included intensifying dialysis therapy, either with increased dialysis duration and/or frequency or transition from peritoneal dialysis to HD, as well as cessation of VKA and use of sodium thiosulphate (STS). Resolution of calciphylaxis was documented in ten patients (female to male ratio 9:1). Twenty-five patients had died by the time of analysis, with a mortality of 50% at 1.6 years [0.2-2.5 years] following diagnosis. Figure 2 shows the Kaplan-Meier survival curve for patients with calciphylaxis. The cause of death was documented in 11 cases, of which four were from septic shock, three from cardiac arrest, three withdrawal from dialysis and one patient had a catastrophic gastrointestinal haemorrhage. Table 2.8 highlights differences between patients who died from calciphylaxis and those who did not. The only statically significant difference was a history of ischaemic heart disease in patients dying from calciphylaxis ($p=0.04$).

Table 2.7 Therapeutic strategies used for calciphylaxis

Treatment strategy	N=47
Intensifying dialysis therapy	
Increase of dialysis duration and/or frequency	12 (26%)
Switch from PD to HD	4 (9%)
Reduction of calcium intake and calcitriol	
Lowering/stopping calcium phosphate binder	10 (22%)
Lowering dialysis bath calcium concentration	7 (15%)
Reduction/stop active vitamin D	8 (17%)
Vitamin K	
Stop VKA	11 (24%)
Give Vitamin K	6 (13%)
Management of SHPT	
Initiate cinacalcet	5 (11%)
Parathyroidectomy	5 (11%)
Specific calcification inhibition	
STS	12 (26%)
FFP/PEX	2 (4%)
Bisphosphonates	1 (2%)
Improve tissue oxygenation	
Hyperbaric oxygen treatment	5 (11%)
Revascularization (surgical and angioplasty)	1 (2%)
Antibiotics	29 (62%)
Surgical wound management (wound debridement, amputation and skin grafting)	23 (50%)
Cessation of intravenous iron	2 (4%)

Results presented as number (%)

Figure 2.2 Kaplan-Meier survival curve for patients with calciphylaxis from time of diagnosis



Time (years)	0	1	2	3	4	5
Subjects at risk	47	25	19	9	5	1

2.5 Discussion

This chapter provides a comprehensive summary of the data collected in the ACR. Specifically evaluating clinical and background information, biochemical markers, applied therapeutic strategies, and importantly, patient outcomes. As expected, most patients had ESKD, with risk factors for calciphylaxis including female gender, diabetes, obesity and administration of VKA. Therapy was multi-faceted, with analgesia and wound care implemented universally, and a combination of other specific therapies including intensifying dialysis, reducing or ceasing medications (calcium, calcitriol and VKA), and also the use of STS in one quarter of cases.

When comparing results from the ACR with those published in case series (Hayashi et al., 2012; Nigwekar et al., 2013; Nigwekar et al., 2016) and the German Calciphylaxis Registry (Brandenburg, Kramann, et al., 2017), there are many similarities. The ACR confirmed previously identified risk factors for calciphylaxis including obesity, diabetes and long dialysis vintage (Nigwekar et al., 2015). Demographic details of patients were similar in the ACR to those reported in the German registry, with a mean age of 66 years compared to 70 years, and a nearly equal gender ratio. The median notification rate for calciphylaxis cases was 8 patients per year, consistent with an annual Australian dialysis patient incidence of 0.3%, given that the seven participating institutions cared for approximately 2,500 dialysis patients. This is also similar to the estimated annual incidence of 0.35% in HD patients reported in the United States (Nigwekar et al., 2016). The registry reported 11% of calciphylaxis cases from patients with CKD not on dialysis, 4% from kidney transplant recipients and one patient (2%) with normal kidney function. These percentages are similar to those of the German Calciphylaxis Registry, where the percentage of patients with normal kidney function, non-dialysis CKD, and functioning kidney transplants were 3%, 7% and 4% respectively (Brandenburg, Kramann, et al., 2017).

Case reports and case series often highlight female gender as a risk factor (Nigwekar et al., 2015) and although this study identified an equal gender ratio, the proportion of female patients with calciphylaxis in the registry remains significantly greater than the overall proportion of female dialysis patients in Australia, at 51% versus 38% (Registry, 2018). Calciphylaxis in the ACR predominantly involved patients of Caucasian race (91% compared to 63% for incident dialysis patients) (Registry, 2018), despite the multiethnic nature of Australian dialysis patients. Whether this reflects an underlying racial predisposition to calciphylaxis, differences in dialysis adherence, BMI, drug regimens, under-reporting or misdiagnosis in other cultural groups requires further evaluation (Nigwekar et al., 2015; Weenig et al., 2007). The median time to diagnosis of calciphylaxis from commencement of dialysis was also similar to previous reports, which range from 30 months in the US and Germany to 105 months in Japan (Brandenburg, Kramann, et al., 2017; Hayashi et al., 2012; Nigwekar et al., 2016).

This chapter also set out to identify biochemical abnormalities of disordered mineral metabolism in ACR patients. Consistent with the German registry, biochemical parameters of CKD-MBD at the time of diagnosis were found to be within suggested KDIGO guideline targets (Ketteler et al., 2017). Hyperphosphataemia was not a significant concern in the current cohort (mean serum phosphate 1.7 ± 0.8 mmol/L at time of diagnosis), although concurrent presence of hypoalbuminaemia (mean albumin 26 ± 6 g/L) may reflect malnutrition or inflammation at the time of diagnosis. ALP levels were at or above the normal reference range (median ALP 147 [113-238 IU]). With higher ALP associated with increased mortality in non-CKD patients (Filipowicz et al., 2013), as well as increased risk of cardiovascular disease (Kabootari et al., 2018). In the current cohort, PTH values were relatively low at the time of diagnosis (median PTH 32 [14-50] pmol/L). This is an important finding, which supports the suggestion that more severe hyperparathyroidism may not be a significant risk factor for calciphylaxis (Floege et al., 2015).

Following withdrawal of government reimbursement for cinacalcet in Australia in 2015, the Nephrology community were concerned about a possible increase in the rate of calciphylaxis. This was supported in part by the subgroup analysis of the EVOLVE trial showing an increased rate of calciphylaxis in participants on placebo compared to cinacalcet (Floege et al., 2015). Analysis of registry data to date shows stable rates over the 5-year observation period. However, given the rare nature of this disease it may take longer to see a potential trend. Cinacalcet, prescribed in Australia through a special access scheme for compassionate use, and/or parathyroidectomy to manage SHPT were reported as part of calciphylaxis treatment in nine patients in the ACR.

With regards to calciphylaxis management, the only universally reported treatments were wound care (including antibiotics and/or surgical wound debridement) and pain management, which were implemented in all cases. Earlier studies have reported approximately 50% of patients with calciphylaxis can be bedridden or wheelchair-bound, and more than 70% may require hospitalization for severe ulcers (Weenig et al., 2007). Wound care is crucial to remove exudate and necrotic tissue and prevent infection, as sepsis from wounds is the commonest cause of death (Nigwekar, Wolf, Sterns, & Hix, 2008; Weenig et al., 2007). In the ACR, 61% of patients required hospitalization within 3 months of calciphylaxis diagnosis. Wound debridement was undertaken in 48% of cases, and retrospective studies have suggested improved survival among patients who undergo operative debridement (McCarthy et al., 2016; Weenig et al., 2007).

Multiple other therapeutic strategies for calciphylaxis were reported in addition to wound care and analgesia. The majority of patients received more than two additional treatment modalities, likely reflecting clinicians' experience of managing this condition, and the lack of an evidence base. The most frequently used treatments included intensifying dialysis therapy, with commencement of HD in the five patients with non-dialysis CKD at the time of calciphylaxis diagnosis and switching to HD/HDF in those on peritoneal dialysis. STS is a common treatment for calciphylaxis and was used in 26% of

patients (compared with 21.5% in the German registry). This therapy has been used more often following the case-series of Nigwekar *et al.,* which reported a complete resolution in 26% of patients with STS and 19% having marked improvement in skin lesions (Nigwekar et al., 2013). Although the exact mechanism of action of STS for calciphylaxis remains unknown, its benefit may lie in direct extracellular inhibition of calcification (W. Charles O'Neill & Hardcastle, 2012) together with antioxidant and vasodilatory properties (A. Pasch et al., 2008).

VKA were stopped in all patients at the time of diagnosis and 60% of patients who ceased VKA were concurrently commenced on vitamin K therapy, perhaps following greater appreciation of the potential clinical benefit in replenishing vitamin K to slow progression of pre-existing vascular calcification (Brandenburg, Reinartz, et al., 2017; Shea et al., 2009). As outlined in Chapter 1, Vitamin K activates matrix Gla protein (MGP) via gamma carboxylation (Cranenburg et al., 2008). Fully active MGP is a prerequisite for maintaining vascular wall integrity by avoiding calcification. Hence, the application of VKA, such as warfarin, is thought to interfere with calcification defense mechanisms and promote VC (Zaragatski et al., 2016).

The major strength of this study is the long-term follow up of cases to identify clinical outcomes, including rates of resolution of calciphylaxis lesions and mortality. Of the 47 patients enrolled in the registry, only 22% had documented resolution of lesions (9 females and 1 male), with a median time to resolution of 8 [5-24] months, highlighting the significant burden of this disease. Mortality in calciphylaxis patients remained high, with 25 deaths over the period of follow up. The mortality rate of 50% at 1.6 [0.2-2.5] years following diagnosis however was more promising when compared to historically published data on outcomes with calciphylaxis (Mazhar et al., 2001).

Despite ambitions for the ACR to be a nationwide project, only seven nephrology centres participated in data collection. Therefore, the true disease incidence in Australia remains uncertain and remains a significant limitation to the work described.

Another limitation is that the ACR may include false-positive cases. This possibility cannot be completely excluded, due to the absence of systematic diagnostic standards for calciphylaxis, and decentralized patient care within the registry. Additionally, despite prospective data collection, laboratory markers at the time of calciphylaxis diagnosis are cross sectional, whereas trends may be more important as contributors to calciphylaxis development.

2.6 Conclusion

In conclusion, this chapter reports real-world management and outcomes of a rare, life-threatening disease. Calciphylaxis is a complex disorder of progressive and unrelenting cutaneous calcification, manifested as painful cutaneous lesions resulting from microvascular calcification. This study contributes to knowledge in the field, particularly confirming outlined risk factors for calciphylaxis as well as supporting biochemical findings of relatively low PTH levels at calciphylaxis diagnosis. Resolution of calciphylaxis is uncommon in the majority of cases despite multi-modal therapy and prognosis is generally poor with significant one-year mortality, although this may not be as high as previously reported.

3. VASCULAR CALCIFICATION IN SKIN AND SUBCUTANEOUS TISSUE IN PATIENTS WITH KIDNEY DISEASE

3.1 Introduction

As outlined in Chapter 1, patients with CKD are at a higher risk of VC compared to the general population, irrespective of age and co-morbidity burden (Goodman et al., 2000). Medial VC is particularly prevalent in dialysis patients, and well described in large- and medium-sized vessels. Pathologically this leads to vessel stiffening and is associated with increased cardiovascular risk (Blacher, Guerin, Pannier, Marchais, & London, 2001; London et al., 2003). A few studies have looked at medial calcification in smaller vessels, these include small arteries and arterioles in malignant breast tissue (W. C. O'Neill & Adams, 2014), as well as subcutaneous tissue from lower limb amputations (Ellis & O'Neill, 2018). VC in these instances was present in association with other risk factors for calcification. The presence of VC in small vessels of the skin and subcutaneous tissue has only been systematically described in calciphylaxis in patients with ESKD and non-uraemic calciphylaxis (Mochel et al., 2013), although recent studies suggest even this may not be a specific finding and VC can be present in patients with peripheral vascular disease (PVD) without the presence of calciphylaxis (Ellis & O'Neill, 2018).

As the largest organ, the skin functions to thermoregulate, create a physical barrier and detect sensory stimuli. It consists of three layers, from the most superficial, the epidermis, through to the middle dermis and deep subcutaneous tissue. The epidermis itself consists of five layers and contains keratinocytes, melanocytes, Langerhans cells (antigen presenting cells), Merkel cells (mechanoreceptors), hair follicles, sebaceous and perieccrine (sweat) glands. Below the epidermis lies the dermis. This region contains irregularly arranged connective tissue and elastic fibres. Other cells in the dermis include dermal muscles, nerve cells and migratory immune cells. Finally, the dermis rests on a loose layer of connective tissue interspersed with adipose tissue.

Adipose tissue functions to thermoregulate as well as disperse force from general impact. Therefore, there is an anatomical variation in the amount of adipose tissue present(Kanitakis, 2002; Prost-Squarcioni, 2006; Yousef, Alhajj, & Sharma, 2020).

The skin is richly supplied with a vascular network of cutaneous vessels branching from underlying vessels that penetrate the subcutaneous fat, enter the deep dermis and ascend vertically to the epidermis. These vessels form the superficial and deep vascular plexus, which is composed of paired arterioles and venules interconnected via capillary loops (Kang S). Control of blood flow through skin micro-vessels plays a critical role in the skin's ability to thermoregulate (Kang S). To date, there is a paucity of literature on the prevalence of VC in the skin of patients with kidney disease, without underlying predisposing conditions (specifically calciphylaxis and skin malignancy). It is also unknown whether peripheral small vessel calcification is prevalent in CKD and if so, at what stage it develops and its relationship to disordered bone and mineral metabolism. Here I hypothesise that peripheral vessel calcification is a feature of CKD-MBD.

3.2 Aims

The aim of this chapter is to:

1. Document the prevalence of peripheral small vessel calcification in the skin of patients with CKD/ESKD compared to a control group without CKD.
2. Identify whether small vessel calcification is a feature of CKD-MBD and evaluate associations between calcification and biochemical markers of bone and mineral metabolism.
3. Examine if any other histological features in the skin of patients with calciphylaxis are also found in patients with CKD/ESKD.

3.3 Materials and methods

3.3.1 Study participants

Between May 2017 and March 2019 patients undergoing a planned surgical procedure performed by the Nephrology Surgical Team at The Royal Melbourne Hospital were approached to participate in this single centre cross-sectional study. Patients enrolled underwent various elective surgical procedures including parathyroidectomy, hernia repair and arteriovenous fistula (AVF) formation. A fasting blood sample was collected at the time of the surgical procedure. Patients over the age of 18 years and able to provide informed consent were included in the study. There were no exclusion criteria. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2017.023) and was conducted in the accordance with the declaration of Helsinki.

3.3.2 Skin tissue samples

Collection of human skin tissue

At the time of surgery, a full-thickness incisional biopsy (~2cm^3) from the surgical site was obtained by the operating surgeon. The tissue was immersed into 10% neutral buffered formalin at a minimum of 10x volume of skin tissue. The section was then refrigerated overnight and processed according to standard histology protocols

Tissue processing

Following overnight immersion fixation of skin tissue, the skin was dehydrated through a series of graded alcohols (50%, 70%, 95%, 100%) and subsequently cleared with two changes of chloroform. The tissue was infiltrated with two changes of Paraplast® at 60°C for a minimum of 4 hours each time then embedded in fresh wax. Finally, the tissue was sectioned (3-5μm) onto silane (APES)-coated slides for histological staining.

Histology staining of tissue sections

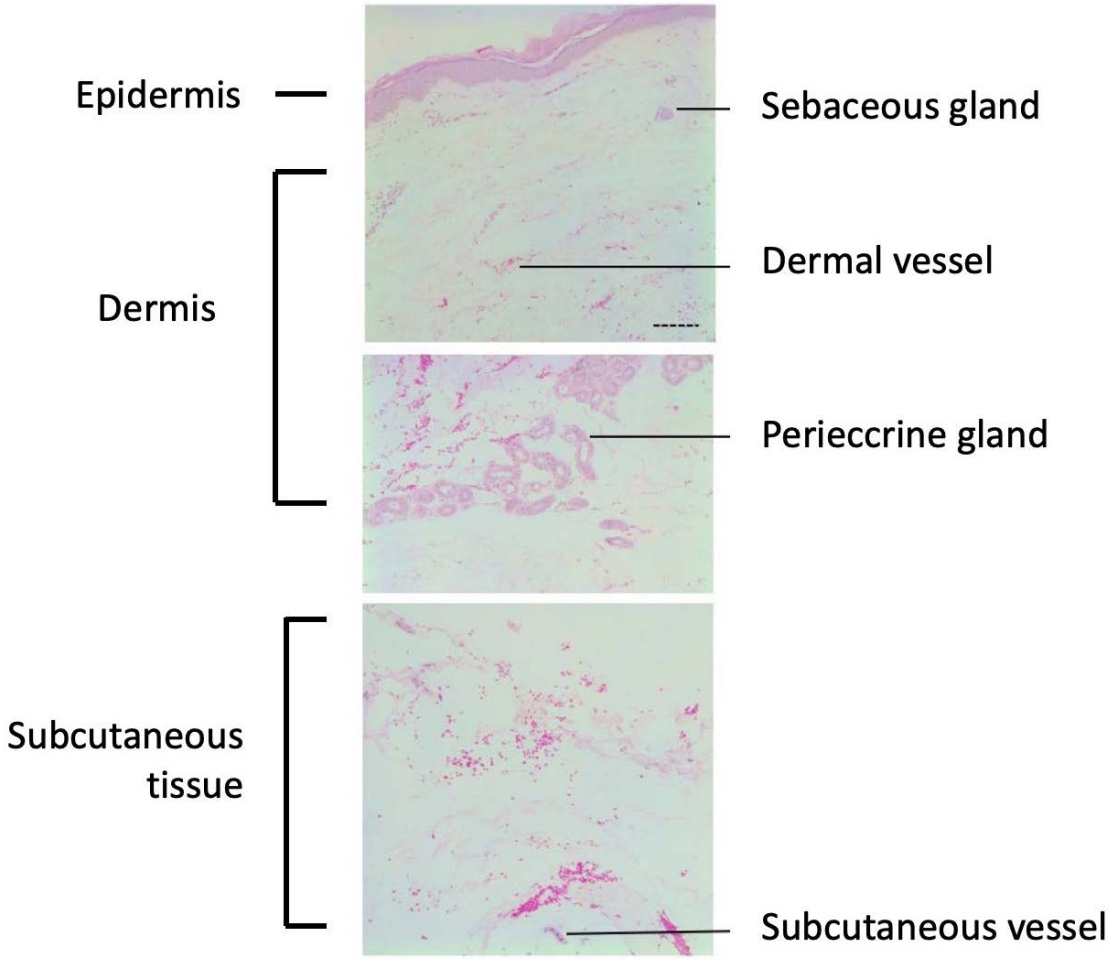
Contiguous tissue sections were first dewaxed then rehydrated through decreasing grades of alcohol to water. For haematoxylin and eosin (H&E) staining, slides were stained with Harris haematoxylin for ten minutes. They were then differentiated in 1% acid alcohol and blued through Scott's solution before being counter stained with Working Eosin Solution for two minutes. Between each step, slides were rinsed in tap water.

Von Kossa staining was used to demonstrate calcium deposits. For this method, slides were immersed in a solution of freshly prepared 5% aqueous silver nitrate. Tissue sections were placed at close range under a bench lamp and exposed to a strong light source for 30 minutes. They were then washed in distilled water. To remove the unreacted silver, slides were covered with 5% sodium thiosulphate for two minutes. This was rinsed in distilled water before being counter stained with working eosin solution for two minutes. For both Von Kossa and H&E stained slides, the sections were then dehydrated before mounting onto glass coverslips using Depex®.

Identification of skin anatomy and vessel counting

Cross-sections of skin and subcutaneous tissue were evaluated in their entirety. Each cross-section contained epidermal, dermal and subcutaneous tissue, with tissue blocks re-cut if they did not contain all three areas (Figure 1). Samples were assessed for the range of vascular and extravascular calcification, as well as the pattern of calcification in affected structures. Counting of vessels in the dermal and subcutaneous tissue was performed on slides stained with H&E due to ease of identifying vessels and differentiating from other structures including perrieccrine, sebaceous glands and hair follicles. Identifying vessels in subcutaneous tissue is more challenging to differentiate without the contrasting haematoxylin staining the nuclei. Typically, arterioles $\leq$50μm in diameter have either absent or partially complete internal elastic lamina (IEL)(E, 2010; Vinay Kumar & with illustrations by James, 2003). As a result, differentiating terminal arterioles from terminal venules is very challenging, therefore all structures with a lumen were defined as vessels. Samples with greater than ten vessels per cross section were considered to have adequate vessel number. H&E sections were examined for evidence of intimal hyperplasia, intimal thrombosis, panniculitis or hyperplasia.

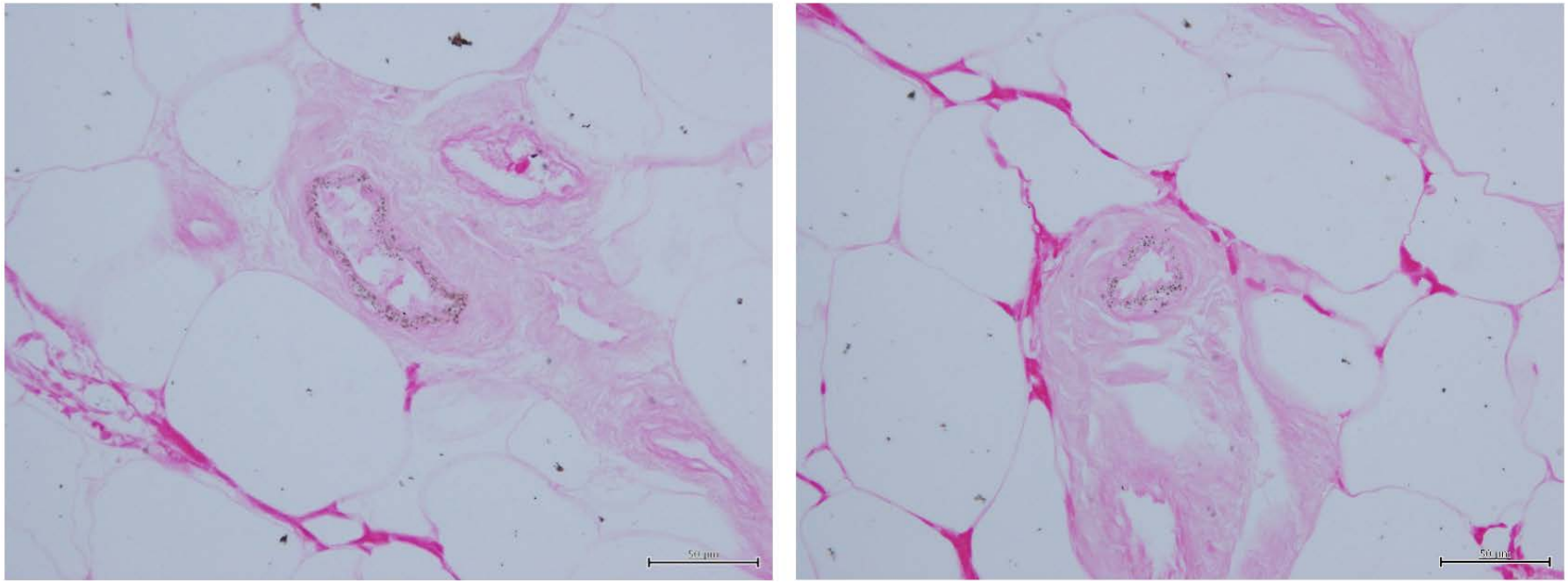
Figure 3.1 Normal skin histology on H&E



All photos taken at 10x magnification; scale bar denoted as dashed line in top panel = 100µm.

Identification of calcification was performed on slides stained with Von Kossa. For each case, the following features were graded as either present or absent: calcification of any sized vessels, presence of extra-vascular calcification (including perrieccrine calcification), internal elastic lamina (IEL) calcification. If VC was present it was then further evaluated for its histological appearance and location within the vessel and whether it was granular, confluent or circumferential. Vessels with presence of IEL calcification were sized using a graticule. Malignant breast tissue was used as a positive control for the Von Kossa stain (Figure 3.2). Histologic sections of all cases were counted independently by two investigators (Irene Ruderman and Belinda Wigg) with results presented as an average of the two observers.

Figure 3.2 Human malignant breast tissue used as positive control for von Kossa stain



Photos taken at 20x magnification; scale bar denoted in each panel = 50µm.

3.3.3 Gene expression analysis

RNA isolation from tissue

Skin tissue stored at -80°C in *RNAlater* solution was removed from *RNAlater* and then disrupted and homogenized using TissueLyser LT (QIAGEN). RNA extraction was performed using RNeasy Fibrous Mini Kit (QIAGEN). Tissue samples were lysed in Buffer RLT and diluted before being treated with proteinase K. Debris was pelleted by centrifugation and the supernatant removed. The supernatant was mixed with ethanol and then centrifuged through a RNeasy Mini® spin column, where RNA binds to the silica membrane. DNAse treatment of the silica membrane was performed to remove any trace DNA contaminants. RNA was then eluted in RNase-free water. Quantification of RNA was performed using the Qubit ®4 Fluorometer and Quant-IT RNA Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instruction.

cDNA synthesis

Reverse transcription of mRNA to cDNA was performed using the iScript® cDNA synthesis kit (BioRad). A 40μL reaction mix was made up using 20μL RNA for each sample together with the reaction mix/buffer and 2μL iScript ® reverse transcriptase. The cDNA synthesis reactions were performed in the C1000 Touch ® Thermal Cycler (BioRad) at the following conditions: 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. The samples were kept at 4°C until they were ready for use.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed to determine specific gene expression in the tissues of interest. Validated PCR primer pairs for *TNAP, RUNX2* and *glyceraldehyde-3-phosphate*

dehydrogenase (*GAPDH,* reference gene) were sourced from Bio-Rad. In each case, a 20μL reaction mixture was made up with the following reagents: SSoAdvanced SYBER® Green supermix, primer pair mix and 50-100ng cDNA. The RT-PCR reactions were performed using the CFX Connect ® Real Time PCR Detection System (Bio-Rad) and the CFSX Manager ® Software (Bio-Rad) with the following conditions: 95 °C for 30 seconds for enzyme activation, 40-50 cycles of denaturation at 95°C for 5 seconds and annealing for 30 seconds at the optimized annealing temperature followed by a melt curve between 65-95°C for 5 seconds in 0.5°C increments.

3.3.4 Statistical analysis

All data were summarised, and results reported as mean (standard deviation [SD]) or median (inter-quartile range [IQR]) for continuous data, and number (percentage, %) for categorical variables. Comparison of unpaired data for variables related to age, BMI and biochemical parameters, between the CKD and control groups and those with and without VC, was performed using unpaired t-tests for parametric data and Mann-Whitney U-tests for skewed data. Chi-square tests were used to investigate associations between various categorical variables, specifically patient demographics and co-morbidities. Two tailed P values of <0.05 were considered to be clinically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPPS, Chicago).

3.4 Results

3.4.1 Demographic and biochemical characteristics of study cohort

Forty-five patients undergoing elective surgical procedures were enrolled in the study. Eleven patients with normal kidney function (mean eGFR 83 $\pm$ 10 mL/min/1.73m^2)

were included in the control group. Two of these underwent donor nephrectomies, five hernia repairs, one laparoscopic cholecystectomy and three total thyroidectomies. Of the 34 patients with advanced CKD and ESKD, 20 underwent AVF creation, superficialization or ligation, seven parathyroidectomies, two kidney transplant nephrectomies, two peritoneal dialysis catheter insertions, two hernia repairs and one total thyroidectomy.

Relevant patient comorbidities and biochemistry are outlined in Table 1. The mean age of the CKD group was 60 $\pm$ 16 years; 76% (n=26) of patients were on haemodialysis, 15% (n=5) on peritoneal dialysis and 9% (n=3) were not on dialysis with CKD stages 4-5. The comorbidity burden was greater in the CKD group compared to the control group; 53% (n=18) of patients were either former or current smokers, 38% (n=13) had a history of diabetes, 70% (n=24) had hypertension, and 12% (n=4) were documented to have PVD. The median BMI of patients was in the overweight range for both the control and CKD cohort. There were significant biochemical differences between the control and CKD cohort, with higher levels of serum PTH ($p<0.001$), phosphate ($p=0.002$) and ALP ($p=0.03$) in patients with CKD. None of the patients were receiving warfarin. Sixteen patients in the CKD group were prescribed calcitriol and 25 prescribed phosphate binders, 12 of whom were on a calcium-based phosphate binder.

Table 3.1 Clinical features of study cohort

Demographics	**CKD (n=34)**	**Control (n=11)**	**P value**
Age, years	60 (16)	62 (9)	0.3
Gender, male	22 (69%)	4 (36%)	
BMI (kg/m^2)	27 [24-33]	27 [24-31]	0.8
Dialysis modality	26 HD, 5 PD, 3 CKD	n/a	
Comorbidities			
Smoker (current)	5 (15%)	2 (18%)	0.6
Smoker (former)	13 (38%)	2 (18%)	0.8
Diabetes	13 (38%)	2 (18%)	0.8
Hypertension	24 (70%)	2 (18%)	0.7
IHD	7 (20%)	0	
PVD	4 (12%)	0	
Parathyroidectomy	4 (12%)	0	
Biochemical parameters			
Corrected calcium (mmol/L)	2.3 (0.2)	2.3 (0.2)	0.65
Phosphate (mmol/L)	1.9 (0.6)	1.1 (0.2)	0.002
ALP (IU/L)	107 [81-149]	74 [64-102]	0.03
PTH (pmol/L)	62 [37-148]	4.6 [0.9-8]	<0.001

Results are mean ± SD, median [IQR] or number (%)

3.4.2 Histological features of participants with VC

Anatomical and histological features of participants

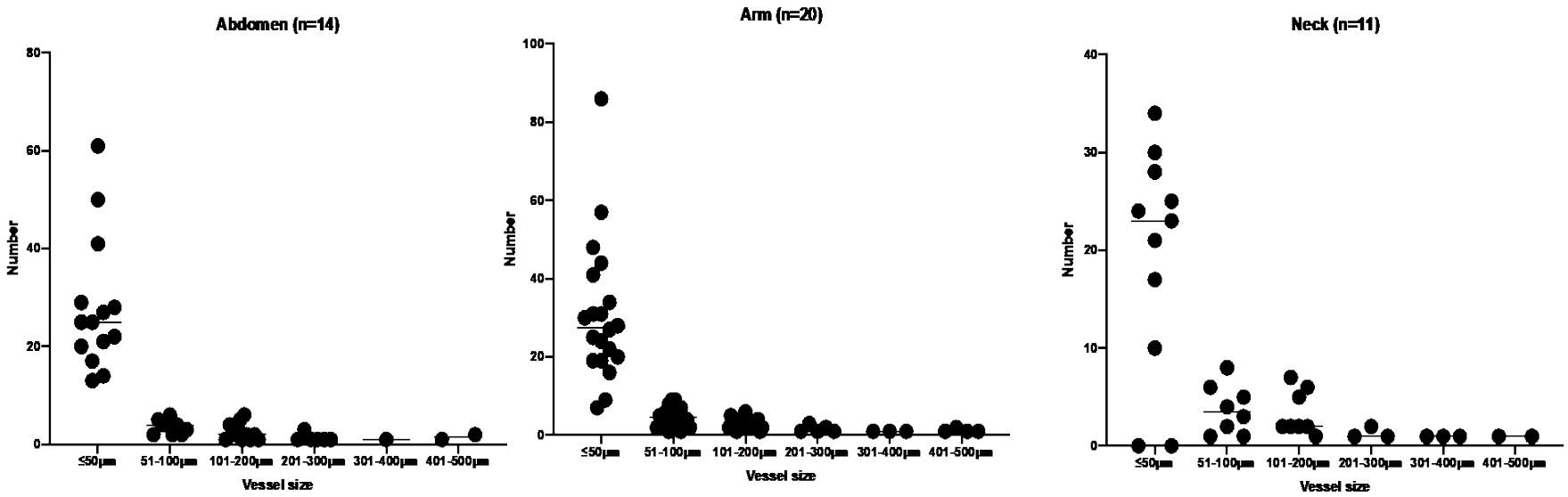
Histologic features of the skin biopsies are summarized in Table 2. The anatomical locations for incisional biopsies were neck (n=8), abdomen (n=14) and arm (n=19). Sections contained a minimum of 10 vessels, and usually many more, with a mean of 41±16 vessels per slide. Sections contained full thickness skin tissue including epidermis, dermis and variable amount of subcutaneous tissue depending on the anatomical location of the sample. The mean number of vessels did not differ significantly across the three anatomical locations (p=0.17) or between groups. Vessels ≤50μm in diameter accounted for 75% of total vessels counted. The largest vessel identified was 500μm in diameter from an arm skin sample. Each sample had a mean of 5 ± 0.6 vessels ≥ 100μm in diameter. Figure 3 demonstrates the number of vessels per vessel diameter in each patient based on anatomical location.

Table 3.2 Anatomical and histological features of skin tissue samples

Histologic features	CKD (n=34)	Control (n=11)
Location, number of samples		
• Neck	8	3
• Abdomen	6	8
• Arm	20	0
Vessel number, mean ± SD		
• All sites	42 ± 17	34 ± 13
• Neck	37 ± 7	47
• Abdomen	63 ± 18	39 ± 15
• Arm	37 ± 14	n/a
Vascular calcification present, number (%)	13 (38%)	2 (18%)
Perieccrine calcification, number (%)	13 (38%)	1 (9%)

Results are mean ± SD, or number (%

Figure 3.3 Number of vessels per vessel diameter in each patient based on anatomical location.

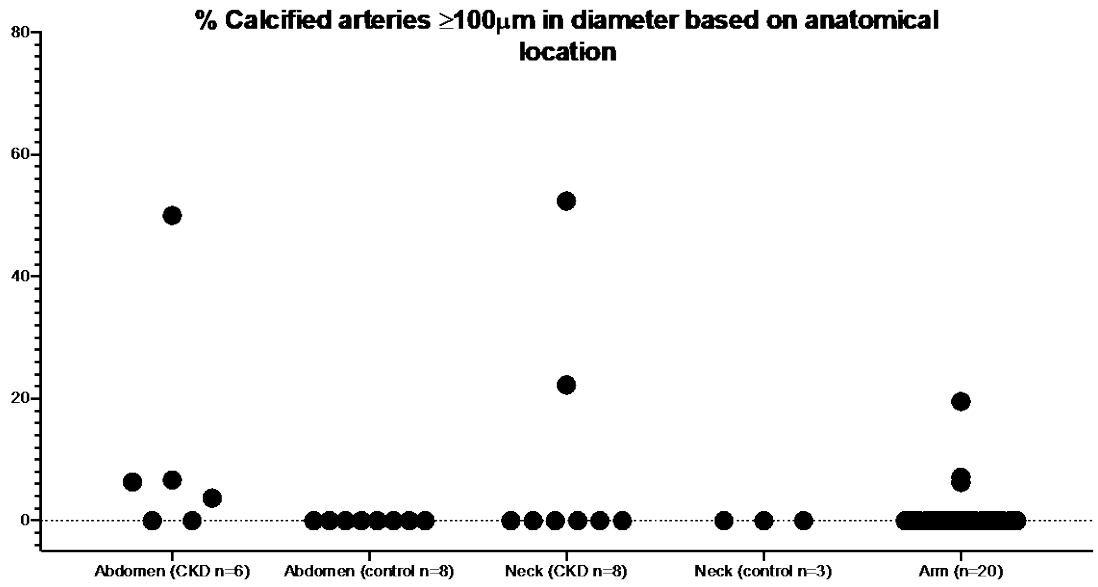


Each dot per column represent individual patient.

CKD/ESKD patients have higher rates of VC than control patients

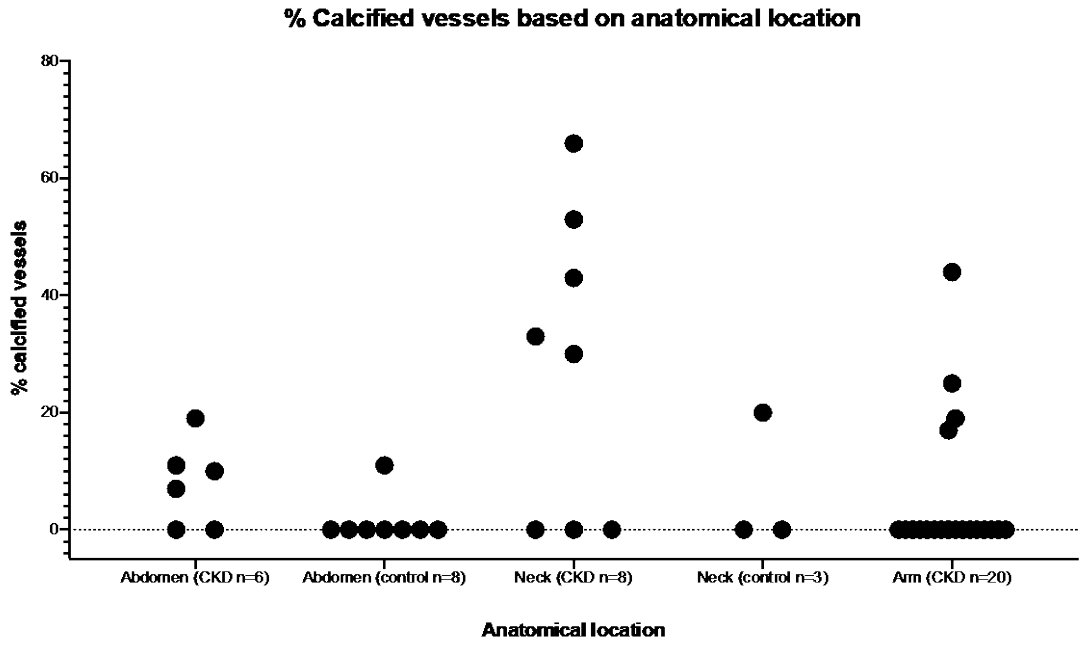
VC was observed on Von Kossa staining in small and medium vessels in 13 CKD/ESKD patients (CKD stage 4 n=1, ESKD n=12) and two control patients. There was equal distribution of calcified vessels in the dermis and subcutaneous tissue in five samples. In the remainder of the samples (n=8), VC was predominantly localized to subcutaneous tissue. In both the dermal and subcutaneous tissue, VC was seen in vessels of all sizes, ranging from 50 to 200 μm in diameter. More calcified vessels were identified in neck skin samples of patients undergoing parathyroidectomy than other surgeries, which may reflect the subset of patients with severe SHPT. VC was identified at each anatomical site in patients with CKD/ESKD and in the abdomen and neck of the control patients. Percentage of calcified vessels based on anatomical location is demonstrated in Figure 4 (vessels $>$ 100 μm in diameter identified as small arteries or arterioles) and Figure 5 (all calcified vessels).

Figure 3.4 Percentage of calcified arteries ≥ 100μm in diameter based on anatomical location.



Each dot per column represents individual patient

Figure 3.5 Percentage of calcified vessels in each patient based on anatomical location



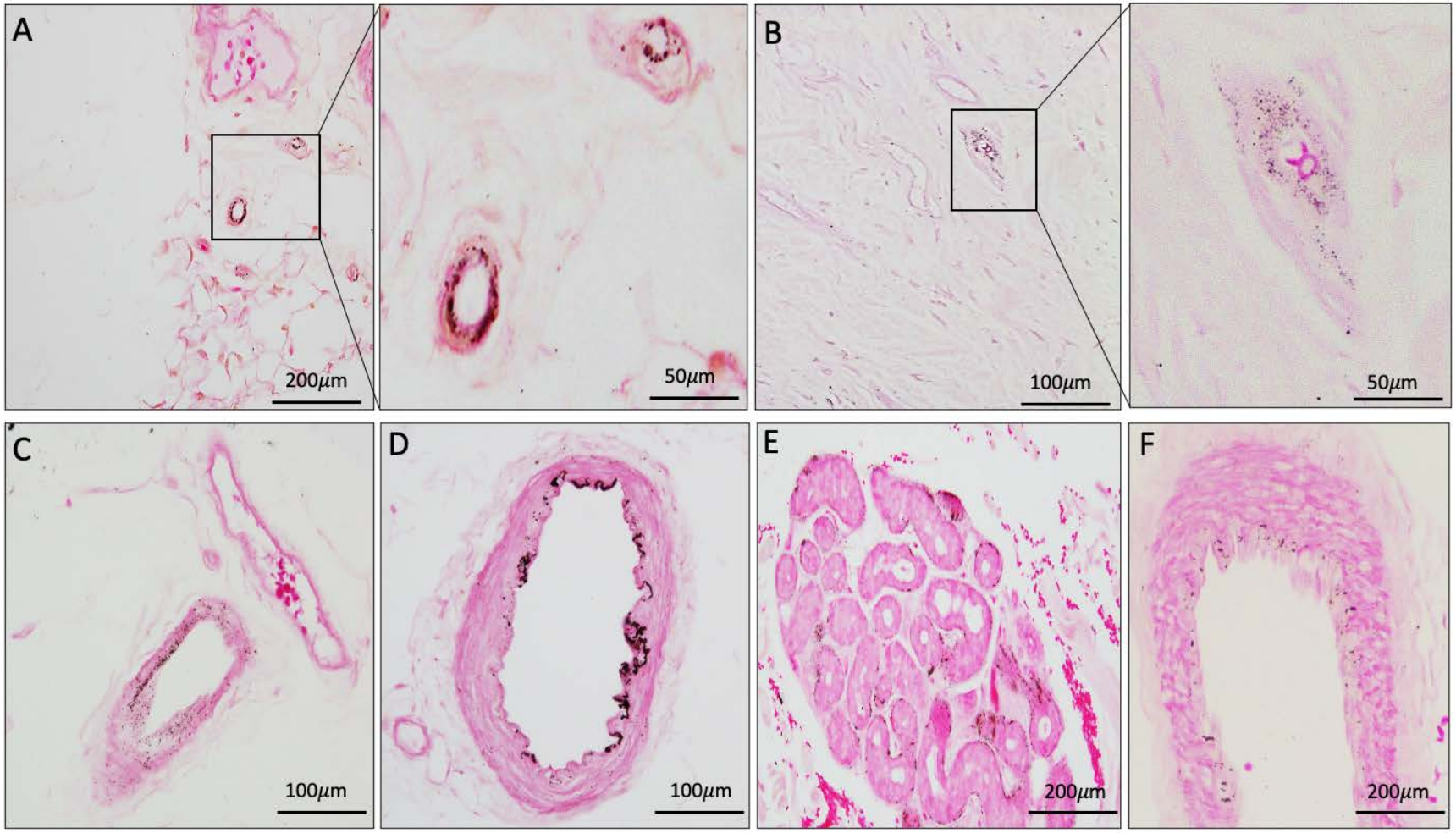
Each dot per column represents individual patient

VC was seen in vessel media and internal elastic lamina

VC of the media was seen in patients with CKD/ESKD, and there was no evidence of intimal calcification. Medial VC was present as granular calcifications in small vessels without evidence of an IEL (Figure 6a). In larger vessels the granular calcification was present alongside the IEL (Figure 6b), and some vessels displayed confluent areas of calcification (Figure 6c). There was no evidence circumferential medial calcification seen, however there was evidence of circumferential linear calcification of the IEL affecting vessels >100μm in diameter in two patients (Figure 6d).

Figure 3.6 Micrographs showing various forms of calcification seen in CKD/ESKD (Figure A-E) and control patient (Figure F).

Facing page: Von Kossa staining shows (A) Vessels <50 µm in diameter with granular medial calcification in subcutaneous tissue and higher magnification insert of vessels highlighted in black box from Panel A. (B) Vessel 100 µm in diameter with granular medial calcification alongside the internal elastic lamina in subcutaneous tissue and higher magnification of vessel highlighted in black box from Panel B (C) Vessel 100 µm in diameter with granular medial calcification in dermal tissue; (D) Confluent internal elastic lamina calcification of vessel > 100 µm in diameter in subcutaneous tissue; (E) Perieccrine calcification in dermal tissue; (F) Subtle and stippled calcification of vessel > 100 µm in diameter in control patient. All photos taken at 20x magnification. Scale bar denoted in each panel.


A
200μm
50μm
B
100μm
50μm
C
100μm
D
100μm
E
200μm
F
200μm

Perrieccrine calcification was concurrently seen in samples with VC

Extra-vascular calcification was identified in perrieccrine glands in 13 participants with CKD and one control participant (Figure 6e). Of the participants with perrieccrine calcification, ten had concurrent VC. There was no intimal hyperplasia or other pathological features consistent with calciphylaxis, including intimal thrombosis, panniculitis or hyperplasia. No other form of extra-vascular calcification was identified, including sub-cutaneous septa.

VC in control group was mild compared to CKD/ESKD group

Two patients in the control group had presence of subtle and stippled medial calcification (Figure 6f). Both patients were female, aged 65 (patient 1) and 71 (patient 2) years of age with no significant co-morbidities and normal biochemistry. Patient 1 underwent a donor nephrectomy. Calcification was present in 11% of vessels in the skin sample and involved large arterioles, without evidence of perrieccrine calcification. Patient 2 underwent a total thyroidectomy. Calcification was present in 20% of vessels in the skin sample, all vessels affected were in the subcutaneous tissue, there was evidence of perieccrine calcification. The pattern of VC in the control group was distinctly different to that in the CKD/ESKD group with no evidence of granular or confluent calcification or involvement of the IEL.

Similar demographic and biochemical markers of mineral metabolism in patients with and without skin and subcutaneous VC

Table 3 describes differences between participants with CKD/ESKD with and without VC. Patients with VC were younger (57 $\pm$ 13 years versus 59 $\pm$ 18 years, p=0.6) and had a shorter dialysis vintage (2.5 [1.7-7.6] years versus 4.2 [2-6.5] years, p=0.2) although these differences did not reach statistical significance. Median BMI (p=0.2) and co-

morbidity profile were similar between groups. There were no statistically significant differences between use of calcium- or non-calcium-based phosphate binders, or activated vitamin D, in the two cohorts. Median PTH in the VC group was 58 [37-184] pmol/L vs 66 [37-123] pmol/L in those without VC (p=0.4). There was no significant difference in calcium, phosphate or ALP between groups. Patients with VC were more likely to have presence of perrieccrine calcification (p=0.003).

Table 3.3 Difference between CKD/ESKD participants with and without VC

Demographic features	VC present (n=13)	VC absent (n=21)	P value
Age, years	57 ± 13	59 ± 18	0.60
Sex (male)	8 (61%)	17 (80%)	0.21
BMI (kg/m^2)	27 ± 4	29 ± 6	0.24
Time on dialysis (if ESKD), years	2.5 [1.77 – 6]	4.2 [2 – 6.5]	0.25
CKD stage			
Pre-dialysis	1 (eGFR 17 ml/min^2)	2 (mean eGFR 20 ± 0.7 ml/min^2)	
PD	4	1	
HD	8 (57%)	18 (86%)	
Diabetes	4 (30%)	9 (42%)	0.55
Hypertension	10 (77%)	14 (67%)	0.52
IHD	3 (23%)	4 (19%)	0.78
PVD	1 (7%)	3 (14%)	0.56
Smoker (current or former)	8 (57%)	15 (50%)	0.66
Previous parathyroidectomy	2 (15%)	2 (9%)	0.64
Calcitriol use	7 (50%)	10 (34%)	0.24
Non-calcium-based binder	7 (50%)	11 (38%)	0.35
Calcium based binder	2 (15%)	10 (47%)	0.06
PTH, pmol/L	58 [37– 184]	66 [37 – 123]	0.41
Total calcium, mmol/L	2.3 ± 0.2	2.4 ± 0.2	0.57
Phosphate, mmol/L	2 ± 0.5	1.9 ± 0.7	0.71
ALP, IU/L	106 [76 – 207]	108 [81 – 139]	0.72
Vessel number	44 ± 19	39 ± 15	0.91
Perieccrine calcification	9 (69%)	4 (19%)	0.003

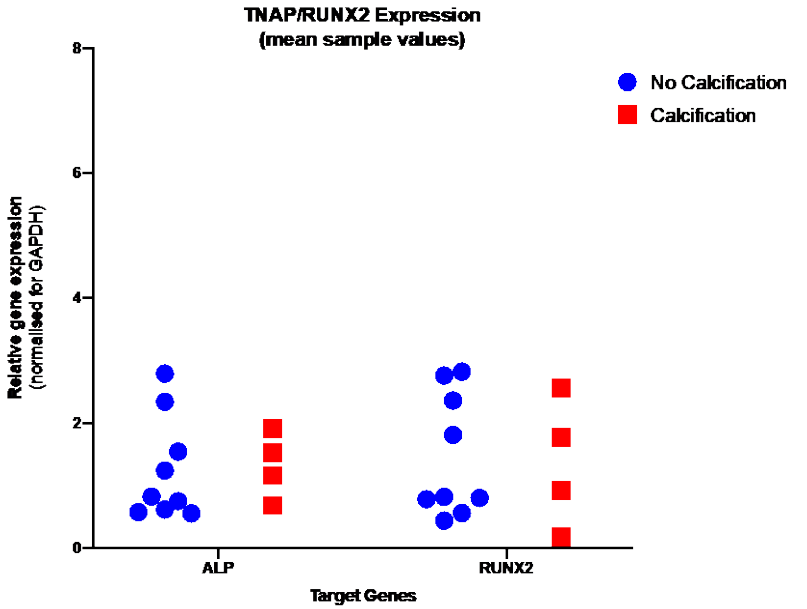
Results are mean ± SD, median [IQR] or number (%)

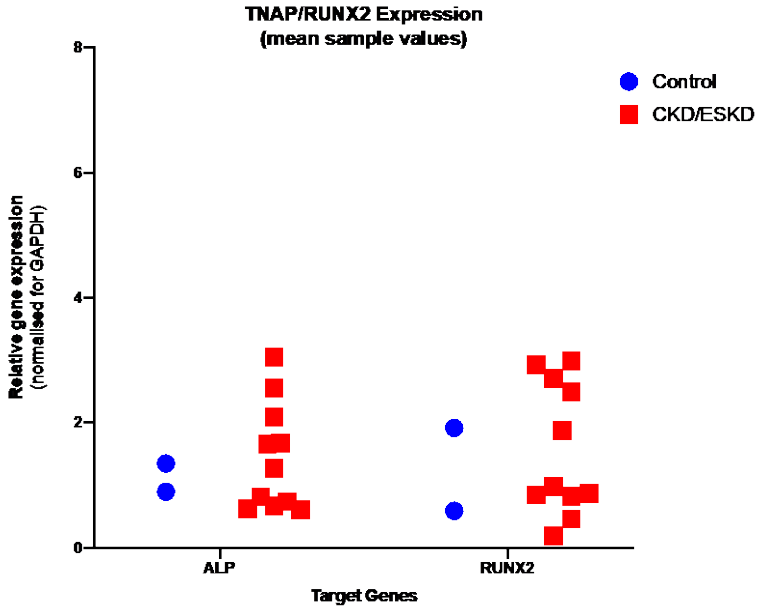
No evidence of upregulation of pro-osteogenic gene expression in patients with ESKD or with VC

De novo expression of pro-osteogenic proteins has been widely implicated as a key driver in the pathogenesis of VC as well as in vascular lesions of patients with calciphylaxis (Kramann et al., 2013). Accordingly, changes in expression of two previously identified pro-calcific factors: TNAP, which cleaves the mineralisation inhibitor pyrophosphate to phosphate, and RUNX2*,* a master transcription factor regulating osteogenic gene programs, were investigated.

Thirteen patients with eligible skin samples were evaluated for mRNA expression. Each skin sample was divided into three segments and tested in triplicate. Results are expressed as mean mRNA expression of each sample. There was minimal variability in gene expression across the three segments of tissue per patient sample. There was no upregulation of gene transcripts in samples with histological evidence of calcification (n=4) compared to those without (n=9) for either *TNAP* or *RUNX2*, p=0.76 and p=0.35 respectively (Figure 7a). When comparing gene expression in patients with CKD/ESKD (n=11) to those with no CKD (n=2), there was also no difference in gene expression for *TNAP* or *RUNX2*, p=0.8 and p=0.98 respectively (Figure 7b).

Figure 3.7 (a) Mean relative expression of TNAP and RUNX2 in participants with and without histological vascular calcification; (b) Relative expression of TNAP and RUNX2 in participants with CKD4/5 compared to those with normal kidney function





3.5 Discussion

The work in this chapter revolves around developing a greater understanding of skin and subcutaneous tissue vasculature in patients with kidney disease. One of the key findings was presence of medial VC in subcutaneous and dermal vessels in multiple anatomical locations in CKD and ESKD patients fit for surgery, without evidence of PVD, malignancy or calciphylaxis. Calcification was present in 38% of CKD/ESKD patient samples and was seen predominantly in the subcutaneous tissue and involving the vessel media as well as the IEL. Perieccrine calcification was identified in 64% of samples with VC and was present mainly in ESKD patients on dialysis. These microvascular calcifications were not related to biochemical markers of mineral metabolism or changes in pro-calcific gene transcripts.

Von Kossa staining is routinely used to identify tissue calcification. Using this stain, two distinct types of VC were identified in skin samples, medial calcification and calcification of the IEL. The medial calcification identified in CKD/ESKD patients was granular and becoming confluent in some of the affected vessels and was distinctly different to the milder medial calcification identified in the two control patients, there was no evidence of intimal calcification. There remains conjecture whether medial VC and calcification of the IEL represent progression of the same disease process. Multiple studies have shown that arterial medial calcification occurs within the tunica media typically starting along the IEL and proceeding in the absence of inflammation (Pai, Leaf, El-Abbadi, & Giachelli, 2011; Shanahan et al., 1999). In contrast, arterial intimal calcification is characterized by focal mineral deposition in highly inflamed and necrotic atherosclerotic lesions.

Development of medial VC occurs initially as irregular deposits of fine granulations, which increase in size and become confluent under the spatial constraints of the vessel wall. Commonly, in muscular and transitional arteries, granular calcifications develop alongside the IEL. These bands of calcium-rich deposits may thicken, becoming solid

plates extending deep into the inner layer of the media. With further progression of the disease, calcifications may distort the junctions of the innermost and outermost layers of the media, or may involve the entire circumference and can lead to intrusions upon the intima of the vessel (Janzen & Vuong, 2001; Lanzer et al., 2014). However, calcification of the IEL may also be a distinct pathological entity. It has previously been described in calcified breast arteries in breast malignancy in ESKD (W. C. O'Neill & Adams, 2014), as well as in coronary arteries of patients with advanced acquired immunodeficiency syndrome (AIDS) (Micheletti, Fishbein, Currier, Singer, & Fishbein, 2008) without the presence of associated medial calcification. In this study cohort, all samples with calcification of the IEL also had presence of medial calcification in other vessels which suggests that this process can occur concurrently even if it is not a continuing entity pathologically.

The high prevalence of perieccrine calcification in patients with VC is novel. There is little literature regarding this histological entity and its clinical significance. Mochel *et al* (Mochel et al., 2013) initially described perieccrine calcification in a retrospective cohort of patients with calciphylaxis, suggesting that this histological feature was highly specific to calciphylaxis. The diagnosis of calciphylaxis in this study was made based on perieccrine calcification alone in 4 out of 56 cases. This histological feature was also identified in a case report of calciphylaxis from a different institution (Dookhan, Ortega, Nayer, & Cho-Vega, 2015). In a recent case series of calciphylaxis patients, perieccrine calcification was not present, however this may have been due to lack of specific staining for calcification (13). The authors suggested that given the subtleness of this histological feature, it is possible that it is routinely missed with standard H&E staining. Perieccrine calcification was identified in two biopsies in patients with low to moderate risk of calciphylaxis, and was not significantly prevalent in biopsies of patients with high clinical suspicion of calciphylaxis (Ellis & O'Neill, 2018). The clinical significance of perieccrine calcification remains unknown, however it may reflect the severity of calcification or progression of co-existent VC.

Calcification of skin tissue is considered to be a precursor to the development of calciphylaxis and is a common finding in skin biopsies in patients with this condition. The specificity of this finding however was recently questioned by Ellis and O'Neil in a study of 38 skin biopsies performed for high clinical suspicion for calciphylaxis, compared with lower limb amputation skin samples in patients with ESKD (Ellis & O'Neill, 2018). All previously documented histopathological features commonly associated with calciphylaxis, including medial calcification, intimal thrombosis and extra-vascular calcification, were prevalent in both high-risk skin lesions and amputation samples. Only the combination of thrombosis and medial calcification was more unique to the high-risk skin biopsy group. Previous studies also identify calcification of subcutaneous deposits and extensive remodelling of the subcutaneous extracellular matrix as prominent finding in calciphylaxis (Kramann et al., 2013), these features were not identified in the current study population. This study confirms that "healthy skin" can have presence of medial VC and perieccrine calcification, and that these features indeed are not specific to calciphylaxis. There was no evidence of vessel thrombosis, perhaps due to the low incidence of PVD in the study cohort. Vessel thrombosis may be more prevalent in patients with significant PVD rather than specific to CKD/ESKD alone.

Surprisingly, there were no significant demographic or biochemical differences in CKD and ESKD patients with and without VC. PTH in patients with VC was similar in both groups, although this may reflect the variability and fluctuations in PTH, as samples used here only capture one time point. There were no significant differences in calcium, phosphate or ALP between the two groups. However, the lack of discriminating factors may in part reflect a small sample size. Schlieper *et al* identified microcalcifications in the media of iliac arteries in uraemic patients (Schlieper et al., 2010) without the presence of intimal calcification or major atherosclerotic plaques. Consistent with the results of this study, the presence of calcification was not associated with abnormalities in serum calcium, phosphate or magnesium, although PTH was not reported. Patients with VC in this study were more likely to be on calcitriol and be prescribed non-calcium-based phosphate binders, although

differences between groups were not statistically different. The median time on dialysis in patients with VC was 2.5 [1.7-7.6] years. Longer dialysis vintage is associated with accelerated VC in patients with CKD (Gross et al., 2007). To what extent VC in the skin and subcutaneous tissue reflects calcification in other vascular beds and whether it is related to more systemic pro-calcific processes affecting larger central arteries remains uncertain.

To better understand the pathophysiology of VC identified on histology, RT-PCR was performed, using pro-osteogenic genes RUNX2 and TNAP. RUNX2, a transcription factor is a master regulator of bone formation and is essential for bone formation and bone remodelling. Some animal studies have shown that RUNX2 expression is required for osteogenic phenotypic change in vascular smooth muscle cells (M. E. Lin, Chen, Leaf, Speer, & Giachelli, 2015). This transcription factor is also reportedly upregulated in human vascular fragments of large vascular territories where calcification is present (Donate-Correa et al., 2019). However, evidence of osteogenesis is not a consistent finding across all studies of human VC. There was no upregulation of *RUNX2* or *TNAP* identified in the subset of patients tested with histological evidence of calcification. Lack of osteogenic gene upregulation may also reflect temporal aspects of calcification (Hortells et al., 2017; W. C. O'Neill & Adams, 2014) or a different underlying mechanism as a culprit. Human studies involving examination of arterial calcification in breast tissue in patients with CKD demonstrated a universal absence of staining for RUNX2 and osteocalcin in early arterial calcification (W. C. O'Neill & Adams, 2014). A previous study identifying upregulation of osteogenic gene expression in skin was in the setting of calciphylaxis with macroscopic evidence of calcification (Kramann et al., 2013). This does not reflect the subtle microscopic calcifications identified in the study cohort, which may have occurred in response to distinct triggers and different mechanisms. Conversely, this may be as a result of technical factors relating to the small sample size, or the sporadic nature of calcification identified on histological examination. Finally, given only a small amount of tissue was processed for PCR, small areas of active calcification may have been missed.

There were some limitations to the work described. The small sample size and single time-point skin biopsy sampling did not allow for the ability to follow up VC progression. It was also not possible to obtain skin samples from control patients to match all anatomical locations and assessment of VC burden at central sites was not performed. This study had many strengths however, including a wide distribution of skin anatomical locations sampled, as well as large incisional biopsy sampling, which allowed for good interpretation of skin architecture and large number of vessels identified. Patients had no underlying skin pathology to confound results including presence of skin malignancy which is commonly associated with VC.

3.6 Conclusion

In conclusion, peripheral small vessel calcification is prevalent and present in patients with CKD and ESKD and can be considered an additional component of the Vascular Calcification Domain of CKD-MBD. Although it was more likely to be identified in patients undergoing parathyroidectomy with severe SHPT, there was no evidence in this small study cohort of any association between presence of medical VC and traditional biochemical makers of CKD-MBD, including PTH. Finally, the presence of medial VC and perieccrine calcification is far more ubiquitous than previously described and these histological findings are not specific for calciphylaxis.

4. BIOCHEMICAL DERANGEMENTS OF CKD-MBD IN DIALYSIS PATIENTS FOLLOWING CINACALCET WITHDRAWAL

4.1 Introduction

Biochemical derangements of CKD-MBD can be identified as early as stage 2 CKD with elevated FGF23 levels (Larsson et al., 2003) as described in Chapter 1. Progression of CKD leads to a cascade of mineral metabolism complications, with SHPT ubiquitous by the time patients reach ESKD. Medical management of rising PTH levels centres around the use of calcimimetics in conjunction with activated vitamin D and control of hyperphosphataemia.

Cinacalcet, an oral calcimimetic, was approved for treatment of SHPT in Australia in 2007, with associated government reimbursement. This medication was widely prescribed in dialysis patients to control biochemical changes associated with moderate to severe SHPT. However, following the publication of the EVOLVE study (Investigators, 2012), which failed to show an effect for cinacalcet versus placebo in an unadjusted intention-to-treat analysis of the primary composite end-point (time to death, myocardial infarction, hospitalization for unstable angina, heart failure, or a peripheral vascular event), the pharmaceutical benefits advisory committee in Australia withdrew its reimbursement. Since 2015, cinacalcet has only been available on private prescription and at a significant cost to patients. As a result of this change, cinacalcet was withdrawn in the majority of patients in whom it was previously prescribed, providing a unique opportunity to study changes in markers of bone and mineral metabolism in patients with SHPT.

Novel biomarkers involved in mineral metabolism such as CPP, identified both *in vitro* and *in vivo*, provide a mechanism for efficient transport and clearance of amorphous calcium phosphate without risk of precipitation. Many studies have demonstrated an

abundance of these biomarkers in serum of CKD patients (Miura et al., 2018; Smith et al., 2013; Smith et al., 2017). *Ex vivo* kinetics of CPP formation (Lorenz et al., 2017; A. Pasch, 2016; A. Pasch et al., 2012; Smith et al., 2014) have also been used to monitor propensity for calcification in the CKD and ESKD cohort.

Higher levels of CPP have been shown to be a predictor of all-cause mortality in patients with CKD (Smith et al., 2014), and have been associated with increased vascular calcification (Smith et al., 2014; Smith et al., 2012). Patients with calciphylaxis have much higher levels of CPP compared to those on dialysis and CKD (M. M. Cai, Smith, Brumby, McMahon, & Holt, 2013). In patients on dialysis, reduction of PTH by parathyroidectomy or calcimimetics also results in a reduction in CPP (Hamano et al., 2010), suggesting abnormal bone metabolism is associated with mineral stress, leading to an imbalance in production and removal of CPP. Detection of elevated levels of CPP in the circulation may therefore serve to identify increased mineral stress and cardiovascular risk.

4.2 Aims

The aims of this study were:

1. To assess the longitudinal changes in CPP over a 12-month period following cinacalcet withdrawal in dialysis patients compared to those in a control group of cinacalcet naïve dialysis patients.
2. To characterize changes in traditional biochemical markers of bone and mineral metabolism, namely PTH, calcium, phosphate and ALP in dialysis patients after withdrawal of cinacalcet.

4.3 Methods and materials

4.3.1 Study design

This single-centre prospective observational study was performed at The Royal Melbourne Hospital. Patient enrollment commenced in August 2015 with completion in March 2016. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2015.180).

4.3.2 Study cohort

Patients receiving dialysis at The Royal Melbourne Hospital or affiliated satellite dialysis units were eligible for recruitment. Inclusion criteria included patients with the ability to provide informed consent, aged over 18 years old and prescribed cinacalcet therapy for clinical features of severe SHPT, not adequately controlled with active vitamin D therapy, and a PTH level greater than nine times the upper limit of normal as per the KDIGO CKD-MBD guidelines (KDIGO, 2009). There were no specific exclusion criteria. Starting doses of cinacalcet were 30 mg/day and doses were titrated to achieve symptom control and a PTH target within the KDIGO guideline suggested range. A detailed medication history was recorded for patients at each visit and the date of cinacalcet cessation was documented in the patient's medical records. All patients provided written informed consent before enrollment, with the study conducted in accordance with the Declaration of Helsinki.

No specific protocol existed for the management of SHPT following cinacalcet withdrawal at The Royal Melbourne Hospital. Treatment was based on usual clinical care for SHPT including reduction of serum phosphate and administration of active vitamin D therapy with avoidance of hypocalcaemia. Patients were referred for surgical

parathyroidectomy if they had a persistently elevated PTH above the KDIGO guidelines, evidence of high turnover bone disease or symptoms of severe SHPT. Dialysate calcium in haemodialysis and peritoneal dialysis patients remained unchanged following cinacalcet withdrawal.

4.3.3 Control cohort

Two control cohorts were used in this study. The first was a historical age-, gender- and dialysis vintage- matched control group who were cinacalcet naïve. Demographic features and biochemical parameters were compared over a 12-month period between the control cohort and the cinacalcet withdrawal group from July 2015 to July 2016. This cohort was identified from the Nephrology database at The Royal Melbourne Hospital, and all patients included had no history of prior cinacalcet use.

The second control group included a subset of thirteen cinacalcet naïve dialysis patients from the historical cohort that were age and dialysis modality matched. These patients had serum samples collected at baseline (June 2017) and after six months for CPP analysis. Management of CKD-MBD in the control cohort was as per usual standard care for SHPT, involving treatment of hyperphosphataemia and use of active vitamin D therapy as indicated by the treating clinician.

4.3.4 Study end points

The primary endpoint was change in CPP following cessation of cinacalcet therapy over a 12-month period. Secondary biochemical outcome measures included serum changes in intact PTH, calcium, phosphate, ALP, ferritin and CRP, as well as haemoglobin.

4.3.5 Biomarker assessment

Serum was collected from study group patients at baseline, whilst still being administered cinacalcet and then following cessation of cinacalcet at 1 month, 6 months and 12 months. Samples were assayed for the following parameters: total CPP, CPP-I, CPP-II, albumin, calcium, phosphate, intact PTH (iPTH), hemoglobin, CRP, ferritin, and ALP. Serum calcium level was adjusted as follows if serum albumin was <40g/L: adjusted serum calcium (mmol/L) = measured serum calcium (mmol/L) + 0.02 (40- serum albumin (g/L)). Results were compared to the same biomarkers in the two control cohorts.

For CPP analysis, blood was collected into 6ml plain tubes using standard phlebotomy techniques. Blood samples were allowed to stand for 60 minutes and then centrifuged at 3000g for 15 minutes at room temperature. Aliquots were stored at -80°C until batched analysis.

4.3.6 CPP evaluation using flow cytometry

A novel method to evaluate CPP using flow cytometry was developed and validated in our laboratory by Dr Edward Smith in 2017 (Smith et al., 2017). This method was used for CPP assessment and analysis as described below.

Sample preparation

Frozen batched serum samples underwent single rapid thaw at 37°C on the day of analysis. Upon thawing, samples were diluted in a 1:4 ratio with sterile-filtered tris buffered saline (TBS) and mixed thoroughly with a pipet. Diluted samples were

centrifuged in safe-locked caped tubes for 15 minutes at 10,000 x g at 4°C to pellet residual cell debris, cryoprecipitates and other large particulate contaminants.

Sample staining

For optimal CPP identification double staining was used in this experiment with fluorescent labelling reagents:

1. OsteoSense® 680EX (PerkinElmer), a bisphosphonate analogue
2. PKH67 (Sigma-Aldrich), a dye to identify and discriminate membrane-bound mineral containing particles from CPP.

OsteoSense® 680EX (PerkinElmer) was diluted with sterile-filtered TBS at 1:50. 200ml of the supernatant was incubated at 4°C with the diluted OsteoSense® 680EX (PerkinElmer) at 1:1 for one hour. Prior to testing PKH67 (Sigma-Aldrich) was prepared by combining the dye and the diluent at 1:150, 25μl was added to each sample.

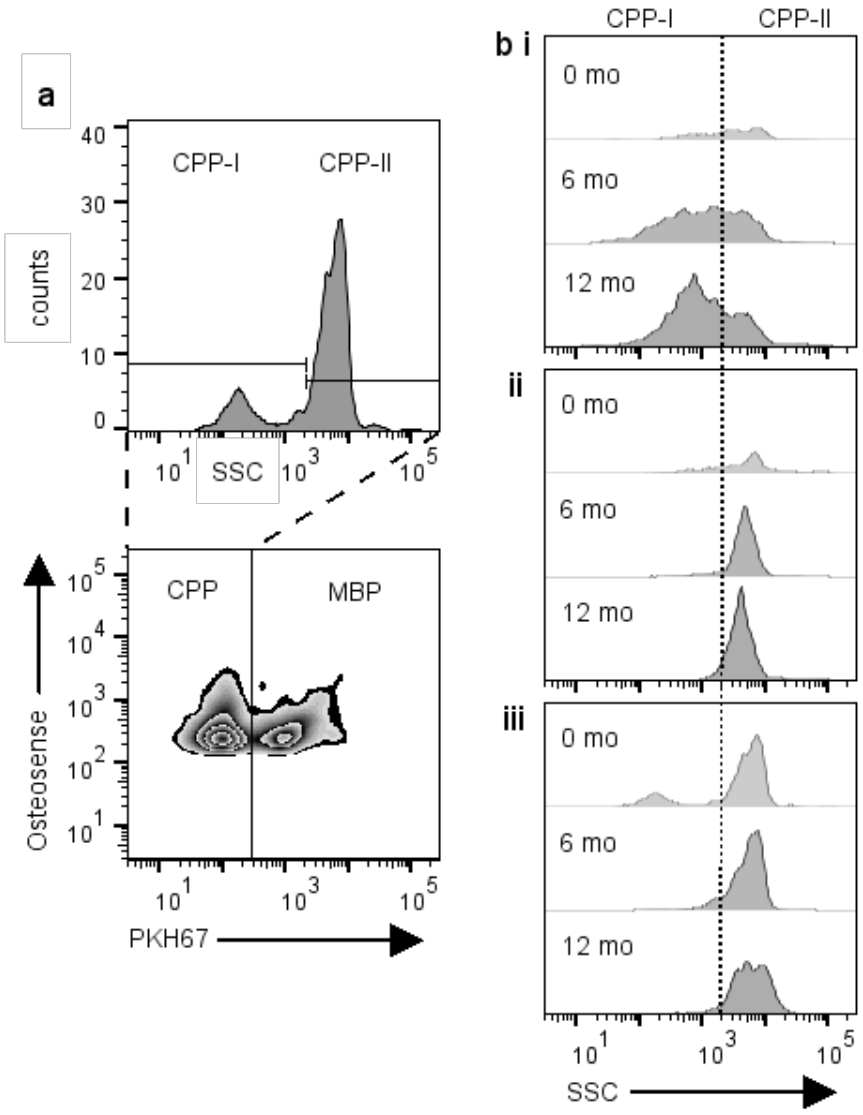
Sample acquisition

Samples were run on a BD FACSVerse flow cytometer using high sensitivity fluidics to allow for better separation of dimly stained particles from background noise. Flow cytometer settings were adjusted at the beginning of the experiment to resolve small particles (100-200nm), thresholding on mineral containing species that bind Osteosense® 680EX (PerkinElmer), and PKH67 (Sigma-Aldrich). Prepared samples were manually loaded on to the flow cytometer. The instrument was operated using BD FACSSuite software (version 1.0.5). OsteoSense® 680EX (PerkinElmer) fluorescence was detected with a red 640nm laser. Acquisition settings were held constant for all samples (60 seconds or 30,000 events).

Sample analysis

Raw FCS files were acquired and imported into FlowJo LLC version 10.1 revision 3 (Ashland, Oregon, USA) for analysis. All measurements were displayed in logarithmic scale and signal stability was assessed in real-time using side scatter vs. time plots. Side scatter intensity was used to differentiate nanoscale particle size. The gating strategy for CPP was set empirically, as described in Figure 4.1.

Figure 4.1 Schematic of flow cytometry gating strategy



(A) Represents cytograms showing dual Osteosense and PKH67 staining on mineral-containing nanoparticles (B) Histograms show gated populations and their respective volumetric measurements (counts/μL). CPP-I, primary calciprotein particle; CPP-II, secondary calciprotein particle

4.3.7 Statistical analysis

Baseline characteristics are reported as mean+/- standard deviation (SD) or when appropriate median [IQR] for continuous variables and as a number and percentage for categorical variables. Paired t test and Wilcoxon signed-rank test were used for between group comparisons. Categorical variables were analyzed with the Chi-square test. Continuous variables were compared with independent samples paired t test if normally distributed or with Mann-Whitney U test if the distribution was skewed.

Mixed linear effect modelling analysis was performed to determine differences within groups over the study period, the variables tested included: CPP, iPTH, phosphate, calcium, albumin, ferritin and ALP. These variables were selected *a priori,* based on known regulatory relationships. CPP and PTH were natural log-transformed (Ln) for the purposes of these analyses. All mixed-effect model analyses were performed allowing for the random effect of different pathology collection times and locations. Two-tailed P values <0.05 were considered statistically significant. Analyses were performed using SPSS software for Macintosh version 21 (IBM SPSS, Chicago, IL). Graphics were created with GraphPad Prism 8 for Macintosh (La Jolla, CA, USA).

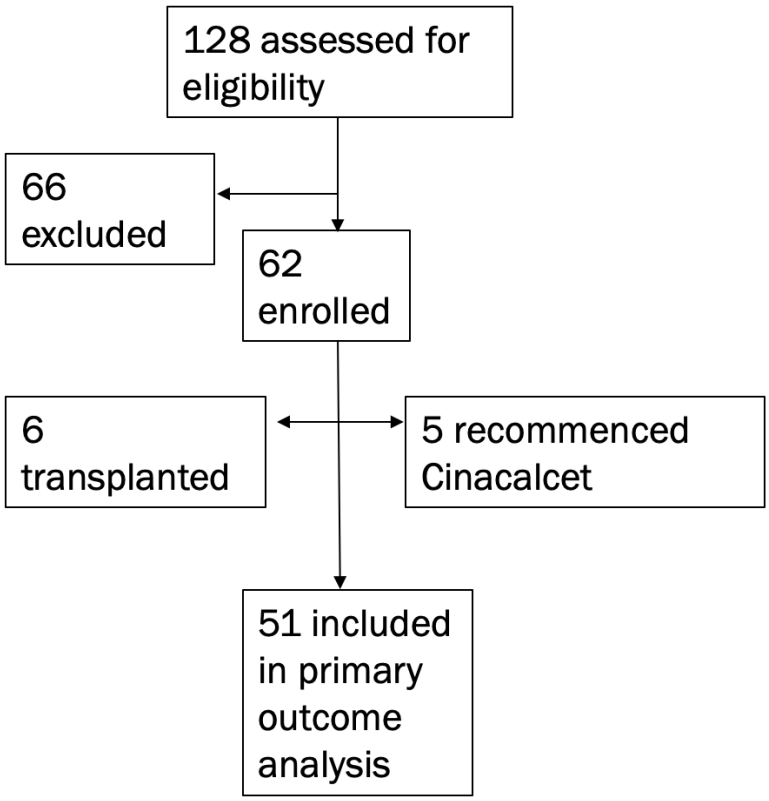
4.4 Results

4.4.1 Demographics and clinical characteristics

One hundred and twenty-eight patients on dialysis were being administered cinacalcet during the recruitment period of August 2015 to March 2016 and were approached to participate in the study. Figure 2 describes the participant flow. Sixty-six were excluded either due to patient or physician preference as many of these patients had a supply of medication remaining and continued on therapy. Sixty-two patients were enrolled in

the study, and their cinacalcet cessation date was documented in medical records. Six patients received a kidney transplant and five re-commenced cinacalcet therapy via an industry sponsored special-access scheme during the 12-month follow up period. Fifty-one patients were included in the primary outcome analysis.

Figure 4.2 Study participant flow diagram



Baseline characteristics of study participants and the contemporaneous age- and gender-matched cinacalcet-naïve dialysis controls are shown in Table 1. Only dialysis modality was significantly different between the two groups, with more patients in the cinacalcet withdrawal cohort on haemodialysis, 86% vs 57%, (p=0.001). The mean age and time on dialysis were 69.9±13.2 years and 7.1±3.6 years respectively in the cinacalcet withdrawal cohort. Over 50% of participants were male, 45% had a history of diabetes and 76% had hypertension. The main etiology of CKD was diabetic nephropathy.

Table 4.1 Patient demographics and clinical characteristics

Demographic	**Cinacalcet withdrawal patients (n=51)**	**Control patients (n=51)**	**p value**
Age, years	69.6 (13.2)	68.6 (12.9)	p=0.08
Gender (male)	28 (55)	28 (55)	p=1
Dialysis modality (HD)	44 (86)	29 (57)	p=0.001
Time on dialysis, years	7.1 (3.6)	6.7 (4.1)	p=0.51
Diabetes	23 (45)	23(45)	p=1
Hypertension	39 (76)	33 (65)	p=0.19
Ischemic heart disease	23 (45)	18 (35)	p=0.23
Peripheral vascular disease	8 (15)	15 (29)	p=0.09
Current or ex-smoker	16 (31)	17 (33)	p=0.83
Previous transplants	4 (8)	2 (4)	p=0.4
Parathyroidectomy	1 (2)	5 (10)	p=0.09
Previous fractures	7 (13)	4 (8)	p=0.34
Aetiology of renal disease			
Diabetic nephropathy	14 (27)	18 (35)	p=0.39
GN	9 (17)	12 (23)	p=0.46
PKD	3 (6)	4 (8)	p=0.69
Other	25	17	p=0.11
Events during follow up			
Cardiac arrest	5	3	p=0.46
Parathyroidectomy and referral for surgery	5	1	p=0.09
Fracture	2	5	p=0.24
Calciphylaxis	1	0	p=0.32
Deaths	13	10	p=0.51
Hypercalcemia at baseline	3	1	p=0.31
Hypercalcemia at 6 months	9	0	p=0.007
Hypercalcemia at 12 months	3	0	p=0.17

Data presented in Table 1 as number (percent) or mean (standard deviation); Hypercalcemia if Ca>2.6mmol/L.

In the 12 months following withdrawal of cinacalcet therapy there were two lower limb fractures, one parathyroidectomy with three patients referred for parathyroid surgery, and one episode of calciphylaxis. Thirteen patients died during the study period, with deaths due to cardiovascular causes (n=5), withdrawal from dialysis (n=3) and other causes including malignancy and infection (n=4). In the control group there were five fractures, one parathyroidectomy and no episodes of calciphylaxis. Of the 10 deaths in the control group, three were due to cardiovascular causes.

4.4.2 Medication changes following cinacalcet withdrawal

A change in prescribing patterns following cinacalcet withdrawal were observed. At baseline, 76% of patients (n=39) were on calcitriol, which reduced to 57% (n=29) by 12 months (p=0.03). Four patients were commenced on calcitriol *de novo*. Calcitriol doses increased over the study period (1.3 mcg/week versus 1.7 mcg/week at baseline and 12 months respectively, p=0.04). Of the 21 patients on a calcium-based phosphate binder at baseline, 12 continued this therapy at 12 months (p=0.05). Seventy-six percent of patients (n=39) were on a non-calcium based phosphate binder at baseline, 59% of patients (n=30) remained on therapy at study completion (p=0.06). No study patients were on magnesium supplementation. There were 12 episodes of hypercalcaemia (serum adjusted calcium >2.60 mmol/L), with nine episodes occurring by 6 months. There were no episodes of hypercalcaemia in the control cohort. The dosage or number of patients taking nutritional vitamin D supplementation did not significantly change over the year (12 at baseline versus 10 at 12 months, p=0.63).

4.4.3 Biochemical outcomes

Baseline levels of iPTH, phosphate, calcium and ALP were similar across the two groups (Table 2). In the cinacalcet withdrawal cohort, there was an increase in PTH from 42.2 pmol/L (27.8-94.6 pmol/L) at baseline to 114.8 pmol/L (83.9-159.1 pmol/L) at 12 months ($p<0.001$). The highest rate of change in PTH occurred at 1 month with a mean iPTH increase of 93% from baseline ($p<0.001$). Serum calcium also increased from 2.31$\pm$0.21 mmol/L to 2.46$\pm$0.14 mmol/L over 12 months ($p<0.001$). There was no change in iPTH or serum calcium in the control group over 12 months. Phosphate remained unchanged in the cinacalcet withdrawal group ($p=0.8$) and the control group ($p=0.6$) over the 12-month study period. Table 2 summarizes baseline, 6- and 12-month values for biochemical outcomes in the control and cinacalcet withdrawal cohorts.

Table 4.2 Biochemical changes over 12-month period

Biochemistry and Haematology	Cinacalcet withdrawal patients (n=51)	Control patients (n=51)	p value
iPTH baseline, (pmol/L)	42.2 [27.8-94.6]	44 [28.5-60.9]	p=0.98
iPTH 6 months, (pmol/L)	103.9 [63.4-122.2]	41 [28.2-67.8]	p<0.005
iPTH 12 months (pmol/L)	114.8 [83.9-159.1]	41.3 [28.5-69.7]	p<0.005
Calcium baseline (mmol/L)	2.31 (0.21)	2.32 (0.15)	p=0.8
Calcium 6 months (mmol/L)	2.48 (0.21)	2.28 (0.18)	p<0.005
Calcium 12 months (mmol/L)	2.46 (0.14)	2.32 (0.14)	p<0.005
Phosphate baseline (mmol/L)	1.72 (0.55)	1.56 (0.42)	p=0.14
Phosphate 6 months (mmol/L)	1.75 (0.47)	1.64 (0.56)	p=0.3
Phosphate 12 months (mmol/L)	1.77 (0.58)	1.6 (0.49)	p=0.14
ALP baseline (IU/L)	141.4 (61)	129.8 (91.8)	p=0.45
ALP 6 months (IU/L)	155.5 (61)	126 (75.5)	p=0.04
ALP 12 months (IU/L)	161.7 (72.9)	121.5 (45)	p=0.003
Albumin baseline (g/L)	35 (4.2)	35.7 (4.3)	p=0.84
Albumin 6 months (g/L)	34 (4.2)	34.9 (4.6)	p=0.97
Albumin 12 months (g/L)	33 (4.5)	34.3 (5)	p=0.94
CRP baseline (mg/L)	6.3 [4.6-19.6]	5 [2-16]	p=0.312

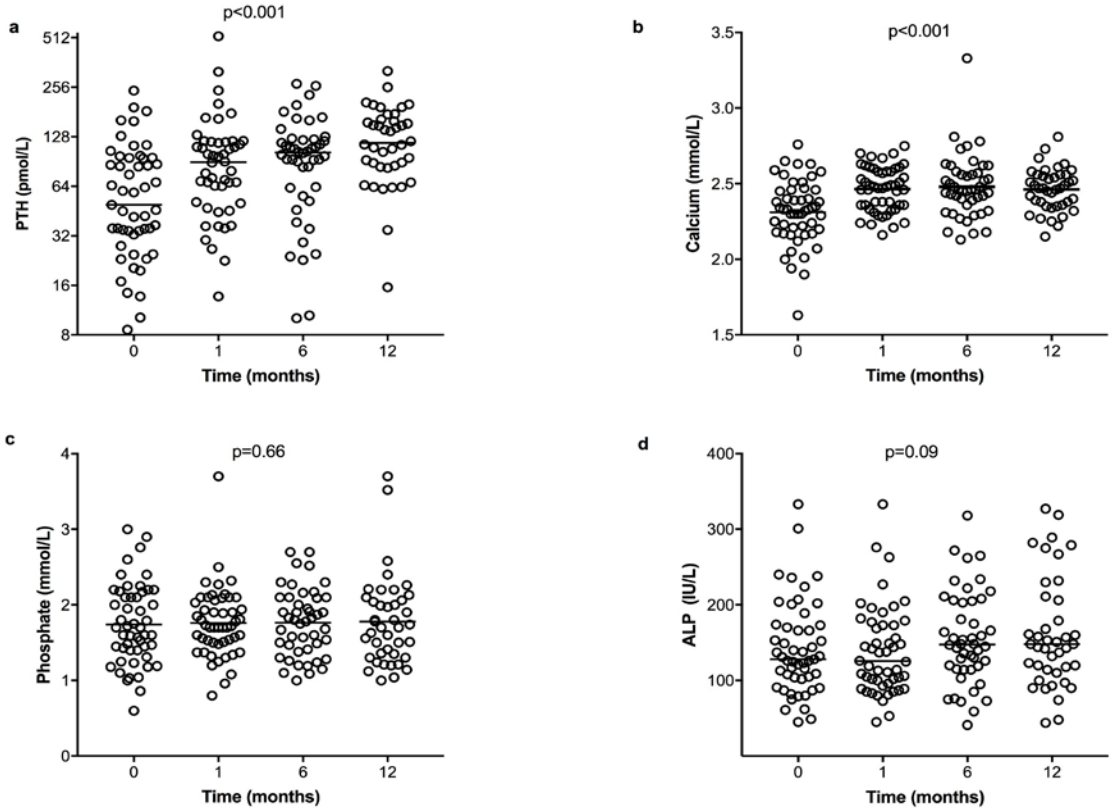
CRP 6 months (mg/L)	7 [3.3-12.3]	4.2 [2-18]	p=0.37
CRP 12 months (mg/L)	7.7 [3-29.5]	6.3 [2-19]	p=0.41
Ferritin baseline (ug/L)	235.5 [102.3-322.3]	229 [140.8-399.3]	p=0.23
Ferritin 6 months (ug/L)	209.5 [80-331.3]	232.5 [114-316]	p=0.86
Ferritin 12 months (ug/L)	274.5 [120-383.5]	221.5 [138.5-322.8]	p=0.64
Haemoglobin baseline (g/L)	112.7 (10.4)	110 (14)	p=0.13
Haemoglobin 6 months (g/L)	111.6 (10.7)	107 (18)	p=0.37
Haemoglobin 12 months (g/L)	109 (14)	114 (14)	p=0.09
Bicarbonate baseline (mmol/L)	23 (3)	24 (3.7)	p=0.53
Bicarbonate 6 months (mmol/L)	23.1 (3)	23.9 (3)	p=0.9
Bicarbonate 12 months (mmol/L)	23.6 (3.2)	24 (3)	p=0.81
25[OH]D baseline (nmol/L/L)	43[33-68]	52[42-60]	p=0.52
25[OH]D 6 months (nmol//L)	49[32-68]	66[51-89]	p=0.1
25[OH]D 12 months (nmol/L)	53[28-70]	60[42-97]	p=0.36

Data presented as number (percent), mean (standard deviation) or median [interquartile range]

A trend towards increased ALP (141.4±61 IU/L at baseline to 161.7±72.9 IU/L at 12 months) was seen in the cinacalcet withdrawal cohort over the study period but did not reach statistical significance (p=0.09). Inflammatory markers including CRP and the

positive acute-phase reactant ferritin remained unchanged over 12 months, however there was a modest decrease in serum albumin from 35±4.2 g/L to 33±4.5 g/L, p=0.03. This trend was not observed in the control group. Blood haemoglobin and serum bicarbonate were also equivalent in both groups over the study period. Changes in biochemical markers in the cinacalcet withdrawal group are outlined in Figure 3

Figure 4.3 Changes in biochemical mineral markers over 12 months following cinacalcet withdrawal



Changes in (a) parathyroid hormone, (b) calcium, (c) phosphate and (d) alkaline phosphatase over a 12-month period summarized in box plots (median, lower and upper quartile, and outliers). Changes between time points measured using mixed linear effect model. P value denotes trend over 12-month period.

4.4.4 CPP assessments in the cinacalcet withdrawal cohort

Forty -seven patients in the cinacalcet withdrawal group were included for CPP analysis. Four out of 51 study patients were excluded from final analysis due to sample instability and unknown analytical interference. Table 3 describes the changes in CPP in the cinacalcet withdrawal group, including patients who received a transplant or re-started therapy during the study period. Total CPP at baseline were $3.3x10^4/\mu L$ (IQR: $1.5x10^4/\mu L$ to $5.7x10^4/\mu L$), predominantly as CPP-II at baseline (61% of total CPP) and 12 months (58% of total CPP), p=0.06.

CPP-I increased significantly over the study period (396% mean increase, p=0.002) in the cinacalcet withdrawal group. Total CPP showed a trend towards increasing levels, but this did not reach statistical significance (p=0.05). Figure 4 depicts cohort and patient-level changes in serial absolute CPP levels over the study period and relative % changes from baseline values.

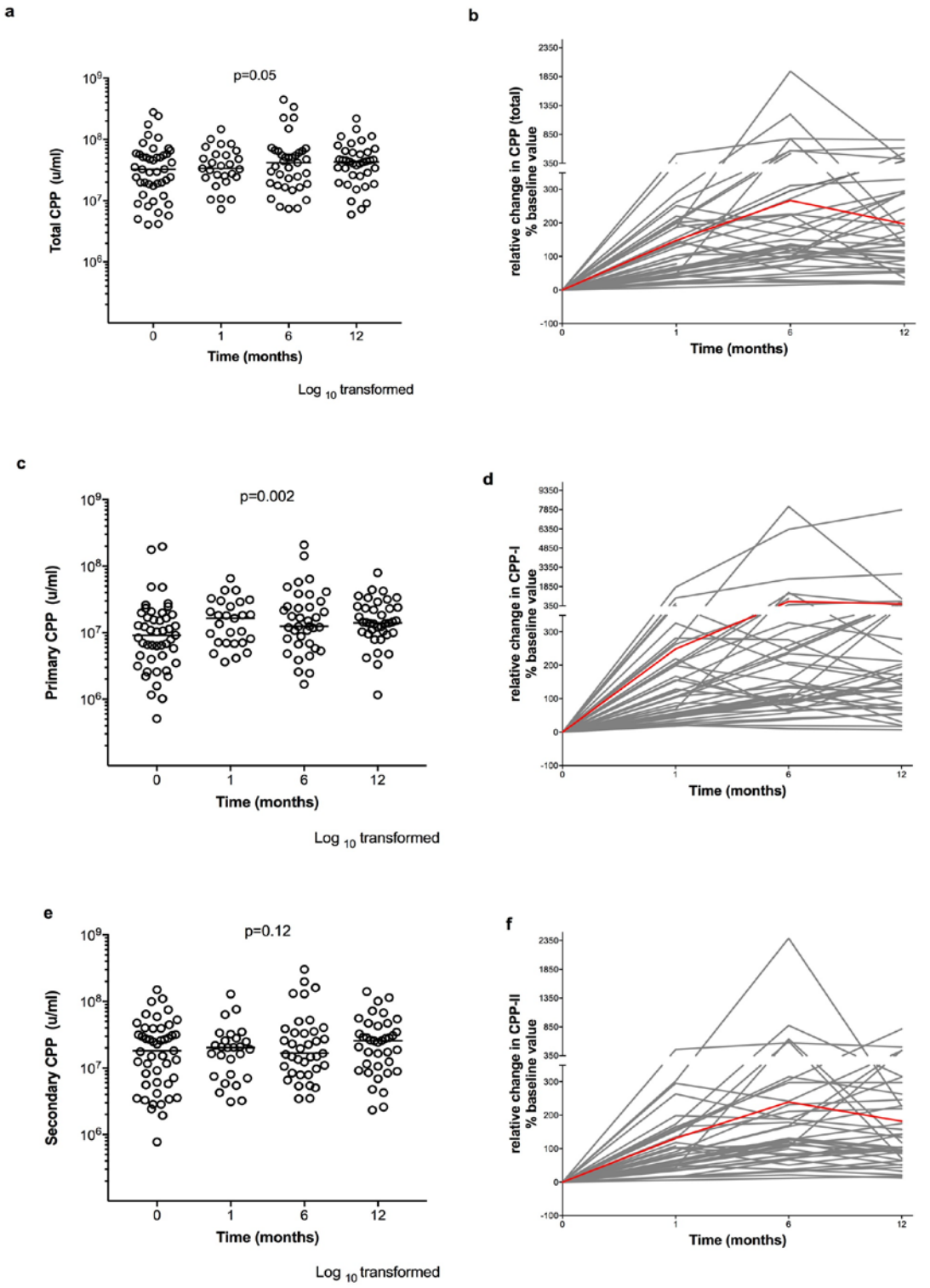
Utilizing a mixed linear effect model, allowing for random effects, changes in total and CPP-I counts were significantly associated with changes in iPTH (p=0.007 and 0.04 respectively). Total CPP, CPP-I and CPP-II counts were all longitudinally associated with changes in serum calcium (p=0.002, 0.02 and 0.001 respectively), albumin (p=0.001, 0.004 and <0.001) and ferritin concentrations (p=0.03, 0.01, and 0.009). Only changes in CPP-II were associated with the change in phosphate (p=0.03) and ALP (p=0.05). In contrast to those in the cinacalcet withdrawal cohort, there was no change in total CPP (p=0.4), CPP-I (p=0.2) or CPP-II (p=0.1) in patients who received a kidney transplant or in those who re-started cinacalcet (total CPP (p=0.15), CPP-I (p=0.2), CPP-II (p=0.2)) over the study period, although numbers were small in these groups.

Table 4.3 CPP levels in study cohort and control dialysis group

CPP/μL	Cinacalcet withdrawal patients (n=47)	Transplanted patients (n=6)	Patients re-started on cinacalcet (n=5)	P values
Total CPP baseline	$3.26x10^4$ [$1.49 x10^4$ -$5.73 x10^4$]	$1.62 x10^4$ [8.85E+03-$7.11 x10^4$]	$3.12 x10^4$ [$1.95 x10^4$ -$6.12 x10^4$]	P=0.31
Total CPP 1 month	$3.36x10^4$ [$2.38 x10^4$ -$5.70 x10^4$]	$1.59 x10^4$ [$1.18 x10^4$-$2.69 x10^4$]	$5.15 x10^4$ [$2.86 x10^4$-$1.47 x10^5$]	P=0.28
Total CPP 6 months	$3.88 x10^4$ [$1.74 x10^4$-$6.44 x10^4$]	$1.84 x10^4$ [$1.08 x10^4$-$1.18 x10^5$]	$7.78 x10^4$ [$2.17 x10^4$-$1.65 x10^5$]	P=0.27
Total CPP 12 months	$4.28 x10^4$ [$2.37 x10^4$-$6.28 x10^4$]	$2.02 x10^4$ [$1.15 x10^4$-$1.83 x10^5$]	$4 x10^4$ [$1.98 x10^4$-$1.42 x10^5$]	P=0.21
CPP-I baseline	$9.18 x10^3$ [$4.01 x10^3$-$1.87 x10^4$]	$7.06 x10^3$ [$4.57 x10^3$-$2.065 x10^4$]	$1.81 x10^4$ [$5.1 x10^3$-$4.15 x10^4$]	P=0.52
CPP-I 1 month	$1.64 x10^4$ [$7.08 x10^3$-$2.91 x10^4$]	$6.03 x10^3$ [$2.92 x10^3$-$4.53 x10^4$]	$4.39 x10^4$ [$1.63 x10^4$-$7.6 x10^4$]	P=0.11
CPP-I 6 months	$1.25 x10^4$ [$6.8 x10^3$-$2.73 x10^4$]	$8.97 x10^3$ [$6.65 x10^3$-$2.82 x10^4$]	$2.78 x10^4$ [$1.28 x10^4$-$5.04 x10^4$]	P=0.53
CPP-I 12 months	$1.41 x10^4$ [$x10^3$-$2.42 x10^4$]	$1.17 x10^4$ [$5.38 x10^3$-$7.04 x10^4$]	$1.47 x10^4$ [$1.26 x10^3$-$8.92 x10^4$]	P=0.14
CPP-II 0 months	$1.81 x10^4$ [$5.78 x10^3$-$3.92 x10^4$]	$9.17 x10^3$ [$3.98 x10^3$-$5.07 x10^4$]	$9.37 x10^3$ [$7 x10^3$-$2.54 x10^4$]	P=0.45
CPP-II 1 month	$2.02 x10^4$ [$7.5 x10^3$-$2.8 x10^4$]	$1.21 x10^4$ [$5.51 x10^3$-$2.25 x10^4$]	$1.23 x10^4$ [$7.67 x10^3$-$7.14 x10^4$]	P=0.81
CPP-II 6 months	$1.63 x10^4$ [$8.27 x10^3$-$3.63 x10^4$]	$9.4 x10^3$ [$4.11 x10^3$-$8.93 x10^4$]	$1.49 x10^4$ [$3.7 x10^3$-$8.94 x10^3$]	P=0.74
CPP-II 12 months	$2.57 x10^4$ [$1.02 x10^4$-$4.39 x10^4$]	$8.5 x10^3$ [$6.11 x10^3$-$1.12 x10^5$]	$1.36 x10^4$ [$9.94 x10^3$-$9.31 x10^4$]	P=0.36

Data presented as median [interquartile range]

Figure 4.4 Changes in calciprotein particles over 12 months following cinacalcet withdrawal

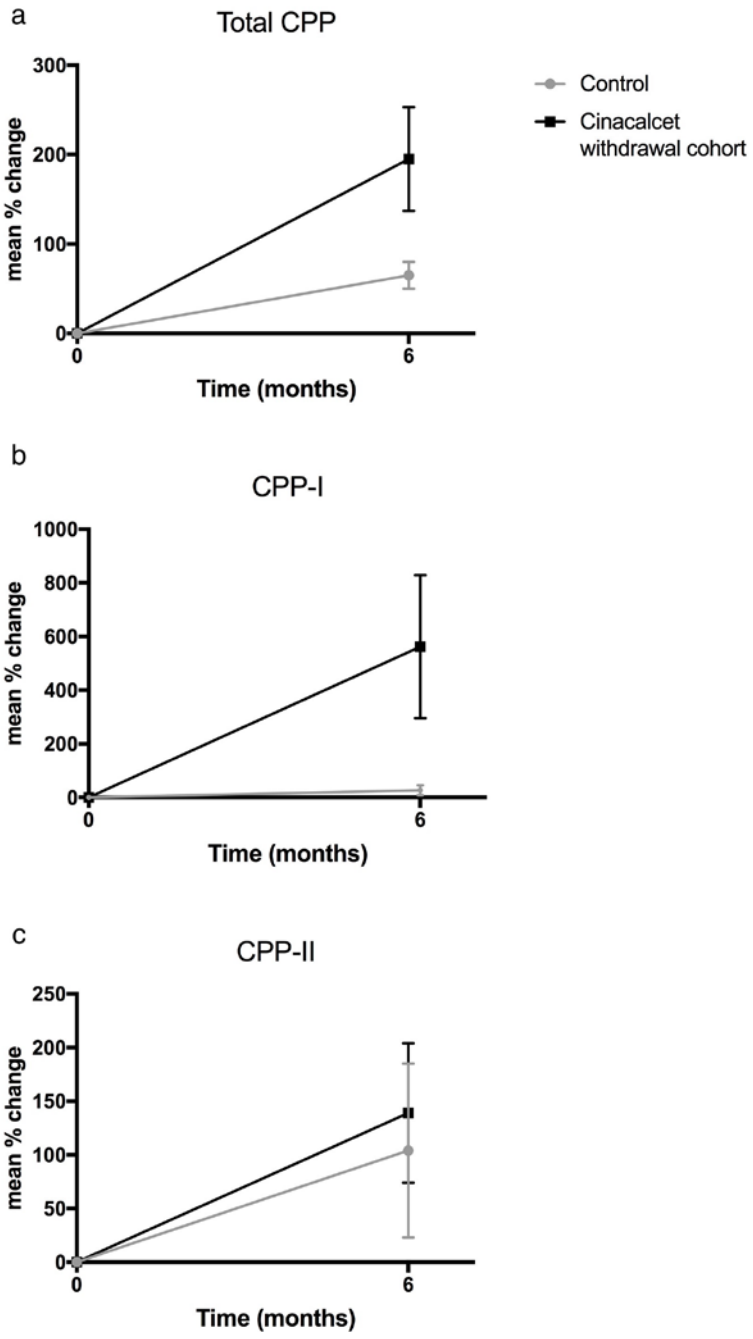


Changes in absolute levels of (a) total CPP, (c) CPP-I and (e) CPP-II over a 12-month time period (median, lower and upper quartile, and outliers). p value denotes trend over 12-month period. Patient-level changes over study period expressed as relative percent change in (b) total CPP, (d) CPP-I and (f) CPP-II from baseline value with mean change highlighted in red over a 12-month period

4.4.5 CPP assessments in the control cohort

Thirteen stable cinacalcet-naïve dialysis patients were used as a control cohort for CPP analysis. Their mean age was 71±13years, 77% were male, with 12 patients on haemodialysis and one on peritoneal dialysis. There were no significant differences in demographic characteristics between the control and the cinacalcet withdrawal cohorts, other than a higher proportion of patients on haemodialysis in the control cohort (p=0.02). There were no hospitalizations or changes in phosphate binder or active vitamin D prescription patterns in the control group. Over a six-month period, there was no significant increase in iPTH (p=0.1), serum calcium (p=0.69), phosphate (p=0.37), ALP (p=0.86) or CPP-I (p=0.08) and CPP-II (p=0.26). In the control cohort of 13 stable cinacalcet naïve patients, CPP were mainly CPP-I (mean 79% CPP-I versus mean 21% CPP-II). The mean percentage increase in total CPP and CPP-I over a six-month period was significantly lower in the control group compared to the cinacalcet withdrawal cohort (p=0.03 and p=0.05 respectively). This effect was not seen for CPP-II (p=0.07). Figure 5 shows the mean percentage changes in total CPP, CPP-I and CPP-II from baseline to six months values in cinacalcet withdrawal patients compared to control patients.

Figure 4.5 Mean percentage change and standard deviation in (A) total CPP, (B) CPP-I and (C) CPP-II over a six-month period in control dialysis cohort versus cinacalcet withdrawal patients.



[A]P<0.001, [B]P<0.001, [C]P=0.24

4.5 Discussion

Withdrawal of government reimbursement for cinacalcet for the medical treatment of SHPT in Australia in 2015 resulted in a unique opportunity to examine emerging biomarkers of CKD-MBD in the Australian dialysis population. The focus in this chapter was to evaluate changes in CPP, a novel marker of mineral stress, which was measured using a recently developed flow cytometry assay. The key findings were an increase in CPP-I over a 12-month period of cinacalcet cessation as well as increase in serum iPTH, calcium, and an associated reduction in serum albumin, without changes in serum phosphate. The rise in CPP (both total and CPP-I) levels was significantly greater in the cinacalcet withdrawal cohort than cinacalcet naïve patients.

There is increasing interest in the role of CPP in the pathogenesis of vascular calcification. CPP detection using a validated fluorescent probe-based flow cytometric assay (Smith et al., 2017), together with blood calcification propensity analysis (A. Pasch, 2016) has shed new light on the potential involvement of these difficult-to-evaluate nanoparticles. As outlined in chapter 1, CPP-I act as mineral chaperones, sequestering mineral that may otherwise seed mineralization at ectopic sites and facilitating transport and clearance from body fluids (Jahnen-Dechent et al., 2008). However, in states of chronic mineral stress, the transition and ripening of CPP-I to pro-inflammatory CPP-II may be enhanced, creating a vicious cycle of inflammation and calcification (Holt & Smith, 2016; Shanahan, 2007).

Increased CPP has been associated with augmented vascular calcification in animal models (Matsui et al., 2009; Shanahan, 2007), and its presence in the serum of adenine-treated rats proceeds the development of vascular calcification (Matsui et al., 2009). Herrmann et al recently demonstrated soft tissue calcification in a mutant mouse model (both fetuin-A deficient and prone to calcification) starting from intravascular precipitation of mineral containing complexes causing

microvasculopathy, rather than osteochondrogenic cell differentiation (Herrmann et al., 2020).

In human studies, higher levels of CPP have been predictive of all-cause mortality in CKD (Smith et al., 2014) as well as being associated with greater coronary artery calcification scores in dialysis patients (Hamano et al., 2010) and increased aortic stiffness measured by pulse wave velocity in pre-dialysis CKD patients (Smith et al., 2012). In this study, the cinacalcet withdrawal group had a 74% one-year survival rate compared to 80% in the control arm. When compared to 87% survival (Registry, 2017) for 65-74 year old Australian dialysis patients (median age of our study population) as reported from ANZDATA, the survival rate in our study is very low, however more research is required to determine whether increases in CPP may predict all-cause of cardiovascular mortality in dialysis patients. One supportive feature to implicate the role of this nanoparticle in mortality is the finding of an increase in CPP over time in dialysis patients, with the percentage change being significantly greater in cinacalcet withdrawal patients compared to stable cinacalcet naïve patients.

The link between CPP and PTH has been reported previously (Hamano et al., 2010; Smith et al., 2017), with cinacalcet or parathyroidectomy treatment reducing CPP in dialysis patients. Although this study is the first to show that a rise in PTH precedes an increase in CPP and is strongly associated with an increase in CPP. Most patients in the cinacalcet withdrawal group had a peak in CPP levels at 6 months with a plateau thereafter, which may reflect attempts to control PTH levels with higher doses of activated vitamin D therapy. Some heterogeneity in the CPP trend was still observed and it is difficult to elicit whether this represents biological variation or the effects of interventions. The observation of a concomitant rise in ALP following cinacalcet withdrawal also contributes to the hypothesis of bone as a possible reservoir for these nanoparticles (Hamano et al., 2010).

This study has several limitations and strengths. The novel method of identifying and

quantifying CPP provides a greater understanding of their role in the mineralization paradigm, although the method of analysis is currently solely used at The Royal Melbourne Hospital. The prospective nature of the study and multiple time point measurements was useful in evaluating CPP trends over a 12-month period in the dialysis cohort and has not been reported to date. Unfortunately, samples for the cinacalcet-naïve control and study cohorts were collected and analysed at two different time points and comparison of absolute total CPP counts between these two populations is not possible due to nature of the analytical technique and inherent changes in laser optics over time. Presently, this limits the use of this assay to a research setting and makes it challenging to generate reference data for these nanoparticles in the dialysis population. However, comparison of relative changes remains valid and perhaps more informative given the order of magnitude differences in absolute levels between individuals. As it is observational in nature, causality of associations found, although likely, cannot be proven, but is useful in further hypothesis generation. This study was conducted in a single centre dialysis population, and therefore the sample size of the study population was small and likely contributed to the reduced magnitude of associations seen over a 12-month period.

4.6 Conclusion

In conclusion, this prospective 12-month study of a dialysis population with SHPT showed cinacalcet withdrawal was associated with an increase in PTH, serum calcium and CPP-I, with stable serum phosphate and a decrease in albumin. Novel biochemical markers of mineral stress-CPP, were greater in cinacalcet withdrawal rather than cinacalcet naïve patients over a six-month period.

To the surprise of the nephrology community in February 2020, the Australian government re-listed cinacalcet on the pharmaceutical benefit scheme after negotiating a financially suitable agreement with another manufacturer of this medication. This now allows dialysis patients in Australia to once again have access to this therapy. Given available published literature on cinacalcet, I suspect the majority

of patients with SHPT will once again be restarted on this therapy, and results of this study are supportive of its benefits on mineral stress.

5. CLINICAL OUTCOMES FOLLOWING CINACALCET WITHDRAWAL IN AUSTRALIAN DIALYSIS PATIENTS

5.1 Introduction

Government reimbursement of cinacalcet in Australia was introduced in 2009, however this was contingent on proof of efficacy from the pivotal EVOLVE trial(Chertow et al., 2012). Following publication of EVOLVE, which showed no change in the unadjusted composite primary endpoint of cardiovascular mortality and morbidity in patients on cinacalcet, government reimbursement for cinacalcet was withdrawn in August 2015 due to the lack of evidence of cost-effectiveness. Despite the disappointing primary outcome, secondary analyses of the study did report benefits, including reduced cardiovascular mortality in patients over 65 years of age (Parfrey et al., 2015), a reduction in the risk of calciphylaxis (Floege et al., 2015) and possibly fracture risk (Moe et al., 2015).

Around the world, cinacalcet continues to be a popular medical alternative to parathyroidectomy and is frequently used as part of the medical management of SHPT in dialysis patients. Economic analysis using predictive Markov models have demonstrated drug cost effectiveness in Italy (Eandi, Pradelli, Iannazzo, Chiroli, & Pontoriero, 2010) and Japan (Komaba et al., 2012), especially for patients unsuitable for parathyroidectomy. In Australia, economic analysis showed benefit in reducing parathyroidectomy rates, as well as clinical benefits in those over 65 years, however given financial feasibility was not met, funding of this medication was not continued.

Chapter 4 reported the traditional and novel biochemical derangements identified in a single-centre cohort of dialysis patients over a 12-month period following cinacalcet withdrawal. The study was limited to a relatively small sample size, making it challenging to comprehensively evaluate clinical consequences of medication

cessation, particularly rates of surgical parathyroidectomy, cardiovascular mortality and morbidity, rates of calciphylaxis and changes in prescribing practices. Given the important role this medication plays in medical management of SHPT, it was hypothesized that drug withdrawal would lead to changes in clinical outcomes, particularly increased parathyroidectomy rates in Australian dialysis patients.

5.2 Aims

The aim of this study was to assess the impact on clinical outcomes and biochemical parameters of mineral metabolism following cinacalcet withdrawal in a national multi-centre study of dialysis patients with SHPT.

5.3 Methods

5.3.1 Study design

This was a retrospective observational study involving maintenance dialysis patients who had ceased cinacalcet therapy after August 2015. The study protocol was approved by the local ethics committee at Melbourne Health (The Royal Melbourne Hospital). Reciprocal ethics and governance approval were arranged at each site involved in the study.

5.3.2 Study population

Eleven nephrology units across Australia (The Royal Melbourne Hospital, Royal Hobart Hospital, Princess Alexandra Hospital, Sunshine Coast Hospital, Canberra Hospital, Westmead Hospital, Blacktown Hospital, Nepean Hospital, St Vincent's Health, Sir

Charles Gairdner Hospital and Western Health) participated in the study. There were no specific exclusion criteria. Each centre provided data regarding patient demographics, changes in prescription practices and biochemical changes over a 12-month period from August 2015. Mortality and morbidity data, including rates of parathyroidectomies, rates of clinically evident vertebral or non-vertebral fractures, and episodes of calciphylaxis were collected at each centre over 24 months following medication cessation.

5.3.3 Study end points

The primary endpoint was change in biochemical outcome measures over a 12-month period. Patients were excluded from final analysis if they underwent a renal transplant, had a parathyroidectomy or restarted cinacalcet, via an industry-sponsored special access scheme, within 12 months of withdrawal of the medication. Comparison analysis between deceased and non-deceased participants was performed to ensure demographics, clinical outcomes and biochemical characteristics were not confounded.

5.3.4 Biomarker assessment

Biochemical data was obtained from each nephrology unit. All hospitals measure intact PTH, although a variety of testing platforms for PTH are currently used by pathology departments. All patients had measurements over the course of the 12-month study period performed at the same centre and on the same platform which reduced intra-sample variability. There remains some variability between different intact PTH platforms currently available (Eddington et al., 2014), however variability between different platforms used across units in different states was unavoidable.

5.3.5 Statistical analysis

All data are summarised and results reported as mean (+/-standard deviation [SD]) or median (inter-quartile range [IQR]) for continuous data and number (percentage, %) for categorical variables. Paired t-test and Wilcoxon signed-rank test were used for between group comparisons. Categorical variables were analyzed with Chi-square test.

Temporal changes in individual biochemical parameters (PTH, calcium, phosphate, ALP, albumin, CRP, ferritin, haemoglobin, and bicarbonate) and changes in prescribing practices were analyzed using either one-way repeated measure ANOVA for multiple comparisons if data was normally distributed or with Friedman test if the data distribution was skewed. Two-tailed P values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPSS, Chicago, IL). Graphics were created with GraphPad Prism 8 for Macintosh (La Jolla, CA, USA).

5.4 Results

5.4.1 Participant demographics and clinical outcomes

The study included 228 patients in whom cinacalcet had been stopped between August and December 2015. This represents 17.7% of dialysis patients across the 11 units. Baseline characteristics are shown in Table 1. The mean age and median time on dialysis were 63$\pm$15 years and 5.7 (IQR 3-8) years respectively. Thirty-seven percent of patients were diabetic, 74% had a history of hypertension and 38% had a history of ischaemic heart disease. Over a 2-year period, 26 patients (11%) underwent parathyroidectomies, 19 of which occurred within the first 12-month period. Based on 2-year data this is equivalent to an 86/1000 person-year parathyroidectomy rate.

There were 3 episodes of calciphylaxis, 8 non-vertebral fractures and 50 deaths (26 in the first 12 months and 24 in the subsequent 12 months), with an annual overall mortality rate of 11%. Deceased patients were older, with a mean age of 69 ±11 years (p=0.005), however the median time on dialysis (6 [IQR3-7] years, p=0.9) and associated comorbidities including hypertension (p=0.70), diabetes (p=0.14) and ischaemic heart disease (p=0.14) were similar in both cohorts.

Table 5.1 Patient demographics and clinical characteristics

Demographic	**Cinacalcet withdrawal patients (n=228)**
Age, years	63 +/- 15
Gender (male)	138 (60%)
Dialysis modality (haemodialysis)	182 (80%)
Time on dialysis, years	5.7 [3-8]
Diabetes	84 (37%)
Hypertension	170 (74%)
Ischaemic heart disease	86 (38%)
Peripheral vascular disease	26 (11%)
Events during follow up (24 months)	
Parathyroidectomy	26 (11%)
Non-vertebral fractures	8 (3.5%)
Calciphylaxis	3 (1.3%)
Death	50 (23%)

Data presented as number (percent), mean +/- standard deviation or median [interquartile range]

5.4.2 Biochemical outcomes

Thirty-five patients were excluded from 12-month analysis of biochemical changes and prescribing practices due to restarting cinacalcet, having a parathyroidectomy or receiving a kidney transplant. Demographics and clinical features of this cohort are

described in Table 2. Nineteen underwent parathyroidectomies, 7 re-commenced cinacalcet therapy, with access to medication via an industry-sponsored special access scheme, and 7 received a kidney transplant.

Table 5.2 Demographics and clinical characteristics of patients who underwent parathyroidectomy and renal transplantation or restarted cinacalcet during the study period

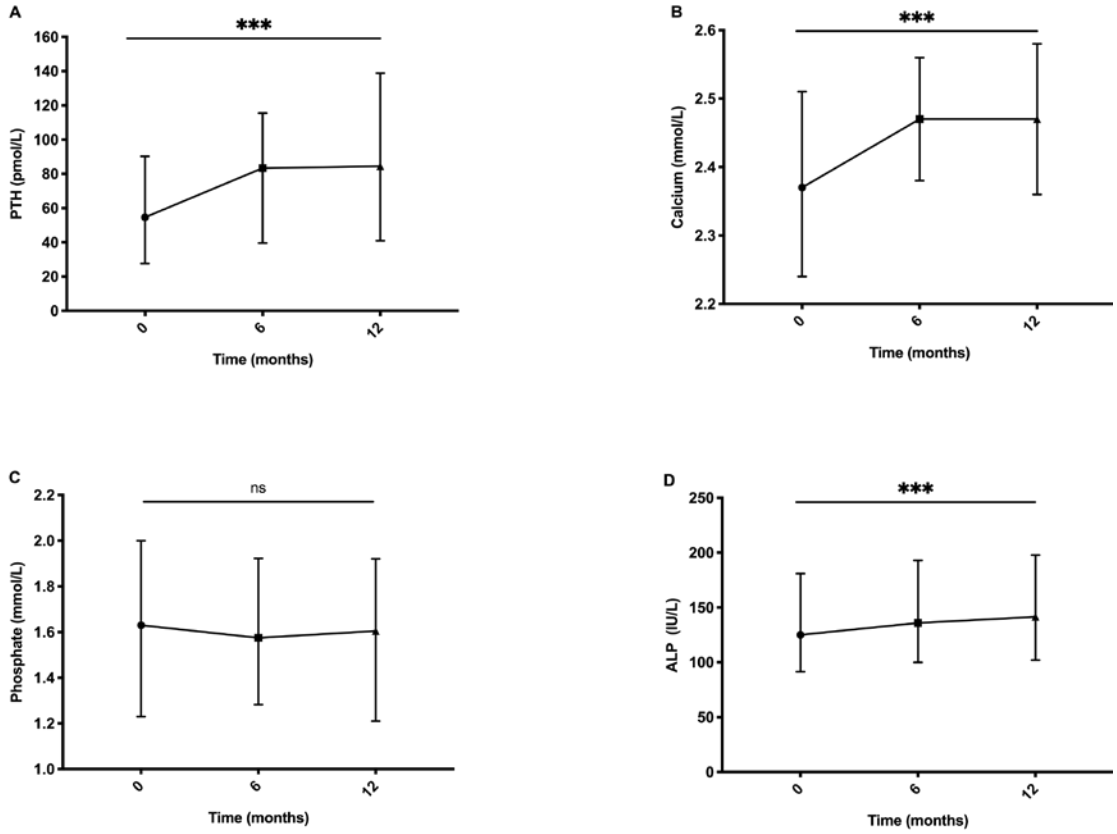
Demographic	Patients excluded from final analysis (n=35)
Age, years	55 +/- 13
Gender (male)	20 (57%)
Dialysis modality (haemodialysis)	22 (63%)
Time on dialysis, years	4 [2-7]
Diabetes	9 (26%)
Hypertension	28 (80%)
Ischaemic heart disease	10 (28%)
Peripheral vascular disease	3 (8%)
Baseline medications	
Calcium-based phosphate binder use	8 (23%)
Non-calcium-based phosphate binder use	23 (66%)
Active vitamin D use	20 (57%)
Nutritional vitamin D use	7 (20%)
Erythropoietin-stimulating agent use	20 (57%)

Data presented as number (percent), mean +/- standard deviation or median [interquartile range]

There was an increase in iPTH from 54 pmol/L (27 -90 pmol/L) to 85 pmol/L (41-139 pmol/L) at 12 months ($p<0.001$), with the greatest change occurring by 6 months ($p<0.001$) (Figure 1). Correspondingly, serum calcium increased from 2.3(±0.2) mmol/L to 2.50(±0.1) mmol/L ($p<0.001$), and phosphate remained unchanged over the

observation period (p=0.84). There were 54 episodes of hypercalcaemia (serum adjusted calcium > 2.6mmol/L) at 6 months, with 31 episodes occurring at 12 months, this was significantly increased from 19 episodes of hypercalcaemia identified at baseline (p=0.002). There was an increase in ALP from 123 IU/L (92-176 IU/L) to 143 IU/L (102-197 IU/L) at 12 months (p<0.001). Inflammatory markers, including CRP and ferritin remained stable over 12-months, and there was a fall in serum albumin from 35.5(±4.6) g/L to 34.5(±4.5) g/L, p=0.01. Table 3 summarizes baseline, 6-month and 12-month values for biochemical outcomes in the study cohort.

Figure 5.1 Changes in biochemical mineral markers over 12 months following cinacalcet withdrawal



Changes in (a) parathyroid hormone, (b) calcium, (c) phosphate and (d) alkaline phosphatase over a 12-month period summarized in line graph (median, lower and upper quartile), *** represent p<0.001

Table 5.3 Biochemical changes over 12-month period following cinacalcet withdrawal

Biochemistry and Haematology	Baseline (n=193)	6 months (n=177)	12 months (n=167)	p value
iPTH, pmol/L	54 [27-90]	83 [40-115]	85 [41-139]	p<0.0001
Calcium, mmol/L	2.3 +/- 0.2	2.5 +/- 0.2	2.5 +/- 0.1	p<0.0001
Phosphate, mmol/L	1.6 +/- 0.58	1.6 +/- 0.47	1.6 +/- 0.57	p=0.84
ALP, IU/L	123 [92-176]	134 [101-184]	143 [102-197]	p<0.0001
Albumin, g/L	35.5 +/- 4.6	35.1 +/- 5.0	34.5 +/- 4.5	p=0.010
CRP, mg/L	7 [3-16]	7 [3-19]	9.5 [4-29]	p=0.09
Ferritin, ug/L	312 [187-496]	309 [159-524]	367 [180-540]	p=0.08
Haemoglobin, g/L	110 +/- 14	111 +/- 14	110 +/- 117	p=0.67
Bicarbonate, mmol/L	23 +/- 3	22 +/- 3	23 +/- 3	p=0.42

Data presented as mean +/- standard deviation or median [interquartile range]

5.4.3 Changes in prescribing practices

The median daily dose of cinacalcet prior to medication cessation was 30mg [30-60mg]. At baseline, 38% of patients (n=74) were on a calcium-based phosphate binder and 66% (n=127) on a non-calcium-based phosphate binder. Twenty-six percent (n=56) were on 2 phosphate binders and 21% percent (n=46) were not taking any phosphate binder. At baseline, active vitamin D use was high at 65% (n=125), and 26% (n=51) were on nutritional vitamin D. By 12 months following cinacalcet withdrawal, phosphate binder use was significantly reduced, with 29% (n=52, p=0.01) on a calcium-based binder and 50% (n=97, p=0.008) on a non-calcium-based binder. Active vitamin D use also reduced

by 12 months to 48% (n=93), p=0.03. However, of patients who remained on active vitamin D, the dose was increased from 1.6 (±0.8) mcg/week to 2.2 (±1.3) mcg/week (p=0.005). There was no change in cholecalciferol administration (p=0.23). Table 4 describes prescribing practice changes following cinacalcet withdrawal. There were no significant changes in prescribing practice results with or without death censoring of data.

Table 5.4 Changes in prescribing practices

Medications (n=193)	**Baseline**	**6 months**	**12 months**	**P value**
Calcium-based phosphate binders	74 (38%)	49 (25%)	52 (29%)	p=0.01
Non-calcium-based phosphate binders	127 (66%)	112 (58%)	97 (50%)	p=0.008
Active vitamin D	125 (65%)	112 (58%)	93 (48%)	p=0.03
Nutritional vitamin D	51 (26%)	33 (17%)	37 (19%)	p=0.23
Erythropoietin-stimulating agent use	127 (66%)	116 (60%)	100 (52%)	p=0.09

Data presented as number (percent)

5.5 Discussion

This chapter examined the clinical and biochemical consequences of cinacalcet withdrawal in a nationwide survey of 228 dialysis patients from 11 nephrology centres across Australia. The key biochemical findings included a rise in intact PTH, adjusted calcium and ALP, no corresponding change in serum phosphate, and a fall in serum albumin. These results consistent with the findings in Chapter 4.

Following cinacalcet withdrawal, there was a statistically significant increase in ALP. A trend to increased ALP was identified in Chapter 4, however given the small population size in that study it was not statistically significant. The increase in ALP is in keeping with known effects of hyperparathyroidism, namely increased bone turnover. Recent publications suggest cinacalcet use may improve bone metabolism and is associated with reduced bone turnover markers including bone specific ALP, osteocalcin and beta-crosslaps (Yuan, Chen, Wang, Li, & Liu, 2018). Cunningham *et al* (Cunningham et al., 2005) demonstrated reduced parathyroidectomy, fracture and cardiovascular hospitalization rates with cinacalcet use in a combined analysis of four clinical trials, and secondary analysis of the EVOLVE trial identified a trend towards reduced rates of fractures with cinacalcet use, particularly in older patients (Moe et al., 2015). The fracture rate in this study was relatively small with only eight fractures during the study period, although fractures in the primary care setting and vertebral fractures may have been missed and underreported by the Nephrology Units.

There was a change in prescribing practices across the country following cinacalcet withdrawal, with reduced use of activated vitamin D from 65% to 48%, which is not unexpected. Cinacalcet use allows clinicians to target lower PTH values by enabling increased dosing with activated vitamin D with a reduced risk of hypercalcaemia. Nevertheless, there was no associated increase in serum phosphate over a 12-month period after cinacalcet withdrawal, suggesting that cinacalcet alone is insufficient to overcome the biochemical effects of hyperphosphataemia resulting from increased calcitriol use. In fact, one consideration is whether cinacalcet availability emphasizes the potential to achieve lower PTH values thereby leading clinicians to prescribe higher doses calcitriol and phosphate binders Since over-suppression of PTH and higher phosphate binder use may have adverse consequences, particularly when calcium-based binders are used, the question must arise as to longer term patient-level benefits of more complex combined therapies that include cinacalcet.

With regards to clinical outcomes, 11% of patients underwent a parathyroidectomy within 2 years of medication cessation, equating to an 86/1000 person-year parathyroidectomy rate. There was no significant increase in calciphylaxis, with three episodes identified in the cohort. The mortality rate of the cohort was 11% per year which is comparable to currently published Australian dialysis mortality data for patients in a similar age group(Registry, 2017). As vascular calcification and associated cardiovascular mortality and morbidity related to SHPT may take many years to develop, this cohort requires further follow up to identify possible longer-term associations with cinacalcet withdrawal.

Parathyroidectomy rates are not currently recorded in the Australian and New Zealand Dialysis and Transplant registry (ANZDATA), unlike other parameters including cardiovascular disease and cancer. Therefore, this study is limited in its ability to identify increased rates of parathyroidectomies. However, at this institution (The Royal Melbourne Hospital), there was a 69% increase in the rate of parathyroidectomies since 2015. Data from Canada following the public formulary listing of cinacalcet showed a significant decrease in parathyroidectomy rates from 11.4/1000 person-years to 3.6/1000 (Lafrance et al., 2013). In this context, the very high cohort rate of rate of 86/1000 person-years post-cinacalcet withdrawal implies a substantial national increase.

Multiple studies have highlighted the short-term risks associated with parathyroidectomies in patients on dialysis (Ishani et al., 2015; Kestenbaum et al., 2004). A review of clinical outcomes following parathyroidectomy using United States Renal Data System (USRDS) data, demonstrated a 2% 30-day mortality and 23.8% re-hospitalisation rate following surgery, together with a 39% increase in hospitalisation rate in the year following parathyroidectomy (Ishani et al., 2015). Long term mortality in younger dialysis patients may be better with parathyroidectomy compared with medical management (Diana Moldovan et al., 2015), however it is difficult to generalize

this potential benefit to our study cohort, given the average age was 63(±15) years, with a higher percentage of co-morbidities including diabetes and ischaemic heart disease.

Limitations of this study include its retrospective observational nature, not all units across Australia were involved in data collection and there was a short duration of follow up. Furthermore, it was not possible to comprehensively collect all morbidity outcomes including fracture rates and types of cardiovascular events given the study's retrospective nature. Strengths of this study include a large data set across 11 Australian centres with varying geographical locations, which provides a unique insight into the effects of cinacalcet withdrawal, perhaps not possible in many another countries. The data collected and presented in this chapter including national biochemical parameters and parathyroidectomy rates in Australia in the era following withdrawal of cinacalcet reimbursement is a comprehensive reflection of current clinical practice up until February 2020.

5.6 Conclusion

In conclusion, for patients in the Australian dialysis population who withdrew from cinacalcet therapy, median values of PTH exceeded the KDIGO CKD-MBD guidelines suggested upper target range of 9-times the upper range of the PTH assay, calcium and ALP values rose, and there was no change in serum phosphate. The parathyroidectomy rate was 86/1000 person-years over the first 24 months from withdrawal and there was no suggestion of an increase in mortality.

6. BONE MICROARCHITECTURE IN PATIENTS UNDERGOING PARATHYROIDECTOMY FOR MANAGEMENT OF SEVERE SHPT

6.1 Introduction

As outlined in Chapter 1, bone abnormalities seen in SHPT result in impaired bone quality and quantity. Affecting both trabecular and cortical bone compartments, SHPT leads to abnormal trabecular connectivity, cortical thinning, and decreased bone mineral density (BMD)(Amling, Grote, Vogel, Hahn, & Delling, 1994; Parfitt, 1998) which increase fracture risk. Fractures, significantly more prevalent in the CKD population, are associated with increased hospitalizations, mortality and morbidity(Alem et al., 2000).

Bone tissue consists of approximately 65% inorganic matrix (minerals, mostly calcium hydroxyapatite crystals), which provides tissue stiffness and 35% organic matrix (type I collagen, proteoglycans, bound water) which provides tissue tensile strength(Deyrup AT, 2016; Gartner LP, 2014). Within bone there are two types of bone tissue, trabecular bone and cortical bone. Trabecular bone is the internal compartment of bone tissue that consists by volume of 25% bone and 75% marrow. On the microstructural level, trabecular bone consists of a complex three-dimensional network of trabecular plates and rods which helps to provide tissue resistance to loading forces. Bone microarchitecture is an important contributor to bone strength independent of bone mass(Dempster, 2003). Cortical bone refers to the dense outer shell of bone tissue, which is 90% bone and 10% pore space by volume and is designed to resist bending torsional and shear forces(Deyrup AT, 2016).

Assessment of bone quality is challenging in patients with CKD as standard serological markers of bone turnover do not reliably distinguish between different renal bone pathologies and, in addition, commonly used biomarkers can have assay limitations

and inconsistent results in the CKD population(Coco & Rush, 2000; Danese et al., 2006; Sprague et al., 2016). Dual-energy X-ray absorptiometry (DXA) is widely used in the general population to diagnose and monitor low BMD as well as provide fracture risk assessment(Kanis, 2002). In the general population there is a strong association between low areal BMD and increased risk of fracture(Marshall, Johnell, & Wedel, 1996). However, DXA has significant limitations in the CKD population and can potentially overestimate vertebral BMD due to the presence of overlying aortic calcification. In recent years, several prospective cohort studies have demonstrated utility of DXA in assessing fracture risk in patients with CKD stages 3-5(S. Iimori et al., 2012; West et al., 2015; Yenchek et al., 2012) and DXA is now recommended in the updated 2017 Kidney Disease Improving Global Outcomes (KDIGO) clinical guidelines to determine BMD and evaluate osteoporosis or renal bone disease in patients with CKD and those on dialysis(Ketteler et al., 2017). DXA, however, provides poor distinction between cortical and trabecular bone, as it is a two-dimensional assessment of BMD.

Bone biopsy remains the gold standard for adequately diagnosing renal bone disease but is rarely performed as biopsy acquisition is invasive, challenging to process and analyse, and fails to permit longitudinal measurements of bone structure at the same location. Bone biopsy is often performed at the iliac crest, a region predominantly composed of trabecular bone with low load and low fracture prevalence. SHPT is associated with loss of predominantly cortical bone in long bones(Osima et al., 2018), which are the commonest site of fracture, therefore bone biopsy of the iliac crest may not provide adequate prognostic information on clinical outcomes. Optimal assessment of renal bone disease is yet to be determined, however new imaging methods may be able to quantitatively assess and monitor bone fragility *in vivo*, including high-resolution magnetic resonance imaging (MRI, or micro-MRI) and high-resolution peripheral quantitative computed tomography (HR-pQCT).

Micro-MRI is a non-invasive technique that provides three-dimensional assessment of bone and can be used for repeat monitoring without exposure to ionising radiation as with HR-pQCT. Micro-MRI of bone trabecular architecture was first described 20 years ago(Jara, Wehrli, Chung, & Ford, 1993) and since then technical advances in image acquisition and analysis have seen its performance rival that of HR-pQCT. Micro-MRI provides high-resolution imaging of bone allowing the evaluation of both cortical and trabecular properties at a scale of 100-200 μm (in-plane resolution)(Singh, Bray, & Hall-Craggs, 2018). Assessment of bone volume fraction and bone topology correlate well with equivalent computed tomography (CT) measurements(Krug et al., 2008).

In addition to analysis of bone microarchitecture, micro-MRI images can also be used for finite element analysis (FEA), which provides a more direct assessment of the mechanical competence of bone. FEA is based on mechanical engineering technology which facilitates virtual stress testing of bone to compute metrics of bone mechanical competence, specifically providing information on bone stiffness and elastic modulus(Chang et al., 2014). In the CKD population, micro-MRI has been used to study bone microarchitecture in patients on dialysis and those pre- and post-kidney transplantation(Leonard et al., 2019; Link et al., 2002; C. S. Rajapakse et al., 2012; C. S. Rajapakse et al., 2017; Sharma et al., 2018; Wehrli, Leonard, Saha, & Gomberg, 2004). In a group of 17 patients on haemodialysis, micro-MRI was used to quantify trabecular and cortical structural parameters, which are traditionally evaluated with bone biopsy(Wehrli et al., 2004), compared to an age- and sex-matched control group, results were consistent with increased bone fragility in HD patients compared to the control group.

To date, micro-MRI to evaluate bone microarchitecture associated with severe SHPT in patients prior to parathyroidectomy has not been reported. A previous study using DXA in a cohort of patients following parathyroidectomy, demonstrated increased BMD post-surgery(Fang et al., 2018), however this was based on retrospective data over a four-year period. HR-pQCT has been evaluated in patients with primary

hyperparathyroidism undergoing a parathyroidectomy with studies reporting significant improvement in total, cortical, and trabecular volumetric bone density as early as 6 months post parathyroidectomy, as well as improved FEA stiffness and failure load(Hansen, Hauge, Rasmussen, Jensen, & Brixen, 2012). Micro-MRI in patients with SHPT undergoing surgery may provide more detailed information regarding bone microarchitecture and bone strength compared to other current imaging techniques. This imaging technology has the capacity to distinguish patients who have evidence of bone disease related to high PTH and those who may not have significant structural bone disease despite high PTH. This information could be valuable to determine who may benefit from parathyroidectomy or who may not, or even who might potentially suffer adverse consequences from parathyroidectomy with risk of developing adynamic bone disease. Static MRI parameters, unlike bone biopsy, provide a representation of the cumulative impact on bone structure due to changes in bone turnover over time.

As discussed in Chapter 3 and 4, the landscape of management for SHPT in Australia significantly changed in August 2015 following withdrawal of government reimbursement for the calcimimetic agent cinacalcet. Over the past five years there has been an increase in the number of patients with severe and progressive SHPT requiring surgical management with a parathyroidectomy, as medical treatment with cinacalcet was not as readily available and other therapies for SHPT, such as calcitriol, are not always effective at suppressing PTH levels. At our institution, the number of parathyroidectomies performed doubled from 2015 to 2019 following withdrawal of funding for cinacalcet. Establishing a non-invasive method to evaluate and monitor underlying bone pathology in SHPT is essential and will assist in future management of this challenging problem, particularly in comparing the effectiveness of medical versus surgical SHPT treatment.

Using a novel, non-invasive and non-ionising imaging technique to evaluate bone microarchitecture in severe SHPT prior to parathyroid surgery may give us significant insight into the natural history of renal bone disease at the time of surgery, as well as

providing a useful way to monitor changes in renal bone disease following intervention such as a parathyroidectomy.

6.2 Aims

The aims of this chapter were to establish baseline micro-MRI parameters of bone microarchitecture in patients undergoing parathyroidectomy and to correlate MRI findings of bone disease with biochemical markers of SHPT at the time of surgery.

6.3 Methods

6.3.1 Study design

This was a single-centre prospective observational study performed at The Royal Melbourne Hospital. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC2019.029). Patient enrolment commenced in March 2019 with completion in October 2019.

6.3.2 Study cohort

Patients undergoing planned surgical parathyroidectomy for SHPT at The Royal Melbourne Hospital or affiliated satellite dialysis units were eligible for recruitment. Inclusion criteria included patients with the ability to provide informed consent and aged over 18 years. Exclusion criteria included patients for whom MRI was contraindicated. A detailed medical history and MRI safety questionnaire was recorded at pre-admission clinic prior to MRI scanning. All patients provided written informed

consent before enrolment and the study was conducted in accordance with the Declaration of Helsinki.

6.3.3 MRI

Micro-MRI of the distal tibia was performed within two weeks of parathyroidectomy with a commercial 3.0-Tesla whole-body imager (Siemens Trio, Erlangen, Germany). Participants were imaged in a feet-first prone position. MRI acquisitions were performed at the distal tibial metaphysis using a three-dimensional (3D) turbo spin echo pulse sequence (Flip angle 180, repetition time/echo time 53/16ms, field-of-view 70mm X 70mm, voxel size 0.273 X 0.273 X 0.6mm^3, 12 signal averages, and echo train length of two). This sequence is commercially available on clinical MRI systems with a scan time of 12 minutes. The MRI scan was undertaken utilising a commercially available 15 channel transmit receive knee coil (Seimens, Erlangen, Germany). The centre of the coil was positioned so that images were obtained 1cm proximal to the midpoint of the medial malleolus. Raw MRI data was pre-processed via bone volume fraction (BVF) mapping and segmentation of the bone into trabecular and cortical compartments for structural measurements and topological analysis using published algorithms(C. S. Rajapakse et al., 2012; Wehrli, 2007).

Segmentation was determined by delineating the periosteal and endosteal boundaries of the bone with an operator-guided semi-automatic algorithm using custom built software(C. S. Rajapakse et al., 2012) and standard microarchitecture parameters were derived for trabecular and cortical bone. Data analysis was performed by Associate Professor Chamith S. Rajapakse from the Department of Radiology and Orthopaedic Surgery at the University of Pennsylvania, PA, USA. Table 1 outlines MRI parameters evaluated in this study.

Table 6.1 Description of MRI parameters evaluated in this study

MRI parameter	Description
Surface to curve ratio (S/C)	Indicates the plate (surface) to rod (curve) ratio of the trabeculae. A higher ratio is a marker of greater trabecular network integrity(Sharma et al., 2018).
Erosion index (EI)	Represents topological parameters expected to increase versus those expected to decrease, with erosion cause by osteoclastic resorption. A higher index is consistent with greater trabecular deterioration(Sharma et al., 2018).
Bone volume/Total volume (BV/TV) (%)	Represents the ratio of bone volume to total volume in the region of interest.
Trabecular thickness (TbTh) (mm)	Represents mean thickness of trabeculae
Trabecular number (TbN) (1/mm)	Represents mean number of trabecular per unit length
Trabecular separation (TbS) (mm)	Represents the bone marrow space between trabeculae
Cortical thickness (CTh) (mm)	Represents mean cortical bone thickness
Elastic Modulus (GPa)	Main component of FEA and a marker of bone mechanical competence

6.3.4 Finite element analysis (FEA)

FEA allows for *in vivo* estimation of bone mechanical properties. Three dimensional micro finite element models generated from acquired images of the distal tibia were subjected to stimulated loading in inferior-superior direction, to compute the elastic modulus of the entire cross-section of the bone(Chang et al., 2017; C. S. Rajapakse et al., 2009; C. S. Rajapakse et al., 2010; C. S. Rajapakse et al., 2014). This data can then be interpreted as bone mechanical competence or strength. The higher the elastic modulus, the greater the strength of the bone.

6.3.5 Biochemical measurements

Serum was collected in all patients within two weeks of parathyroidectomy. Samples were collected to measure the following parameters: PTH, calcium, phosphate, ALP, CRP, albumin. Serum calcium level 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)).

6.3.6 Statistical analysis

Results are presented as mean (± standard deviation) for normally distributed variables and as median (and interquartile range) for variables with non-parametric distribution. Relationships were studied using Pearson or Spearman correlation, depending on the distribution of variables. Two-tailed P values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPSS, Chicago, IL). Graphics were created with GraphPad Prism 8 for Macintosh (La Jolla, CA, USA).

6.4 Results

6.4.1 Demographics and biochemical outcomes

Twenty-three patients underwent surgical parathyroidectomy at The Royal Melbourne Hospital from January 2019 to February 2020. All patients were approached to participate in the study. Three patients were excluded due to MRI being contraindicated (claustrophobia, n=2; foreign material on orbital x-ray, n=1). Twenty patients were enrolled in the study, with micro-MRI performed within two weeks of

surgery (2.5 [1.3 – 4] days). Sixteen patients were on dialysis, three patients had a functioning kidney transplant (mean time post-transplant 14 ± 5 months) and one patient was pre-dialysis with CKD stage 5. Mean serum creatinine for patients with a kidney transplant was 90 ± 35 umol/L and the patient with CKD stage 5 had a serum creatinine of 560 umol/L. All patients had biochemical evidence of SHPT and were therefore included in the study to maximise study sample size. Participant demographics are outlined in Table 2 and biochemistry outlined in Table 3.

Table 6.2 Demographics and clinical characteristics

Demographics	Participants (n=20)
Age, years	48 ± 12
Gender, male	15
Stage of CKD and/or dialysis modality	
- CKD stage 5 (non-dialysis)	1
- HD (satellite)	9
- HD (home)	2
- PD	5
- Kidney transplant recipient	3
Time on dialysis, years (n=16)	2.9 [1 – 5]
Aetiology of CKD	
- Diabetic nephropathy	2
- Glomerulonephritis	9
- Reflux nephropathy	4
- Other	5
Co-morbidities	
- Previous parathyroidectomy	1
- Previous fracture	3
- Cinacalcet use in past 6 months	0
- Diabetes mellitus	4
- Cardiovascular disease	5
- Hypertension	13
- History of osteoporosis	1
- Failed transplant	6
- Smoker/ex-smoker	6

Data presented as number, mean ± standard deviation or median [interquartile range]

Table 6.3 Biochemistry at time of parathyroidectomy

Biochemistry	Participants (n=20)	Normal range
PTH, pmol/L	138.5 [39.6 – 186.7]	1.7 – 10.0
Adjusted calcium, mmol/L	2.5 ± 0.2	2.1 – 2.6
Phosphate, mmol/L	1.7 ± 0.6	0.75 – 1.50
ALP, IU/L	176 [103 – 274]	30 – 120
CRP mg/L	2 [1 – 5]	<3
Albumin g/L	31 ± 1.5	32 – 45

Data presented as mean ± standard deviation or median [interquartile range]

6.4.2 MRI findings

MRI of participants showed trabecular bone volume (BV/TV) of 10.8 ± 2.9 % and surface-to-curve ratio (S/C) of 5.4 ± 2.3. Representative MRI images illustrating trabecular and cortical compartments of a study participant are shown in Figure 1. Mean values of trabecular and cortical structural parameters assessed at the distal tibia by MRI in the whole cohort, as well as divided into combined dialysis patients and pre-dialysis CKD stage 5 patient (n=17) compared with patients with a functioning kidney transplant (n=3), and by gender (15 male, 5 female), are presented in Table 4 and Table 5 respectively. There were no statistically significant differences in trabecular or cortical parameters between CKD stage 5 patients (dialysis and non-dialysis) compared to patients with a kidney transplant. Female patients with SHPT had lower trabecular bone volume (BV/TV, 9 ± 21.1 % versus 11.5 ± 3 %, p=0.04) and trabecular thickness (TbTh, 0.12 ± 0.002 mm versus 0.13 ± 0.008 mm, p=0.01) compared to male patients, there was no difference in cortical thickness between genders (p=0.17). Table 6 outlines MRI parameters at the distal tibia in previously published cohorts compared to our current study cohort. Micro-MRI remains an emerging imaging modality therefore no normal reference ranges for the distal tibia exist at present. Micro-MRI parameters from two publications with non-CKD patients (Benito et al., 2003; Wehrli et al., 2004) are used as predicted normal ranges. As part of

the MRI validation process at the Royal Melbourne Hospital, Sharma et al(Sharma et al., 2018) scanned eight healthy volunteers in 2016, these results are unpublished. Bone microarchitecture in healthy control participants compared to SHPT study cohort are presented in Table 7.

Figure 6.1 (a) Anatomic site of MRI image of distal tibia and fibula; (b) High-resolution MRI image through distal tibia showing trabecular and cortical microarchitecture

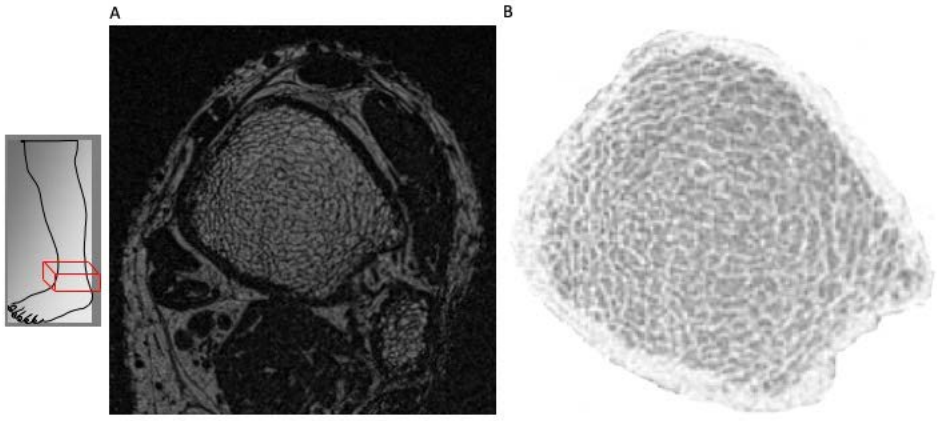


Table 6.4 MRI parameters in patients with SHPT at time of parathyroidectomy

MRI parameter	All patients (n=20)	CKD stage 5 patients (n=17; dialysis n=16, non-dialysis, n=1)	Renal transplant patients (n=3)	p value
S/C	5.4 ± 2.3	5.3±2.4	5.2±1.5	0.96
EI	1.01 ± 0.3	1.02±0.3	0.98±0.27	0.93
BV/TV (%)	10.8 ± 2.9	10.9±3	10.4±2.7	0.88
TbTh (mm)	0.13 ± 0.007	0.128±0.007	0.127±0.006	0.86
TbN (1/mm)	0.84 ± 0.17	0.84±0.16	0.81±0.17	0.89
TbS (mm)	1.11 ± 0.22	1.09±0.2	1.16±0.3	0.89
CTh (mm)	2.7 ± 0.63	2.8+0.63	2.3±0.5	0.46

Data presented as mean ± standard deviation

Table 6.5 MRI parameters in patients with SHPT at time of parathyroidectomy based on gender

MRI parameter	Male (n=15)	Female (n=5)	p value
S/C	5.8±2.4	3.9±0.77	0.06
EI	0.9±0.3	1.2±0.26	0.14
BV/TV (%)	11.5±3	9±21.1	**0.04**
TbTh (mm)	0.13±0.008	0.12±0.002	**0.01**
TbN (1/mm)	0.88±0.17	0.72±0.08	0.08
TbS (mm)	1.05±0.2	1.3±0.2	0.08
CTh (mm)	2.8±0.66	2.3±0.3	0.17
Elastic modulus (GPa)	2.04±0.43	2.12±0.47	0.8

Data presented as mean ± standard deviation

Table 6.6 Comparison of demographic and distal tibial MRI parameters to published studies

Parameter	Study cohort (n=20)	Benito *et al*(Benito et al., 2003) (n=10)	Benito *et al* (Benito et al., 2003) (n=10)	Sharma *et al*(Sharma et al., 2018) (n=14)	Wehrli *et al*(Wehrli et al., 2004) (n=17)	Wehrli *et al*(Wehrli et al., 2004) (n=17)
Population	Dialysis patients with SHPT	Eugonadal men	Hypogonadal men	CKD pre kidney transplant	HD patients	Control group
Age	48 ± 12	53.7 ± 13.2	53.1 ± 13.4	46 ± 11.3	40.3 ± 6.4	40.2 ± 6.7
Gender, male	15 (75%)	10 (100%)	10 (100%)	9 (62.5%)	9 (53%)	9 (53%)
CKD stage	CKD5 on dialysis	No CKD	No CKD	CKD pre transplant	CKD5 on dialysis	No CKD
S/C	5.4 ± 2.3	10.8 ± 2.4	6.9 ± 1.8	5.51 ± 1.36	5.6 ± 1.5	6.4 ± 0.6
EI	1.01 ± 0.3	0.89 ± 0.13	1.21 ± 0.24	0.91 ± 0.25	1.17 ± 0.45	0.97 ± 0.21
BV/TV (%)	10.8 ± 2.9	14.3 ± 1.1	12 ± 1.6	10.4 ± 2.2	12 ± 2.2	13.3 ± 2
TbTh (mm)	0.128 ± 0.007			0.126 ± 0.005	0.151 ± 0.019	0.156 ± 0.013
TbN (1/mm)	0.84 ± 0.17			0.82 ± 0.14		
TbS (mm)	1.107 ± 0.219			1.120 ± 0.023		
CTh (mm)	2.698 ± 0.630			2.632 ± 0.545		

Data presented as mean ± standard deviation

Table 6.7 Comparison of distal tibial MRI parameters of study participants with SHPT to health controls

Parameter	Study cohort (n=20)	Control group (n=8)	p value
Age	48 ± 12	43±8	0.27
Gender, male	15 (75%)	6 (75%)	1
S/C	5.4 ± 2.3	7.4±1.7	**0.004**
EI	1.01 ± 0.3	0.7±0.15	**0.005**
BV/TV (%)	10.8 ± 2.9	13.6±0.02	**0.005**
TbTh (mm)	0.128 ± 0.007	0.133±0.008	**0.06**
TbN (1/mm)	0.84 ± 0.17	0.82±0.12	0.95
TbS (mm)	1.107 ± 0.219	0.86±0.12	**0.003**
CTh (mm)	2.698 ± 0.630	2.7±0.44	0.95

Data presented as mean ± standard deviation

6.4.3 Correlation between biochemical variables and structural MRI parameters

There was a weak correlation between ALP and trabecular parameters specifically BV/TV (r=0.5, p=0.025) and TbTh (r=0.5, p=0.026) (Figure 2). Unlike previous published literature by Sharma *et al*(Sharma et al., 2018), which showed that PTH correlated with both S/C and EI, in our study there was only a correlation between PTH and cortical thickness (r=0.46, p=0.04) (Figure 2). Spearman correlations are outlined in Table 7.

Figure 6.2 Spearman correlations (r) between trabecular and cortical topological parameters derived by MRI and bone turnover markers (PTH and ALP)

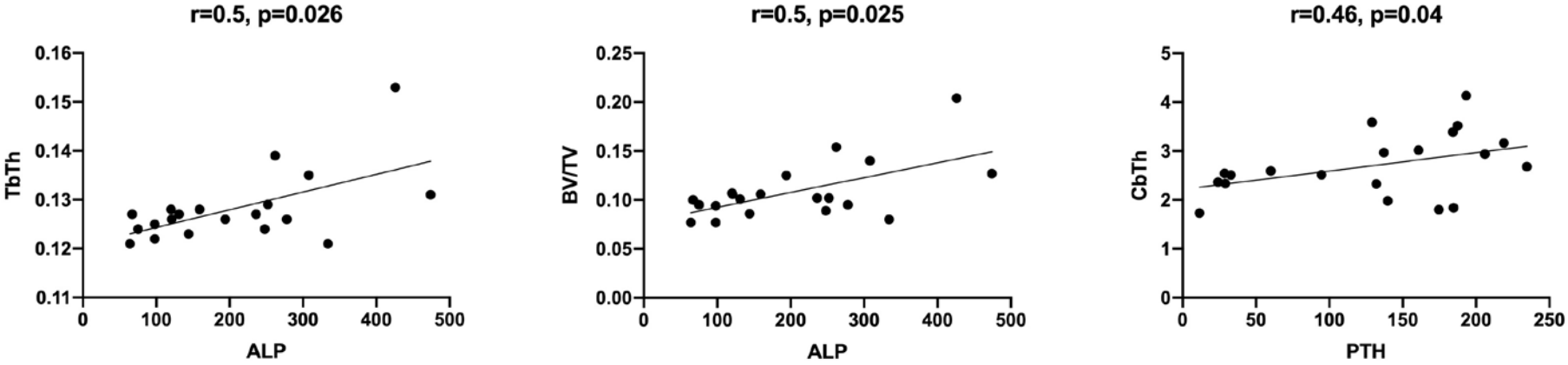


Table 6.8 Spearman correlations (r) between trabecular and cortical microarchitecture parameters (determined by MRI) and bone turnover markers

MRI Parameter	ALP	PTH
S/C	0.34	0.23
EI	-0.32	-0.12
BV/TV (%)	**0.5***	0.27
TbTh (mm)	**0.5***	0.32
TbN (1/mm)	0.44	0.22
TbS (mm)	-0.44	-0.22
CTh (mm)	0.1	**0.46***

*$p \leq 0.05$

6.4.4 FEA results

Elastic modulus in the study cohort ranged between 1.31 and 2.80 (mean 2.07 ± 0.44). Two patients with a history of calcaneal and neck of femur fractures had low elastic modulus of 1.31 and 1.65 respectively. There was a negative correlation between age and elastic modulus (r=-0.48, p=0.03), and there was no correlation between dialysis vintage or gender and elastic modulus.

6.5 Discussion

This chapter examines bone microarchitecture of the distal tibia in patients with SHPT at the time of parathyroidectomy using the novel imaging technique of micro-MRI. The key findings include significant trabecular topological abnormalities with low S/C ratio, BV/TV and elevated EI consistent with greater trabecular deterioration and lower trabecular integrity and bone volume in patients with SHPT compared to published cohorts of individuals with no kidney disease. There was also evidence of reduced

cortical thickness and low elastic modulus in keeping with poor bone mechanical competence. Although there were statistically significant correlations with ALP, PTH and some trabecular and cortical microarchitecture parameters, these associations were weak and highlight the imprecision of using bone turnover markers alone to identify and monitor renal bone disease.

Renal osteodystrophy is commonly seen in patients with CKD stage 5 on HD(Trombetti et al., 2013), with bone abnormalities contributing to increased fracture risk(West et al., 2015) as well as an associated increased risk of vascular calcification(D. Moldovan et al., 2011). The majority of studies evaluating BMD and fracture in patients with CKD have been performed using DXA or HR-pQCT. West *et al* demonstrated in pre-dialysis CKD patients that hip BMD, calculated by either DXA or HR-pQCT, was significantly lower in those with a history of incident fractures compared to those without(West et al., 2015). A major concern with DXA in the CKD population is the inability to accurately analyse bone microstructure. Differentiation between cortical and trabecular bone is very challenging and can be influenced by artefactual vascular calcification projections particularly at the lumbar spine.

Alterations of bone microarchitecture contribute to skeletal fragility, independent of areal BMD, and are equally important to evaluate fracture risk(Boutroy, Bouxsein, Munoz, & Delmas, 2005; Sornay-Rendu, Boutroy, Munoz, & Delmas, 2007). Negri *et al* imaged the distal radius and tibia with HR-pQCT in patients on HD and showed that significantly deceased cortical and trabecular parameters correlated with severity of SHPT in women(Negri et al., 2012). Of note, the medium serum intact PTH was 63 pmol/L [4.3 – 295.5] in this group, which is much lower than the median serum PTH in our current study participants (138.5 pmol/L [39.6 – 186.7]). Elevated PTH has previously been associated with reduced cortical volumetric BMD, increased cortical porosity and reduced cortical thickness(Osima et al., 2018). This study found a weak positive correlation between PTH and cortical thickness (r=0.46, p=0.04). This was a surprising finding, as it would have been expected to see a negative association

between PTH and cortical thickness. Larger studies using micro-MRI are required to explore this association further. Although PTH is routinely used to monitor SHPT in patients with CKD, it is not a maker of bone turnover per se, as it does not accurately reflect osteoblastic or osteoclastic action in the bone. Additionally, not all circulating PTH in patients with advanced CKD is biologically active, with a proportion of PTH undergoing oxidation thereby losing biological activity(Hocher et al., 2013), and circulating PTH has an extremely short half-life (minutes), therefore PTH levels fluctuate significantly and a trend rather than a single value is more useful to monitor SHPT progression. PTH alone is also insufficient to differentiate the type of renal bone disease present, as suggested by the weak correlations in the study cohort.

Similar findings to this study were identified in a study by of 74 patients on HD (mean PTH 355 pg/ml) with female dialysis patients having markedly impaired bone microarchitecture when assessed by HR-pQCT compared to a gender- and race-matched healthy control group (Cejka et al., 2011). Addition of FEA to bone microarchitecture analysis of the distal radius and tibia by HR-pQCT was evaluated by Trombetti *et al*(Trombetti et al., 2013) in patients on HD and findings were consistent with previous studies. Addition of FEA, unsurprisingly, showed that stiffness and predicted load failure were lower at both the distal radius and tibia in women with CKD but not in men, compared with age-matched controls.

Few studies have assessed the utility of micro-MRI in evaluating bone microarchitecture in CKD patients. Sharma *et al*(Sharma et al., 2018) compared bone microarchitecture assessment using DXA, pQCT and micro-MRI with 'gold standard' bone biopsy in 14 patients with CKD prior to undergoing surgery (13 renal transplantation, one parathyroidectomy). Micro-MRI changes in this study correlated well with bone histomorphometry and DXA, with significant correlations observed between histomorphometric mineralisation and turnover indices and various MRI parameters. MRI-derived trabecular parameters were also significantly related to femoral neck BMD. Importantly, micro-MRI parameters were comparable to results in

our study and corroborate findings of severe renal osteodystrophy in the current cohort, both in dialysis patients and a small sample of kidney transplant recipients, with bone loss and deranged microarchitecture in relatively young and mostly male patients. Another study of high-resolution MRI in patients with CKD was performed by Links *et al*(Link et al., 2002), investigating structural measures of the calcaneus compared to BMD in the lumbar spine using DXA in kidney transplant recipients, pre- and post-transplantation. This study reported significant differences in trabecular MRI parameters and BMD in patients with fractures compared to those without.

Micro-MRI has emerged as a novel tool to accurately and safely assess both trabecular and cortical bone without risk of radiation exposure. The ability to incorporate FEA provides useful information regarding bone strength which could be used to monitor treatment effect. Micro-MRI was used in a cohort of hypogonadal and eugonadal males to assess trabecular architecture of the distal tibia(Benito et al., 2003). Benito *et al* showed that the S/C ratio was 36% lower (p=0.004) and the EI was 36% higher (p=0.003) in hypogonadal men, suggesting that male hypogonadism was associated with marked deterioration of trabecular architecture. When comparing these results to male patients in the current study, our patients with severe SHPT have lower S/C ratio and BV/TV than both hypogonadal and eugonadal males with normal kidney function. Testosterone levels were not measured in the study population, although it is likely that a significant proportion of male patients would have hypogonadism given the prevalence of this endocrinological issue in kidney disease is reported to be greater than 50%(Albaaj et al., 2006; Gungor et al., 2010).

Peak BMD or BV/TV is a genetic predisposition influenced by multiple environmental factors and women typically have a lower peak BV/TV compared to males. In an exclusively female population, micro-MRI was used to assess trabecular structural bone parameters of the distal radius in a postmenopausal osteopaenic cohort pre- and post-treatment with bisphosphonates(Folkesson et al., 2011; Greenspan et al., 2010). To date, however, there is no published literature of bone microarchitecture

parameters in premenopausal women with normal kidney function. In this study there were statistically significant reductions in both BV/TV and TbTh in females compared to males with SHPT, despite no significant differences in age (p=0.93) or dialysis vintage (p=0.38).

Similar to the study by Benito *et al*, FEA analysis of the distal tibia with micro-MRI was performed in another cohort of eugonadal and hypogonadal males to assess the effects of testosterone treatment. Zhang *et al*(X. H. Zhang et al., 2008) showed improved elastic moduli of the tibial trabecular bone by increased trabecular plate thickness following testosterone therapy in hypogonadal males. These studies highlight the usefulness of FEA derived from micro-MRI for monitoring treatment response. FEA using specialised MRI algorithms have been validated for the distal tibia(Chamith S. Rajapakse, Kobe, Batzdorf, Hast, & Wehrli, 2018) and more recently for the femur(C. S. Rajapakse & Chang, 2018; C. S. Rajapakse et al., 2020). FEA of the femur is especially useful for evaluating fracture risk in the hip as it can stimulate forces experienced during a sideways fall, which accounts for the main orientation in which hip fractures occur(Keyak et al., 2011). Using FEA derived from micro-MRI images in patients with SHPT could enable targeted therapies to be comprehensively evaluated with the effect on bone microarchitecture, and ultimately risk of fracture, established.

There are some limitations to this study including the small sample size and cross-sectional nature of the study which does not allow assessment of bone changes over time and evaluation as to whether surgical treatment may lead to improvement in bone microarchitecture. A 12-month follow up study is planned for the cohort of study patients to assess this question, where each patient can be used as their own control. Given the heterogeneity of renal bone disease and fluctuations in PTH measurements in dialysis patients, a control dialysis group was not selected for this study. There was also no concurrent DXA scan evaluation performed, although the predictive validity of fracture of this imaging modality in dialysis patients has previously been described in

the literature(Soichiro Iimori et al., 2011). One strength of this study is the use of a commercially available, routinely used RF coil and sequence acquisition for MRI scanning, which will hopefully allow this imaging modality to be more widely accessible. Also, the use of FEA in this patient cohort is novel and likely to become a tool for monitoring treatment in the future.

6.6 Conclusions

In conclusion, using a novel, non-invasive and ionising-radiation free imaging modality, patients with severe SHPT prior to parathyroidectomy showed evidence of significant bone microarchitecture changes with trabecular deterioration, reduced trabecular bone integrity, low trabecular and cortical bone volume, and reduced mechanical competence of bone as identified on FEA. Commonly used bone turnover markers in CKD, including PTH and ALP, correlated poorly with trabecular and cortical topological parameters and confirm the need for high quality imaging to be used as a diagnostic tool in renal bone disease. The use of micro-MRI in this population has the potential to guide treatment strategies in the future.

7. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

CKD-MBD remains a significant clinical burden and treatment challenge and is broadly defined by KDIGO as 1) disturbances in mineral metabolism, 2) abnormal bone remodelling and 3) accelerated extraosseous calcification leading to increased mortality and morbidity(KDIGO, 2009). Due to the complex and multifactorial nature of this condition, there remains many unanswered questions. A better understanding of the pathophysiology and clinical outcomes of CKD-MBD is crucial for management of this important clinical complication.

7.1 Objectives and Aims

The objectives of this thesis were to explore the pathophysiology, clinical outcomes and novel assessment strategies of the three domains of CKD-MBD. This is an ongoing area of research interest particularly given the significant burden of disease attributed to accelerated vascular calcification in patients with CKD and ESKD on dialysis (Blacher et al., 2001; Goodman et al., 2000; London et al., 2003). Calciphylaxis can be regarded as an accelerated template for VC, with life-threatening complications of wound sepsis and uncontrollable disease burden, and is associated with a 12-month mortality rate reported as high as 45-80% (Fine & Zacharias, 2002; Nigwekar et al., 2015; Weenig et al., 2007). Due to the rare nature of this disease, RCT are almost impossible to perform, consequently data on risk factors, prognosis, treatment and disease progression are challenging to investigate. Real-world registry data becomes an extremely valuable tool to better understand this disease. Therefore, the first objective of the thesis was to evaluate the first five years of Australian calciphylaxis outcomes and how these compare with published literature from international registries and databases.

Well-described histological findings in calciphylaxis include vascular calcification, intimal thrombosis, with progression to panniculitis and ultimately tissue

necrosis(Chen, Lehman, Gibson, Lohse, & El-Azhary, 2017; Mochel et al., 2013) . However, little is documented on whether vascular calcification is present in small vessels of the skin and subcutaneous tissue in patients with kidney disease, without calciphylaxis. Therefore, to further focus on the vascular consequences of CKD-MBD, my aim was to develop a greater understanding of the changes in skin and subcutaneous tissue in patients with different stages of CKD and ESKD, and determine whether they were associated with biochemical markers of SHPT.

In 2017, when I commenced this thesis, government funding for cinacalcet had been withdrawn in Australia and this medication, which is widely prescribed around the world for the medical management of SHPT, was no longer reimbursed and available to the majority of Australian dialysis patients. This led to changes in clinical practice including acceptance of higher PTH levels in dialysis patients and increased number of patients undergoing parathyroidectomies. Cessation of calcimimetic treatment allowed a unique opportunity to develop a greater understanding of the disturbances in mineral metabolism that occur in CKD-MBD. The impact of cinacalcet withdrawal in severe SHPT on clinical and biochemical consequences were specific aims of this thesis, both at a single centre and national level.

Together, with evaluating traditional biochemical markers of CKD-MBD in cinacalcet withdrawal, I also wanted to focus on novel biomarkers currently under investigation. CPP play a critical role in normal mineral handling, and in states of chronic mineral stress the transition of CPP-I to pro-inflammatory CPP-II may be enhanced, creating a vicious cycle of inflammation and calcification(Holt & Smith, 2016; Shanahan, 2007). My specific aim was to understand the temporal relationship between CPP and biochemical markers of CKD-MBD in dialysis patients following cinacalcet withdrawal.

Renal bone disease encompasses the third domain of CKD-MBD. Cinacalcet withdrawal resulted in a greater number of patients referred for parathyroidectomy due to refractory SHPT. To develop a greater understanding of bone health in this patient

population, my aim was to evaluate bone microarchitecture of the distal tibia prior to surgery, using a non-invasive and novel imaging modality, micro-MRI.

7.2 Outcomes of calciphylaxis in Australian patients with kidney disease

Although the pathogenesis of calciphylaxis is complex, in many respects it represents an accelerated process of calcification that can occur as part of the spectrum of CKD-MBD. Prospective data collection under the auspices of a registry framework is a well establish methodology to gain knowledge and ascertain clinical outcomes. Consistent with this, Chapter 2 focused on interpretation of the first five years of data collection from the *Australian Calciphylaxis Registry*. This involved identifying well recognised risk factors for calciphylaxis in the Australian population, namely preponderance for Caucasian race, female gender, obesity, diabetes and VKA use(Brandenburg, Kramann, et al., 2017; Nigwekar et al., 2015; Nigwekar, Thadhani, & Brandenburg, 2018). Despite longstanding opinion among some researchers and clinicians that severe SHPT is a significant risk factor for the development of calciphylaxis(Floege et al., 2015; Mohammed et al., 2007; Velasco et al., 2006), elevated PTH levels were not a consistent finding in this registry, with PTH levels at the time of and within 12-months of calciphylaxis diagnosis within the suggested range of the international KDIGO clinical guidelines(Ketteler et al., 2017). Elevated PTH levels may not be a significant risk factor for the development of calciphylaxis, and this key finding, supports similar results from the German Calciphylaxis registry(Brandenburg, Kramann, et al., 2017).

Not all circulating PTH in patients with ESKD may be biological active. *In vivo* animal work suggests oxidised PTH loses its PTH receptor stimulating properties(Galceran, Lewis-Finch, Martin, & Slatopolsky, 1984; Horiuchi, 1988; Vogt, 1995). Work by Hocher *et al* (Hocher et al., 2013) confirms this finding in kidney disease and renal transplantation. Oxidised PTH undergoes a three-dimensional conformational change altering protein folding and PTH-receptor interaction. As a result, oxidised PTH loses its biological activity. Hocher *et al* demonstrated a greater ratio of oxidised to non-

oxidised PTH in patients with ESKD(Hocher et al., 2013). Currently used second generation intact PTH assays do not measure PTH oxidation. Therefore, rather than describing PTH hormone status it may also reflect oxidative stress in patients with CKD(Hocher et al., 2013).

The second important observation from this study was the mortality for patients with calciphylaxis in the Australian population. Although still carrying a very poor prognosis, compared to published world literature,(Mazhar et al., 2001) Australian calciphylaxis patients in our registry had better outcomes, with a 50% mortality at 1.6 years following diagnosis. Given the high burden of this condition, these findings serve as a reminder of the importance for risk factor modification, particularly in respect to VKA use in dialysis patients. Conversely, aggressive PTH suppression for the sole purposes of modifying calciphylaxis risk is probably unwarranted and may even be counterproductive.

The ongoing search for successful and targeted treatment options for calciphylaxis remains elusive. The current management strategy revolves around diversifying treatment to maximise a patient's chance of survival. Fortunately, on the horizon are multiple trials looking at both targeted novel agents, such as crystallization inhibitor SN472 (now in a phase 3 double-blind RCT)("Phase 3 Study of SNF472 for Calciphylaxis (Calciphyx). Clinical Trials Identifier: NCT04195906,") and different treatment approaches (incorporating treatment with vitamin K2, magnesium and sodium thiosulphate, which all have varying degrees of evidence for the management of vascular calcification(Ruderman, Holt, Hewitson, Smith, & Toussaint, 2018)) with the pragmatic clinical trials such as BEAT-Calci("Better Evidence And Translation in Calciphylaxis Trial (BEAT-Calci). Trial number: AKTN. 17.01,") assessing the impact of wound healing, amputation rates and mortality.

Now, more than ever, calciphylaxis registries remain extremely useful in providing ongoing real-world information and experience in this rare condition. As of 2020,

calciphylaxis as a co-morbidity has been added as a new data collection component to Australia and New Zealand Dialysis and Transplant Registry (ANZDATA). This will ensure greater capture of calciphylaxis cases and will enable a stronger link to the Australian Calciphylaxis Registry with more comprehensive outcome data in the future.

7.3 The incidence of vascular calcification in the skin of patients with CKD and ESKD

While the presence of calcified deposits in small arteries and arterioles in the skin is considered a vital component in the diagnosis of calciphylaxis(Mochel et al., 2013), it is not pathognomonic of disease. VC in subcutaneous tissues has now been described outside the calciphylaxis setting, in cutaneous breast tissue(W. C. O'Neill & Adams, 2014) and amputated lower limbs in patients with ESKD(Ellis & O'Neill, 2018). These studies were the catalyst for the study in chapter 3, where it was thought that the presence of peripheral VC in the skin would imply a more widespread pathological process in CKD-MBD than has been assumed.

Results from this chapter show for the first time the presence of medial VC and perieccrine calcification in skin and subcutaneous tissue in multiple anatomical locations in patients with stage 4 CKD and ESKD undergoing elective surgery, without the presence of malignancy, PVD or calciphylaxis. Interestingly, VC was identified in the majority of neck skin samples of patients undergoing parathyroidectomy. However, the presence of VC itself was not associated with high PTH levels. This may be as a result of small sample size or variability and fluctuations in PTH levels, or perhaps reflect alternate pathophysiological mechanisms for VC. Additionally, these histological findings were not associated with changes in pro-calcific gene transcripts (specifically RUNX2 and BMP2). This may be due to early arterial calcification, a finding identified by O'Neill *et al*, demonstrating universal absence of RUNX2 and osteocalcin staining in breast tissue with early arterial calcification(W. C. O'Neill & Adams, 2014). However,

even if they do occur, osteogenic changes may not be the primary step in early calcification and occur down the track as a later reactive phenomenon.

Medial VC and perieccrine calcification are not specific histological findings for calciphylaxis; despite previous studies suggesting these histopathological features, in particular perieccrine calcification, are unique to this condition(Dookhan et al., 2015; Mochel et al., 2013). The bigger question now is the clinical relevance and clinical significance of these histopathological findings which is difficult to answer based on this study alone. One possible hypothesis is that these findings reflect a greater VC burden and suggest more aggressive modification of both traditional and non-traditional cardiovascular risk factors should be implemented. An important limitation is the single time point evaluated. To understand the progression of VC in skin and subcutaneous tissue, a longitudinal study is warranted and should also correlate small vessel skin calcification with more widespread VC of large central vessels, which is considered to be more clinically significant(Lehto et al., 1996; London et al., 2003). The second question is whether patients with subcutaneous VC might be more prone to calciphylaxis, particularly if exposed to other risk factors, such as VKA use. Given the prevalence of subcutaneous calcification in the study (as well as presence of VC in a control patient without CKD), it is unlikely that one trigger alone would be sufficient to cause calciphylaxis development and more probable that calciphylaxis occurs as a result of cascading triggers in an at-risk population group.

7.4 Changes in traditional and novel biochemical markers of mineral stress in dialysis patients after cinacalcet withdrawal

Chapter 4 and 5 focused on the traditional and novel biochemical changes and clinical outcomes of cinacalcet withdrawal in dialysis patients with SHPT. In Chapter 4 it was hypothesised that withdrawal of cinacalcet in patients with ESKD would increase mineral stress and cardiovascular risk. Cinacalcet withdrawal in a single-centre study of dialysis patients with SHPT, resulted in significant increases in CPP-I, PTH and serum calcium, without associated increases in CPP-II and serum phosphate. The second

important finding was a significant increase in CPP-I in cinacalcet withdrawal compared to cinacalcet-naïve dialysis patients, over a 6-month period. These findings suggest that increased CPP-I is a novel biochemical feature of worsening CKD-MBD.

Over the past 3 years, significant work has gone into further identifying the role and structure of these nanoparticles, using the flow cytometry method described here, which was developed and validated in our laboratory(Smith et al., 2017; Smith et al., 2018). There has been a particular focus on identifying the physiochemical composition of CPP as well as biological transformation of CPP-I to CPP-II in patients with kidney disease. Fetuin A remains an abundant protein present in the CPP shell, however a raft of other mineral binding proteins, lipoproteins, immunoglobulins and fragments of DNA are also present depending on the biological environment form which they are isolated(Smith et al., 2018).

CPP-I, containing protein bound amorphous calcium phosphate, play an important role in normal mineral trafficking. Recent evidence suggests CPP-I is involved in FGF-23 homeostasis, by direct access to osteoblasts to induce FGF-23 excretion following phosphate ingestion(Akiyama et al., 2020). CPP-I can undergo transition to larger particles containing calcium phosphate in crystalline phase, known as CPP-II. *In vitro* evidence suggests CPP-II have a pathogenic role in the body and may provide a mechanistic link to explain the mineralization paradox that occurs in CKD-MBD. Cai *et al* demonstrated combining CPP-II with a high phosphate environment works synergistically to mineralise VSMC and reduce osteoblast-like mineralisation(M. M. X. Cai et al., 2017). In Chapter 4, CPP-I, not CPP-II were significantly increased in patients following cinacalcet withdrawal, however there was a trend in increased CPP-II. Certain physiochemical factors, for example number of freeze-thaw cycles or composition of buffer used for sample dilution, may promote CPP aggregation(Miura et al., 2018), thereby increasing *in vitro* levels of CPP-II which may not be present in *in vivo*. It is likely that the *in vivo* circulating pool of native CPP-II is very low and accumulation of

CPP-I in ESKD can in itself overwhelm physiological mineral handling and contribute to pro-inflammation and pro-calcific milieu.

It therefore follows that CPP reduction may represent a therapeutic target. Using our laboratory's assay, Bressendorff *et al* have demonstrated reduction in CPP formation following treatment with high dialysate magnesium(Bressendorff et al., 2019). This was the first therapy shown to lower measured CPP although prior less specific assays have previously shown a reduction in fetuin-A-mineral particles, thought to represent CPP, with parathyroidectomy or cinacalcet use in a small group of HD patients(Hamano et al., 2010). Future studies comparing CPP levels in patients treated with cinacalcet versus those undergoing parathyroidectomy could be informative in identifying potential biochemical benefits of one intervention versus the other, however given the inherent differences in the two populations, adequate matching to avoid confounding of results will be challenging.

The search for novel biomarkers to further evaluate deranged mineral metabolism in CKD-MBD is an ongoing area of research. CPP perhaps deserve to be the next novel biomarker in this area, particularly given the growing evidence of their contribution to mineral stress(M. M. X. Cai et al., 2017) and association with increase VC risk (Smith et al., 2012). Consistent with this, multiple assays are being developed to study CPP and calcification propensity both directly and indirectly. In addition to our groups assay, this includes the T50 assay (as described in chapter 1) and more recently use of gel filtration to isolate Osteosense-bound mineral particles(Gatate et al., 2020). So far, application of these assays has been confined to basic research and clinical trial analyses. Direct quantitation of CPP by these assays along with measurement of serum calcification propensity using the T50 method(Smith et al., 2014) may offer future generalisability and widespread clinical application(Smith et al., 2014).

7.5 Clinical outcomes in dialysis patients after cinacalcet withdrawal

Chapter 5 expands on single-centre data to include results from eleven nephrology centres across Australia following cinacalcet withdrawal in dialysis patients with SHPT. This study confirmed findings from chapter 4, such as rebound increases in PTH, serum calcium and ALP with no change in serum phosphate. Cinacalcet withdrawal leads to biochemical changes which, in the short term, may not translate to increased cardiovascular mortality and morbidity, fracture rates or calciphylaxis for patients with severe SHPT. Longer term follow up of this cohort of patients without either cinacalcet or parathyroidectomy treatment is required to assess cardiovascular mortality and morbidity given the likely long lead time.

It was anticipated that cinacalcet withdrawal would result in increased rates of parathyroidectomy. Although the rate of parathyroidectomy across participating centres was likely higher, it is difficult to definitively confirm this as parathyroid surgery is not recorded as part of the ANZDATA registry. Importantly for the first time, this study was able to capture Australian parathyroidectomy rates, with a rate of 86/1000 person years, which is much greater than countries such as Canada (parathyroidectomy rates from 11.4/1000 persons-years to 3.6/1000(Lafrance et al., 2013)), where cinacalcet remains commercially available.

The loss of ready access to cinacalcet in the Australian nephrology community over the past five years has translated to changes in prescribing practice. There is more reliance on and higher doses of calcitriol used and clinicians have become desensitized to the grossly elevated PTH levels, well above KDIGO clinical guidelines(Ketteler et al., 2017). However, an important change occurred in February 2020 when cinacalcet was re-listed on the Pharmaceutical Benefits Scheme after the government negotiated a financially suitable agreement with another manufacturer of this medication. Given the long period of time this medication has been infrequently used in Australia, together with the lack of efficacy in the pre-specified intention to treat analysis, in

reducing cardiovascular mortality and morbidity demonstrated in the EVOLVE trial(Chertow et al., 2012), more time is required to see whether clinicians will embrace this therapeutic option. Based on the subgroup analysis of the EVOLVE trial(Parfrey et al., 2015), cinacalcet should perhaps be considered for dialysis patients with SHPT over the age of 65. Secondary analysis of the EVOVLE trial also demonstrated a reduced incidence of calciphylaxis in dialysis patients on cinacalcet (n=6) compared to the placebo (n=18)(Floege et al., 2015). Although the placebo group had multiple additional risk factors for the development of calciphylaxis, such as increased BMI and greater VKA use. Given the presence of other significant calciphylaxis risk factors, the results of the secondary analysis are not conclusive enough to suggest cinacalcet use to lower the rate of calciphylaxis.

7.6 Bone microarchitecture evaluated with micro-MRI in patients with SHPT

Renal osteodystrophy affects all components of bone including mechanical competence, strength, microarchitecture and density. Clinically, this translates into increased fracture risk. Current imaging techniques, fracture risk assessment tools and bone turnover markers do not adequately assess bone microarchitecture in ESKD and CKD, and safe and novel imaging techniques are required to better evaluate bone pathology in this population.

The study in chapter 6 demonstrated that patients with severe SHPT prior to surgery have evidence of global bone microarchitecture changes including trabecular deterioration, low trabecular and cortical volumes as well as reduced mechanical competence of bone on FEA. FEA, is becoming a common engineering method for virtual stress testing of bone and has been used to assess fracture risk(Keyak et al., 2011), as it can stimulate forces experienced during a fall. The second significant finding is the poor correlation of MRI findings with traditionally used bone turnover markers in CKD, namely ALP and PTH. As a result, it is challenging to make accurate decisions to minimise fracture risk, consider suitable therapeutic options and monitor

bone disease progression without better bone imaging. A limiting factor in the study was use of total not bone-specific ALP measurement, however given the population studied had no underlying liver disease, there is evidence to suggest total ALP correlates well with bone-specific ALP (Woitge, Seibel, & Ziegler, 1996).

High-resolution imaging modalities such as HRpQCT and micro-MRI are becoming useful tools as 'virtual bone biopsies' and will allow for more accurate diagnosis and monitoring of renal bone disease. Micro-MRI of the distal tibia at the time of parathyroidectomy in patients with severe SHPT allows us to better understand this complex bone disease. The long-term aim would be to promote widespread utilisation of this imaging modality. MRI has several advantages over HRpQCT including lack of ionising radiation and commercial availability, as well as routinely used RF coils and sequence acquisition for MRI scanning. The significant barrier at present is lack of widespread radiological training and expertise in this field to allow accurate and rapid imaging interpretation and analysis. Additionally, as scan time varies with anatomical location, longer scan times are required for more proximal locations such as the proximal femur. At present, distal locations such as the wrist and tibia are used to extrapolate bone findings for the rest of the skeleton. Methods to address this issue are currently being evaluated, with FEA of the proximal femur using micro-MRI successfully performed for the first time this year by Rajapakse et al(C. S. Rajapakse et al., 2020). FEA to evaluate bone competence has the potential to become a useful fracture risk assessment tool.

Micro-MRI has the capacity to distinguish patients who have evidence of bone disease related to high PTH and those who do not have evidence of bone problems despite high PTH. In the future this information can be valuable in deciding who might benefit from parathyroidectomy and who might not require any intervention; or perhaps even who might be harmed by intervention with a parathyroidectomy in terms of developing adynamic bone disease. Future studies are planned to investigate changes in bone microarchitecture 12 months following parathyroid surgery to evaluate for any improvement following definitive treatment of severe SHPT.

7.7 Concluding remarks

Improving clinical outcomes in CKD-MBD is contingent on better understanding of the pathological drivers of this disease, as well as adequate diagnostic tools to assess different domains of this syndrome. In summary, this thesis presents insights into the vascular, biochemical and bone domains of CKD-MBD with both clinical and basic science research focusing on pathophysiology, novel biochemical markers and imaging techniques. The novel findings obtained in this thesis have broadened the understanding of all three domains of CKD-MBD. With regard to vascular complications of CKD-MBD, it has expanded our knowledge by supporting the lack of an association of SHPT as a significant risk factor for the development of calciphylaxis and also identified small vessel and perieccrine calcification in fat and subcutaneous tissue in patients with CKD and ESKD without evidence of calciphylaxis. In terms of biochemical complications of CKD-MBD, the studies presented here have identified CPP as a potential novel biomarker for CKD-MBD and demonstrated higher levels of CPP-I in patients with severe SHPT, together with a parallel rise in CPP-I and PTH following cinacalcet withdrawal. Finally, the bone complications of CKD-MBD presented here outline the trabecular and cortical bone derangement present in patients with significant SHPT prior to parathyroidectomy, which correlate poorly with traditionally used bone turnover markers and identify a need for high-resolution imaging to better understand and manage renal bone disease. Further clinical and basic science studies will be required to validate findings and to further explore some concepts established in this thesis, however, the data presented here establishes important concepts and hypotheses for future research in this field.

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APPENDIX A: THE AUSTRALIAN CALCIPHYLAXIS REGISTRY DATA COLLECTION FORM

Definition: Calciphylaxis defined as skin breakdown / necrosis associated with calcification of arteries and skin.

Baseline at time of diagnosis

PATIENT CONSENT

1. Has patient consent to study been obtained……yes / no

A. BACKGROUND INFORMATION	
A.1 Abstractor's initials and email address:	A.2 Date completed __ / ___/ __ DD MM YY
A.3 Patient's sex 0=Female 1=Male	A.4 Patient's race (choose one number) 21. White/Caucasian 22. Black/Afro Caribbean 23. Asian (South East Asian) 25. Indian Subcontinent 24. Indigenous (e.g.Aboriginal/Torres Strait Islander)
A.5 Indicate patient's renal status at the time of first symptoms of calciphylaxis:	

1. Haemodialysis/filtration	4. Functioning renal transplant
2. Peritoneal dialysis	5. No kidney disease
3. Chronic kidney disease not on dialysis	
(***if 1 or 2, activates appropriate data entry fields)***	

A.6 Date of first ever regular dialysis for chronic kidney disease. (At least once weekly regardless of hospital or facility setting).

__ / ___/ __

DD MM YY

A 7 If at end-stage renal disease, please provide a chronological history of the patient's treatment modalities from start of end-stage renal disease to the enrolment date (reference date). Give as much information as is available (e.g. give year only if month or date are unknown).

Start date (DD / MM / YYYY)	**End date (DD / MM / YYYY**	**Modality if relevant (Use code number provided below)**	
/ /	/ /		1. Haemodialysis
/ /	/ /		2. Haemodiafiltration
/ /	/ /		3. Haemofiltration
/ /	/ /		4. Peritoneal Dialysis
/ /	/ /		5. Transplant
/ /	/ /		6. Chronic kidney disease
/ /	/ /		7. Unknown

C. PSYCHOSOCIAL EVALUATION			
Complete this section from the most recent psychosocial evaluation on or before the patient's enrolment date.			
C.1	Able to eat independently? (Choose one)	0=No	1=Yes
C.2	Able to walk without assistance / assistive device? (Choose one)	0=No	1=Yes
C.3	Walks with assistance (e.g. person, cane, walker)? (Choose one)	0=No	1=Yes
C.4	Requires wheelchair? (Choose one)	0=No	1=Yes
C.5	Able to transfer independently (e.g. without assistive device or help)? (Choose one)	0=No	1=Yes

D. MEDICAL HISTORY ON OR BEFORE ENROLMENT DATE			
D.1	Height (if not measured, ask the patient) (centimetres)	Cm:	
D.2	Was the patient a bilateral amputee (if	0=No	1=Yes

	yes, give ***original*** height above)			
D.3	Primary cause of renal disease (use enclosed Diagnosis Code Sheet)			
D.4	Other contributing or secondary cause of end stage renal disease, if applicable (use enclosed diagnosis code sheet):			
D.5	Is the patient anephric (e.g. no native kidneys)? (Choose one)	0=No	1=Yes	
D.6	Cigarette smoking status at enrolment date (Choose one number)			
	1. Active (still smoking) 2. Former, stopped <1 year ago	3. Former, stopped >1 year ago 4. Smoker, current status unknown	5. Non smoker 6. Unknown	
D.7	Was the patient hospitalised in the three months prior to the enrolment date?	0=No	1=Yes	
Coronary Heart Disease / Coronary Artery Disease on or before Enrolment Date		**Choose the best answer**		
D.8	Prior diagnosis of coronary heart	0=No	1=Yes	2=Suspected

	disease / coronary artery disease?			
D.9	History of angina (at any time)	0=No	1=Yes	2=Suspected
D.10	Angina associated with exertion or dialysis within 12 months of the enrolment date	0=No	1=Yes	2=Suspected
D.11	Angina at rest within 12 months of enrolment date	0=No	1=Yes	2=Suspected
D.12	Myocardial infarction ever	0=No	1=Yes	2=Suspected
D.13	Myocardial infarction within three months of enrolment date	0=No	1=Yes	2=Suspected
D.14	Coronary angiography (if no, skip the next question)	0=No	1=Yes	2=Suspected
D.15	Was coronary angiography normal?	0=No	1=Yes	2=Suspected
D.16	Coronary bypass surgery ever	0=No	1=Yes	2=Suspected

D.17	Coronary angioplasty ever	0=No	1=Yes	2=Suspected
D.18	Arterial Calcification seen on X-ray or CT	0=No	1=Yes	2=Suspected
Other Cardiovascular Disease on or before Enrolment Date		**Choose the best answer**		
D.19	Cardiac arrest ever	0=No	1=Yes	2=Suspected
D.20	Chronic atrial fibrillation	0=No	1=Yes	2=Suspected
D.21	Paroxysmal / recurrent atrial fibrillation	0=No	1=Yes	2=Suspected
D.22	Other arrhythmias	0=No	1=Yes	2=Suspected
D.23	Permanent pacemaker implanted	0=No	1=Yes	2=Suspected
D.24	Automatic implanted cardiac defibrillator (AICD)	0=No	1=Yes	2=Suspected
D.25	Congestive heart failure	0=No	1=Yes	2=Suspected
D.26	Congestive heart failure requiring	0=No	1=Yes	2=Suspected

	hospitalisation within the past 12 months			
D.28	Left Ventricular ejection fraction (if known)			%
D.29	Cardiomegaly by X-ray	0=No	1=Yes	2=Suspected
D.30	History of Pulmonary oedema	0=No	1=Yes	2=Suspected
D.31	Left ventricular hypertrophy by electrocardiogram	0=No	1=Yes	2=Suspected
D.32	Left ventricular hypertrophy by echocardiography	0=No	1=Yes	2=Suspected
D.33	Left ventricular hypertrophy, mode of diagnosis unspecified	0=No	1=Yes	2=Suspected
D.34	History of Pericarditis	0=No	1=Yes	2=Suspected
D.35	Valvular heart disease by echocardiogram or heart catheterisation	0=No	1=Yes	2=Suspected
D.36	Prosthetic heart valve (s/p valve replacement)	0=No	1=Yes	2=Suspected

D.37	Diagnosis of hypertension	0=No	1=Yes	2=Suspected
D.38	Hyperlipidaemia	0=No	1=Yes	2=Suspected
Cerebrovascular Disease on or before Enrolment Date			**Choose the best answer**	
D.39	Cerebrovascular accident (stroke) ***without*** major residual neurologic deficit	0=No	1=Yes	2=Suspected
D.40	Cerebrovascular accident (stroke) ***with*** major residual neurologic deficit (e.g. weakness, impaired speech)	0=No	1=Yes	2=Suspected
D.41	Carotid endarterectomy	0=No	1=Yes	2=Suspected
D.42	Transient ischemic attacks	0=No	1=Yes	2=Suspected
Aortic or Peripheral Vascular Disease on or before Enrolment Date			**Choose the best answer**	
D.43	Prior diagnosis of peripheral vascular disease	0=No	1=Yes	2=Suspected

D.44	Aortic Aneurysm	0=No	1=Yes	2=Suspected
D.45	Arterial bypass surgery for peripheral vascular disease or surgical repair of aortic aneurysm	0=No	1=Yes	2=Suspected
D.46	Claudication (pain in extremities with exertion due to peripheral vascular disease)	0=No	1=Yes	2=Suspected
D.47	Rest pain of extremities due to peripheral vascular disease	0=No	1=Yes	2=Suspected
D.48	Recurrent cellulitis, skin infection, gangrene	0=No	1=Yes	2=Suspected
D.49	Amputation due to peripheral vascular disease	0=No	1=Yes	2=Suspected
D.50	Limb amputation (for reason other than peripheral vascular disease)	0=No	1=Yes	2=Suspected

D.51	Previous episode of calciphylaxis	0=No	1=Yes	2=Suspected
D.52	History of deep vein thrombosis	0=No	1=Yes	2=Suspected
Diabetes on or before Enrolment Date			**Choose the best answer**	
D.53	Does the patient have a diagnosis of diabetes (even if not the primary cause of end stage renal disease)? **If no, skip the next three questions.**	0=No	1=Yes	2=Suspected
D.54	Insulin therapy (any time prior to enrolment)	0=No	1=Yes	2=Suspected
D.55	Oral hypoglycaemic agents (any time prior to enrolment)	0=No	1=Yes	2=Suspected
D.56	Diabetic gastroparesis	0=No	1=Yes	2=Suspected
Lung Disease on or before Enrolment Date			**Choose the best answer**	
D.57	Chronic Obstructive Pulmonary Disease (COPD, chronic	0=No	1=Yes	2=Suspected

	bronchitis, emphysema)			
D.58	Use of home oxygen	0=No	1=Yes	2=Suspected
D.59	Dyspnoea (shortness of breath) at rest or with minimal exertion (other than with excessive weight gain)	0=No	1=Yes	2=Suspected
D.60	Diagnosis of sleep apnoea on or before the enrolment date? **If no, skip the next question.**	0=No	1=Yes	2=Suspected
D.61	If yes, is patient being treated with Continuous Positive Airway Pressure (CPAP) at night?	0=No	1=Yes	2=Suspected
Neurologic and Psychiatric Disease on or before Enrolment Date		**Choose the best answer**		
D.62	Seizure disorder	0=No	1=Yes	2=Suspected
D.63	Dementia	0=No	1=Yes	2=Suspected
D.64	Cognitive impairment other than dementia	0=No	1=Yes	2=Suspected

D.65	Peripheral neuropathy (diabetic or other)	0=No	1=Yes	2=Suspected
D.66	Parkinson's disease	0=No	1=Yes	2=Suspected
D.67	Depression or bipolar disorder (schizophrenia, psychosis) within the past 12 months	0=No	1=Yes	2=Suspected
D.68	Substance abuse within the past 12 months	0=No	1=Yes	2=Suspected
Skeletal, Arthritis and Musculoskeletal Disease on or before Enrolment Date		**Choose the best answer**		
D.69	History of hip fractures since starting dialysis	0=No	1=Yes	2=Suspected
D.70	Carpal tunnel syndrome	0=No	1=Yes	2=Suspected
D.71	Surgery for carpal tunnel syndrome	0=No	1=Yes	2=Suspected
D.72	Beta-2 microglobulin disease (dialysis-	0=No	1=Yes	2=Suspected

	associated amyloidosis)			
D.73	Parathyroid surgery (parathyroidectomy)	0=No	1=Yes	2=Suspected
	Gastrointestinal / Liver Disease on or before Enrolment Date		**Choose the best answer**	
D.74	Peptic ulcer disease	0=No	1=Yes	2=Suspected
D.75	Gastrointestinal bleed within the past 12 months	0=No	1=Yes	2=Suspected
D.76	Ascites (cirrhosis of the liver) within the past 12 months	0=No	1=Yes	2=Suspected
D.77	Diagnosis of Hepatitis B	0=No	1=Yes	2=Suspected
D.78	Diagnosis of Hepatitis C	0=No	1=Yes	2=Suspected
	Cancer on or before Enrolment Date		**Choose the best answer**	
D.79	History of cancer other than skin cancer. **If no, skip the next question**	0=No	1=Yes	2=Suspected

D.80	Year of most recent cancer diagnosis (YYYY)	Year:			
Ophthalmologic Conditions on or before Enrolment Date				**Choose the best answer**	
D.81	Diabetic retinopathy				
D.82	Legally blind				
Viral Infections on or before Enrolment Date				**Choose the best answer**	
D.83	HIV Status	0=Negative	1=Positive	2=Unknown	3=Can't disclose
D.84	AIDS diagnosis	0=Negative	1=Positive	2=Unknown	3=Can't disclose
D.85	Did the patient have some residual renal function with urine output greater than 200 ml / day or one cup / day on or before the enrolment date?	0=No	1=Yes	2=unknown	

E. LABORATORY DATA
Enter the most recent lab values taken <u>on or before</u> the requested date(s) below. If any of the measurements entered for this patient are in units other than those

reported on your Laboratory Reference Sheet, please write in the correct units after the value.				
		On or Before the Reference Date		
		DD	**MM**	**YY**
E.1	Date Majority of Measurements are taken	/	/	
E.2	Creatinine (µmol/L)	/	/	
E.3	Sodium (mmol/L)	/	/	
E.4	Potassium (mmol/L)	/	/	
E.5	Chloride (mmol/L)	/	/	
E.6	Bicarbonate or (or Total CO^2) (mmol/L)	/	/	
E.7	Calcium (Total corrected; indicate if uncorrected calcium provided) (mmol/L)	/	/	
E.8	Calcium (Ionized) (mmol/L)	/	/	
E.9	Phosphate (mmol/L)	/	/	
E.10	Total Protein (g/L)	/	/	
E.11	Albumin (g/L)	/	/	
E.12	Alkaline Phosphatase Total (U/L)	/	/	
E.13	Bone Specific Alkaline Phosphatase	/	/	

	Other bone turnover marker? (indicate units used)		
E.14	Uric Acid (mmol/L)	/	/
E.15	Glucose (mmol/L)	/	/
E.16	Magnesium (mmol/L)	/	/
E.17	Total Cholesterol (mmol/L)	/	/
E.18	HDL Cholesterol (mmol/L)	/	/
E.19	LDL Cholesterol (mmol/L)	/	/
E.20	Triglycerides (mmol/L)	/	/
E.21	Glycosylated Haemoglobin (HbA1c) (indicate mmol/mmol OR %)	/	/
E.22	Intact PTH (iPTH) (pmol/L OR indicate other units used)	/	/
E.23	Bioactive intact PTH (Bi PTH) (Whole PTH) (pmol/L OR indicate other units used)	/	/
E.24	C-Reactive Protein (mg/L); (indicate if high-sensitive CRP)	/	/
E.25	Interleukin-6 (IL-6); indicate units used	/	/
E.26	Homocysteine (µmol/L)	/	/

E.27	Haemoglobin (g/L)		/	/
E.28	MCV		/	/
E.29	Haematocrit		/	/
E.30	White blood cell count		/	/
E.31	Neutrophils		/	/
E.32	Lymphocytes		/	/
E.33	Serum Iron (µmol/L)		/	/
E.34	Total Iron Binding Capacity (µmol/L)		/	/
E.35	Ferritin (µg/L)		/	/
E.36	Transferrin Saturation (%)		/	/
E.37	Hepatitis B Surface Antigen (Choose one)	0=Negative	1=Positive	2=Unknown
E.38	Hepatitis B Antibody (Choose one)	0=Negative	1=Positive	2=Unknown
E.39	Hepatitis C Antibody (Choose one)	0=Negative	1=Positive	2=Unknown

F. HAEMODIALYSIS PRESCRIPTION & DELIVERED DOSE

Enter the most recent prescribed values on or before the Reference Date for this questionnaire

F.1	Prescribed length of treatment in total minutes (e.g. 3 ½ hrs = 210 min)	: minutes
F.2	Prescribed number of dialysis sessions per week	: sessions / week
F.3	Blood Urea Reduction Ratio	
F.4	Kt/V (if determined)_____1=single pool spKt/V 2= double pool, eKt/V 3=unsure	
F.5	What is the predominant base / buffer used in the dialysate? (Choose one)	1=Bicarbonate 2=Acetate
F.6	Prescribed dialysate base / buffer concentration as sum of acetate and bicarbonate if combined (enter value in mmol/l or mEq/l	_____ ___
F.7	Prescribed dialysate calcium concentration (check units used)	---------- □ mEql/l □ mmol/l □mg/dl
F.8	Type of dialysis anticoagulation: 1. Heparin 2. Low molecular heparin (name)_____ 3. Other heparin (e.g. danaparoid) 4. No anticoagulant Dose or load dose of anticoagulant (1, 2, or 3 from above): _____ □ units □ ml □ mg	

F.9	Heparin infusion (pump) rate in units / hr	: units / hour
F.10	Duration of heparin infusion	: hours

J. PERITONEAL DIALYSIS PRESCRIPTION & DELIVERED DOSE

Enter the most recent prescribed values on or before the Reference Date for this questionnaire

J.1	Which therapy is the patient receiving	1.Continuous Ambulatory Peritoneal dialysis (CAPD) 2. Automated peritoneal dialysis (APD)
J.2	Prescribed number of exchanges if APD	
J.3	Manufacturer, dialysis fluid, volume used per 24 hours eg Fresenius 11 x2 x 2L, 12 x1 x1L, extraneal x1 x1L	
J4	Total weekly creatinine clearance	L/wk/1.73 m(2)
J5	Total weekly Kt/V urea	
J6	Please indicate transporter status	1=high / high-average transporter

		2=low / low-average transporter 3=unknown		

N. RENAL MEDICATIONS PRIOR TO ONSET OF CALCIPHYLAXIS				
N.1	a)	Did the patient receive any <u>intravenous</u> vitamin D analogs for the control of PTH OR bone disease during the **6 months** prior to the questionnaire Reference Date?	0=No	1=Yes
	b)	If yes, what is the drug name, dose, frequency of administration and length of prescription eg calcitriol 1 microgram x3 week for 2 months? (Choose all that apply) 1. Calcitriol _____ 2. Paricalcitol (Zemplar)_____ 3. Other intravenously (specify): - _____		
N.2	a)	Did the patient receive any <u>oral</u> drugs (e.g. Vitamin D analogs or other agents for the control of PTH – bone disease) during the **6 months** prior to the questionnaire Reference Date?	0=No	1=Yes

b) If yes, what is the drug name, dose, frequency of administration and length of prescription eg calcitriol 0.25 microgram daily for 6 months. (Choose all that apply)

1. Calcitrol (Rocaltrol)	3. Cinacalcet (Sensipar)	5. Alphacalcidol (One Alfa)
2. Doxercalciferol (Hectoral)	4. Other oral (specify)	6. Paricalcitol (Zemplar)

	Did the patients receive 'nutritional vitamin D'; cholecalciferol or ergocalciferol in the 6 months prior to the qustionnaire?	Yes/no Daily dose
N.3	Did the patient receive phosphate binders within the **6 months** prior to the questionnaire Reference Date? (Choose one) **If no, skip next question.**	0=No 1=Yes

N.4 If yes, what is the drug name, dose, frequency of administration and length of prescription eg Caltrate 6 months. (Choose all that apply)

1. Calcium Carbonate (Calcichew, Cal-sup, Caltrate)	4. Aluminium hydroxide (Alu-tabs)	7. Colestimide
2. Calcium Acetate (Phos-ex)	5. Magnesium	8. Niceritrol
3. Sevelamer (Renagel)	6. Lanthanum carbonate (Fosrenol)	9. Other_____ _____

N.5	Did the patient receive a water soluble vitamin preparation (such as Vitamin C, B, folic acid) during the **6 months** prior to the questionnaire Reference Date? (Choose one) If yes, what is the drug name, dose, frequency of administration and length of prescription _____ _____	0=No	1=Yes
N.6	Was Erythropoietin (Epo) administered during the 6 months prior to the questionnaire Reference Date? (Choose one) **If no, skip the next two questions.**	0=No	1=Yes
N.7	Was warfarin (Coumadin or Marveran) administered during the 36 months prior to the questionnaire Reference Date? (Choose one) **If yes, please outline dose and duration.**	0=No	1=Yes
N.8	What ESA preparation was used and which route of administration ie intravenous (IV) or subcutaneous (SC)? 1. Epoetin alfa (Eprex) 2. Darbepoetin alfa (Aranesp) 3. Other (specify) _____ _____ 4. Epoetin beta (NeoRecormon)		

N.9	Did the patient receive intravenous iron during the 6 months prior to the questionnaire Reference Date? **If no, skip next question.**	0=No	1=Yes

N.10	What iron preparation was used? (Choose one number)		
	1. Iron Sorbital (Jectofer)	4. Ferric or Ferrous Gluconate (Ferrlecit, Ferlixit)	7. Chondroitin Sulfate/Iron Clloid
	2. Iron sucrose (Venofer)	5. Ferric Hydroxide Polymaltose (Maltofer)	8. Ferric Oxide Saccharated
	3. Iron Dextran	6. Other _____	9. Cideferron

O. OTHER MEDICATIONS

Begin a medications worksheet for this patient, filling in the columns for the 'Medication Name', 'Strength', 'Medication Order and the column for the current questionnaire reference for all medications that the patient is taking as of the reference date.

Please list all medications being given to the patient during dialysis sessions or prescribed for the patient to be taken at home (include both prescription and non-prescription medications).

Write the medication names and respective strength and medication order, of these medications in the table below.

Note Examples are provided

1	2	3	4
	Medication Name	**Strength**	**Medication Order**
EX 1:	**Ibuprofen**	**400 mg**	**1-2 PO PRN**
EX 2:	**Caltrate**	**600 mg**	**2 TABS tds with MEALS**
EX 3:	**Simple linctus**	**15 mg / 5 ml**	**2 TSP PO once daily**
EX 4:	**Clarithromycin**	**500 mg**	**bd for 2 weeks**
EX 5:	**Heparin**	**5000 units / ml**	**IV 4 hourly**
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			

CALCIPHYLAXIS DETAILS	
P1. Date of onset of first lesions (month / year)	
P2. Potentially contributing event at site of lesion(s): If yes, indicate the event:	yes / no / unknown 1 trauma 2 injection 3 other_____
P3. Time from onset of lesions to diagnosis of calciphylaxis (weeks)	
P4. Diagnosis made by: (check all that apply)	1 clinical impression 2 wound / skin biopsy 3 radiograph of soft tissue 4 nuclear medicine “bone” scan 5 transcutaneous oxygen tension assessment 6 other _____

P5. Could the diagnosis more likely be tumoral calcinosis (instead of calciphylaxis)?	Yes / no
P6. Location of lesions: (check all that apply)	1 abdomen 2 thighs 3 buttock 4 penis / vulvar area 5 breasts 6 lower extremities (calves, legs) 7 feet / toes 8 back 9 arms 10 hands / fingers 11 other _____
P7. Which of the following medical interventions were used to treat the calciphylaxis? (check all that apply)	1 calcium containing phosphate binders stopped 2 Calcitriol or calcitriol analogue therapy)stopped 3 calcimimetics administered 4 dialysate calcium concentration reduced 5 dialysis frequency increased 6 dialysis modality changed, if yes describe _____

	7 warfarin/coumadin stopped 8 iron therapy stopped 9 bisphosphonates given – if yes name, dose, route, length of administration_____ _____ 10 sodium thiosulfate If yes dose given and length of administration 11 hyperbaric oxygen treatment if yes, prescription _____ _____ 12 oral antibiotics 13 intravenous antibiotics 14 maggots 15 antioxidants 16 other e.g. Vitamin K, Cholecalciferol (nutritional vitamin D) Name, dose and frequency: _____ _____
P8. Which of the following surgical	1 parathyroidectomy

interventions were done? (check all that apply)	if yes, date_____ _____ 2 wound debridement in operating room 3 wound debridement outside of operating room (i.e. clinic, home) 4 amputation 5 revascularisation / angioplasty 6 skin graft 7 other _____
P9. Were any other consultants/teams involved in the management of patient? (check all that apply)	1 surgical 2 vascular 3 general 4 plastic 5 wound care specialist other than plastic surgeon 6 dermatology 7 infectious disease 8 rheumatology 9 haematology 10 pain specialist
P10. Did the patient develop bacteraemia?	yes / no

P11. Did skin lesions resolve? If yes approximate date of full healing_____ _____	yes / no
P12 If the patient died, what was the primary cause of death?	
P13. If not the primary cause, was calciphylaxis considered a secondary cause of death?	yes/ no
P14. Did the patient withdraw from dialysis prior to death?	yes / no
[repeat E and P5-13 in 6 months}	

APPENDIX B: THE AUSTRALIAN CALCIPHYLAXIS REGISTRY: REPORTING CLINICAL FEATURES AND OUTCOMES OF PATIENTS WITH CALCIPHYLAXIS

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The Australian Calciphylaxis Registry: reporting clinical features and outcomes of patients with calciphylaxis

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ABSTRACT

Background. Calciphylaxis is a rare disease, predominantly affecting patients with chronic kidney disease (CKD) and associated with significant morbidity and mortality due to progressive cutaneous calcification, necrotic ulceration and infection. Clinical registries have been established to better understand the risk factors, optimal treatments and disease outcomes of calciphylaxis.

Methods. We established a prospective, Internet-based clinical registry for the online notification of calciphylaxis cases in Australia. Seven institutions participated, with data recorded on patient characteristics, biochemical parameters, treatments and disease outcomes.

Results. Between 2014 and 2019, 47 cases of calciphylaxis were registered. The mean patient age was 66 ± 11 years and body mass index was 35 ± 9 kg/m^2, with a higher proportion of females (51%). Eighty-seven percent of patients had end-stage kidney disease (ESKD), with 61% on hemodialysis or hemodiafiltration, with a median dialysis vintage of 4.8 [interquartile range (IQR) 1.7–7.4)] years. Five patients had CKD not requiring dialysis and two were kidney transplant recipients. Diabetes was present in 76% of patients and the cause of ESKD in 60%; 34% received vitamin K antagonists (VKAs) before diagnosis. The median parathyroid hormone level at diagnosis was 32 (IQR 14–50) pmol/L. The most common site of calciphylaxis was the lower limbs (63%), with 19% of patients having more than one area involved. Ten patients (22%) had a resolution of calciphylaxis and 25 died, with 50% mortality at a median of 1.6 (IQR 0.2–2.5) years from diagnosis.

Conclusions. The Australian Calciphylaxis Registry highlights risk factors for calciphylaxis, including diabetes, obesity and VKA use. Resolution of calciphylaxis is uncommon despite multimodal therapy and mortality from calciphylaxis in the first year following diagnosis remains high.

Keywords: calcific uremic arteriolopathy, calciphylaxis, chronic kidney disease, end-stage kidney disease, mineral metabolism

INTRODUCTION

Calciphylaxis is a rare disease predominately affecting patients with chronic kidney disease (CKD), especially those with end-stage kidney disease (ESKD) on dialysis, although it has also been described in patients with normal kidney function and following kidney transplantation [1]. Calciphylaxis presents with painful subcutaneous lesions that can develop into necrotic ulcers. Due to the progressive and unrelenting nature of cutaneous calcification, calciphylaxis is associated with significant morbidity and mortality due to extreme pain, non-healing wounds and lengthy hospitalizations. One-year mortality is reported as high as 45–80%, mainly due to complications of wound sepsis, adverse effects of treatment and treatment withdrawal due to uncontrollable disease burden [1–3].

Calciphylaxis was first described in 1898; however, the term ‘calciphylaxis’ was coined in 1961 by Hans Selye after laboratory experiments induced generalized subcutaneous soft tissue calcification in rats using a two-step process of sensitization and application of a challenging agent (such as local trauma) [4–6]. In humans, the pathogenesis of calciphylaxis is not considered to be a hypersensitivity reaction, but rather accelerated vascular and extraosseous calcification with evidence of osteogenic transformation of vascular smooth muscle cells,

together with vessel thrombosis and fibrointimal hyperplasia [7]. This results in vascular endothelial dysfunction and panniculitis leading to skin breakdown and impaired wound healing [7, 8]. The term calcific uremic arteriolopathy is perhaps a more accurate description of the histological disease process, although calciphylaxis remains the most popular nomenclature, particularly as it infrequently occurs in patients with normal kidney function.

Due to the rare nature of calciphylaxis, there are currently no completed, randomized controlled trials to guide treatment, with evidence largely based on case series or observational studies that are predominantly retrospective. There are also no uniform criteria for diagnosis, which is most commonly based on clinical impression, supporting features and suggestive risk factors. Occasionally, imaging or skin biopsy is performed for confirmation, although clinicians are often fearful that the latter will worsen skin lesions.

Calciphylaxis registries have been established in several countries to provide real-world information regarding risk factors, diagnosis and management of this disease. The German Calciphylaxis Registry (http://www.calciphylaxie.de) was the first online registry and has reported a calciphylaxis incidence of 4/10 000 patients on dialysis in Germany [9]. This registry also highlighted the high use of vitamin K antagonists (VKAs) in patients with calciphylaxis and suggested that low rather than high serum parathyroid hormone (PTH) values may be a key risk factor for calciphylaxis, with 45% of dialysis patients having PTH values below the recommended target range. This finding was in contrast to previous case reports showing an association with high PTH [10, 11] and results from the Evaluation of Cinacalcet Hydrochloride Therapy to Lower Cardiovascular Events (EVOLVE) trial, which demonstrated that the calcimimetic cinacalcet decreased PTH levels and was associated with a reduced incidence of calciphylaxis [12].

Registries for calciphylaxis may provide important insights and information to increase our knowledge about the epidemiology and clinical aspects of calciphylaxis and may help to optimize therapeutic management. The German Calciphylaxis Registry [9] was limited to collecting data at a single time point and did not collect data on longer-term outcomes, including mortality. The Australian Calciphylaxis Registry (ACR) became active in 2013, with the aim of developing a large database of cases of calciphylaxis in Australia that could provide a resource for research into causes and evidence-based management. The registry would also evaluate the safety and efficacy of therapies and assess overall patient care and outcomes with prospective, longitudinal data collection.

Following the publication of the EVOLVE trial, which showed no change in the unadjusted composite primary endpoint of cardiovascular mortality and morbidity in patients on cinacalcet, government reimbursement for cinacalcet in Australia was withdrawn in 2015. Therefore we were also interested in identifying whether cinacalcet withdrawal would lead to an increased rate of calciphylaxis in the Australian dialysis population. The aim of this analysis is to provide a detailed and comprehensive summary of the first 5 years of data collection within the ACR.

MATERIALS AND METHODS

Registry design and development

The ACR is accessible at http://www.calciphylaxis.org.au and was established in association with investigators involved in the development of the German Calciphylaxis Registry. The registry is non-interventional, prospective and observational, and participants provide informed consent for de-identified data collection and storage. The registry received ethical approval from the Western Sydney Local Health District Human Research Ethics Committee in New South Wales, Australia, with reciprocal ethics and governance arranged at each additional involved site. Registry activities are supported by the Australian and New Zealand Society of Nephrology and by an unrestricted grant from Amgen Australia (2013–17). Advertising of the registry was initially undertaken by investigators to increase awareness and for case recruitment, which commenced in 2014.

Registry data collection

The registry includes a comprehensive database similar to the German Calciphylaxis Registry with questions covering >70 parameters and items regarding patient-related and laboratory data, clinical background and presentation as well as therapeutic strategies and outcomes of treatment. The online registry questionnaire provides drop-down boxes for item selection and includes free text options for questions regarding dialysis prescription, medications and treatment outcomes. Photos of skin lesions can also be uploaded online to support the diagnosis.

The registry also records biochemical and haematological parameters of serum creatinine, urea, potassium, bicarbonate, calcium, albumin, total protein, phosphate, magnesium, uric acid, alkaline phosphatase (ALP), PTH, C-reactive protein (CRP), haemoglobin, white cell count and platelets. For each parameter, standard procedures were applied according to the local participating laboratory. For serum albumin levels <40 g/L, the corrected serum calcium was calculated as calcium (mmol/L) + 0.02(40 − serum albumin).

Recruitment to the ACR is performed by treating physicians at involved centres. Given the rare nature of the condition, all patients admitted with a clinical suspicion of calciphylaxis are eligible for enrolment. Diagnosis of calciphylaxis is made clinically by the treating physician and with the use of skin biopsy and imaging in cases where confirmation is necessary. As there is no centralized adjudication process, there may be some false-positive cases included and equally some cases of calciphylaxis may be missed.

Missing or ambiguous registry data for this summary report were clarified with either the treating physician or research nurse at the participating centre. In the event that implausible data could not be clarified, such data or the entire dataset were excluded from analysis. To assess patient outcomes in the ACR, following the initial 5-year period of data collection, all treating units were contacted for patient follow-up data, including calciphylaxis resolution and mortality.

Table 1. Patient demographics, divided by gender

Characteristics	Total ($n = 47$)	Female ($n = 24$)	Male ($n = 23$)
Age (mean ± SD), years	66 ± 11	64 ± 11	68 ± 12
Gender (female), *n* (%)	24 (51)	–	–
Race (Caucasian), *n* (%)	43 (91)	22 (92)	21 (91)
BMI (kg/m^2)	35 (9)	36 (10)	33 (5)
CKD/ESKD/dialysis modality, *n* (%)			
Normal kidney function	1	0	1
CKD, non-dialysis	5	3	2
Kidney transplant recipient	2	0	2
PD	10	5	5
HD or HDF	29	15	13
Time on dialysis (years), median (IQR)	4.8 (1.7–7.4)	4.9 (1.8–7)	4.0 (1.6–8.3)
Etiology of CKD, *n* (%)			
Diabetic nephropathy	28 (60)	16	12
Renovascular disease	5 (11)	2	3
Glomerulonephritis	7 (15)	1	4
Polycystic kidney disease	1 (2)	0	1
Reflux nephropathy	4 (8)	3	1
Other	2 (4)	1	1
Hospitalized within 3 months of diagnosis, *n* (%)	28 (60)	13	15

Statistical analysis

Descriptive statistics were performed for the entire cohort and for subgroups stratified by gender. All data are summarized and results reported as mean ± SD or median [interquartile range (IQR)] for continuous data and number (%) for categorical variables. Paired *t*-tests and Wilcoxon signed-rank tests were used for between-group comparisons. Categorical variables were analysed using the chi-square test. Temporal changes in PTH were analysed with a Friedman test, given skewed data distribution. Two-tailed P-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (IBM, Armonk, NY, USA).

RESULTS

Participant characteristics

From May 2014 to May 2019, seven participating Australian nephrology units provided data to the registry and recorded 50 calciphylaxis cases. Three patients were excluded from the final analysis due to missing data ($n = 2$) or an alternative diagnosis to calciphylaxis being established ($n = 1$), leaving 47 patients diagnosed with calciphylaxis. The mean patient age was 66 ± 11 years and 51% were female. The majority of patients were Caucasian (93%) and the mean body mass index (BMI) was 35 ± 9 kg/m^2. The median notification rate was 8 cases per year in complete years (minimum 5–12) across the seven centres. Baseline patient demographics are outlined in Table 1.

Calciphylaxis cases predominantly involved patients with ESKD (83%), with haemodialysis (HD) and haemodiafiltration (HDF) the most common dialysis modality (61%) and 10 patients (21%) on peritoneal dialysis (PD) at the time of diagnosis. Diabetic nephropathy was the most common cause of CKD (60%), although 76% of all patients had diabetes. Calciphylaxis was seen in five patients with CKD not on dialysis and in two patients with a functioning kidney transplant. One patient was reported to have calciphylaxis with normal kidney function.

Table 2. Patient comorbidities ($N = 47$)

Comorbidities	*n* (%)
Ischaemic heart disease	25 (54)
Arrhythmia	21(45)
Valvular disease	9 (20)
Hypertension	37 (80)
Diabetes	35 (76)
Hyperlipidemia	22 (48)
Peripheral vascular disease	25 (54)
Peripheral neuropathy	14 (30)
Chronic obstructive airways disease	5 (11)
Previous fracture	5 (11)
Gastrointestinal/peptic ulcer disease	7 (15)
Malignancy	10 (22)
Current or ex-smoker	17 (37)
Previous calciphylaxis	4 (9)

Sixty percent of patients had been hospitalized within 3 months prior to the diagnosis of calciphylaxis.

Patients with calciphylaxis had a high comorbidity burden, including cardiovascular disease, arrhythmias and metabolic syndrome (Table 2). Compared with incident dialysis patients in Australia, as recorded by the Australian and New Zealand Dialysis and Transplant Registry (ANZDATA) in the 2017 report [13], calciphylaxis patients in the registry were more likely to be female and to have a history of diabetes as well as vascular and coronary artery disease (Table 3). VKAs were used by 16 patients (34%) prior to diagnosis (female:male ratio 14:2) and other commonly administered medications at diagnosis included active vitamin D (calcitriol) and calcium-based phosphate binders (Table 4). Biochemical parameters at diagnosis are listed in Table 5. The median PTH was 32 (IQR 14–50) pmol/L and was within the target range recommended by the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines [14]. PTH levels in the 12 months prior to and following diagnosis of calciphylaxis were analysed. There was no significant change in PTH within 12 months prior to

diagnosis (P = 0.18), although there was a statistically significant increase in PTH >12 months following calciphylaxis (P = 0.04) (Figure 1).

Four patients had a prior history of calciphylaxis (female:male ratio 3:1). All patients were on HD, with a median BMI of 34 (IQR 30–49) kg/m^2. Three of these patients had a history of diabetes mellitus on insulin therapy and all patients had peripheral vascular disease. The median PTH at the most recent calciphylaxis diagnosis was 45 (IQR 4.3–52.5) pmol/L. All patients were prescribed calcitriol, two patients were on calcium carbonate and one patient was on warfarin. The initial episode of calciphylaxis had been diagnosed in the lower limbs ($n = 2$), abdomen ($n = 1$) and buttock ($n = 1$). Only one patient had complete resolution of their initial calciphylaxis lesion prior to recurrence in the same anatomical site 3 years later.

Diagnosis and site of calciphylaxis

Calciphylaxis was diagnosed by clinical impression in 31 patients, by skin biopsy in 21 patients and by both in 11 patients (Table 6). Twelve patients were documented to have a suspected contributing event that may have precipitated calciphylaxis, including trauma (such as at injection sites) ($n = 10$) and dialysis non-compliance ($n = 2$). The lower limb was the most common site of calciphylaxis ($n = 31$) and nine patients had more than one affected area. Females had a predilection to calciphylaxis affecting the abdomen and breasts, compared with feet, lower limbs and thighs as more common sites for males.

Treatment and outcome of calciphylaxis

The treatment of calciphylaxis was multimodal and multidisciplinary (Table 7). In all cases, analgesia and wound care were provided, and antibiotics were prescribed in 29 cases (62%). Specific treatments for calciphylaxis are outlined in Table 7, and in all but two cases, more than one treatment option was administered. The most common treatment options included intensifying dialysis therapy, either with increased dialysis duration and/or frequency or transition from PD to HD, as well as cessation of VKA and the use of sodium thiosulphate (STS). Resolution of calciphylaxis was documented in 10 patients (female:male ratio 9:1). Twenty-five patients had died by the time of analysis, with a mortality of 50% at 1.6 (IQR 0.2–2.5) years following diagnosis. Figure 2 shows the Kaplan–Meier survival curve for patients with calciphylaxis. The median survival for patients on dialysis at the time of diagnosis of calciphylaxis was 1.6 (IQR 0.2–2.8) years compared with 1.9 (IQR 0.4–2.2) years for those not on dialysis (P = 0.4). The cause of death was documented in 11 cases, of which 4 were from septic shock, 3 from cardiac arrest, 3 from withdrawal from dialysis and 1 patient had a catastrophic gastrointestinal haemorrhage. Table 8 highlights and compares the demographic and comorbid features of patients with calciphylaxis in the ACR who died and those who had not at the time of follow-up. The only statistically significant difference was a history of ischaemic heart disease in patients dying from calciphylaxis (P = 0.04).

Table 3. Comparison of demographic details between patients with calciphylaxis and incident dialysis patients from the ANZDATA registry (2017 report)

Demographics	ACR ($N = 47$), n (%)	ANZDATA ($N = 3056$), n (%)
HD (satellite)	60	51
PD	21	19
Gender (female)	50	38
Race (Caucasian)	91	63
Diabetes	76	50
Coronary artery disease	54	29
Peripheral vascular disease	54	14
Chronic lung disease	11	2
ESA use	86	77

Table 5. Serum laboratory parameters at time of diagnosis of calciphylaxis

Laboratory parameter	$n = 47$	Reference range
Albumin, g/L	26 ± 6	35–55
Corrected calcium, mmol/L	2.56 ± 0.22	2.20–2.70
Phosphate, mmol/L	1.7 ± 0.8	1.12–1.45
Magnesium, mmol/L	0.86 ± 0.15	0.60–1.10
PTH, pmol/L	32 (14–50)	10–65
ALP, U/L	147 (113–238)	44–147
Hemoglobin, g/L	95 ± 15	103–180 (males) 120–160 (females)
CRP, mg/L	36 (12–97)	<5
Bicarbonate, mmol/L	23 ± 4	23–30

Results presented as mean ± SD, median (IQR).

Table 4. Medication prescribed at the time of diagnosis of calciphylaxis divided by dialysis modality. No dialysis includes patients with CKD ($n = 5$), renal transplantation ($n = 2$) and normal renal function ($n = 1$)

Medication	PD ($N = 10$), n (%)	HD/HDF ($N = 29$), n (%)	No dialysis ($N = 8$), n (%)	Total ($N = 47$), n (%)
Cinacalcet	0 (0)	2 (7)	2 (25)	4 (9)
VKA (warfarin)	2 (20)	10 (34)	4 (50)	16 (34)
Active vitamin D (calcitriol)	4 (40)	15 (52)	0 (0)	19 (41)
Phosphate binder				
Calcium-based binder	3 (30)	15 (15)	2 (25)	20 (43)
Sevelamer	3 (30)	8 (27)	0 (0)	11 (24)
Lanthanum	0 (0)	7 (24)	0 (0)	7 (15)
Aluminum-based binder	3 (30)	0 (0)	0 (0)	3 (7)
Magnesium-based binder	0 (0)	5 (17)	0 (0)	5 (11)
ESA	4 (40)	25 (86)	0 (0)	29 (63)
Intravenous iron	3 (30)	26 (89)	0 (0)	29 (63)

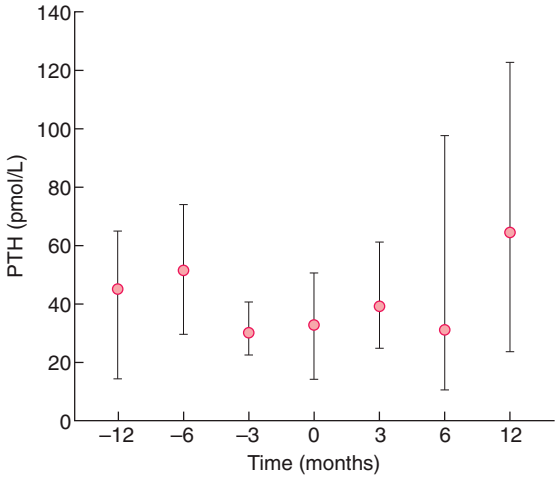

FIGURE 1: PTH levels 12 months prior to and following diagnosis of calciphylaxis (time = 0). Error bars refer to median with IQR.

DISCUSSION

This study represents 5 years of data collection by units participating in the nationwide, online ACR. The data include clinical and background information, biochemical markers, applied therapeutic strategies and, importantly, patient outcomes. As expected, most patients had ESKD, with risk factors for calciphylaxis including female gender, diabetes, obesity and administration of VKA. Therapy was multifaceted, with analgesia and wound care implemented universally, and a combination of other specific therapies including intensifying dialysis, reducing or ceasing medications (calcium, calcitriol and VKA) and the use of STS in one-quarter of cases.

The median notification rate was eight patients per year, consistent with an annual Australian dialysis patient incidence of 0.3%, given that the seven participating institutions cared for approximately 2500 dialysis patients. This is also similar to the estimated annual incidence of 0.35% in HD patients reported in the USA [15]. We acknowledge there may be unreported cases from participating centres, and the numbers we report may further underestimate the development of calciphylaxis by patients with CKD not on dialysis or those with kidney transplants in participating units. Nevertheless, the registry reported 11% of calciphylaxis cases from patients with CKD not on dialysis, 4% from kidney transplant recipients and one patient (2%) with normal kidney function. These percentages are similar to those of the German Calciphylaxis Registry, where the percentage of patients with normal kidney function, non-dialysis CKD and functioning kidney transplants were 3, 7 and 4%, respectively [9].

There are many similarities between the current data and those published in case series [15–17] and the German Calciphylaxis Registry [9]. The ACR confirmed previously identified risk factors for calciphylaxis, including obesity, diabetes and long dialysis vintage [1]. Demographic details of patients were similar in the ACR to those reported in the German registry, with a mean age of 66 years compared with 70 years and a nearly equal gender ratio. Case reports and case series often highlight female gender as a risk factor [1], and

Table 6. Characteristics of calciphylaxis lesions, divided by gender

Clinical features	Total ($n = 47$)	Female ($n = 24$)	Male ($n = 23$)
Contributing event to calciphylaxis, *n*			
Trauma to site	8	1	7
Injection to site	2	2	0
Non-compliance with dialysis	2	1	1
No precipitating event	11	6	5
Not recorded	23	12	11
Site of calciphylaxis, *n*			
Thighs and calves	33	13	21
Feet	9	4	5
Hands	1	0	1
Abdomen	8	7	1
Buttocks	2	1	1
Breast	3	3	0
Penis	1	0	1
More than one location involved	9	3	6
Diagnosis of calciphylaxis, *n*			
Clinical impression	31	15	16
Skin biopsy	21	8	13
Radiograph	4	2	2
Nuclear medicine	1	0	1
More than one modality used for diagnosis	11	3	8
Outcome			
Resolution of lesion, *n*	10	9	1
Time to resolution (months), median (IQR)	8 (5–24)	6.7 (4.8–15)	32
Deceased, *n*	25	12	13

although we report an equal gender ratio, the proportion of female patients with calciphylaxis in our registry is significantly greater than the overall proportion of female dialysis patients in Australia, at 51% versus 38% [13]. Calciphylaxis in the ACR predominantly involved patients of Caucasian race (91% compared with 63% for incident dialysis patients) [13], despite the multiethnic nature of Australian dialysis patients. Whether this reflects an underlying racial predisposition to calciphylaxis, differences in dialysis adherence, BMI, drug regimens, underreporting or misdiagnosis in other cultural groups need further evaluation [1, 3]. The median time to diagnosis of calciphylaxis from commencement of dialysis was also similar to that in previous reports, ranging from 30 months in the USA and Germany to 105 months in Japan [9, 15, 17].

Also consistent with the German registry, biochemical parameters of CKD–mineral and bone disorder at the time of diagnosis of calciphylaxis were found to be within suggested KDIGO guideline targets [14]. Hyperphosphatemia was not a significant concern in the current cohort (mean serum phosphate 1.7 ± 0.8 mmol/L at time of diagnosis), although concurrent presence of hypoalbuminemia (mean albumin 26 ± 6 g/L) may reflect malnutrition or inflammation at the time of diagnosis. ALP levels were at or above the normal reference range [median ALP 147 (IQR 113–238) IU], and higher ALP has been associated with increased mortality in non-CKD patients [18] as well as increased risk of cardiovascular disease [19]. In the current cohort, PTH values were relatively low at the time of diagnosis [median 32 (IQR 14–50) pmol/L] and in the preceding 12 months [median 45 (IQR 14–65) pmol/L], supporting the

Table 7. Therapeutic strategies used for calciphylaxis patients ($N = 47$)

Treatment strategy	*n* (%)
Intensifying dialysis therapy	
Increase of dialysis duration and/or frequency	12 (26)
Switch from PD to HD	4 (9)
Reduction of calcium intake and calcitriol	
Lowering/stopping calcium phosphate binder	10 (22)
Lowering dialysis bath calcium concentration	7 (15)
Reduction/stop active vitamin D	8 (17)
Vitamin K	
Stop VKA	11 (24)
Give vitamin K	6 (13)
Management of secondary hyperparathyroidism	
Initiate cinacalcet	5 (11)
Parathyroidectomy	5 (11)
Specific calcification inhibition	
STS	12 (26)
FFP/PEX	2 (4)
Bisphosphonates	1 (2)
Improve tissue oxygenation	
Hyperbaric oxygen treatment	5 (11)
Revascularization (surgical and angioplasty)	1 (2)
Antibiotics	29 (62)
Surgical wound management (wound debridement, amputation and skin grafting)	23 (50)
Cessation of intravenous iron	2 (4)

FFP, fresh frozen plasma; PEX, plasma exchange.

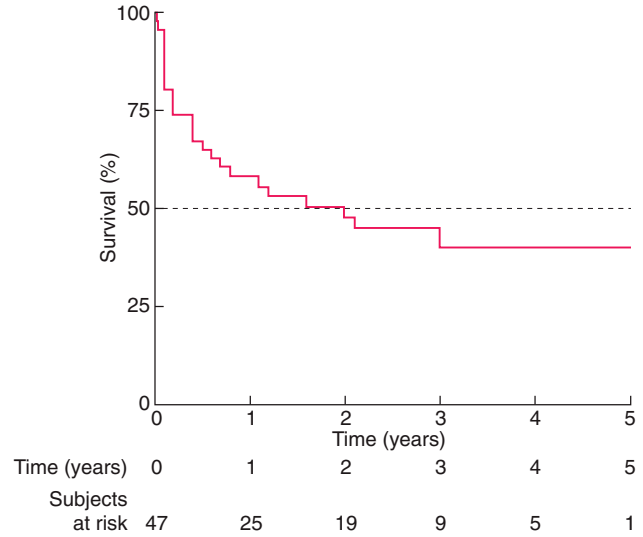

FIGURE 2: Kaplan–Meier survival curve for patients with calciphylaxis from time of diagnosis.

suggestion that more severe hyperparathyroidism may not be a significant risk factor [12]. There was an increase in PTH in the 12 months following diagnosis of calciphylaxis, which may have related to cessation of calcitriol therapy. Interestingly, subgroup analysis of the EVOLVE trial showed an increased rate of calciphylaxis in participants on placebo compared with cinacalcet [12]. Following withdrawal of government reimbursement for cinacalcet in Australia in 2015, there was concern for a possible increase in the rate of calciphylaxis, although registry data to date show stable rates over the 5-year observation period. However, given the rare nature of this disease, it may take longer to see a potential trend. Cinacalcet, prescribed in Australia through a special access scheme for compassionate use, and/or parathyroidectomy to manage secondary hyperparathyroidism was reported to the registry in nine patients with calciphylaxis.

High use of erythropoiesis-stimulating agents (ESAs) has previously been associated with an increased risk of calciphylaxis [20]. Anaemia management in our cohort of patients on HD at the time of diagnosis of calciphylaxis involved 86 and 89% of patients administered ESA and intravenous iron, respectively. ESA use in these patients was slightly higher than the rate of ESA use in Australian HD patients (77%) based on data from the ANZDATA registry [13]. Administration of intravenous iron is not routinely recorded in ANZDATA; however, based on Australian data from the Dialysis Outcome and Practice Patterns Study (DOPPS), iron use was recorded in 64% of HD patients with low transferrin saturation [21]. Increased ESA and iron use in our HD cohort may be explained by a state of acute inflammation and relative iron resistance that can occur with calciphylaxis, potentially requiring escalation of therapy for anemia.

The only universally reported treatments were wound care (including antibiotics and/or surgical wound debridement) and pain management, which were implemented in all cases of calciphylaxis. Earlier studies have reported that ∼50% of patients with calciphylaxis can be bedridden or wheelchair-bound and >70% may require hospitalization for severe ulcers [3]. Wound care is crucial to remove exudate and necrotic tissue and prevent infection, as sepsis from wounds is the most common cause of death [3, 22]. The ACR reported that 61% of patients required hospitalization within 3 months of calciphylaxis diagnosis. Wound debridement was undertaken in 48% of cases, and retrospective studies have suggested improved survival among patients who undergo operative debridement [3, 23].

Multiple other therapeutic strategies for calciphylaxis were reported in addition to wound care and analgesia. The majority of patients received more than two additional treatment modalities, likely reflecting clinicians' experience in managing this condition, and the lack of an evidence base. The most frequently used treatments included intensifying dialysis therapy, with commencement of HD in the five patients with non-dialysis CKD at the time of calciphylaxis diagnosis and switching to HD/HDF in those on PD. STS is a common treatment for calciphylaxis and was used in 26% of patients (compared with 21.5% in the German registry). This therapy has been used more often following the case series of Nigwekar *et al.* [16], which reported complete resolution in 26% of patients with STS and 19% having marked improvement in skin lesions. Although the exact mechanism of action of STS for calciphylaxis remains unknown, its benefit may lie in direct extracellular inhibition of calcification [24] together with antioxidant and vasodilatory properties [25].

VKA therapy was stopped in all patients at the time of diagnosis and 60% of patients who ceased VKA therapy were concurrently commenced on vitamin K therapy, perhaps following greater appreciation of the potential clinical benefit in replenishing

Table 8. Characteristics of deceased compared with living patients in the ACR

Clinical features	Deceased ($n = 25$)	Living ($n = 22$)	P-value
Demographics			
Age (years), mean ± SD	68 ± 10	63 ± 4	0.2
Gender (female), *n*	12	11	0.8
BMI (kg/m^2), median (IQR)	33 (27–40)	35 (27–40)	0.7
CKD/ESKD/dialysis modality, *n*			
Normal kidney function	0	1	0.5
CKD, non-dialysis	4	1	0.2
Kidney transplant recipient	1	1	0.9
PD	4	6	0.32
HD or HDF	16	13	0.64
Time on dialysis (years), median (IQR)	4 (1.5–6.2)	5.2 (2–8.2)	0.41
Comorbidities, *n*			
Diabetes	21	14	0.17
Ischaemic heart disease	17	8	0.04
Hypertension	20	17	0.93
Hyperlipidemia	10	12	0.24
Peripheral vascular disease	14	11	0.8
VKA use	9	7	0.59
Hospitalized within 3 months of diagnosis	15	13	0.9
>1 location of calciphylaxis	5	13	0.93

vitamin K to slow the progression of pre-existing vascular calcification [26, 27]. Vitamin K activates matrix Gla protein (MGP) via gamma carboxylation [28]. Fully active MGP is a prerequisite for maintaining vascular wall integrity by avoiding calcification. Hence the application of VKA such as warfarin is thought to interfere with calcification defence mechanisms and promote vascular calcification [29], and patients with ESKD already have a high prevalence of extra-skeletal calcification.

The strength of the ACR is the long-term follow-up of cases to identify clinical outcomes, including rates of resolution of calciphylaxis lesions and mortality. Of the 47 patients enrolled in the registry, only 22% had documented resolution of lesions (9 females and 1 male), with a median time to resolution of 8 (IQR 5–24) months, highlighting the significant burden of this disease. Mortality in calciphylaxis patients remained high, with 25 deaths over the period of follow-up. The mortality rate of 50% at 1.6 (IQR 0.2–2.5) years following diagnosis, however, was more promising when compared with historically published data on outcomes with calciphylaxis [20]. Further controlled clinical trials to investigate therapeutic strategies are desperately needed for this disease.

Although the ACR was developed as a nationwide registry, only seven nephrology centres were involved, with the vast majority of centres not participating in data collection. This is a significant study limitation in determining the true disease incidence in Australia. Another limitation is that the registry may include false-positive cases. This possibility cannot be completely excluded, due to the absence of systematic diagnostic standards for calciphylaxis and decentralized patient care within the registry. Additionally, despite prospective data collection, laboratory markers at the time of calciphylaxis diagnosis are cross-sectional, whereas trends may be more important as contributors to calciphylaxis development. Despite these limitations, the registry is able to collect a significant amount of patient data on a rare condition, and we endeavor to continue data collection via the ACR.

In summary, the ACR reports real-world management and outcomes of a rare, life-threatening disease. Calciphylaxis is a complex disorder manifested as painful cutaneous lesions resulting from microvascular calcification. We highlight risk factors for calciphylaxis, including a higher percentage of females than in the background dialysis population, as well as diabetes, obesity and VKA use. Resolution of calciphylaxis is uncommon in the majority of cases despite multimodal therapy, and the ACR highlights the need for more targeted therapy. The prognosis is generally poor, with significant 1-year mortality, although this may not be as high as previously reported.

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CONFLICT OF INTEREST STATEMENT

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APPENDIX C: VASCULAR CALCIFICATION IN SKIN AND SUBCUTANEOUS TISSUE IN PATIENTS WITH CHRONIC AND END-STAGE KIDNEY DISEASE

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RESEARCH ARTICLE Open Access

Vascular calcification in skin and subcutaneous tissue in patients with chronic and end-stage kidney disease

Irene Ruderman[1,2*], Tim D. Hewitson[1,2], Edward R. Smith[1,2], Stephen G. Holt[1,2], Belinda Wigg[1] and Nigel D. Toussaint[1,2]

Abstract

Background: Vascular calcification (VC) is well described in large- and medium-sized vessels in patients with chronic kidney disease (CKD), especially in those with end-stage kidney disease (ESKD) on dialysis. Medial calcification is particularly prevalent in this population and contributes to arterial stiffness and increased cardiovascular mortality and morbidity. Apart from in the setting of calciphylaxis, few studies have assessed skin and subcutaneous calcification and associations with abnormalities of bone and mineral metabolism in patients with CKD.

Methods: We performed a single-centre observational study to evaluate incisional skin tissue samples from three anatomical sites in patients with different stages of CKD undergoing elective surgery. We compared these samples to skin samples of a control cohort without CKD. Staining for calcification was performed with von Kossa method. A subgroup of skin samples were assessed by RT-PCR for upregulation of pro-calcific gene transcripts for tissue non-specific alkaline phosphatase (*TNAP)* and Runt-related transcription factor 2 (*RUNX2*).

Results: Forty-five patients were evaluated, 34 with CKD (including ESKD) and 11 control patients. VC was identified in 15 skin samples (13 CKD/ESKD and 2 controls). VC was present in the dermal and subcutaneous tissues of the neck, abdomen and arm samples. Two different histological types of VC were identified: speckled medial calcification and internal elastic lamina calcification. Presence of perieccrine calcification was identified in 14 samples, 10 with concurrent VC. There were no significant differences in serum parathyroid hormone, phosphate or calcium in patients with or without VC. Expression of *TNAP* or *RUNX2* was not increased in samples from patients with ESKD or those with histological evidence of calcification.

Conclusion: This study reports the novel finding of dermal and subcutaneous calcification in multiple anatomical locations in 38% of patients with advanced CKD/ESKD undergoing elective surgery but free from calciphylaxis.

Keywords: Vascular calcification, Chronic kidney disease, End-stage kidney disease, Calcific uraemic arteriolopathy

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Background

Vascular calcification (VC) is prevalent and well described in large- and medium-sized vessels of patients with chronic kidney disease (CKD), especially in those with end-stage kidney disease (ESKD) on dialysis [1, 2]. Patients with CKD are at higher risk of VC compared to the general population, irrespective of age and comorbidity burden [3]. Medial calcification is particularly prevalent in the CKD population and is associated with arterial stiffening and higher cardiovascular morbidity and mortality [4, 5]. Medial arterial calcification has been identified in multiple anatomical locations in patients with ESKD, including normal breast tissue from mastectomy samples [6], as well as subcutaneous tissue of lower limb amputations [7]. In both locations, VC has been identified in vessels in the presence of other patient risk factors for calcification, including active malignancy or peripheral vascular disease.

Conversely, the presence of VC of small vessels in the skin and subcutaneous tissue has only been systemically described in ESKD patients with calciphylaxis [8], although recent studies suggest even this may not be a specific finding and VC can be present in patients with peripheral vascular disease without the presence of calciphylaxis [7]. Calciphylaxis is considered an accelerated template of widespread VC. It is characterized by severe vessel ischaemia accompanied by vessel thrombosis and surrounding panniculitis [9], ultimately resulting in painful skin lesions which typically break down to non-healing ulcers that are prone to infection, with accompanying high mortality [10, 11]. However, calciphylaxis is a rare condition and only occurs in a very small minority of patients with ESKD with approximately 3.5 cases per 1000 patient years in those receiving chronic haemodialysis therapy [12] To date, there is little information about the prevalence of calcification in skin and subcutaneous tissue in patients with advanced CKD and ESKD outside the setting of calciphylaxis, and if present, whether it is related to disordered bone and mineral metabolism.

The aim of the present study was to characterize VC changes in the skin and subcutaneous tissue of patients with CKD compared to a control group. We also aimed to evaluate associations between skin calcification and biochemical markers of deranged bone and mineral metabolism and identify any other histological features present that are typically described in calciphylaxis.

Methods

Patients

Between May 2017 and March 2019 patients undergoing a planned surgical procedure performed by the Nephrology Surgical Team at The Royal Melbourne Hospital were approached to participate in this single-centre cross-sectional study. Patients enrolled underwent elective surgical procedures including parathyroidectomy, hernia repair and arteriovenous fistula (AVF) formation. A fasting blood sample was collected at the time of surgical procedure. Patients over the age of 18 and able to provide informed consent were included in the study. There were no exclusion criteria.

Ethics approval and consent to participate

The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2017.023) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained for this study.

Histology

A full-thickness incisional skin sample was collected by the operating surgeon from the surgical site. Tissue samples were fixed in neutral buffered formalin and processed to paraffin wax for histology. Serial paraffin embedded skin sections were stained with haematoxylin and eosin (H&E) and von Kossa. Cross-sections of skin and subcutaneous tissue were evaluated in their entirety.

Counting of vessels was performed on slides stained with H&E due to ease of identifying vessels. Each cross section contained epidermal, dermal and subcutaneous tissue, with tissue blocks re-cut if they did not contain all three areas. Vessel size was estimated using a graticule. Typically, arterioles ≤50 μm in diameter have either absent or partially complete internal elastic lamina (IEL) [13, 14]. As a result, differentiating terminal arterioles from terminal venules is very challenging, therefore all structures with a lumen were defined as vessels. Samples with greater than 10 vessels per cross section were considered to have adequate vessel number. H&E sections were examined for evidence of intimal hyperplasia, intimal thrombosis, pannicultis or hyperplasia.

Identification of calcification was performed on slides stained with von Kossa. In brief, sections were dewaxed and rehydrated before being immersed in a solution of freshly prepared 5% Aqueous Silver Nitrate. Tissue sections were placed at close range under a bench lamp and exposed to a strong light source for 30 min. They were then washed in distilled water. To remove the unreacted silver, slides were covered with 5% Sodium Thiosulphate for 2 minutes. This was rinsed in distilled water before being counter stained with Working Eosin solution for 2 minutes. For each case, the following features were graded as either present or absent: calcification of any sized vessels, presence of extra-vascular calcification, IEL calcification. Results were expressed as a % of all vessels in the section. Histologic sections of all cases were counted independently by two investigators (IR and BW)

with results presented as an average of the two observers. Malignant necrotic breast tissue was used as a positive control for the von Kossa stain.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from a subgroup (13 out of 43) of patient samples stored in RNAlater solution (Invitrogen, Carlsbad, CA, USA) using the RNEeasy Fibrous Mini Kit (QIAGEN, Hilden, Germany) in accordance with manufacturer's instructions. To minimise sampling error, triplicate 30 mg portions of tissue from each sample were processed. Quantification of RNA was performed using the Qubit 4 Fluorometer and Quant-IT RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction with 1 μg used as a template for cDNA synthesis using the iScript RT supermix kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed in triplicate in a CFX96 cycler (Bio-Rad) using equal volumes of cDNA (2 μL) and the SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and validated PCR Prime assay primer pairs (Bio-Rad): *TNAP* (Unique Assay ID:qHsaCID0010031); *Runx2* (Unique Assay ID: qHsaCID006726); and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Unique Assay ID: qHsaCED0038674). PCR conditions were set according to the manufacturer's instructions. Melt curve analysis was performed to verify the purity and specificity of the amplicons. Threshold cycles were calculated using CFX Manager Software (Bio-Rad). The mRNA level of target genes was normalised to the house keeping gene *Gapdh*, and expressed relative to an appropriate control using the 2^(−ΔΔCt) method. Results are presented as the mean of all three samples.

Statistical analysis

A sample size estimation for this cross-sectional study was 50 patients based on feasibility with the number of patients undergoing elective surgery at our institution over the recruitment period as well as a larger enough cohort to allow for heterogeneity of skin sample sites and different stages of CKD. All data were summarised, and results reported as mean (standard deviation [SD]) or median (inter-quartile range [IQR]) for continuous data and number (percentage, %) for categorical variables. Comparison of the values of continuous variables between groups was made using an unpaired t test or Wilcoxon signed-rank test as appropriate. Chi-squared and Fisher's exact tests were used to investigate associations between various categorical variables. Two-tailed *P* values of < 0.05 were considered to be significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPPS, Chicago, IL). Graphics were created with GraphPad Prism 8 for Macintosh (la Jolla, CA, USA).

Results

Demographic and biochemical characteristics

Forty-five patients undergoing elective surgical procedures were enrolled in the study. Eleven patients with normal kidney function (mean eGFR 83 ± 10 mL/min/1.73m^2) were included in the control group. Of these 11, 2 underwent donor nephrectomies, 5 hernia repairs, 1 laparoscopic cholecystectomy and 3 total thyroidectomies. Of the 34 patients with advanced CKD (mean eGFR 21 ± 0.5 ml/min/1.73m^2) and ESKD, 20 underwent AVF creation, superficialization or ligation, 7 parathyroidectomies, 2 kidney transplant nephrectomies, 2 peritoneal dialysis catheter insertions, 2 hernia repairs and 1 total thyroidectomy.

Relevant patient comorbidities and biochemistry are summarised in Table 1. The mean age of the CKD group was 60 ± 16 years; 76% (n = 26) of patients were on haemodialysis, 15% (n = 5) on peritoneal dialysis and 9% (n = 3) had CKD stages 4 and 5 not on dialysis. The comorbidity burden was greater in the CKD group

Table 1 Clinical features of study cohort

Demographics	CKD (***n*** = 34)	Control (***n*** = 11)	***P*** value
Age, years	60 ± 16	62 ± 9	0.3
Gender, male	22 (69%)	4 (36%)	
BMI (kg/m^2)	27 [24–33]	27 [24–31]	0.8
Dialysis modality	26 HD, 5 PD, 3 CKD	n/a	
Time on dialysis (years)	4 [2.8–9.8]		
Comorbidities			
Smoker (current)	5 (15%)	2 (18%)	0.6
Smoker (former)	13 (38%)	2 (18%)	0.8
Diabetes	13 (38%)	2 (18%)	0.8
Hypertension	24 (70%)	2 (18%)	0.7
IHD	7 (20%)	0	
PVD	4 (12%)	0	
Parathyroidectomy	4 (12%)	0	
Biochemical parameters			
Corrected calcium (mmol/L)	2.3 ± 0.2	2.3 ± 0.2	0.65
Phosphate (mmol/L)	1.9 ± 0.6	1.1 ± 0.2	0.002
ALP (IU/L)	107 [81–149]	74 [64–102]	0.03
PTH (pmol/L)	62 [37–148]	4.6 [0.9–8]	< 0.001

Results are mean ± SD, median [IQR] or number (%)
Abbreviations: *ALP* alkaline phosphatase, *BMI* body mass index, *HD* haemodialysis, *IHD* ischaemic heart disease, *PD* peritoneal dialysis, *PTH* parathyroid hormone, *PVD* peripheral vascular disease

Table 2 Anatomical and histological features of skin tissue samples

Histologic features	CKD (***n*** = 34)	Control (***n*** = 11)
Location, number of samples		
• Neck	8	3
• Abdomen	6	8
• Arm	20	0
Vessel number, mean ± SD		
• All sites	42 ± 17	34 ± 13
• Neck	37 ± 7	47
• Abdomen	63 ± 18	39 ± 15
• Arm	37 ± 14	n/a
Vascular calcification present, number (%)	13 (38%)	2 (18%)
Perieccrine calcification, number (%)	13 (38%)	1 (9%)

Results are mean ± SD, or number (%)

compared to the control group; 53% (n = 18) of patients were either former or current smokers, 38% (n = 13) had a history of diabetes, 70% (n = 24) had hypertension, and 12% (n = 4) were documented to have peripheral vascular disease. The median body mass index (BMI) of patients was in the overweight range for both the control and CKD cohort. As expected, there were significant differences in mineral biochemistry between the control and CKD cohort, with higher levels of serum parathyroid hormone (PTH) (p < 0.001), phosphate (p = 0.002) and alkaline phosphatase (ALP) (p = 0.03) in patients with CKD. None of the patients were receiving warfarin. Sixteen patients in the CKD group were prescribed calcitriol and 25 prescribed phosphate binders, 12 of who were on a calcium-based phosphate binder.

Anatomical and histological features of participants

Anatomical location and histologic features of the skin samples are summarized in Table 2. All sections contained a minimum of 10 vessels, with a mean of 41 ± 16 and 34 ± 17 vessels per slide in the CKD/ESKD and control groups, respectively. All sections contained full thickness skin tissue including epidermis, dermis and variable amount of subcutaneous tissue depending on the anatomical location of the sample. The mean number of vessels did not differ significantly across the three anatomical locations (p = 0.17) or between groups. Vessels ≤50 μm in diameter accounted for 75% of total vessels counted. The largest vessel identified was 500 μm in diameter from an arm skin sample. Each sample had a mean of 5 ± 0.6 vessels ≥100 μm in diameter. Figure 1 demonstrates the number of vessels per vessel diameter in each patient based on anatomical location.

CKD/ESKD patients have high rates of VC

Calcification was observed on von Kossa staining in small (typical diameter 50–100 μm lumen) and medium-sized (typical diameter > 100 μm lumen) vessels in 13 CKD/ESKD patients (38%) (CKD stage 4 n = 1, ESKD n = 12) and 2 control patients (18%). The difference in proportion of patients with calcification did not differ between CKD/ESKD patients and the control cohort (p = 0.28), however this is likely due to the small study sample size. In the majority of samples (n = 8), VC was predominantly localized to subcutaneous tissue, whereas in 5 samples there was an equal distribution of calcified vessels in the dermis and subcutaneous tissue. Irrespective of the tissue layer affected, VC was observed in vessels of all sizes, ranging from 50 to 200 μm in diameter. We identified more calcified vessels in neck skin samples of patients undergoing parathyroidectomy than other surgeries, which may reflect the subset of patients with severe secondary hyperparathyroidism. VC was identified at each of the three anatomical sites in patients with CKD/ESKD and in both sites sampled in the controls. Percentage of calcified vessels based on anatomical location is demonstrated in Fig. 2 (vessels > 100 μm in diameter identified as small arteries or arterioles) and Fig. 3 (all calcified vessels). The percentage of calcified vessels in this study however likely underestimates total arterial calcification since the count denominator includes veins and venules.

VC was seen in vessel media and internal elastic lamina

To better understand the nature of VC, we further evaluated its histological appearance and location within the vessel and whether it was granular, confluent or circumferential in nature. VC of the media was seen in patients with CKD/ESKD, with no evidence of intimal calcification. Medial VC was present as circumferential and granular calcifications in small vessels, although we were unable to determine if this involved the IEL (Fig. 4a). In larger vessels the granular calcification was noted within the vessel media and alongside the IEL (Fig. 4b), and some vessels displayed confluent areas of calcification (Fig. 4c). There was no evidence of circumferential medial calcification seen, however there was evidence of circumferential linear calcification of the IEL affecting vessels > 100 μm in diameter in two patients with ESKD (Fig. 4d).

Concurrence of perieccrine calcification in samples with medial VC

Extra-vascular calcification was identified surrounding sweat glands (perieccrine) in 13 participants with CKD and one control participant (Fig. 4e). Of the participants with perieccrine calcification, 10 also had VC. There was no intimal hyperplasia or other pathological features

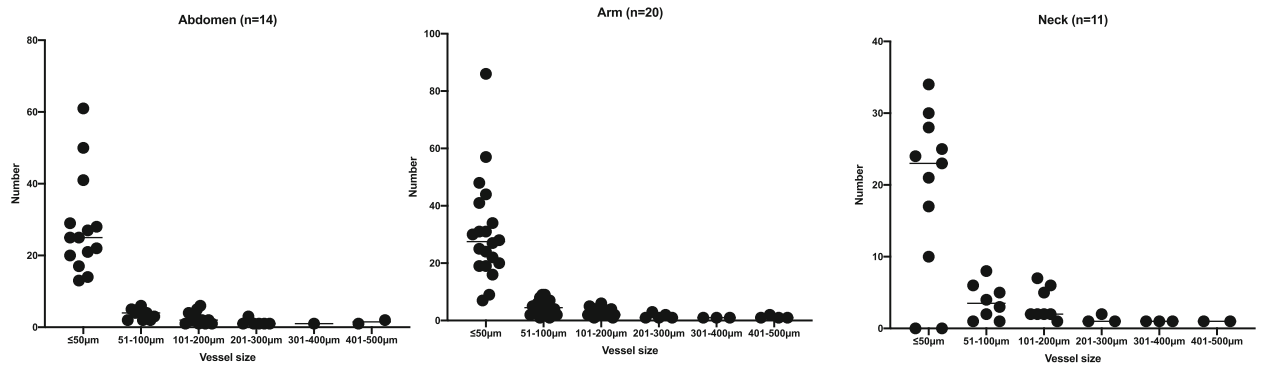

Fig. 1 Number of vessels per vessel diameter in each patient based on anatomical location. Each dot per column represent individual patient

consistent with calciphylaxis, including intimal thrombosis, panniculitis or hyperplasia. No other form of extra-vascular calcification was identified, including subcutaneous septa.

VC in the control group was mild compared to the CKD/ESKD group

Two patients in the control group had presence of subtle and stippled medial calcification (Fig. 4f). Both patients were female, aged 65 and 71 years of age with no significant co-morbidities and normal biochemistry. One patient underwent a donor nephrectomy and had calcification present in 11% of vessels, mostly involving large arterioles. The second patient underwent a total thyroidectomy and was found to have calcification in 20% of vessels, all within subcutaneous tissue. The pattern of VC in the control group appeared distinct to that in the CKD/ESKD group with no evidence of granular or confluent calcification, or involvement of the IEL. There was evidence of perieccrine calcification in one patient in the control cohort.

Similar demographic and biochemical markers of mineral metabolism in patients with and without skin and subcutaneous VC

In order to determine if the presence of VC was related to demographic and clinical features, we compared the specific characteristics of CKD/ESKD patients with and without VC (Table 3). Patients in both groups were of a similar age ($p = 0.6$) and dialysis vintage ($p = 0.2$).

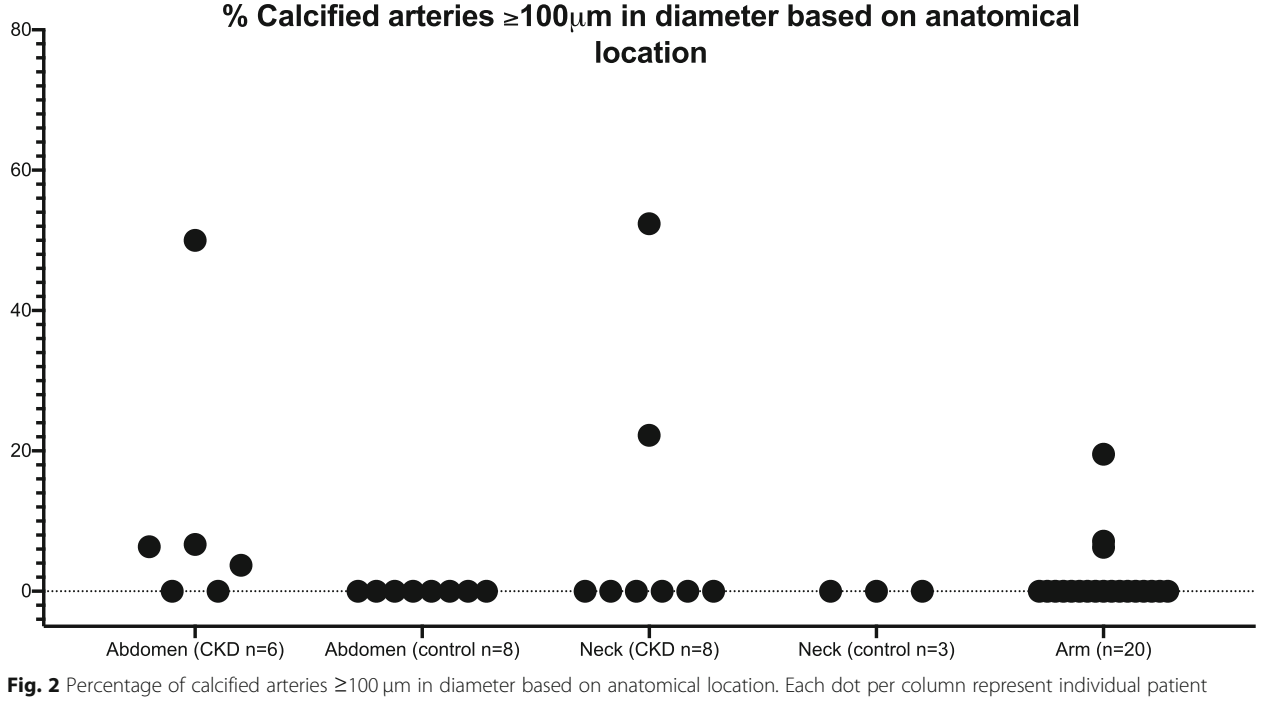

Fig. 2 Percentage of calcified arteries ≥100 μm in diameter based on anatomical location. Each dot per column represent individual patient

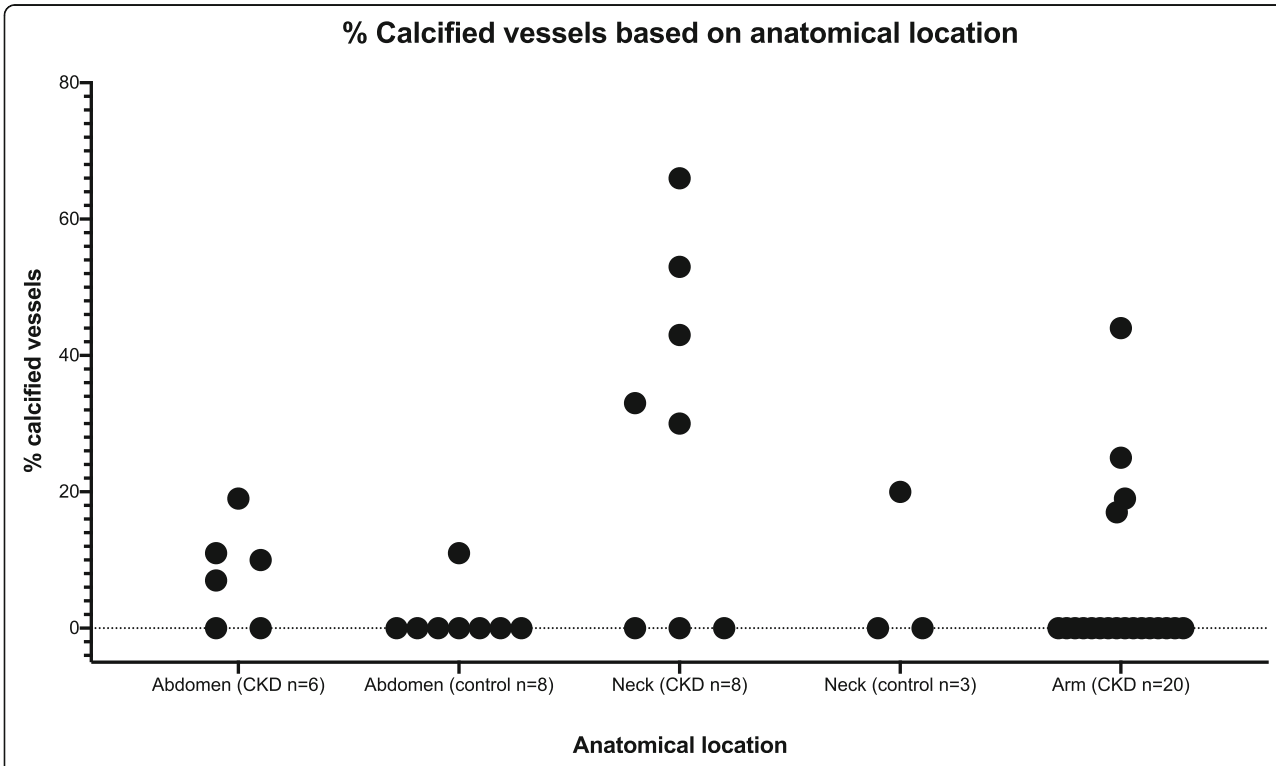

Fig. 3 Percentage of calcified vessels in each patient based on anatomical location

Median BMI was similar between both groups ($p = 0.2$), as was the co-morbidity profile. With respect to systemic biochemical measures of mineral metabolism and medical therapy, there were no differences in serum calcium, phosphate, ALP or PTH, or in the use of calcium- or non-calcium-based phosphate binders or activated vitamin D between groups.

No evidence of upregulation of pro-osteogenic gene expression in patients with ESKD or with VC

De novo expression of pro-osteogenic proteins has been widely implicated as a key driver in the pathogenesis of VC as well as in vascular lesions of patients with calciphylaxis [15]. Accordingly, we looked for changes in expression of two previously identified pro-calcific factors: tissue non-specific alkaline phosphatase (TNAP), which cleaves the mineralisation inhibitor pyrophosphate to phosphate, and RUNX2, a master transcription factor regulating osteogenic gene programs. Thirteen patients with eligible skin samples were evaluated for mRNA expression. Each skin sample was divided into three segments and tested in triplicate. Results are expressed as mean mRNA expression of each sample. There was minimal variability in gene expression across the three segments of tissue per patient sample. There was no upregulation of gene transcripts in samples with histological evidence of calcification ($n = 4$) compared to those without ($n = 9$) for either *TNAP* or *RUNX2*, $p = 0.76$ and $p = 0.35$ respectively (Fig. 5a). When comparing gene expression in patients with CKD/ESKD ($n = 11$) to those with no CKD ($n = 2$), there was also no difference in gene expression for *TNAP* or *RUNX2*, $p = 0.8$ and $p = 0.98$ respectively (Fig. 5b).

Discussion

To our knowledge, this is the first study to show evidence of medial VC in subcutaneous and dermal vessels in multiple anatomical locations in patients with advanced CKD and ESKD undergoing elective surgery, without peripheral vascular disease or calciphylaxis. Calcification was present in 38% of samples from patients with CKD/ESKD, predominantly in the subcutaneous tissue and involving the vessel media as well as the IEL. Perieccrine calcification was identified in 64% of samples with VC, present mainly in patients with ESKD on dialysis. These microvascular calcifications were not related to biochemical markers of mineral metabolism or changes in pro-calcific gene transcripts.

Von Kossa staining is routinely used to identify tissue calcification [2]. Using this stain, two distinct types of VC were identified in our skin samples, medial calcification and calcification of the IEL. The medial calcification identified in patients with CKD/ESKD was mostly granular but becoming confluent in some of the affected vessels. Calcification identified in the two control patients was more diffuse and milder in nature, which may

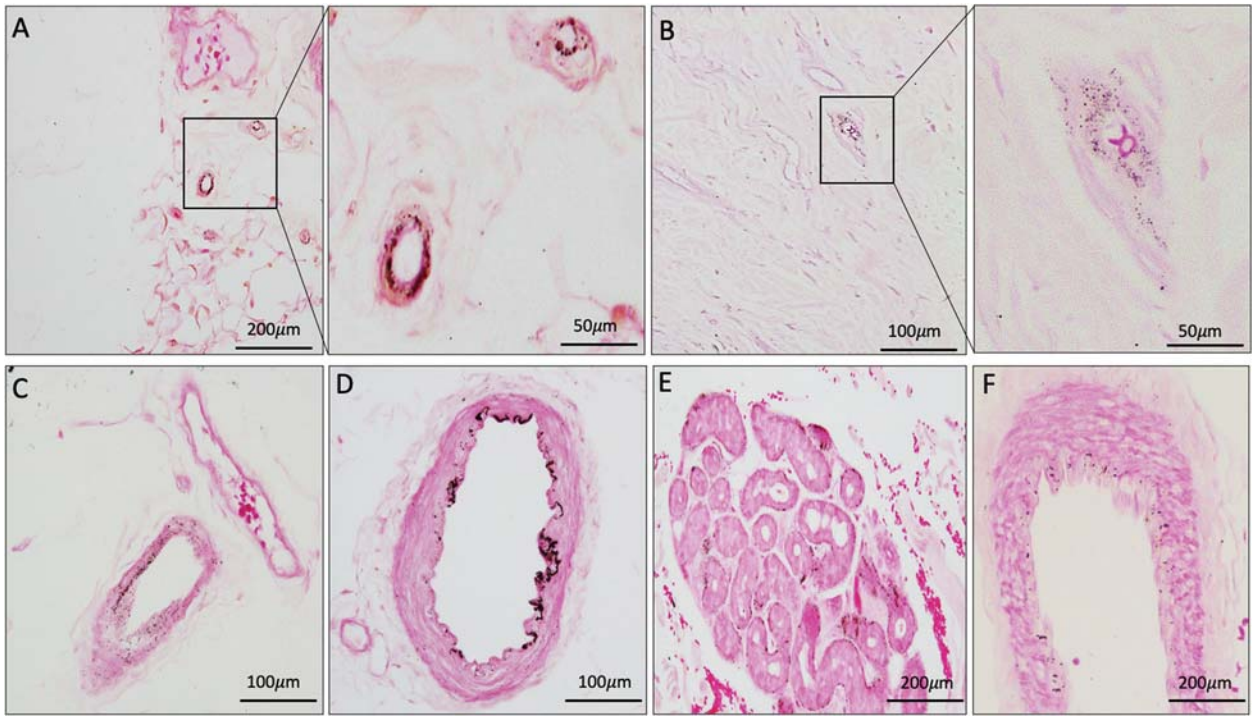

Fig. 4 Micrographs showing various forms of calcification seen in CKD/ESKD (Fig. **a**-**e**) and control patient (Fig. **f**). Von Kossa staining shows (**a**) Vessels < 50 μm in diameter with granular and circumferential medial calcification in subcutaneous tissue and higher magnification insert of vessels highlighted in black box from Panel A. **b** Vessel 100 μm in diameter with granular calcification throughout the media and alongside the internal elastic lamina in subcutaneous tissue and higher magnification of vessel highlighted in black box from Panel B (**c**) Vessel 100 μm in diameter with granular medial calcification in dermal tissue; **d** Confluent internal elastic lamina calcification of vessel > 100 μm in diameter in subcutaneous tissue; **e** Perieccrine calcification in dermal tissue; **f** Subtle and stippled calcification of vessel > 100 μm in diameter in control patient. All photos taken at 20x magnification. Scale bar denoted in each panel

represent a less advanced stage of VC progression or perhaps a different pathophysiological process. There was no evidence of intimal calcification in our study and there remains conjecture as to whether medial VC and calcification of the IEL represent progression of the same disease process. Multiple studies have shown that arterial medial calcification typically starts along the IEL and in the absence of inflammation [16, 17]. In contrast, arterial intimal calcification is characterized by focal mineral deposition in highly inflamed and necrotic atherosclerotic lesions.

The high prevalence of perieccrine calcification in patients with VC is a novel finding in our study. There is little literature regarding this histological entity and its clinical significance. Mochel et al. [8] initially described perieccrine calcification in a retrospective cohort of patients with calciphylaxis, suggesting that this histological feature was highly specific to calciphylaxis - the diagnosis of calciphylaxis being made based on this histological feature alone in 4 out of 56 cases. It was also identified in a case report of calciphylaxis [18]. In a recent case series of patients with calciphylaxis (13), perieccrine calcification was not present, however this may have been due to lack of specific staining for calcification. The authors argued that given the subtleness of this histological feature, it may be possible it is routinely missed with standard H&E staining. In further case series, perieccrine calcification was identified in two biopsies in patients with low to moderate risk of calciphylaxis, and was not significantly prevalent in biopsies of patients with high clinical suspicion of calciphylaxis [7]. The clinical significance of perieccrine calcification remains unknown and it may simply reflect the severity or progression of coexistent VC.

Calcification of skin tissue is considered to be a precursor to the development of calciphylaxis and is a common finding in skin biopsies in patients with this pathology [8]. The specificity of this finding was recently questioned by Ellis and O'Neill in a study of 38 skin biopsies taken for high clinical suspicion for calciphylaxis and compared with lower limb amputation skin samples in patients with ESKD [7]. All previously documented histopathological features commonly associated with calciphylaxis, including medial calcification, intimal thrombosis and extravascular calcification, were prevalent in both high-risk skin lesions and amputation samples.

Table 3 Differences between CKD/ESKD participants with and without VC

Demographic features	VC present (***n*** = 13)	VC absent (***n*** = 21)	***P*** value
Age, years	57 ± 13	59 ± 18	0.60
Sex (male)	8 (61%)	17 (80%)	0.21
BMI (kg/m^2)	27 ± 4	29 ± 6	0.24
Time on dialysis (if ESKD), years	2.5 [1.77–6]	4.2 [2–6.5]	0.25
CKD stage			
Pre-dialysis	1 (eGFR 17 ml/min^2)	2 (mean eGFR 20 ± 0.7 ml/min^2)	
PD	4	1	
HD	8 (57%)	18 (86%)	
Diabetes	4 (30%)	9 (42%)	0.55
Hypertension	10 (77%)	14 (67%)	0.52
IHD	3 (23%)	4 (19%)	0.78
PVD	1 (7%)	3 (14%)	0.56
Smoker (current or former)	8 (57%)	15 (50%)	0.66
Previous parathyroidectomy	2 (15%)	2 (9%)	0.64
Calcitriol use	7 (50%)	10 (34%)	0.24
Non-calcium-based binder	7 (50%)	11 (38%)	0.35
Calcium based binder	2 (15%)	10 (47%)	0.06
PTH, pmol/L	58 [37–184]	66 [37–123]	0.41
Total calcium, mmol/L	2.3 ± 0.2	2.4 ± 0.2	0.57
Phosphate, mmol/L	2 ± 0.5	1.9 ± 0.7	0.71
ALP, IU/L	106 [76–207]	108 [81–139]	0.72
Vessel number	44 ± 19	39 ± 15	0.91
Perieccrine calcification	9 (69%)	4 (19%)	0.003

Results are mean ± SD, median [IQR] or number (%)
Abbreviations: *ALP* alkaline phosphatase, *BMI* body mass index, *HD* haemodialysis, *IHD* ischaemic heart disease, *PD* peritoneal dialysis, *PTH* parathyroid hormone, *PVD* peripheral vascular disease

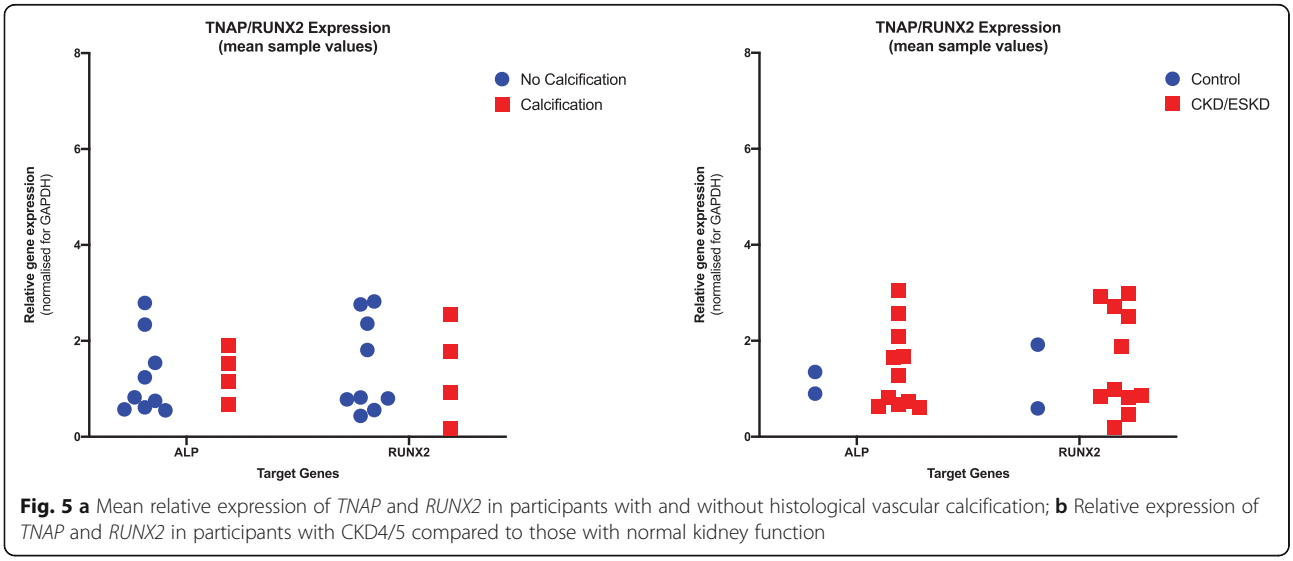

Fig. 5 a Mean relative expression of *TNAP* and *RUNX2* in participants with and without histological vascular calcification; **b** Relative expression of *TNAP* and *RUNX2* in participants with CKD4/5 compared to those with normal kidney function

Only the combination of thrombosis and medial calcification was unique to the high-risk skin biopsy group. Our study confirms that medial vascular calcification and perieccrine calcification can be present in "healthy skin" taken from three different anatomical locations, and that these features indeed are not specific to calciphylaxis. We could not identify any evidence of vessel thrombosis, likely due to the low incidence of peripheral vascular disease in our CKD/ESKD cohort. This histological finding may be more prevalent in patients with significant peripheral vascular disease rather than specific to CKD alone.

There were no significant demographic or biochemical differences in patients with CKD/ESKD with and without VC. PTH in patients with VC was similar to those without, but highly variable perhaps reflecting the variability and fluctuations in PTH, as the samples used here only capture one time point. We also identified no significant differences in serum calcium, phosphate or ALP between the two groups. However, the apparent lack of discriminating factors between the two groups may in part reflect the small sample size. It is recognised that medial VC is accelerated in patients with CKD [19]. Schlieper et al identified microcalcifications in the media of iliac arteries in uraemic patients [20] without the presence of intimal calcification or major atherosclerotic plaques. Consistent with our results, the presence of calcification was not associated with abnormalities in serum calcium, phosphate or magnesium, although PTH was not evaluated. Despite these similarities, it is uncertain to what extent VC in skin and subcutaneous tissue reflects calcification in other vascular beds and whether it is related to more systemic pro-calcific processes affecting the larger central arteries.

RUNX2, a transcription factor, is a master regulator of bone formation and is essential for bone formation and bone remodelling. Some animal studies have shown that RUNX2 expression is required for osteogenic phenotypic change in smooth muscle cells [21]. This transcription factor is also reportedly upregulated in human vascular fragments of large vascular territories where calcification is present [22]. However, evidence of osteogenesis is not a consistent finding across all studies of human VC. We did not see upregulation of *RUNX2* or *TNAP* in the subset of patients tested with histological evidence of calcification. Although osteogenic transformation of vascular smooth muscle cells is one proposed hypothesis for development of medial arterial calcification, other mechanisms have been implicated including elastin degradation, smooth muscle apoptosis and more passive biochemical processes [23]. Lack of osteogenic gene upregulation in our study may also reflect temporal aspects of calcification [6, 24] or a different underlying mechanism as a culprit. Human studies involving examination of arterial calcification in breast tissue in patients with CKD demonstrated a universal absence of staining for RUNX2 and osteocalcin in early arterial calcification [6]. A previous study identifying upregulation of osteogenic gene expression in skin was in the setting of calciphylaxis with macroscopic evidence of calcification [15]. This is not reflective of the subtle microscopic calcification identified in our participant cohort that is likely to occur in response to distinct triggers and mechanisms. On the other hand, this may be as a result of technical factors relating to the small sample size, or the sporadic nature of calcification identified on histological examination. Finally, given only a small amount of tissue was processed for PCR, small areas of active calcification may have been missed.

This study was limited by a small sample size and its cross-sectional design with a single skin incisional sample at one time point, without the ability to prospectively follow up VC progression, resolution or patient outcomes. It was not possible to obtain skin samples from control patients to match all anatomical locations and a future case-controlled study may better evaluate the impact of anatomical site on the presence of calcification. Assessment of VC burden at more central sites was also not undertaken. This study had many strengths, however, including a wide distribution of skin anatomical locations sampled as well large incisional biopsy sampling, which allowed for good interpretation of skin architecture and large number of vessels (mean of 40 vessels per slide). Also, patients had no underlying skin pathology to confound results of the study including presence of skin malignancy which is commonly associated with VC.

Conclusion

In conclusion, this study confirms the presence of medial VC in dermal and subcutaneous tissues in patients with advanced CKD and ESKD. VC was not associated with PTH levels, however, was more likely to be present in patients undergoing parathyroidectomy. The presence of medial VC and perieccrine calcification is not a specific histological finding for calciphylaxis.

Abbreviations

ALP: alkaline phosphatase; CKD: chronic kidney disease; ESKD: end-stage kidney disease; PTH: parathyroid hormone; VC: vascular calcification

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Authors' contributions

IR: contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work. TH: contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work. ES: contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work. SH: contributed to critical appraisal of paper and drafting of the work. BW:

contributed data interpretation. NT: contributed to the conception, design, critical appraisal of paper and drafting of the work. All authors approved final copy of paper.

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Availability of data and materials
Comprehensive data presented in manuscript, tables and figures. Additional data available on request from the corresponding author.

Ethics approval and consent to participate
Research performed in accordance with the Declaration of Helsinki. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2017.023). Written informed consent was obtained for all study participants.

Consent for publication
Not applicable to current manuscript.

Competing interests
ERS has received research funding from Sanofi, Baxter, and Amgen and holds stock in Calciscon AG, which specializes in performing the T_{50}-test. SGH has received research funding, teaching and travel support from AstraZenica, Amgen, Baxter, Sanofi.
NDT has received honoraria, travel support and research funding from Amgen, Shire and Sanofi. TDH, BW and IR declare that they have no competing interests.

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Publisher's Note

Background

Vascular calcification (VC) is prevalent and well described in large- and medium-sized vessels of patients with chronic kidney disease (CKD), especially in those with end-stage kidney disease (ESKD) on dialysis [1, 2]. Patients with CKD are at higher risk of VC compared to the general population, irrespective of age and comorbidity burden [3]. Medial calcification is particularly prevalent in the CKD population and is associated with arterial stiffening and higher cardiovascular morbidity and mortality [4, 5]. Medial arterial calcification has been identified in multiple anatomical locations in patients with ESKD, including normal breast tissue from mastectomy samples [6], as well as subcutaneous tissue of lower limb amputations [7]. In both locations, VC has been identified in vessels in the presence of other patient risk factors for calcification, including active malignancy or peripheral vascular disease.

Conversely, the presence of VC of small vessels in the skin and subcutaneous tissue has only been systemically described in ESKD patients with calciphylaxis [8], although recent studies suggest even this may not be a specific finding and VC can be present in patients with peripheral vascular disease without the presence of calciphylaxis [7]. Calciphylaxis is considered an accelerated template of widespread VC. It is characterized by severe vessel ischaemia accompanied by vessel thrombosis and surrounding panniculitis [9], ultimately resulting in painful skin lesions which typically break down to non-healing ulcers that are prone to infection, with accompanying high mortality [10, 11]. However, calciphylaxis is a rare condition and only occurs in a very small minority of patients with ESKD with approximately 3.5 cases per 1000 patient years in those receiving chronic haemodialysis therapy [12] To date, there is little information about the prevalence of calcification in skin and subcutaneous tissue in patients with advanced CKD and ESKD outside the setting of calciphylaxis, and if present, whether it is related to disordered bone and mineral metabolism.

The aim of the present study was to characterize VC changes in the skin and subcutaneous tissue of patients with CKD compared to a control group. We also aimed to evaluate associations between skin calcification and biochemical markers of deranged bone and mineral metabolism and identify any other histological features present that are typically described in calciphylaxis.

Methods

Patients

Between May 2017 and March 2019 patients undergoing a planned surgical procedure performed by the Nephrology Surgical Team at The Royal Melbourne Hospital were approached to participate in this single-centre cross-sectional study. Patients enrolled underwent elective surgical procedures including parathyroidectomy, hernia repair and arteriovenous fistula (AVF) formation. A fasting blood sample was collected at the time of surgical procedure. Patients over the age of 18 and able to provide informed consent were included in the study. There were no exclusion criteria.

Ethics approval and consent to participate

The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2017.023) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained for this study.

Histology

A full-thickness incisional skin sample was collected by the operating surgeon from the surgical site. Tissue samples were fixed in neutral buffered formalin and processed to paraffin wax for histology. Serial paraffin embedded skin sections were stained with haematoxylin and eosin (H&E) and von Kossa. Cross-sections of skin and subcutaneous tissue were evaluated in their entirety.

Counting of vessels was performed on slides stained with H&E due to ease of identifying vessels. Each cross section contained epidermal, dermal and subcutaneous tissue, with tissue blocks re-cut if they did not contain all three areas. Vessel size was estimated using a graticule. Typically, arterioles ≤50 μm in diameter have either absent or partially complete internal elastic lamina (IEL) [13, 14]. As a result, differentiating terminal arterioles from terminal venules is very challenging, therefore all structures with a lumen were defined as vessels. Samples with greater than 10 vessels per cross section were considered to have adequate vessel number. H&E sections were examined for evidence of intimal hyperplasia, intimal thrombosis, pannicultis or hyperplasia.

Identification of calcification was performed on slides stained with von Kossa. In brief, sections were dewaxed and rehydrated before being immersed in a solution of freshly prepared 5% Aqueous Silver Nitrate. Tissue sections were placed at close range under a bench lamp and exposed to a strong light source for 30 min. They were then washed in distilled water. To remove the unreacted silver, slides were covered with 5% Sodium Thiosulphate for 2 minutes. This was rinsed in distilled water before being counter stained with Working Eosin solution for 2 minutes. For each case, the following features were graded as either present or absent: calcification of any sized vessels, presence of extra-vascular calcification, IEL calcification. Results were expressed as a % of all vessels in the section. Histologic sections of all cases were counted independently by two investigators (IR and BW)

with results presented as an average of the two observers. Malignant necrotic breast tissue was used as a positive control for the von Kossa stain.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from a subgroup (13 out of 43) of patient samples stored in RNAlater solution (Invitrogen, Carlsbad, CA, USA) using the RNEeasy Fibrous Mini Kit (QIAGEN, Hilden, Germany) in accordance with manufacturer's instructions. To minimise sampling error, triplicate 30 mg portions of tissue from each sample were processed. Quantification of RNA was performed using the Qubit 4 Fluorometer and Quant-IT RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction with 1 μg used as a template for cDNA synthesis using the iScript RT supermix kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed in triplicate in a CFX96 cycler (Bio-Rad) using equal volumes of cDNA (2 μL) and the SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and validated PCR Prime assay primer pairs (Bio-Rad): *TNAP* (Unique Assay ID:qHsaCID0010031); *Runx2* (Unique Assay ID: qHsaCID006726); and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Unique Assay ID: qHsaCED0038674). PCR conditions were set according to the manufacturer's instructions. Melt curve analysis was performed to verify the purity and specificity of the amplicons. Threshold cycles were calculated using CFX Manager Software (Bio-Rad). The mRNA level of target genes was normalised to the house keeping gene *Gapdh*, and expressed relative to an appropriate control using the 2^(−ΔΔCt) method. Results are presented as the mean of all three samples.

Statistical analysis

A sample size estimation for this cross-sectional study was 50 patients based on feasibility with the number of patients undergoing elective surgery at our institution over the recruitment period as well as a larger enough cohort to allow for heterogeneity of skin sample sites and different stages of CKD. All data were summarised, and results reported as mean (standard deviation [SD]) or median (inter-quartile range [IQR]) for continuous data and number (percentage, %) for categorical variables. Comparison of the values of continuous variables between groups was made using an unpaired t test or Wilcoxon signed-rank test as appropriate. Chi-squared and Fisher's exact tests were used to investigate associations between various categorical variables. Two-tailed *P* values of < 0.05 were considered to be significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPPS, Chicago, IL). Graphics were created with GraphPad Prism 8 for Macintosh (la Jolla, CA, USA).

Results

Demographic and biochemical characteristics

Forty-five patients undergoing elective surgical procedures were enrolled in the study. Eleven patients with normal kidney function (mean eGFR 83 ± 10 mL/min/1.73m^2) were included in the control group. Of these 11, 2 underwent donor nephrectomies, 5 hernia repairs, 1 laparoscopic cholecystectomy and 3 total thyroidectomies. Of the 34 patients with advanced CKD (mean eGFR 21 ± 0.5 ml/min/1.73m^2) and ESKD, 20 underwent AVF creation, superficialization or ligation, 7 parathyroidectomies, 2 kidney transplant nephrectomies, 2 peritoneal dialysis catheter insertions, 2 hernia repairs and 1 total thyroidectomy.

Relevant patient comorbidities and biochemistry are summarised in Table 1. The mean age of the CKD group was 60 ± 16 years; 76% (n = 26) of patients were on haemodialysis, 15% (n = 5) on peritoneal dialysis and 9% (n = 3) had CKD stages 4 and 5 not on dialysis. The comorbidity burden was greater in the CKD group

Table 1 Clinical features of study cohort

Demographics	CKD (***n*** = 34)	Control (***n*** = 11)	***P*** value
Age, years	60 ± 16	62 ± 9	0.3
Gender, male	22 (69%)	4 (36%)	
BMI (kg/m^2)	27 [24–33]	27 [24–31]	0.8
Dialysis modality	26 HD, 5 PD, 3 CKD	n/a	
Time on dialysis (years)	4 [2.8–9.8]		
Comorbidities			
Smoker (current)	5 (15%)	2 (18%)	0.6
Smoker (former)	13 (38%)	2 (18%)	0.8
Diabetes	13 (38%)	2 (18%)	0.8
Hypertension	24 (70%)	2 (18%)	0.7
IHD	7 (20%)	0	
PVD	4 (12%)	0	
Parathyroidectomy	4 (12%)	0	
Biochemical parameters			
Corrected calcium (mmol/L)	2.3 ± 0.2	2.3 ± 0.2	0.65
Phosphate (mmol/L)	1.9 ± 0.6	1.1 ± 0.2	0.002
ALP (IU/L)	107 [81–149]	74 [64–102]	0.03
PTH (pmol/L)	62 [37–148]	4.6 [0.9–8]	< 0.001

Results are mean ± SD, median [IQR] or number (%)
Abbreviations: *ALP* alkaline phosphatase, *BMI* body mass index, *HD* haemodialysis, *IHD* ischaemic heart disease, *PD* peritoneal dialysis, *PTH* parathyroid hormone, *PVD* peripheral vascular disease

Table 2 Anatomical and histological features of skin tissue samples

Histologic features	CKD (***n*** = 34)	Control (***n*** = 11)
Location, number of samples		
• Neck	8	3
• Abdomen	6	8
• Arm	20	0
Vessel number, mean ± SD		
• All sites	42 ± 17	34 ± 13
• Neck	37 ± 7	47
• Abdomen	63 ± 18	39 ± 15
• Arm	37 ± 14	n/a
Vascular calcification present, number (%)	13 (38%)	2 (18%)
Perieccrine calcification, number (%)	13 (38%)	1 (9%)

Results are mean ± SD, or number (%)

compared to the control group; 53% (n = 18) of patients were either former or current smokers, 38% (n = 13) had a history of diabetes, 70% (n = 24) had hypertension, and 12% (n = 4) were documented to have peripheral vascular disease. The median body mass index (BMI) of patients was in the overweight range for both the control and CKD cohort. As expected, there were significant differences in mineral biochemistry between the control and CKD cohort, with higher levels of serum parathyroid hormone (PTH) (p < 0.001), phosphate (p = 0.002) and alkaline phosphatase (ALP) (p = 0.03) in patients with CKD. None of the patients were receiving warfarin. Sixteen patients in the CKD group were prescribed calcitriol and 25 prescribed phosphate binders, 12 of who were on a calcium-based phosphate binder.

Anatomical and histological features of participants

Anatomical location and histologic features of the skin samples are summarized in Table 2. All sections contained a minimum of 10 vessels, with a mean of 41 ± 16 and 34 ± 17 vessels per slide in the CKD/ESKD and control groups, respectively. All sections contained full thickness skin tissue including epidermis, dermis and variable amount of subcutaneous tissue depending on the anatomical location of the sample. The mean number of vessels did not differ significantly across the three anatomical locations (p = 0.17) or between groups. Vessels ≤50 μm in diameter accounted for 75% of total vessels counted. The largest vessel identified was 500 μm in diameter from an arm skin sample. Each sample had a mean of 5 ± 0.6 vessels ≥100 μm in diameter. Figure 1 demonstrates the number of vessels per vessel diameter in each patient based on anatomical location.

CKD/ESKD patients have high rates of VC

Calcification was observed on von Kossa staining in small (typical diameter 50–100 μm lumen) and medium-sized (typical diameter > 100 μm lumen) vessels in 13 CKD/ESKD patients (38%) (CKD stage 4 n = 1, ESKD n = 12) and 2 control patients (18%). The difference in proportion of patients with calcification did not differ between CKD/ESKD patients and the control cohort (p = 0.28), however this is likely due to the small study sample size. In the majority of samples (n = 8), VC was predominantly localized to subcutaneous tissue, whereas in 5 samples there was an equal distribution of calcified vessels in the dermis and subcutaneous tissue. Irrespective of the tissue layer affected, VC was observed in vessels of all sizes, ranging from 50 to 200 μm in diameter. We identified more calcified vessels in neck skin samples of patients undergoing parathyroidectomy than other surgeries, which may reflect the subset of patients with severe secondary hyperparathyroidism. VC was identified at each of the three anatomical sites in patients with CKD/ESKD and in both sites sampled in the controls. Percentage of calcified vessels based on anatomical location is demonstrated in Fig. 2 (vessels > 100 μm in diameter identified as small arteries or arterioles) and Fig. 3 (all calcified vessels). The percentage of calcified vessels in this study however likely underestimates total arterial calcification since the count denominator includes veins and venules.

VC was seen in vessel media and internal elastic lamina

To better understand the nature of VC, we further evaluated its histological appearance and location within the vessel and whether it was granular, confluent or circumferential in nature. VC of the media was seen in patients with CKD/ESKD, with no evidence of intimal calcification. Medial VC was present as circumferential and granular calcifications in small vessels, although we were unable to determine if this involved the IEL (Fig. 4a). In larger vessels the granular calcification was noted within the vessel media and alongside the IEL (Fig. 4b), and some vessels displayed confluent areas of calcification (Fig. 4c). There was no evidence of circumferential medial calcification seen, however there was evidence of circumferential linear calcification of the IEL affecting vessels > 100 μm in diameter in two patients with ESKD (Fig. 4d).

Concurrence of perieccrine calcification in samples with medial VC

Extra-vascular calcification was identified surrounding sweat glands (perieccrine) in 13 participants with CKD and one control participant (Fig. 4e). Of the participants with perieccrine calcification, 10 also had VC. There was no intimal hyperplasia or other pathological features

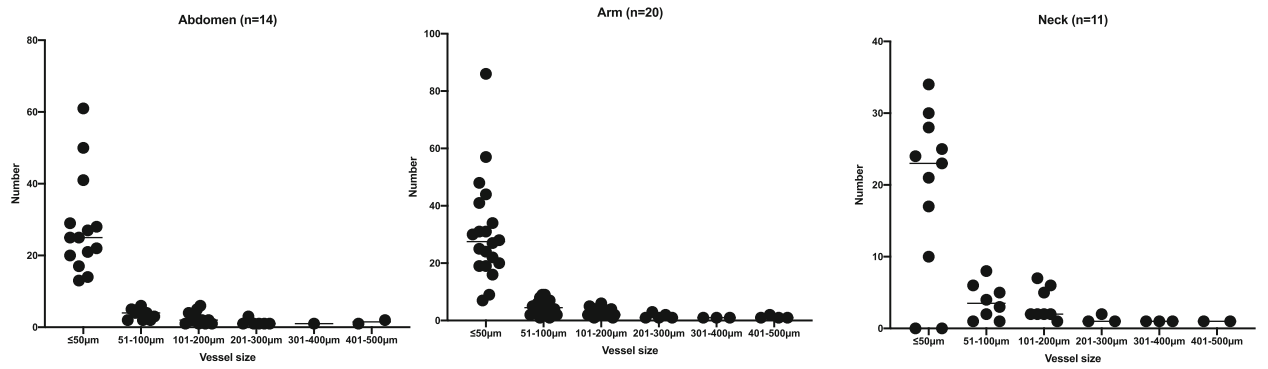

Fig. 1 Number of vessels per vessel diameter in each patient based on anatomical location. Each dot per column represent individual patient

consistent with calciphylaxis, including intimal thrombosis, panniculitis or hyperplasia. No other form of extra-vascular calcification was identified, including subcutaneous septa.

VC in the control group was mild compared to the CKD/ESKD group

Two patients in the control group had presence of subtle and stippled medial calcification (Fig. 4f). Both patients were female, aged 65 and 71 years of age with no significant co-morbidities and normal biochemistry. One patient underwent a donor nephrectomy and had calcification present in 11% of vessels, mostly involving large arterioles. The second patient underwent a total thyroidectomy and was found to have calcification in 20% of vessels, all within subcutaneous tissue. The pattern of VC in the control group appeared distinct to that in the CKD/ESKD group with no evidence of granular or confluent calcification, or involvement of the IEL. There was evidence of perieccrine calcification in one patient in the control cohort.

Similar demographic and biochemical markers of mineral metabolism in patients with and without skin and subcutaneous VC

In order to determine if the presence of VC was related to demographic and clinical features, we compared the specific characteristics of CKD/ESKD patients with and without VC (Table 3). Patients in both groups were of a similar age ($p = 0.6$) and dialysis vintage ($p = 0.2$).

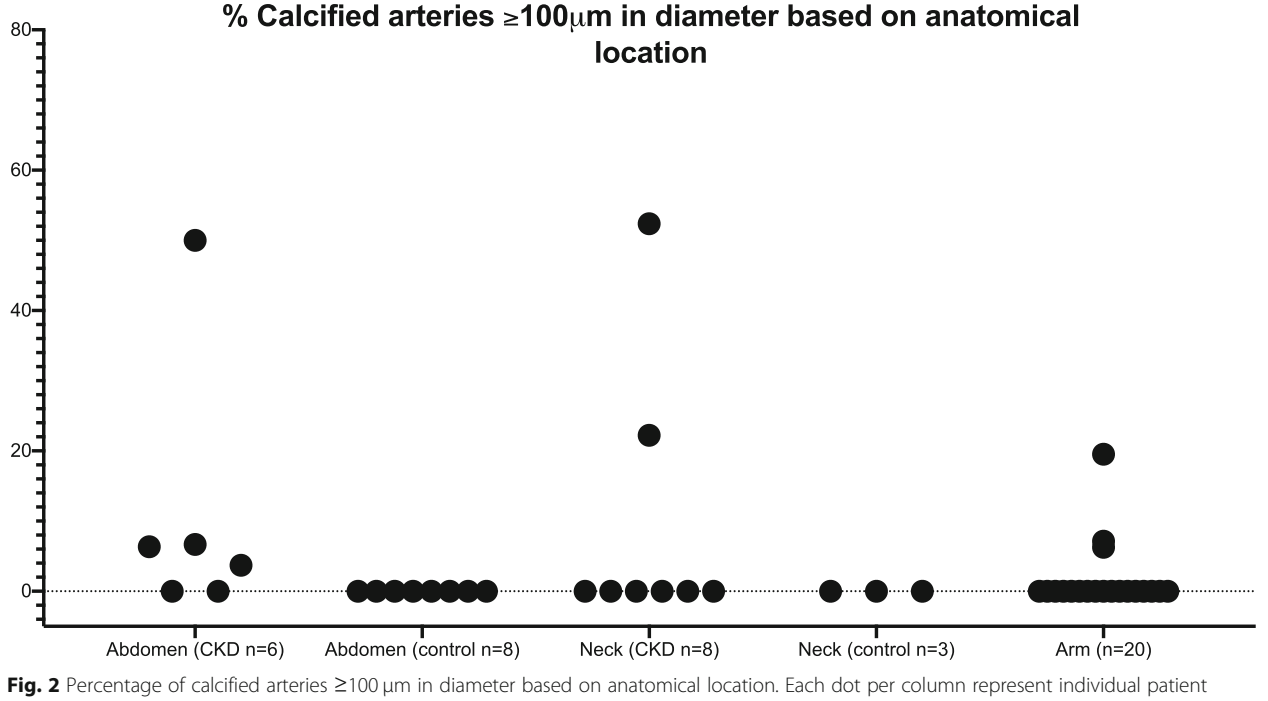

Fig. 2 Percentage of calcified arteries ≥100 μm in diameter based on anatomical location. Each dot per column represent individual patient

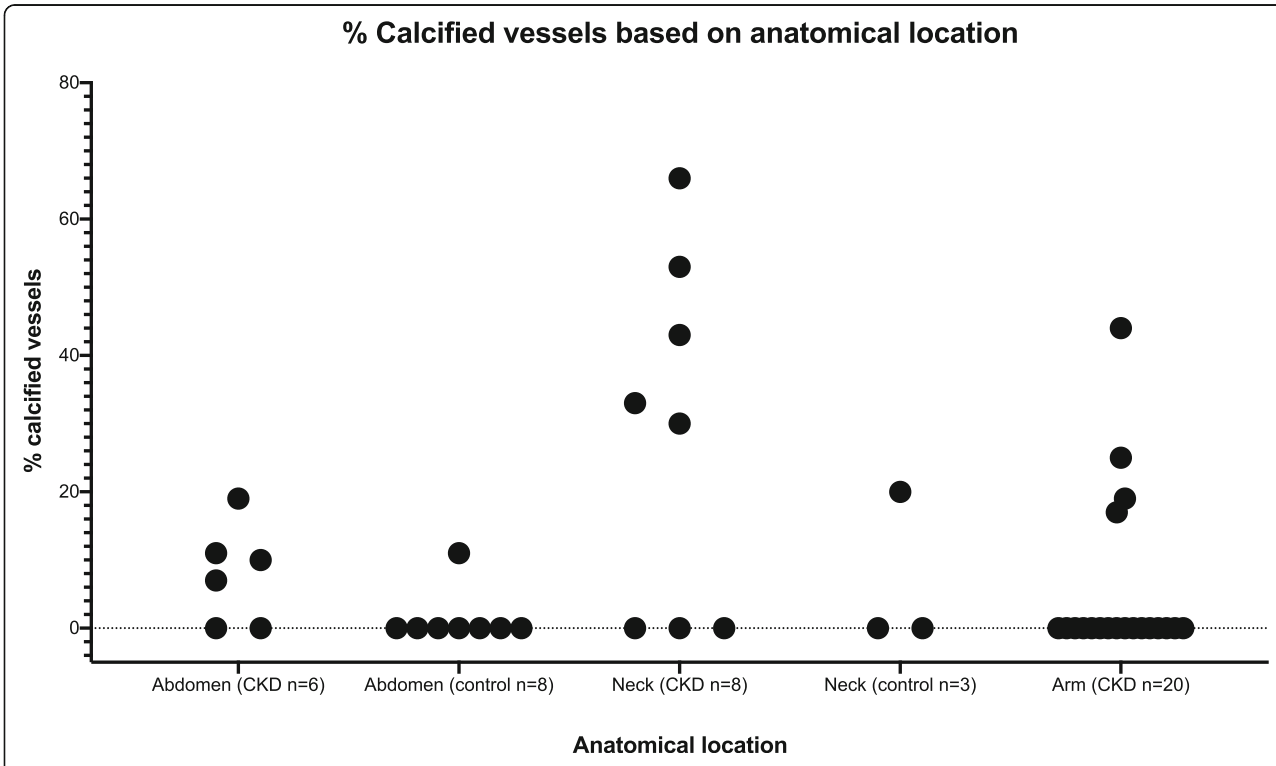

Fig. 3 Percentage of calcified vessels in each patient based on anatomical location

Median BMI was similar between both groups ($p = 0.2$), as was the co-morbidity profile. With respect to systemic biochemical measures of mineral metabolism and medical therapy, there were no differences in serum calcium, phosphate, ALP or PTH, or in the use of calcium- or non-calcium-based phosphate binders or activated vitamin D between groups.

No evidence of upregulation of pro-osteogenic gene expression in patients with ESKD or with VC

De novo expression of pro-osteogenic proteins has been widely implicated as a key driver in the pathogenesis of VC as well as in vascular lesions of patients with calciphylaxis [15]. Accordingly, we looked for changes in expression of two previously identified pro-calcific factors: tissue non-specific alkaline phosphatase (TNAP), which cleaves the mineralisation inhibitor pyrophosphate to phosphate, and RUNX2, a master transcription factor regulating osteogenic gene programs. Thirteen patients with eligible skin samples were evaluated for mRNA expression. Each skin sample was divided into three segments and tested in triplicate. Results are expressed as mean mRNA expression of each sample. There was minimal variability in gene expression across the three segments of tissue per patient sample. There was no upregulation of gene transcripts in samples with histological evidence of calcification ($n = 4$) compared to those without ($n = 9$) for either *TNAP* or *RUNX2*, $p = 0.76$ and $p = 0.35$ respectively (Fig. 5a). When comparing gene expression in patients with CKD/ESKD ($n = 11$) to those with no CKD ($n = 2$), there was also no difference in gene expression for *TNAP* or *RUNX2*, $p = 0.8$ and $p = 0.98$ respectively (Fig. 5b).

Discussion

To our knowledge, this is the first study to show evidence of medial VC in subcutaneous and dermal vessels in multiple anatomical locations in patients with advanced CKD and ESKD undergoing elective surgery, without peripheral vascular disease or calciphylaxis. Calcification was present in 38% of samples from patients with CKD/ESKD, predominantly in the subcutaneous tissue and involving the vessel media as well as the IEL. Perieccrine calcification was identified in 64% of samples with VC, present mainly in patients with ESKD on dialysis. These microvascular calcifications were not related to biochemical markers of mineral metabolism or changes in pro-calcific gene transcripts.

Von Kossa staining is routinely used to identify tissue calcification [2]. Using this stain, two distinct types of VC were identified in our skin samples, medial calcification and calcification of the IEL. The medial calcification identified in patients with CKD/ESKD was mostly granular but becoming confluent in some of the affected vessels. Calcification identified in the two control patients was more diffuse and milder in nature, which may

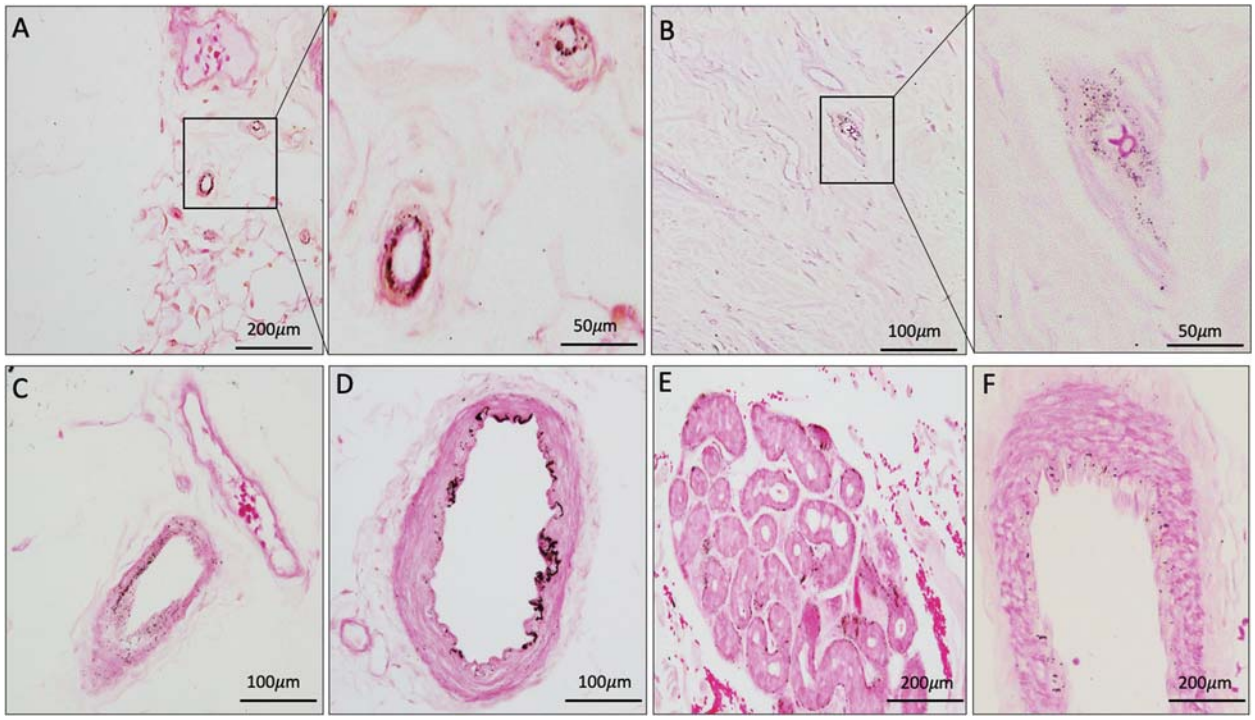

Fig. 4 Micrographs showing various forms of calcification seen in CKD/ESKD (Fig. **a**-**e**) and control patient (Fig. **f**). Von Kossa staining shows (**a**) Vessels < 50 μm in diameter with granular and circumferential medial calcification in subcutaneous tissue and higher magnification insert of vessels highlighted in black box from Panel A. **b** Vessel 100 μm in diameter with granular calcification throughout the media and alongside the internal elastic lamina in subcutaneous tissue and higher magnification of vessel highlighted in black box from Panel B (**c**) Vessel 100 μm in diameter with granular medial calcification in dermal tissue; **d** Confluent internal elastic lamina calcification of vessel > 100 μm in diameter in subcutaneous tissue; **e** Perieccrine calcification in dermal tissue; **f** Subtle and stippled calcification of vessel > 100 μm in diameter in control patient. All photos taken at 20x magnification. Scale bar denoted in each panel

represent a less advanced stage of VC progression or perhaps a different pathophysiological process. There was no evidence of intimal calcification in our study and there remains conjecture as to whether medial VC and calcification of the IEL represent progression of the same disease process. Multiple studies have shown that arterial medial calcification typically starts along the IEL and in the absence of inflammation [16, 17]. In contrast, arterial intimal calcification is characterized by focal mineral deposition in highly inflamed and necrotic atherosclerotic lesions.

The high prevalence of perieccrine calcification in patients with VC is a novel finding in our study. There is little literature regarding this histological entity and its clinical significance. Mochel et al. [8] initially described perieccrine calcification in a retrospective cohort of patients with calciphylaxis, suggesting that this histological feature was highly specific to calciphylaxis - the diagnosis of calciphylaxis being made based on this histological feature alone in 4 out of 56 cases. It was also identified in a case report of calciphylaxis [18]. In a recent case series of patients with calciphylaxis (13), perieccrine calcification was not present, however this may have been due to lack of specific staining for calcification. The authors argued that given the subtleness of this histological feature, it may be possible it is routinely missed with standard H&E staining. In further case series, perieccrine calcification was identified in two biopsies in patients with low to moderate risk of calciphylaxis, and was not significantly prevalent in biopsies of patients with high clinical suspicion of calciphylaxis [7]. The clinical significance of perieccrine calcification remains unknown and it may simply reflect the severity or progression of coexistent VC.

Calcification of skin tissue is considered to be a precursor to the development of calciphylaxis and is a common finding in skin biopsies in patients with this pathology [8]. The specificity of this finding was recently questioned by Ellis and O'Neill in a study of 38 skin biopsies taken for high clinical suspicion for calciphylaxis and compared with lower limb amputation skin samples in patients with ESKD [7]. All previously documented histopathological features commonly associated with calciphylaxis, including medial calcification, intimal thrombosis and extravascular calcification, were prevalent in both high-risk skin lesions and amputation samples.

Table 3 Differences between CKD/ESKD participants with and without VC

Demographic features	VC present (***n*** = 13)	VC absent (***n*** = 21)	***P*** value
Age, years	57 ± 13	59 ± 18	0.60
Sex (male)	8 (61%)	17 (80%)	0.21
BMI (kg/m^2)	27 ± 4	29 ± 6	0.24
Time on dialysis (if ESKD), years	2.5 [1.77–6]	4.2 [2–6.5]	0.25
CKD stage			
Pre-dialysis	1 (eGFR 17 ml/min^2)	2 (mean eGFR 20 ± 0.7 ml/min^2)	
PD	4	1	
HD	8 (57%)	18 (86%)	
Diabetes	4 (30%)	9 (42%)	0.55
Hypertension	10 (77%)	14 (67%)	0.52
IHD	3 (23%)	4 (19%)	0.78
PVD	1 (7%)	3 (14%)	0.56
Smoker (current or former)	8 (57%)	15 (50%)	0.66
Previous parathyroidectomy	2 (15%)	2 (9%)	0.64
Calcitriol use	7 (50%)	10 (34%)	0.24
Non-calcium-based binder	7 (50%)	11 (38%)	0.35
Calcium based binder	2 (15%)	10 (47%)	0.06
PTH, pmol/L	58 [37–184]	66 [37–123]	0.41
Total calcium, mmol/L	2.3 ± 0.2	2.4 ± 0.2	0.57
Phosphate, mmol/L	2 ± 0.5	1.9 ± 0.7	0.71
ALP, IU/L	106 [76–207]	108 [81–139]	0.72
Vessel number	44 ± 19	39 ± 15	0.91
Perieccrine calcification	9 (69%)	4 (19%)	0.003

Results are mean ± SD, median [IQR] or number (%)
Abbreviations: *ALP* alkaline phosphatase, *BMI* body mass index, *HD* haemodialysis, *IHD* ischaemic heart disease, *PD* peritoneal dialysis, *PTH* parathyroid hormone, *PVD* peripheral vascular disease

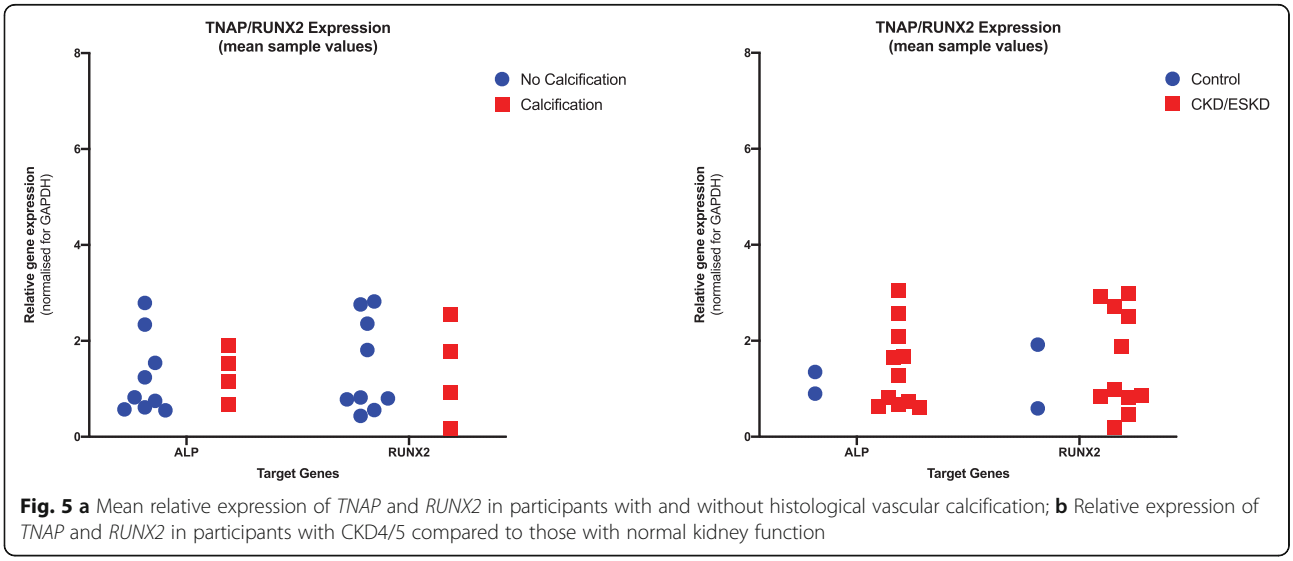

Fig. 5 a Mean relative expression of *TNAP* and *RUNX2* in participants with and without histological vascular calcification; **b** Relative expression of *TNAP* and *RUNX2* in participants with CKD4/5 compared to those with normal kidney function

Only the combination of thrombosis and medial calcification was unique to the high-risk skin biopsy group. Our study confirms that medial vascular calcification and perieccrine calcification can be present in "healthy skin" taken from three different anatomical locations, and that these features indeed are not specific to calciphylaxis. We could not identify any evidence of vessel thrombosis, likely due to the low incidence of peripheral vascular disease in our CKD/ESKD cohort. This histological finding may be more prevalent in patients with significant peripheral vascular disease rather than specific to CKD alone.

There were no significant demographic or biochemical differences in patients with CKD/ESKD with and without VC. PTH in patients with VC was similar to those without, but highly variable perhaps reflecting the variability and fluctuations in PTH, as the samples used here only capture one time point. We also identified no significant differences in serum calcium, phosphate or ALP between the two groups. However, the apparent lack of discriminating factors between the two groups may in part reflect the small sample size. It is recognised that medial VC is accelerated in patients with CKD [19]. Schlieper et al identified microcalcifications in the media of iliac arteries in uraemic patients [20] without the presence of intimal calcification or major atherosclerotic plaques. Consistent with our results, the presence of calcification was not associated with abnormalities in serum calcium, phosphate or magnesium, although PTH was not evaluated. Despite these similarities, it is uncertain to what extent VC in skin and subcutaneous tissue reflects calcification in other vascular beds and whether it is related to more systemic pro-calcific processes affecting the larger central arteries.

RUNX2, a transcription factor, is a master regulator of bone formation and is essential for bone formation and bone remodelling. Some animal studies have shown that RUNX2 expression is required for osteogenic phenotypic change in smooth muscle cells [21]. This transcription factor is also reportedly upregulated in human vascular fragments of large vascular territories where calcification is present [22]. However, evidence of osteogenesis is not a consistent finding across all studies of human VC. We did not see upregulation of *RUNX2* or *TNAP* in the subset of patients tested with histological evidence of calcification. Although osteogenic transformation of vascular smooth muscle cells is one proposed hypothesis for development of medial arterial calcification, other mechanisms have been implicated including elastin degradation, smooth muscle apoptosis and more passive biochemical processes [23]. Lack of osteogenic gene upregulation in our study may also reflect temporal aspects of calcification [6, 24] or a different underlying mechanism as a culprit. Human studies involving examination of arterial calcification in breast tissue in patients with CKD demonstrated a universal absence of staining for RUNX2 and osteocalcin in early arterial calcification [6]. A previous study identifying upregulation of osteogenic gene expression in skin was in the setting of calciphylaxis with macroscopic evidence of calcification [15]. This is not reflective of the subtle microscopic calcification identified in our participant cohort that is likely to occur in response to distinct triggers and mechanisms. On the other hand, this may be as a result of technical factors relating to the small sample size, or the sporadic nature of calcification identified on histological examination. Finally, given only a small amount of tissue was processed for PCR, small areas of active calcification may have been missed.

This study was limited by a small sample size and its cross-sectional design with a single skin incisional sample at one time point, without the ability to prospectively follow up VC progression, resolution or patient outcomes. It was not possible to obtain skin samples from control patients to match all anatomical locations and a future case-controlled study may better evaluate the impact of anatomical site on the presence of calcification. Assessment of VC burden at more central sites was also not undertaken. This study had many strengths, however, including a wide distribution of skin anatomical locations sampled as well large incisional biopsy sampling, which allowed for good interpretation of skin architecture and large number of vessels (mean of 40 vessels per slide). Also, patients had no underlying skin pathology to confound results of the study including presence of skin malignancy which is commonly associated with VC.

Conclusion

In conclusion, this study confirms the presence of medial VC in dermal and subcutaneous tissues in patients with advanced CKD and ESKD. VC was not associated with PTH levels, however, was more likely to be present in patients undergoing parathyroidectomy. The presence of medial VC and perieccrine calcification is not a specific histological finding for calciphylaxis.

Abbreviations

ALP: alkaline phosphatase; CKD: chronic kidney disease; ESKD: end-stage kidney disease; PTH: parathyroid hormone; VC: vascular calcification

Acknowledgments

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Authors' contributions

IR: contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work. TH: contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work. ES: contributed to the conception, design, data interpretation, critical appraisal of paper and drafting of the work. SH: contributed to critical appraisal of paper and drafting of the work. BW:

contributed data interpretation. NT: contributed to the conception, design, critical appraisal of paper and drafting of the work. All authors approved final copy of paper.

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Availability of data and materials
Comprehensive data presented in manuscript, tables and figures. Additional data available on request from the corresponding author.

Ethics approval and consent to participate
Research performed in accordance with the Declaration of Helsinki. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2017.023). Written informed consent was obtained for all study participants.

Consent for publication
Not applicable to current manuscript.

Competing interests
ERS has received research funding from Sanofi, Baxter, and Amgen and holds stock in Calciscon AG, which specializes in performing the T_{50}-test. SGH has received research funding, teaching and travel support from AstraZenica, Amgen, Baxter, Sanofi.
NDT has received honoraria, travel support and research funding from Amgen, Shire and Sanofi. TDH, BW and IR declare that they have no competing interests.

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APPENDIX D: LONGITUDINAL CHANGES IN BONE AND MINERAL METABOLISM AFTER CESSATION OF CINACALCET IN DIALYSIS PATIENTS WITH SECONDARY HYPERPARATHYROIDISM

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Longitudinal changes in bone and mineral metabolism after cessation of cinacalcet in dialysis patients with secondary hyperparathyroidism

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Abstract

Background: The calcimimetic agent cinacalcet is effective for the management of secondary hyperparathyroidism (SHPT) in dialysis patients. Changes to reimbursement of cinacalcet in Australia provided an opportunity to assess effects of medication cessation on biochemical and clinical outcomes in dialysis patients, including changes to novel biomarkers such as calciprotein particles (CPP). CPP are nanoparticles of mineral and protein in the circulation associated with increased vascular calcification in patients with chronic kidney disease.

Methods: Dialysis patients from a single center who ceased cinacalcet between August 2015 and March 2016 were included in a prospective observational study. Bloods were taken at the time of cessation of cinacalcet and at 1, 6 and 12 months. Clinical and biochemical outcomes were compared with an age- and gender-matched cohort of cinacalcet-naïve dialysis patients.

Results: Sixty-two patients participated in the study. Mean age was 69.6 ± 13.2 years. Biochemical changes over 12 months following cessation of cinacalcet included an increase in serum parathyroid hormone (PTH) (42.2 [IQR 27.8–94.6] pmol/L to 114.8 [83.9–159.1] pmol/L [$p < 0.001$]), serum calcium (2.31 ± 0.21 mmol/L to 2.46 ± 0.14 mmol/L [$p < 0.001$]) and primary CPP (CPP-I) ($p = 0.002$). Changes in CPP were associated with an increase in PTH ($p = 0.007$), calcium ($p = 0.002$) and ferritin ($p = 0.02$) but a reduction in serum albumin ($p = 0.001$). Over the 12-month period, there were two fractures, five cardiovascular events, one episode of calciphylaxis, and one parathyroidectomy, with a mortality rate of 19% ($n = 13$).

Conclusion: Uniquely we report the effects of cinacalcet withdrawal in a real world setting with demonstrated increases in PTH, serum calcium and CPP subsets, novel CKD-MBD related factors, over a 12-month period.

Keywords: End-stage kidney disease, Secondary hyperparathyroidism, Dialysis, Calciprotein particles, Parathyroid hormone, Cinacalcet

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Background

Abnormalities in bone and mineral metabolism, encompassed by the term 'chronic kidney disease - mineral and bone disorder' (CKD-MBD) [1], play a significant role in vascular calcification and increased cardiovascular risk in patients with chronic kidney disease (CKD) [2]. Progressive changes in mineral homeostasis with disruption of normal calcium and phosphate balance are associated with changes in the phosphaturic hormones like fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH), and abnormalities in vitamin D metabolism, and these result in complications of secondary hyperparathyroidism (SHPT) [3]. SHPT has been linked with pathology [4] including; bone disease [5], hip fractures [6], myocardial hypertrophy and dysfunction [7], disturbances in lipid [8] and glucose [9] metabolism, anemia [4] and vascular calcification [10].

Vascular calcification in patients with CKD results from several interlinked mechanisms that involve aberrant bone metabolism, inflammatory pathways and dysregulation of endogenous calcification inhibitors [11]. One potent inhibitor of endogenous calcification is fetuin A, a highly conserved and ubiquitous phosphoprotein present in all mammals which forms nanoparticles with calcium and phosphate in the circulation forming calciprotein particles (CPP). CPP may provide an important pathway for transporting mineral nanocrystals around the body and clearance in the circulation is by macrophages [12]. Initially these nano-sized particles are present in amorphous calcium-phosphate form (primary CPP or CPP-I), however chronic dysregulation of mineral metabolism may result in accumulation and ripening of these particles into larger crystalline calcium phosphate (secondary CPP or CPP-II), possibly resulting in toxicity [13–15]. Levels of CPP are significantly higher in patient cohorts known to develop premature ageing and arterial calcification, such as patients with CKD (especially those on dialysis) [14, 16] and those with inflammatory diseases [16], compared to healthy controls.

Higher levels of CPP are a predictor of all-cause mortality in patients with CKD [17], and have been associated with increased vascular calcification [18] and calciphylaxis in this population [16, 19]. In patients on dialysis, reduction of PTH by parathyroidectomy or calcimimetics also results in a reduction in CPP [20], suggesting that abnormal bone metabolism is associated with mineral stress, leading to an imbalance in production and removal of CPP. Detection of elevated levels of CPP in the circulation in pathology may therefore serve as a novel biomarker of mineral stress and cardiovascular risk.

The calcimimetic agent cinacalcet is commonly used in the management of SHPT, with multiple studies reporting clinical efficacy of this therapy to reduce PTH levels [21, 22]. This medication was approved for treatment of SHPT in Australia in 2007, with associated government reimbursement, and has been widely prescribed to control biochemical changes associated with moderate to severe SHPT in patients on dialysis. Following the publication of the Evaluation of Cinacalcet Hydrochloride Therapy to Lower Cardiovascular Events (EVOLVE) study [23] which failed to show an effect for cinacalcet vs. placebo in unadjusted intention-to-treat analysis of the primary composite end-point (time to death, myocardial infarction, hospitalization for unstable angina, heart failure, or a peripheral vascular event), the pharmaceutical benefits advisory committee in Australia withdrew reimbursement for this medication. Cinacalcet is now available only on private prescription and at a significant cost to the patient. The effect of this change meant that cinacalcet was withdrawn in the majority of patients previously prescribed, and this provided a unique opportunity to study changes in markers of bone and mineral metabolism, including CPP, in patients with SHPT.

Methods

Study design

This was a single-center prospective observational study performed at The Royal Melbourne Hospital. The aim was to assess the impact of withdrawal of cinacalcet in patients on dialysis over a 12-month period. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2015.180) and patient enrollment commenced in August 2015 with completion in March 2016.

Study cohort

Patients receiving dialysis at The Royal Melbourne Hospital or affiliated satellite dialysis units were eligible for recruitment, and written informed consent was obtained from all participants at study commencement. Inclusion criteria included patients with the ability to provide informed consent, aged over 18 years old and prescribed cinacalcet therapy for clinical features of severe SHPT, not adequately controlled with active vitamin D therapy, and a PTH level greater than nine times the upper limit of normal as per the Kidney Disease: Improving Global Outcome (KDIGO) CKD-MBD guidelines [1]. There were no specific exclusion criteria. Starting doses of cinacalcet were 30 mg/day and doses had been titrated to achieve symptom control and a PTH target within the KDIGO guideline suggested range. A detailed medication history was recorded for patients at each visit, and date of cinacalcet cessation was documented in the patient's medical records. All patients provided written informed consent before enrollment and the study was conducted in accordance with the Declaration of Helsinki.

No specific protocol existed for the management of SHPT following cinacalcet withdrawal. Treatment was

based on usual clinical care for SHPT including reduction of serum phosphate and administration of active vitamin D therapy with avoidance of hypocalcemia. Patients were referred for surgical parathyroidectomy if they had a persistently elevated PTH above the KDIGO guidelines, evidence of high turnover bone disease or symptoms of severe PTH. Dialysate calcium in hemodialysis and peritoneal dialysis patients remained unchanged following cinacalcet withdrawal.

Control cohorts

Two control cohorts were used in this study to the potential effects of temporal changes unrelated to drug cessation. The first was a historical age-, gender- and dialysis vintage- matched control cohort who were cinacalcet naïve. We compared demographic features and biochemical parameters over a 12-month period between the control cohort and the cinacalcet withdrawal cohort from July 2015 to July 2016. This control cohort was identified from the Nephrology database at The Royal Melbourne Hospital, and all patients included had no history of prior cinacalcet use. The second control group included a subset of thirteen cinacalcet naïve dialysis patients from the historical control cohort that were age and dialysis modality matched, who had serum samples collected at baseline (June 2017) and after 6 months for CPP analysis. The purpose of this control group was to assess whether a rise in CPP observed in the intervention group reflected a response to drug cessation or a more generic rise in CPP that would be observed in any group of chronic dialysis patients with progressive disease over time. Management of CKD-MBD in the control cohort was as per usual standard care for SHPT, involving treatment of hyperphosphatemia and use of active vitamin D therapy as indicated by the treating clinician.

Study end points

The primary endpoint was change in CPP following cessation of cinacalcet therapy over a 12-month period. Secondary biochemical outcome measures included serum changes in PTH, calcium, phosphate, alkaline phosphatase (ALP), ferritin and C-reactive protein (CRP), as well as hemoglobin.

Biomarker assessment

Serum was collected in all patients at baseline, whilst still being administered cinacalcet and then following cessation of cinacalcet at 1 month, 6 months and 12 months. Samples were collected to measure the following parameters: total CPP, CPP-I, CPP-II, albumin, calcium, phosphate, PTH, hemoglobin, CRP, ferritin, and ALP. Serum calcium level was adjusted as follows if serum albumin was < 40 g/L: corrected serum calcium (mmol/L) = measured serum calcium (mmol/L) + 0.02 x (40 - serum albumin (g/L)). For CPP analysis blood was collected into 6 ml plain tubes using standard phlebotomy techniques. Blood samples were allowed to stand for 60 min and then centrifuged at 3000 g for 15 min at room temperature. Aliquots were stored at − 80 C until batched analysis.

CPP evaluation using flow cytometry

A novel method to evaluate CPP using flow cytometry has recently been published by our group [15]. Briefly, batched patient samples were run on a BD FACSVerse flow cytometer using high sensitivity fluidics. The instrument was operated using BD FACSSuite software (version 1.0.5). Raw FCS files were acquired and imported into FlowJo LLC version 10.1 revision 3 (Ashland, Oregon, USA) for analysis. OsteoSense 680EX fluorescence was detected with a red 640 nm laser. Acquisition settings were held constant for all samples (60 s or 30,000 events). All measurements were displayed in logarithmic scale and signal stability was assessed in real-time using SSC-H vs. time plots. The gating strategy for CPP was set empirically, as described in Fig. 1.

Statistical analysis

Baseline characteristics are reported as mean+/− standard deviation (SD) or, when appropriate, median (interquartile range [IQR]) for continuous variables and as a number and percentage for categorical variables. Paired t test and Wilcoxon signed-rank test were used for between group comparisons. Categorical variables were analyzed with the chi-square test. Continuous variables were compared with independent samples paired t test if normally distributed or with Mann-Whitney U test if the distribution was skewed. Mixed linear effect modelling was used to determine differences within groups over the study period, with the variables selected a priori, based on known regulatory relationships. CPP and PTH were natural log-transformed (Ln) for the purposes of these analyses. All mixed-effect model analyses were performed allowing for the random effect of different pathology collection times and locations. Two-tailed P values < 0.05 were considered statistically significant. Analyses were performed using SPSS software for Macintosh version 21 (IBM SPSS, Chicago, IL).

Results

Demographics and clinical characteristics

One hundred and twenty-eight patients on dialysis were being administered cinacalcet during the recruitment period of August 2015 to March 2016 and were approached to participate in the study. Figure 2 describes the participant flow. Sixty-six were excluded either due to patient or physician preference as many of

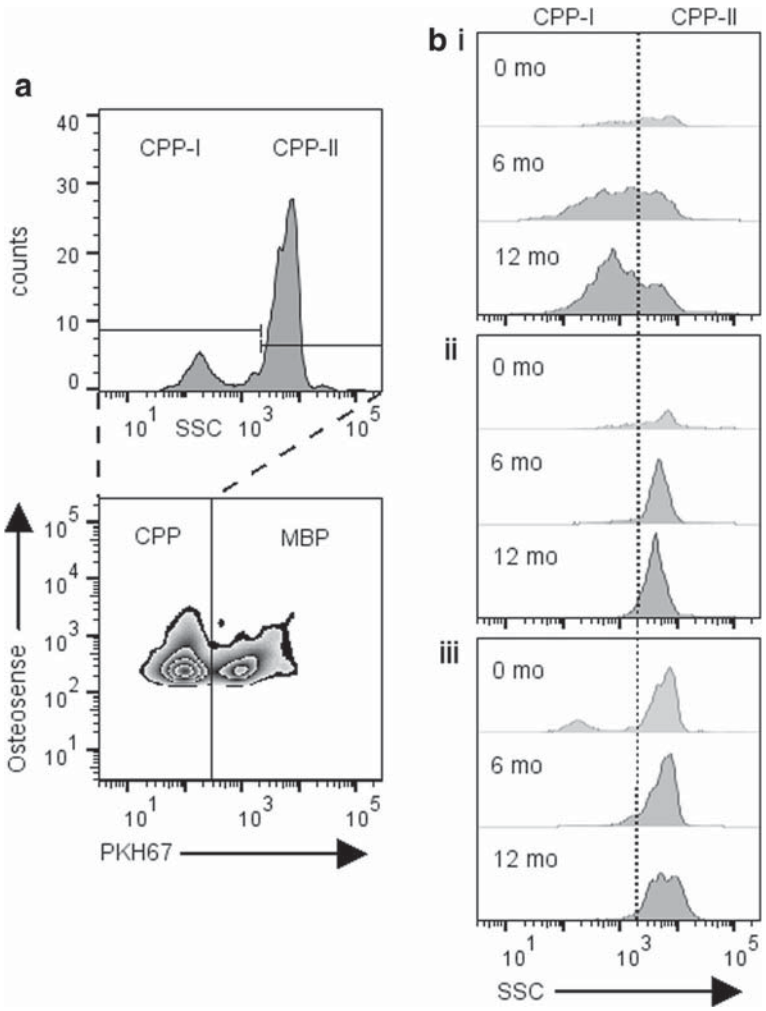

Fig. 1 Schematic of flow cytometry gating strategy. **a** Represents cytograms showing dual Osteosense and PKH67 staining on mineral-containing nanoparticles (**b**) Histograms show gated populations and their respective volumetric measurements (counts/μL). CPP-I, primary calciprotein particle; CPP-II, secondary calciprotein particle

these patients had a supply of medication remaining and continued on therapy. Sixty-two patients were enrolled in the study, and their cinacalcet cessation date was documented in medical records. Six patients received a kidney transplant and five re-commenced cinacalcet therapy via an industry sponsored special-access scheme during the 12-month follow up period. Fifty-one patients were included in the primary outcome analysis.

Baseline characteristics of study participants and the contemporaneous age- and gender-matched cinacalcet-naïve dialysis controls are shown in Table 1. Only dialysis modality was significantly different between the two groups, with more patients in the cinacalcet withdrawal cohort on hemodialysis, 86% vs 57%, ($p = 0.001$). The mean age and time on dialysis were 69.9±13.2 years and 7.1±3.6 years respectively in the cinacalcet withdrawal cohort. Over 50% of participants were male, 45% had a history of diabetes and 76% had hypertension. The main etiology of CKD was diabetic nephropathy.

In the 12 months following withdrawal of cinacalcet therapy there were two lower limb fractures, one parathyroidectomy with three patients referred for parathyroid surgery, and one episode of calciphylaxis. Thirteen patients died during the study period, with deaths due to cardiovascular causes ($n = 5$), withdrawal from dialysis ($n = 3$) and other causes including malignancy and infection ($n = 4$). In the control group there were five fractures, one parathyroidectomy and no episodes of calciphylaxis. Of the 10 deaths in the control group, three were due to cardiovascular causes.

A change in prescribing patterns following cinacalcet withdrawal were observed. At baseline 76% of patients ($n = 39$) were on calcitriol, which reduced to 57% ($n = 29$) by 12 months ($p = 0.03$). Four patients were commenced on calcitriol de novo. Calcitriol

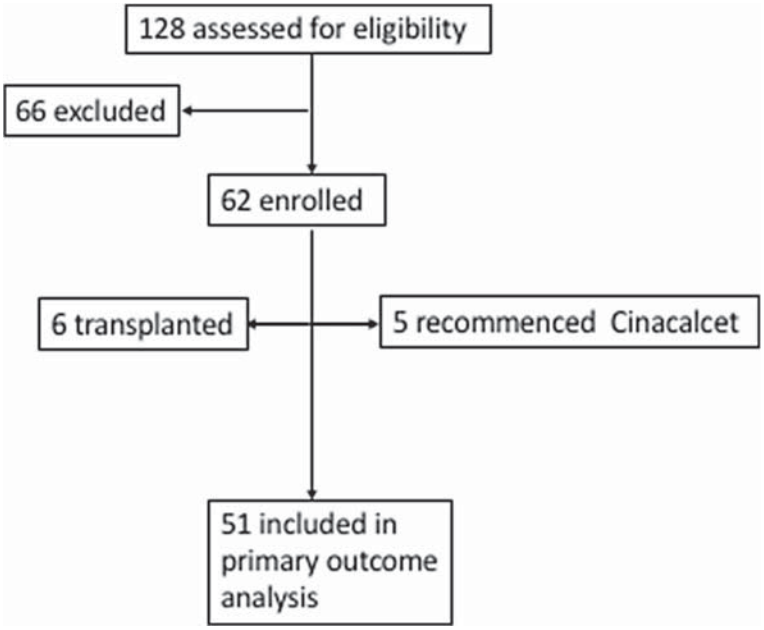

Fig. 2 Participant flow diagram

doses increased over the study period (1.3 mcg/week versus 1.7 mcg/week at baseline and 12 months respectively, $p = 0.04$). Of 21 patients on a calcium-based phosphate binder at baseline, 12 continued this therapy at 12 months ($p = 0.05$). Seventy-six percent of patients ($n = 39$) were on a non-calcium based phosphate binder at baseline, 59% of patients ($n = 30$) remained on therapy at study completion ($p = 0.06$). No patients were on magnesium supplementation. There were 12 episodes of hypercalcemia (serum corrected calcium > 2.60 mmol/L), with 9 episodes occurring by 6 months. There were no episodes of hypercalcemia in the control arm. The dosage or number of patients taking nutritional vitamin D supplementation did not significantly change over the year (12 at baseline versus 10 at 12 months, $p = 0.63$).

Biochemical outcomes

Baseline levels of PTH, phosphate, calcium and ALP were similar across the two groups (Table 2). In the cinacalcet withdrawal cohort, there was an increase in PTH from 42.2 pmol/L (27.8–94.6 pmol/L) at baseline to 114.8 pmol/L (83.9–159.1 pmol/L) at 12 months ($p < 0.001$). The highest rate of change in PTH occurred at 1 month with a mean PTH increase of 93% from baseline ($p < 0.001$). Serum calcium also increased from 2.31±0.21 mmol/L to 2.46±0.14 mmol/L over 12 months ($p < 0.001$). There was no change in PTH or serum calcium in the control group over 12 months. Phosphate remained unchanged in the cinacalcet withdrawal group ($p = 0.8$) and the control group ($p = 0.6$) over the 12-month study period. Table 2 summarizes baseline, 6- and 12-month values for biochemical outcomes in the control and cinacalcet withdrawal cohorts.

A trend towards increased ALP (141.4±61 IU/L at baseline to 161.7±72.9 IU/L at 12 months) was seen in the cinacalcet withdrawal cohort over the study period but did not reach statistical significance ($p = 0.09$). Inflammatory markers including CRP and the positive acute-phase reactant ferritin remained unchanged over 12 months, however there was a modest decrease in serum albumin from 35±4.2 g/L to 33±4.5 g/L, $p = 0.03$. This trend was not observed in the control group. Hemoglobin and serum bicarbonate were also equivalent in both groups over the study period. Changes in biochemical markers in the cinacalcet withdrawal group are presented in Fig. 3.

CPP assessments

CPP assessment and characterization was performed using a novel flow cytometry method published by our group [15]. Four out of 51 study patients were excluded from final CPP analysis due to sample instability and unknown analytical interference. Table 3 describes the changes in CPP in the cinacalcet withdrawal group, including patients who received a transplant or re-started therapy during the study period. Total CPP at baseline were $3.3 \times 10^4/\mu L$ (IQR: $1.5 \times 10^4/\mu L$ to $5.7 \times 10^4/\mu L$), predominantly as CPP-II at baseline (61% of total CPP) and 12 months (58% of total CPP), $p = 0.06$.

CPP-I increased significantly over the study period (396% mean increase, $p = 0.002$). Total CPP showed a trend towards increasing levels, but this did not reach

Table 1 Patient demographics and clinical characteristics

Demographic	Cinacalcet withdrawal patients ($n = 51$)	Control patients ($n = 51$)	p value
Age, years	69.6 (13.2)	68.6 (12.9)	ns
Gender (male)	28 (55)	28 (55)	ns
Dialysis modality (HD)	44 (86)	29 (57)	$p = 0.001$
Time on dialysis, years	7.1 (3.6)	6.7 (4.1)	ns
Diabetes	23 (45)	23(45)	ns
Hypertension	39 (76)	33 (65)	ns
Ischemic heart disease	23 (45)	18 (35)	ns
Peripheral vascular disease	8 (15)	15 (29)	ns
Current or ex-smoker	16 (31)	17 (33)	ns
Previous transplants	4 (8)	2 (4)	ns
Parathyroidectomy	1 (2)	5 (10)	ns
Previous fractures	7 (13)	4 (8)	ns
Aetiology of renal disease			
Diabetic nephropathy	14 (27)	18 (35)	ns
GN	9 (17)	12 (23)	ns
PKD	3 (6)	4 (8)	ns
Other	25	17	ns
Events during follow up			
Cardiac arrest	5	3	ns
Parathyroidectomy and referral for surgery	5	1	ns
Fracture	2	5	ns
Calciphylaxis	1	0	ns
Deaths	13	10	ns
Hypercalcemia at baseline	3	1	ns
Hypercalcemia at 6 months	9	0	$p = 0.007$
Hypercalcemia at 12 months	3	0	ns

Data presented as number (percent), or mean (standard deviation)
Hypercalcemia if Ca > 2.6 mmol/L
Abbreviations: *HD* hemodialysis, *GN* glomerulonephritis, *PKD* polycystic kidney disease

statistical significance ($p = 0.05$). Figure 4 depicts cohort and patient-level changes in serial absolute CPP levels over the study period and relative % changes from baseline values.

Utilizing a mixed linear effect model, allowing for random effects, changes in total and CPP-I counts were significantly associated with changes in PTH ($p = 0.007$ and 0.04 respectively). Total CPP, CPP-I and CPP-II counts were all longitudinally associated with changes in serum calcium ($p = 0.002$, 0.02 and 0.001 respectively), albumin ($p = 0.001$, 0.004 and < 0.001) and ferritin concentrations ($p = 0.03$, 0.01, and 0.009). Only changes in CPP-II were associated with the change in phosphate ($p = 0.03$) and ALP ($p = 0.05$). In contrast to those in the cinacalcet withdrawal cohort, there was no change in total CPP ($p = 0.4$), CPP-I ($p = 0.2$) or CPP-II ($p = 0.1$) in patients who received a kidney transplant or in those who re-started cinacalcet (total CPP ($p = 0.15$), CPP-I ($p = 0.2$), CPP-II ($p = 0.2$)) over the study period, although numbers were small.

Thirteen stable cinacalcet-naïve dialysis patients were used as a control cohort for CPP analysis, mean age 70.8±13years, 77% male, with 12 patients on hemodialysis and one on peritoneal dialysis. There were no significant differences in demographic characteristics between the control and the cinacalcet withdrawal cohorts, other than a higher proportion of patients on hemodialysis in the CPP analysis cohort ($p = 0.02$). There were no hospitalizations or changes in phosphate binder or active vitamin D prescription patterns in the control group. Over a six-month period, there was no significant increase in PTH ($p = 0.1$), serum calcium ($p = 0.69$), phosphate ($p = 0.37$), ALP ($p = 0.86$) or CPP-I ($p = 0.08$) and CPP-II ($p = 0.26$). In the control cohort of 13 stable cinacalcet naïve patients, CPP were mainly CPP-I (mean 79% CPP-I versus mean 21% CPP-II). The mean percentage increase in total CPP and CPP-I over a six-month period was significantly lower in the control group compared to the cinacalcet withdrawal cohort ($p = 0.03$ and $p = 0.05$ respectively). This effect was not seen for CPP-II ($p = 0.07$). Figure 5 shows the mean percentage changes in total CPP, CPP-I and CPP-II from baseline to 6 months values in cinacalcet withdrawal patients compared to control patients.

Discussion

Whilst the EVOLVE study was inconclusive as to its effects on cardiovascular mortality overall, a major issue with EVOLVE was that it was underpowered to detect mortality in the age group recruited because of the low event rate in the control group [24]. For patients over the age of 65, where the parathyroidectomy and transplantation rates were much lower, the EVOLVE study did appear to derive a cardiovascular benefit from cinacalcet therapy. Nevertheless, whilst cinacalcet has been shown to be effective in controlling metabolic parameters, it cannot be recommended to improve survival without a better understanding of its link with cardiovascular mortality [25]. It would therefore be desirable to have a biomarker that could more directly illustrate the link between cardiovascular disease and SHPT. CPP

Table 2 Biochemical changes over 12-month period

Biochemistry	Cinacalcet withdrawal patients ($n = 51$)	Control patients ($n = 51$)	p value
PTH baseline, (pmol/L)	42.2 [27.8–94.6]	44 [28.5–60.9]	$p = 0.98$
PTH 6 months, (pmol/L)	103.9 [63.4–122.2]	41 [28.2–67.8]	$p < 0.005$
PTH 12 months (pmol/L)	114.8 [83.9–159.1]	41.3 [28.5–69.7]	$p < 0.005$
Calcium baseline (mmol/L)	2.31 (0.21)	2.32 (0.15)	$p = 0.8$
Calcium 6 months (mmol/L)	2.48 (0.21)	2.28 (0.18)	$p < 0.005$
Calcium 12 months (mmol/L)	2.46 (0.14)	2.32 (0.14)	$p < 0.005$
Phosphate baseline (mmol/L)	1.72 (0.55)	1.56 (0.42)	$p = 0.14$
Phosphate 6 months (mmol/L)	1.75 (0.47)	1.64 (0.56)	$p = 0.3$
Phosphate 12 months (mmol/L)	1.77 (0.58)	1.6 (0.49)	$p = 0.14$
ALP baseline (IU/L)	141.4 (61)	129.8 (91.8)	$p = 0.45$
ALP 6 months (IU/L)	155.5 (61)	126 (75.5)	$p = 0.04$
ALP 12 months (IU/L)	161.7 (72.9)	121.5 (45)	$p = 0.003$
Albumin baseline (g/L)	35 (4.2)	35.7 (4.3)	$p = 0.84$
Albumin 6 months (g/L)	34 (4.2)	34.9 (4.6)	$p = 0.97$
Albumin 12 months (g/L)	33 (4.5)	34.3 (5)	$p = 0.94$
CRP baseline (mg/L)	6.3 [4.6–19.6]	5 [2–16]	$p = 0.312$
CRP 6 months (mg/L)	7 [3.3–12.3]	4.2 [2–18]	$p = 0.37$
CRP 12 months (mg/L)	7.7 [3–29.5]	6.3 [2–19]	$p = 0.41$
Ferritin baseline (ug/L)	235.5 [102.3–322.3]	229 [140.8–399.3]	$p = 0.23$
Ferritin 6 months (ug/L)	209.5 [80–331.3]	232.5 [114–316]	$p = 0.86$
Ferritin 12 months (ug/L)	274.5 [120–383.5]	221.5 [138.5–322.8]	$p = 0.64$
Hemoglobin baseline (g/L)	112.7 (10.4)	110 (14)	$p = 0.13$
Hemoglobin 6 months (g/L)	111.6 (10.7)	107 (18)	$p = 0.37$
Hemoglobin 12 months (g/L)	109 (14)	114 (14)	$p = 0.09$
Bicarbonate baseline (mmol/L)	23 (3)	24 (3.7)	$p = 0.53$
Bicarbonate 6 months (mmol/L)	23.1 (3)	23.9 (3)	$p = 0.9$
Bicarbonate 12 months (mmol/L)	23.6 (3.2)	24 (3)	$p = 0.81$
25[OH]D baseline (nmol/L/L)	43[33–68]	52[42–60]	$p = 0.52$
25[OH]D 6 months (nmol//L)	49[32–68]	66[51–89]	$p = 0.1$
25[OH]D 12 months (nmol/L)	53[28–70]	60[42–97]	$p = 0.36$

Data presented as number (percent), mean (standard deviation) or median [interquartile range]
Abbreviations: *ALP* alkaline phosphatase, *CRP* C-reactive protein, *PTH* parathyroid hormone, *25[OH]D* 25-hydroxy vitamin D

appear to be such an emerging biomarker and therefore ideal for testing potential effects of cinacalcet removal.

Withdrawal in Australia of reimbursement for cinacalcet for the medical treatment of SHPT has enabled us to examine potentially important changes in this emerging biomarker associated with CKD-MBD. Cinacalcet cessation resulted in increased PTH, serum calcium, and CPP-I, which was associated with a reduction in serum albumin but no change in serum phosphate. Longitudinal changes in CPP were associated with an increase in PTH, calcium, ALP, ferritin and decrease in albumin.

There is mounting interest in the potential role of CPP in the pathogenesis of CKD-MBD. Recently our group published a novel validated method for CPP detection and quantitation [15] using a fluorescent probe-based flow cytometric assay, and this together with blood calcification propensity analysis [26] has shed new light on the potential involvement of these difficult-to-evaluate nanoparticles. The primary role of CPP-I is likely to act as a mineral chaperone, sequestering mineral that may otherwise seed mineralization at ectopic sites and facilitating transport and clearance from body fluids [27]. However, in states of chronic mineral stress, the transition and ripening of CPP-I to pro-inflammatory CPP-II may be enhanced, creating a vicious cycle of inflammation and calcification [13, 28].

Increased CPP has been associated with increased vascular calcification in animal models, and its presence in

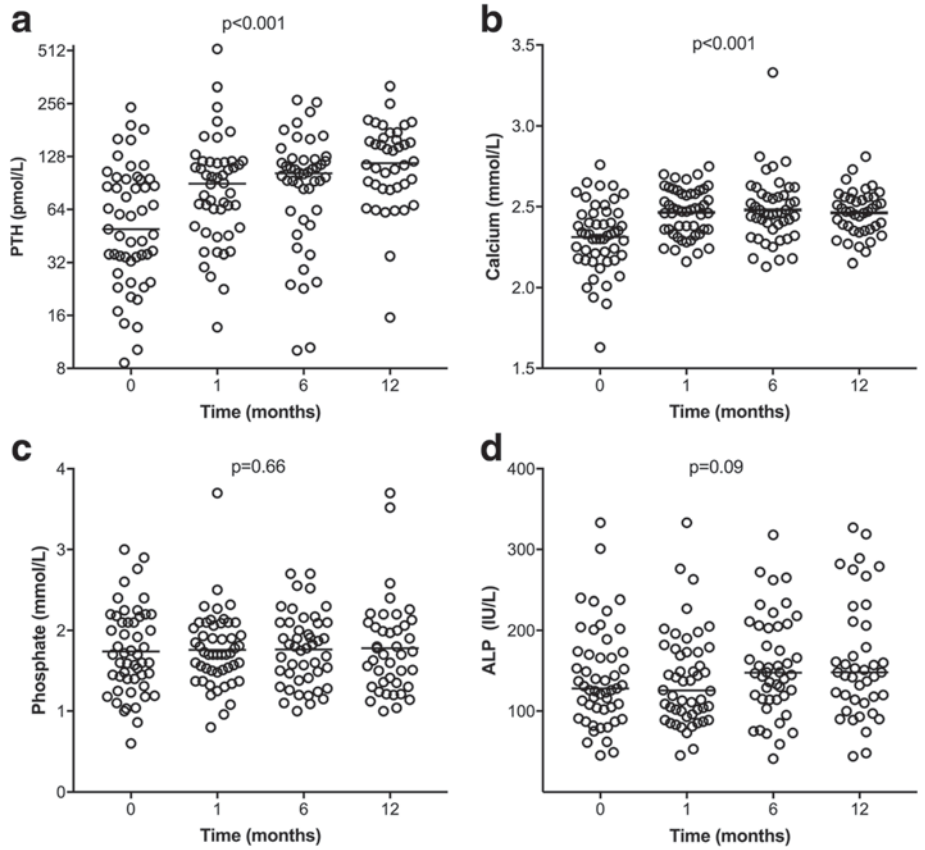

Fig. 3 Changes in biochemical mineral markers over 12 months following cinacalcet withdrawal. Changes in (**a**) parathyroid hormone, (**b**) calcium, (**c**) phosphate and (**d**) alkaline phosphatase over a 12-month period summarized in box plots (median, lower and upper quartile, and outliers). Changes between time points measured using mixed linear effect model. *P* value denotes trend over 12-month period

Table 3 CPP levels in study cohort and control dialysis group

CPP/μL	Cinacalcet withdrawal patients ($n = 47$)	Transplanted patients ($n = 6$)	Patients re-started on cinacalcet ($n = 5$)	*P* values
Total CPP baseline	3.26×10^4 [1.49×10^4–5.73×10^4]	1.62×10^4 [8.85E + 03–7.11×10^4]	3.12×10^4 [1.95×10^4–6.12×10^4]	$P = 0.31$
Total CPP 1 month	3.36×10^4 [2.38×10^4–5.70×10^4]	1.59×10^4 [1.18×10^4–2.69×10^4]	5.15×10^4 [2.86×10^4–1.47×10^5]	$P = 0.28$
Total CPP 6 months	3.88×10^4 [1.74×10^4–6.44×10^4]	1.84×10^4 [1.08×10^4–1.18×10^5]	7.78×10^4 [2.17×10^4–1.65×10^5]	$P = 0.27$
Total CPP 12 months	4.28×10^4 [2.37×10^4–6.28×10^4]	2.02×10^4 [1.15×10^4–1.83×10^5]	4×10^4 [1.98×10^4–1.42×10^5]	$P = 0.21$
CPP-I baseline	9.18×10^3 [4.01×10^3–1.87×10^4]	7.06×10^3 [4.57×10^3–2.065×10^4]	1.81×10^4 [5.1×10^3–4.15×10^4]	$P = 0.52$
CPP-I 1 month	1.64×10^4 [7.08×10^3–2.91×10^4]	6.03×10^3 [2.92×10^3–4.53×10^4]	4.39×10^4 [1.63×10^4–7.6×10^4]	$P = 0.11$
CPP-I 6 months	1.25×10^4 [6.8×10^3–2.73×10^4]	8.97×10^3 [6.65×10^3–2.82×10^4]	2.78×10^4 [1.28×10^4–5.04×10^4]	$P = 0.53$
CPP-I 12 months	1.41×10^4 [× 10^3–2.42×10^4]	1.17×10^4 [5.38×10^3–7.04×10^4]	1.47×10^4 [1.26×10^3–8.92×10^4]	$P = 0.14$
CPP-II 0 months	1.81×10^4 [5.78×10^3–3.92×10^4]	9.17×10^3 [3.98×10^3–5.07×10^4]	9.37×10^3 [7×10^3–2.54×10^4]	$P = 0.45$
CPP-II 1 month	2.02×10^4 [7.5×10^3–2.8×10^4]	1.21×10^4 [5.51×10^3–2.25×10^4]	1.23×10^4 [7.67×10^3–7.14×10^4]	$P = 0.81$
CPP-II 6 months	1.63×10^4 [8.27×10^3–3.63×10^4]	9.4×10^3 [4.11×10^3–8.93×10^4]	1.49×10^4 [3.7×10^3–8.94×10^3]	$P = 0.74$
CPP-II 12 months	2.57×10^4 [1.02×10^4–4.39×10^4]	8.5×10^3 [6.11×10^3–1.12×10^5]	1.36×10^4 [9.94×10^3–9.31×10^4]	$P = 0.36$

Data presented as median [interquartile range]
Abbreviations: *CPP* calciprotein particles, *CPP-I* primary calciprotein particles, *CPP-II* secondary calciprotein particles

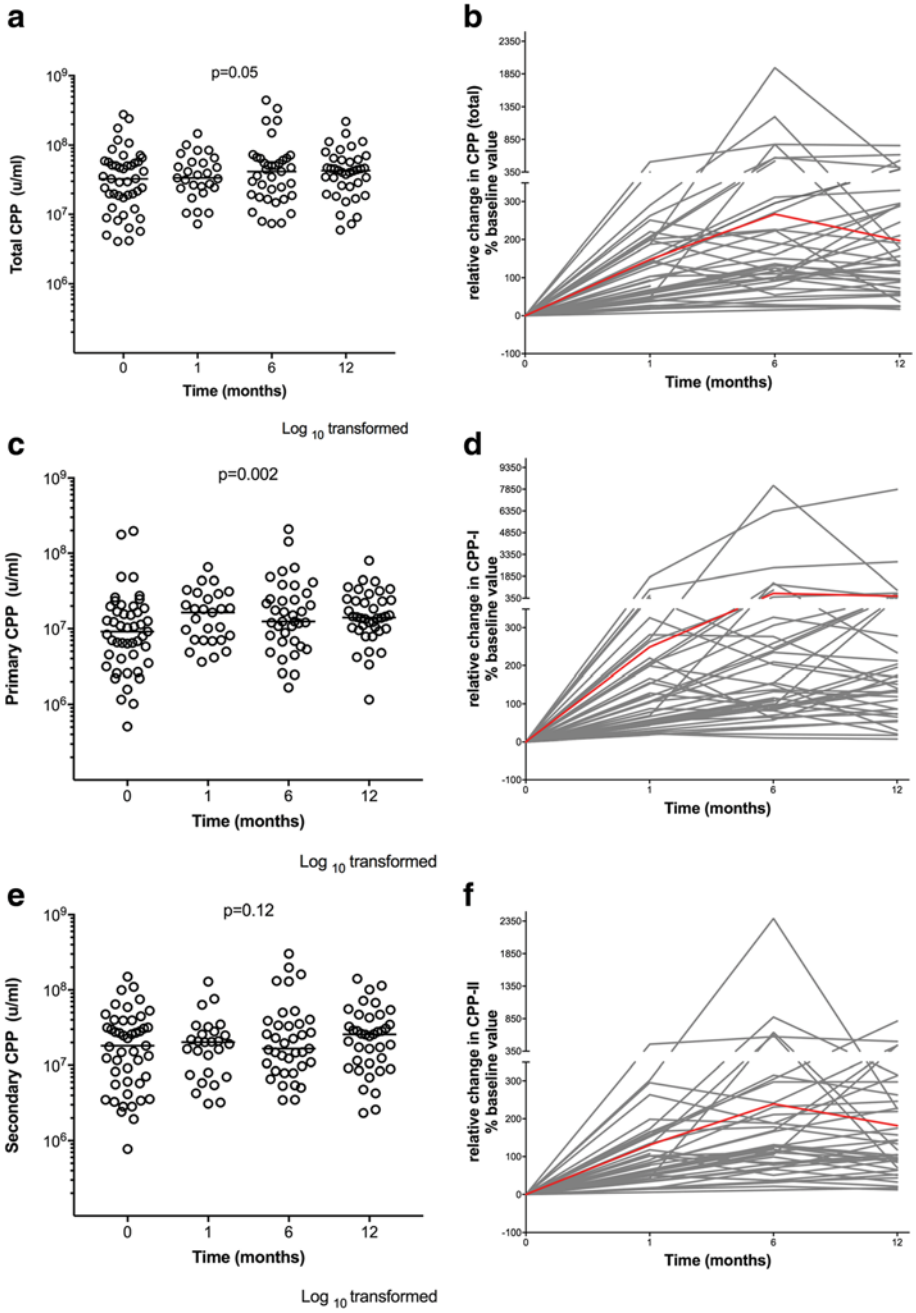

Fig. 4 Changes in calciprotein particles over 12 months following cinacalcet withdrawal. Changes in absolute levels of (**a**) total CPP, (**c**) CPP-I and (**e**) CPP-II over a 12-month time period (median, lower and upper quartile, and outliers). P value denotes trend over 12-month period. Patient-level changes over study period expressed as relative percent change in (**b**) total CPP, (**d**) CPP-I and (**f**) CPP-II from baseline value with mean change highlighted in red over a 12-month period

the serum of adenine-treated rats proceeds the developmental of vascular calcification [28]. CPP have also been identified in multiple other animal models including 5/6 nephrectomized rats, where there was an increase in CPP over a 10-week period of renal failure [14].

CPP have also been identified in humans [16, 20], with higher levels of CPP being predictive of all-cause mortality in CKD patients [17] as well as being associated with greater coronary artery calcification scores in dialysis patients [20] and increased aortic stiffness measured by pulse wave velocity in pre-dialysis CKD patients [18]. In our cinacalcet withdrawal group, we observed a 74% one-year survival rate compared to 80% in our control arm. While these survival rates are in keeping with

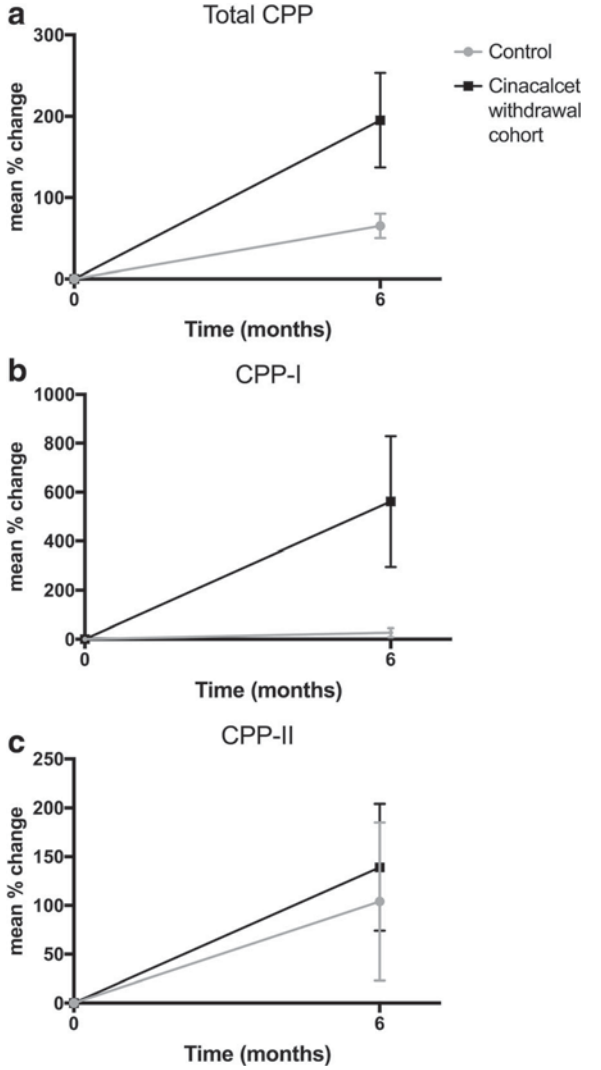

Fig. 5 Mean percentage change and standard deviation in (**a**) total CPP, (**b**) CPP-I and (**c**) CPP-II over a six-month period in control dialysis cohort versus cinacalcet withdrawal patients. $^{a}P < 0.001$, $^{b}P < 0.001$, $^{c}P = 0.24$

the Australian and New Zealand Dialysis and Transplantation Registry (ANZDATA) reported one-year survival for the 65–74 year old age (the median age of our study population) of 87% [29], sample size and short follow-up preclude formal comparison of survival characteristics in these cohorts.

Whilst more research is needed to determine whether increases in CPP, and which subfraction, may be linked to outcome, we were able to show that although CPP increased over time in dialysis patients, the percentage change was significantly greater in cinacalcet withdrawal patients compared to stable cinacalcet naïve patients. We interpret the finding of enhanced CPP-I as indicative of increased mineral flux, potentially resulting from changes in bone turnover following cinacalcet cessation. Indeed, previous studies in rats suggest that bone is a likely source of circulating particles [30]. The strongly correlated changes of CPP-I with PTH and ALP over time, certainly support such a hypothesis. Presently, the potential clinical significance of this marked elevation in CPP-I is uncertain but since CPP-I transform into CPP-II under the influence of the uremic environment, higher levels of CPP-I may eventually engender increased CPP-II levels if persistent. Studies with longer follow-up are therefore needed to assess these effects. This may be important with respect to their putative pathogenicity as in vitro data clearly points to disparate effects of CPP-I and CPP-II on vascular smooth muscle cells [31].

The link between CPP and PTH has also been reported previously [14, 20]. Therapeutic interventions of cinacalcet or parathyroidectomy for the treatment of SHPT were shown to reduce fetuin-A mineral complexes (CPP) in dialysis patients. Our data is the first to report a rise in PTH is strongly associated with an increase in CPP. Most patients in the cinacalcet withdrawal group had a peak in CPP levels at 6 months with a plateau thereafter, which may well reflect attempts to control PTH levels with higher doses of activated vitamin D therapy. Some heterogeneity in the CPP trend was still observed and it is difficult to elicit whether this represents biological variation or the effects of interventions. The observation of a concomitant rise in ALP following cinacalcet withdrawal also contributes to the hypothesis of bone as a possible reservoir for these nanoparticles [20].

There was a reduction in calcium-based phosphate binder use in our study ($p = 0.05$) and a reduction in non-calcium-based phosphate binder use ($p = 0.06$) following cinacalcet withdrawal. One explanation for this may have been the concurrent reduction in calcitriol use following cinacalcet cessation ($p = 0.03$) as a result of rebound hypercalcemia. The reduction in calcitriol use may have contributed to a fall in phosphate absorption allowing for reduction in phosphate binder use, although this was an observed association and the rationale for this association is speculative.

Our study has several limitations and strengths. The novel method of identifying and quantifying CPP provides a greater understanding of their role in the mineralization paradigm. The methodology of CPP analysis in this study is novel and, although currently undertaken only by our team, this methodology is validated and published [15]. The prospective nature of the study and multiple time point measurements was useful in evaluating CPP trends over a 12-month period in the dialysis cohort and has not been performed to date. Unfortunately, samples for the cinacalcet-naïve control and study cohorts were collected and analysed at two different time points and comparison of absolute total CPP counts between these two populations is not possible

due to nature of the analytical technique and inherent changes in laser optics over time. Presently, this limits the use of this assay to a research setting and makes it challenging to generate reference data for these nanoparticles in the dialysis population. However, comparison of relative changes remains valid and perhaps more informative given the order of magnitude of differences in absolute levels between individuals.

As our study is observational in nature, causality of the associations found, although likely, cannot be proven, but is useful in further hypothesis generation. This study was conducted in a single-center dialysis population, and therefore the sample size of the study population was small and likely contributed to the reduced magnitude of associations seen over a 12-month period. Unlike many observational studies that are cross-sectional in nature however, the strength of the current analysis is its longitudinal design allowing us to assess the evolution of changes in CPP and their inter-relationship with other biochemical parameters over time. Importantly despite the small sample size, we show significant temporal effects on CPP subsets and biochemically relevant co-correlations in serial comparisons.

Conclusion

In conclusion, cinacalcet withdrawal in a dialysis population with SHPT was associated with an increase in PTH, serum calcium and CPP-I. Assessment of CPP using the novel fluorescent probe-based flow cytometric assay as described here, may provide further insight into the biochemical abnormalities associated with the CKD-MBD. Longer follow up of this population will allow us to determine if cinacalcet withdrawal is associated with increased rates of parathyroidectomies and higher cardiovascular mortality.

Abbreviations

ALP: Alkaline phosphatase; CKD: Chronic kidney disease; CKD-MBD: Chronic kidney disease mineral bone disorder; CPP: Calciprotein particles; CRP: C-reactive protein; FGF23: Fibroblast growth factor 23; PTH: Parathyroid hormone; SHPT: Secondary hyperparathyroidism

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Availability of data and materials

Comprehensive data presented in manuscript, tables and figures. Additional data available on request from the corresponding author.

Authors' contributions

IR - contributed to data and statistical analysis, data interpretation, critical appraisal of the paper and drafting of the work. ES - contributed to data and statistical analysis, data interpretation, critical appraisal of the paper and drafting of the work. NT - contributed to conception and study design, critical appraisal of the paper and drafting of the work. TH - contributed to critical appraisal of the paper and drafting of the work. SH - contributed to conception and study design, data interpretation, critical appraisal of the paper and drafting of the work. All authors approved final copy of paper.

Ethics approval and consent to participate

Research performed in accordance with the Declaration of Helsinki. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC 2015.180). Written informed consent was obtained from all participants at study commencement.

Competing interests

ERS owns stock in Calciscon. ERS received research funding from Amgen, Baxter and Sanofi. NDT has received honoraria, travel support and research funding from Amgen, Shire and Sanofi. SGH has research funding, honoraria, travel support and from Amgen, Astra-Zenica, Baxter and Sanofi. TDH and IR declares that they have no competing interests.

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APPENDIX E: OUTCOMES OF CINACALCET WITHDRAWAL IN AUSTRALAIN DIALYSIS PATIENTS

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Outcomes of cinacalcet withdrawal in Australian dialysis patients

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Key words
end-stage kidney disease, secondary hyperparathyroidism, dialysis, parathyroid hormone, cinacalcet.

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Abstract

Background: Secondary hyperparathyroidism (SHPT) in chronic kidney disease is associated with cardiovascular and bone pathology. Measures to achieve parathyroid hormone (PTH) target values and control biochemical abnormalities associated with SHPT require complex therapies, and severe SHPT often requires parathyroidectomy or the calcimimetic cinacalcet. In Australia, cinacalcet was publicly funded for dialysis patients from 2009 to 2015 when funding was withdrawn following publication of the EVOLVE study, which resulted in most patients on cinacalcet ceasing therapy. We examined the clinical and biochemical outcomes associated with this change at Australian renal centres.

Aim: To assess changes to biochemical and clinical outcomes in dialysis patients following cessation of cinacalcet.

Methods: We conducted a retrospective study of dialysis patients who ceased cinacalcet after August 2015 in 11 Australian units. Clinical outcomes and changes in biochemical parameters were assessed over a 24- and 12-month period, respectively, from cessation of cinacalcet.

Results: A total of 228 patients was included (17.7% of all dialysis patients from the units). Patients were aged 63 ± 15 years with 182 patients on haemodialysis and 46 on peritoneal dialysis. Over 24 months following cessation of cinacalcet, we observed 26 parathyroidectomies, 3 episodes of calciphylaxis, 8 fractures and 50 deaths. Eight patients recommenced cinacalcet, meeting criteria under a special access scheme. Biochemical changes from baseline to 12 months after cessation included increased levels of serum PTH from 54 (interquartile range 27–90) pmol/L to 85 (interquartile range 41–139) pmol/L ($P < 0.0001$), serum calcium from 2.3 ± 0.2 mmol/L to 2.5 ± 0.1 mmol/L ($P < 0.0001$) and alkaline phosphatase from 123 (92–176) IU/L to 143 (102–197) IU/L ($P < 0.0001$).

Conclusion: Significant increases in serum PTH, calcium and alkaline phosphatase occurred over a 12-month period following withdrawal of cinacalcet. Longer-term follow up will determine if these biochemical and therapeutic changes are associated with altered rates of parathyroidectomies and cardiovascular mortality and morbidity.

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Conflict of interest: I. Ruderman declares she has no competing interests. N. D. Toussaint and G. J. Elder have received honoraria, travel support and research funding from Amgen, Shire and Sanofi. S. G. Holt has research funding, honoraria and travel support from Amgen, Astra-Zenica, Baxter and Sanofi. R. Krishnasamy has received honoraria and travel support from Shire and Amgen.

Introduction

Secondary hyperparathyroidism (SHPT) begins early in the course of chronic kidney disease (CKD) and is characterised by progressive parathyroid gland hyperplasia, excessive parathyroid hormone (PTH) secretion and abnormalities in calcium and phosphate metabolism. As renal function declines SHPT becomes almost ubiquitous and results in bone and endocrine abnormalities, together with extra-osseous calcification. SHPT has been associated with left ventricular dysfunction,[1] myocardial fibrosis,[2] dyslipidaemia,[3] peripheral neuropathy[4] and anaemia as a result of reduced red blood cell survival and bone marrow fibrosis.[5] SHPT is a significant component of the well-recognised broader clinical syndrome encompassing mineral, bone and cardiovascular abnormalities that develop as a complication of CKD – CKD-mineral and bone disorder (CKD-MBD).

Traditional management of SHPT is aimed at controlling phosphate by means of restricting intake, optimising dialysis and administering phosphate binders, as well as modulating calcium balance and suppressing PTH release with activated vitamin D. Despite traditional management, patients with severe and progressive disease may require surgical management with a parathyroidectomy. Although parathyroidectomies improve biochemical parameters and clinical symptoms related to severe SHPT, they are associated with an increased 30-day mortality and risk of re-hospitalisation with hypocalcaemia.[6] Surgical management also may not be suitable for older patients with complex comorbidities, which encompasses a large proportion of dialysis patients in the Western world. In some cases, the effect of parathyroidectomy is also transient, with recurrence in up to 14%.[7]

The only oral calcimimetic available to date is cinacalcet, which provides a medical alternative to parathyroidectomy and acts as a positive allosteric modulator of the calcium-sensing receptor reducing PTH secretion. Cinacalcet reduces PTH[8–11] and, in patients on dialysis, cinacalcet plus low doses of activated vitamin D reduces progression of coronary artery calcification score volume when compared to higher activated vitamin D therapy.[12] In Australia, government reimbursement for cinacalcet was introduced in 2009, but was contingent on proof of efficacy and cost-effectiveness from the pivotal Effect of Cinacalcet on Cardiovascular Disease in Patients Undergoing Dialysis (EVOLVE) trial.[13]

Following publication of EVOLVE, which showed no change in the unadjusted composite primary end-point of cardiovascular mortality and morbidity in patients on cinacalcet, government reimbursement for cinacalcet in Australia was withdrawn in August 2015 due to the lack of evidence of cost-effectiveness. Secondary analyses of the study did report benefits, including reduced cardiovascular mortality in patients over 65 years of age,[14] a reduction in the risk of calciphylaxis[15] and possibly fracture risk.[16] Globally, predictive Markov models were able to demonstrate cost-effectiveness in Italy[17] and Japan,[18] especially for patients unsuitable for parathyroidectomy. Economic analysis in Australia showed benefit in reducing parathyroidectomy rates, as well as clinical benefits in those over 65 years; however, given financial feasibility was not met, funding of this medication was not continued. The hypothesis of our present study was that withdrawal of cinacalcet in dialysis patients with SHPT in Australia may be associated with changes to biochemical and clinical outcomes, including parathyroidectomy rates and prescribing practices.

Methods

Study design

We performed a retrospective observational study involving maintenance dialysis patients who had ceased cinacalcet therapy after August 2015. The aim was to assess the impact on clinical outcomes and biochemical parameters of mineral metabolism after withdrawal from cinacalcet in patients on dialysis. The study protocol was approved by the local ethics committee at Melbourne Health (The Royal Melbourne Hospital). Reciprocal ethics and governance approval was arranged at each site involved in the study.

Study population

Eleven nephrology units across Australia (The Royal Melbourne Hospital, Royal Hobart Hospital, Princess Alexandra Hospital, Sunshine Coast Hospital, Canberra Hospital, Westmead Hospital, Blacktown Hospital, Nepean Hospital, St Vincent's Health, Sir Charles Gairdner Hospital and Western Health) participated in the study. There were no specific exclusion criteria. Each centre provided data regarding patient demographics, changes in prescription practices and biochemical changes over a 12-month period from August 2015. Mortality and morbidity data, including rates of parathyroidectomies, rates of clinically evident vertebral or non-vertebral fractures and episodes of calciphylaxis, were collected at each centre over 24 months following medication cessation.

Study end-points

The primary end-point was change in biochemical outcome measures over a 12-month period. Patients were excluded from final analysis if they underwent a renal

transplant, had a parathyroidectomy or restarted cinacalcet, through an industry-sponsored special access scheme, within 12 months of withdrawal of the medication. Comparison analysis between deceased and non-deceased participants was performed to ensure demographics, clinical outcomes and biochemical characteristics were not confounded.

Biomarker assessment

Biochemical data were obtained from each nephrology unit. All hospitals measure intact PTH, although a variety of testing platforms for PTH are currently used by pathology departments. All patients had measurements over the course of 12-month study period performed at the same centre and on the same platform, which reduced intra-sample variability. There remains some variability between different intact PTH platforms currently available[19]; however, variability between different platforms used across units in different states was unavoidable.

Statistical analysis

All data are summarised and results reported as mean (±standard deviation (SD)) or median (interquartile range (IQR)) for continuous data and number (%) for categorical variables. Paired *t*-test and Wilcoxon-signed rank test were used for between-group comparisons. Categorical variables were analysed with Chi-squared test. Continuous variables were compared with one-way repeated measure analysis of variance if normally distributed or with Freidman test if the distribution was skewed. Two-tailed P values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPSS, Chicago, IL, USA).

Results

Demographics and clinical characteristics

The study included 228 patients in whom cinacalcet had been ceased between August and December 2015. This represents 17.7% of dialysis patients across the 11 units. Baseline characteristics are shown in Table 1. The mean age and median time on dialysis were 63 ± 15 years and 5.7 (IQR 3–8) years respectively. Thirty-seven percent of patients had diabetes, 74% had a history of hypertension and 38% had a history of ischaemic heart disease.

Clinical outcomes

Over a 2-year period, 26 patients underwent parathyroidectomies, 19 of which occurred within the first 12-month period. There were three episodes of calciphylaxis, eight non-vertebral fractures and 50 deaths (26 in the first 12 months and 24 in the subsequent 12 months), with an annual overall mortality rate of 11.0%. Deceased patients were older, with a mean age of 69 ± 11 years ($P = 0.005$); however, the median time on dialysis (6 (IQR 3–7) years, $P = 0.9$) and associated comorbidities including hypertension ($P = 0.70$), diabetes ($P = 0.14$) and ischaemic heart disease ($P = 0.14$) were similar in both cohorts.

Table 1 Patient demographics and clinical characteristics

Demographic	Cinacalcet withdrawal patients (n = 228)
Age, mean ± SD (years)	63 ± 15
Gender (male), *n* (%)	138 (60)
Dialysis modality (haemodialysis), *n* (%)	182 (80)
Time on dialysis, median (IQR) (years)	5.7 (3–8)
Diabetes, *n* (%)	84 (37)
Hypertension, *n* (%)	170 (74)
Ischaemic heart disease, *n* (%)	86 (38)
Peripheral vascular disease, *n* (%)	26 (11)
Events during follow up (24 months)	
Parathyroidectomy, *n* (%)	26 (11)
Non-vertebral fractures, *n* (%)	8 (3.5)
Calciphylaxis, *n* (%)	3 (1.3)
Deaths, *n* (%)	50 (23)

IQR, interquartile range; SD, standard deviation.

Biochemical outcomes

Thirty-five patients were excluded from 12-month analysis of biochemical changes and prescribing practices.

Table 2 Demographics and clinical characteristics of patients who underwent parathyroidectomy and renal transplantation or restarted cinacalcet during the study period

Demographic	Patients excluded from final analysis (n = 35)
Age, mean ± SD (years)	55 ± 13
Gender (male), *n* (%)	20 (57)
Dialysis modality (haemodialysis), *n* (%)	22 (63)
Time on dialysis, median (IQR) (years)	4 (2–7)
Diabetes, *n* (%)	9 (26)
Hypertension, *n* (%)	28 (80)
Ischaemic heart disease, *n* (%)	10 (28)
Peripheral vascular disease, *n* (%)	3 (8)
Baseline medications	
Calcium-based phosphate binder use, *n* (%)	8 (23)
Non-calcium-based phosphate binder use, *n* (%)	23 (66)
Active vitamin D use, *n* (%)	20 (57)
Nutritional vitamin D use, *n* (%)	7 (20)
Erythropoietin-stimulating agent use, *n* (%)	20 (57)

IQR, interquartile range; SD, standard deviation.

Demographics and clinical features of this cohort are described in Table 2. Nineteen underwent parathyroidectomies, eight recommenced cinacalcet therapy, with access to medication through an industry-sponsored special access scheme, and eight received a kidney transplant.

There was an increase in PTH from 54 pmol/L (27–90 pmol/L) to 85 pmol/L (41–139 pmol/L) at 12 months ($P < 0.001$), with the greatest change occurring by 6 months ($P < 0.001$) (Fig. 1). Correspondingly, serum calcium increased from 2.30 ± 0.2 mmol/L to 2.50 ± 0.1 mmol/L ($P < 0.001$), and phosphate remained unchanged over the observation period ($P = 0.84$). There were 54 episodes of hypercalcaemia (serum corrected calcium >2.6 mmol/L) at 6 months, with 31 episodes occurring at 12 months; this was significantly increased from 19 episodes of hypercalcaemia identified at baseline ($P = 0.002$). There was an increase in alkaline phosphatase (ALP) from 123 IU/L (92–176 IU/L) to 143 IU/L (102–197 IU/L) at 12 months ($P < 0.001$). Inflammatory markers, including C-reactive protein and ferritin remained stable over 12 months, and there was a fall in serum albumin from 35.5 ± 4.6 g/L to 34.5 ± 4.5 g/L, $P = 0.01$. Table 3 summarises baseline, 6-month and 12-month values for biochemical outcomes in the study cohort.

Changes in prescribing practices

The median daily dose of cinacalcet prior to medication cessation was 30 mg (30–60 mg). At baseline, 38% of patients ($n = 74$) were on a calcium-based phosphate binder and 66% ($n = 127$) on a non-calcium-based phosphate binder; 26% ($n = 56$) were on two phosphate binders and 21% ($n = 46$) were not taking any phosphate binder. At baseline, active vitamin D use was high at 65% ($n = 125$) and 26% ($n = 51$) were on nutritional vitamin D. By 12 months following cinacalcet withdrawal, phosphate binder use was significantly reduced, with 29% ($n = 52$, $P = 0.01$) on a calcium-based binder and 50% ($n = 97$, $P = 0.008$) on a non-calcium-based binder. Active vitamin D use also reduced by 12 months to 48% ($n = 93$), $P = 0.03$. However, of the patients who remained on active vitamin D their dose was increased from 1.6 ± 0.8 μg/week to 2.2 ± 1.3 μg/week ($P = 0.005$). There was no change in cholecalciferol administration ($P = 0.23$). Table 4 describes prescribing

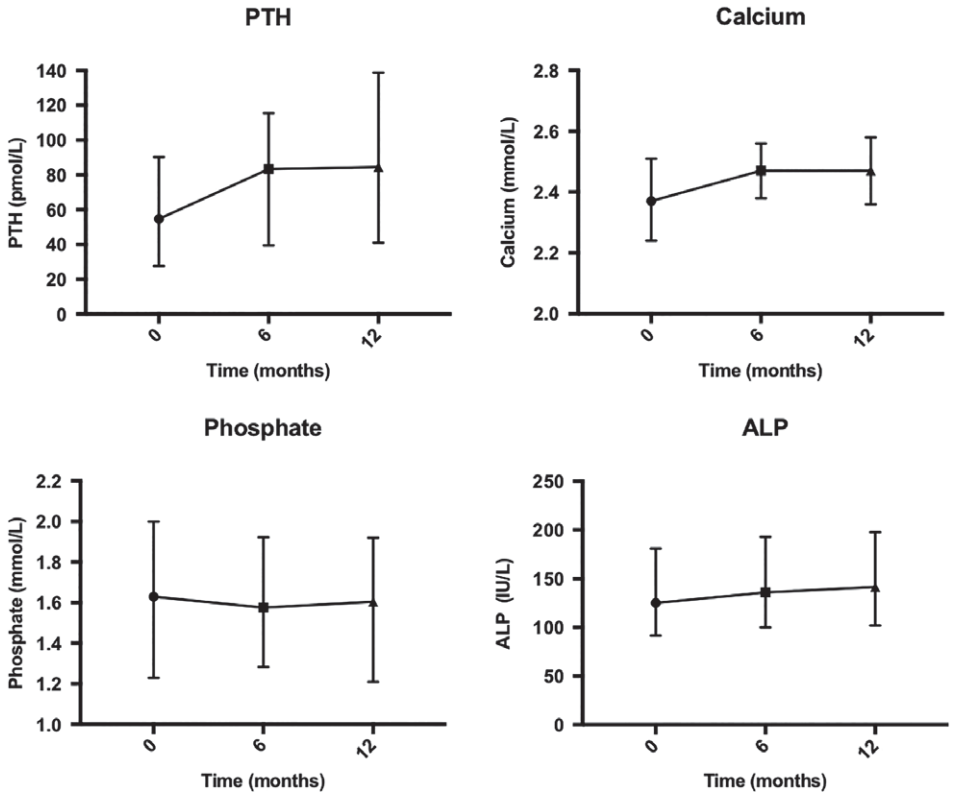

Figure 1 Changes in biochemical mineral markers over 12 months following cinacalcet withdrawal. Changes in (A) parathyroid hormone (PTH), (B) calcium, (C) phosphate and (D) alkaline phosphatase (ALP) over a 12-month period summarised in line graph (median, lower and upper quartile).

Table 3 Biochemical changes over 12-month period following cinacalcet withdrawal

Biochemistry	Baseline (*n* = 193)	6 months (*n* = 177)	12 months (*n* = 167)	*P* value
PTH, median (IQR) (pmol/L)	54 (27–90)	83 (40–115)	85 (41–139)	<0.0001
Calcium, mean ± SD (mmol/L)	2.3 ± 0.2	2.5 ± 0.2	2.5 ± 0.1	<0.0001
Phosphate, mean ± SD (mmol/L)	1.6 ± 0.58	1.6 ± 0.47	1.6 ± 0.57	0.84
ALP, median (IQR) (IU/L)	123 (92–176)	134 (101–184)	143 (102–197)	<0.0001
Albumin, mean ± SD (g/L)	35.5 ± 4.6	35.1 ± 5.0	34.5 ± 4.5	0.010
CRP, median (IQR) (mg/L)	7 (3–16)	7 (3–19)	9.5 (4–29)	0.09
Ferritin, median (IQR) (μg/L)	312 (187–496)	309 (159–524)	367 (180–540)	0.08
Haemoglobin, mean ± SD (g/L)	110 ± 14	111 ± 14	110 ± 117	0.67
Bicarbonate, mean ± SD (mmol/L)	23 ± 3	22 ± 3	23 ± 3	0.42

ALP, alkaline phosphatase; CRP, C-reactive protein; IQR, interquartile range; PTH, parathyroid hormone; SD, standard deviation.

practice changes following cinacalcet withdrawal. There were no significant changes in prescribing practice results with or without death censoring of data. There was no change to time on dialysis following cinacalcet withdrawal or any change in dialysis modality among individuals in the cohort.

Discussion

Our study of 228 dialysis patients from 11 nephrology centres across Australia is the first to show clinical and biochemical outcomes of cinacalcet withdrawal in a large cohort. We report that cinacalcet cessation, when previously used for the management of SHPT, was not associated with increased clinical outcomes after 2 years from withdrawal when compared to reported outcomes in the Australian dialysis population, apart from the rate of parathyroidectomy. Associated biochemical changes, however, included a rise in PTH, calcium and ALP, no corresponding change in serum phosphate and a fall in serum albumin in the first 12 months after medication withdrawal.

Following cinacalcet withdrawal, there was a statistically significant increase in ALP, which may represent increased bone turnover. Recent publications suggest cinacalcet use may improve bone metabolism and is associated with reduced bone turnover markers including bone-specific ALP, osteocalcin and beta-crosslaps.[20] Cunningham *et al*.[21] reported reduced parathyroidectomy, fracture and cardiovascular hospitalisation rates with cinacalcet use in a combined analysis of four clinical trials, and secondary analysis of the EVOLVE trial identified a trend towards reduced rates of fractures with cinacalcet use, particularly in older patients.[16] The fracture rate in our cohort was relatively small with only eight clinically evident fractures during the study period, although the incidence of fracture may have been under-reported by units. Fractures seen in primary care settings and asymptomatic vertebral fractures may have potentially not been included.

With regards to clinical outcomes, 11% of patients underwent a parathyroidectomy within 2 years of medication cessation, equating to an 86/1000 persons-year parathyroidectomy rate. There was no observed increase in calciphylaxis, with three episodes identified in the cohort over the 24-month study period. The mortality rate of the cohort was 11% per year, which is comparable to current published Australian dialysis mortality data for patients in a similar age group.[22] As vascular calcification and associated cardiovascular mortality and morbidity related to SHPT may take many years to develop, this cohort requires further follow up to identify if there are potentially longer-term associations with cinacalcet withdrawal.

Parathyroidectomy rates are not currently recorded in the Australian and New Zealand Dialysis and Transplant registry (ANZDATA), unlike other parameters including cardiovascular disease and cancer. Therefore, this study is limited in being able to identify increased rates of parathyroidectomy since reimbursement of cinacalcet ceased. Certainly, within one centre at The Royal Melbourne Hospital, we have seen a 69% increase in the rate of parathyroidectomies since 2015. Data from Canada following the public formulary listing of cinacalcet showed

Table 4 Changes in prescribing practices

Medications (*n* = 193)	Baseline, *n* (%)	6 months, *n* (%)	12 months, *n* (%)	*P* value
Calcium-based phosphate binders	74 (38)	49 (25)	52 (29)	0.01
Non-calcium-based phosphate binders	127 (66)	112 (58)	97 (50)	0.008
Active vitamin D	125 (65)	112 (58)	93 (48)	0.03
Nutritional vitamin D	51 (26)	33 (17)	37 (19)	0.23
Erythropoietin-stimulating agent use	127 (66)	116 (60)	100 (52)	0.09

a significant decrease in parathyroidectomy rates from 11.4/1000 persons-year to 3.6/1000 persons-year.[23]

Multiple studies have highlighted the short-term risks associated with parathyroidectomies in patients on dialysis.[6,24] A review of clinical outcomes following parathyroidectomy using US Renal Data System (USRDS) data, demonstrated a 2% 30-day mortality and 23.8% re-hospitalisation rate following surgery, together with a 39% increase in hospitalisation rate in the year following parathyroidectomy.[24] Long-term mortality in younger dialysis patients may be better with parathyroidectomy compared with medical management[25]; however, it is difficult to generalise this potential benefit to our study cohort, given the average age in our study cohort was 63 years, with a higher percentage of comorbidities including diabetes and ischaemic heart disease.

Patient preference in regards to treatment of SHPT with cinacalcet versus parathyroidectomy has not been assessed to our knowledge. The impact on quality of life with parathyroidectomy versus cinacalcet has recently been examined in a systematic review of eight studies.[26] Although a direct comparison between the two interventions could not be made, parathyroidectomy was associated with improvement in short- and long-term quality of life, including improvement in itch, joint pain and muscle weakness, whereas no difference was seen in quality of life with the use of cinacalcet. It is possible that reduction in PTH with surgery compared to medical suppression is more beneficial; however, it is unlikely that a head-to-head comparison will ever be undertaken.

Reduced use of activated vitamin D from 65% to 48% was not unexpected, because the use of cinacalcet allows clinicians to target lower PTH values by enabling increased dosing with activated vitamin D with a reduced risk of hypercalcaemia. Nevertheless, there was no associated increase in serum phosphate over a 12-month period after cinacalcet withdrawal, suggesting that cinacalcet alone was insufficient to overcome the biochemical effects of hyperphosphataemia resulting from increased calcitriol use. In fact, one consideration is whether cinacalcet availability emphasises the potential to achieve lower PTH values, leading to higher calcitriol and phosphate binder requirements. As over-suppression of PTH and higher phosphate binder use may have adverse consequences, particularly when calcium-based binders are used, the question arises as to longer-term patient-level benefits of more complex combined therapies that include cinacalcet.

Limitations of our study include its retrospective observational nature and therefore we do not have a control group to compare identified clinical and biochemical changes. Comparing mortality and morbidity data in our cohort to published data in the general Australian dialysis population involves selection bias as we have studied a cohort of individuals previously prescribed cinacalcet based on clinical indication as determined by their treating clinicians. Also, not all units across Australia were involved in data collection and there was a short duration of follow up. Furthermore, we were unable to collect comprehensively all morbidity outcomes, including fracture rates and types of cardiovascular events, as well as changes in dosing of phosphate binders in all study participants, given the study's retrospective nature. Another limitation is that given the variability between different PTH analysis platforms, it is possible we have over- or under-estimated the change in PTH over the study period. Strengths of our study include a large data set across 11 Australian centres with varying geographical locations, which provides a unique insight into the effects of cinacalcet withdrawal, perhaps not possible in many another countries. We have also collected comprehensive national biochemical parameters and parathyroidectomy rates in Australia in the era following withdrawal of cinacalcet reimbursement.

Conclusion

For patients in the Australian dialysis population who withdrew from cinacalcet therapy, median values of PTH exceeded the Kidney Disease Improving Global Outcomes (KDIGO) CKD-mineral and bone disorder guidelines-suggested upper target range of 9 times the upper range of the PTH assay, calcium and ALP values rose and there was no change in serum phosphate. The parathyroidectomy rate was 86/1000 patient-years over the first 24 months from withdrawal. Longer-term follow up will allow greater insight into associated future cardiovascular morbidity, mortality and fracture rates.

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APPENDIX F: BONE MICROARCHITECTURE IN PATIENTS UNDERGOING PARATHYROIDECTOMY FOR MANAGEMENT OF SECONDARY HYPERPARATHYROIDISM

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Bone microarchitecture in patients undergoing parathyroidectomy for management of secondary hyperparathyroidism

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ABSTRACT

Background: Secondary hyperparathyroidism (SHPT) in patients with chronic kidney disease (CKD) leads to complex bone disease, affecting both trabecular and cortical bone, and increased fracture risk. Optimal assessment of bone in patients with CKD is yet to be determined. High-resolution magnetic resonance imaging (MRI) can provide three-dimensional assessment of bone microarchitecture, as well as determination of mechanical strength with finite element analysis (FEA).

Methods: We conducted a single-centre, cross-sectional study to determine bone microarchitecture with MRI in CKD patients with SHPT undergoing parathyroidectomy. Within two weeks of surgery, MRI was performed at the distal tibia and biochemical markers of SHPT (parathyroid hormone [PTH] and alkaline phosphatase [ALP]) were collected. Trabecular and cortical topological parameters as well as bone mechanical competence using FEA were assessed. Correlation of MRI findings of bone was made with biochemical markers.

Results: Twenty patients with CKD (15 male, 5 female) underwent MRI at the time of parathyroidectomy (16 on dialysis, 3 with functioning kidney transplant, one pre-dialysis with CKD stage 5). Median PTH at the time of surgery was 138.5 pmol/L [39.6–186.7 pmol/L]. MRI parameters in patients were consistent with trabecular deterioration, with erosion index (EI) 1.01 ± 0.3, and trabecular bone volume (BV/TV) 10.8 ± 2.9%, as well as poor trabecular network integrity with surface-to-curve ratio (S/C) 5.4 ± 2.3. There was also evidence of reduced cortical thickness, with CTh 2.698 ± 0.630 mm, and FEA demonstrated overall poor bone mechanical strength with mean elastic modulus of 2.07 ± 0.44.

Conclusion: Patients with severe SHPT requiring parathyroidectomy have evidence of significant changes in bone microarchitecture with trabecular deterioration, low trabecular and cortical bone volume, and reduced mechanical competence of bone.

1. Introduction

Bone abnormalities seen in patients with chronic kidney disease (CKD) complicated by secondary hyperparathyroidism (SHPT) result in impaired bone quality and quantity, affecting both trabecular and cortical bone compartments. SHPT leads to abnormal trabecular connectivity, cortical thinning, and decreased bone mineral density (BMD) (Amling et al., 1994; Parfitt, 1998) which increase fracture risk. Fractures are significantly more prevalent in patients with CKD and SHPT and are associated with increased hospitalisations, mortality and morbidity (Alem et al., 2000).

Assessment of bone quality is challenging in patients with CKD as standard serological markers of bone turnover do not reliably distinguish between different renal bone pathologies and, in addition, commonly used biomarkers can have assay limitations and inconsistent results in the CKD population (Coco and Rush, 2000; Sprague et al., 2016; Danese et al., 2006). Dual-energy X-ray absorptiometry (DXA) is widely used in the general population to diagnose and monitor low BMD as well as provide fracture risk assessment (Kanis, 2002). In the general population there is a strong association between low areal BMD

Abbreviations: MRI, magnetic resonance imaging; HRpQCT, high-resolution peripheral quantitative computed tomography; DXA, dual-energy X-ray absorptiometry; BMD, bone mineral density; ALP, alkaline phosphatase; CKD, chronic kidney disease; PTH, parathyroid hormone; SHPT, secondary hyperparathyroidism

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and increased risk of fracture (Marshall et al., 1996). However, DXA has significant limitations in the CKD population and can potentially overestimate vertebral BMD due to the presence of overlying aortic calcification. In recent years, several prospective cohort studies have demonstrated utility of DXA in assessing fracture risk in patients with CKD stages 3–5 (Yenchek et al., 2012; West et al., 2015; Iimori et al., 2011) and DXA is now recommended in the updated 2017 Kidney Disease Improving Global Outcomes (KDIGO) clinical guidelines to determine BMD and evaluate osteoporosis or renal bone disease in patients with CKD and those on dialysis (Ketteler et al., 2017). DXA, however, provides poor distinction between cortical and trabecular bone, as it is a two-dimensional (2D) assessment of BMD.

Bone biopsy remains the gold standard for adequately diagnosing renal bone disease but is rarely performed as biopsy acquisition is invasive, challenging to process and analyse, and fails to permit longitudinal measurements of bone structure at the same location. Bone biopsy is often performed at the iliac crest, a region predominantly composed of trabecular bone with low load and low fracture prevalence. SHPT is associated with loss of predominantly cortical bone in long bones (Osima et al., 2018), which are the commonest site of fracture, therefore bone biopsy of the iliac crest may not provide adequate prognostic information on clinical outcomes. Optimal assessment of renal bone disease is yet to be determined, however new imaging methods may be able to quantitatively assess and monitor bone fragility *in vivo*, including high-resolution magnetic resonance imaging (MRI, or micro-MRI) and high-resolution peripheral quantitative computed tomography (HR-pQCT).

Micro-MRI is a non-invasive technique that provides three-dimensional (3D) assessment of bone and can be used for repeat monitoring without exposure to ionising radiation as with HR-pQCT. Micro-MRI of bone trabecular architecture was first described 20 years ago (Jara et al., 1993) and since then technical advances in image acquisition and analysis have seen its performance rival that of HR-pQCT. Micro-MRI provides high-resolution imaging of bone allowing the evaluation of both cortical and trabecular properties at a scale of 100–200 μm (in-plane resolution) (Singh et al., 2018). Assessment of bone volume fraction and bone topology correlates well with equivalent computed tomography (CT) measurements (Krug et al., 2008).

In addition to analysis of bone microarchitecture, micro-MRI images can also be used for finite element analysis (FEA), which provides a more direct assessment of the mechanical competence of bone. FEA is based on mechanical engineering technology which facilitates virtual stress testing of bone to compute metrics of bone mechanical competence, specifically providing information on bone stiffness and elastic modulus (Chang et al., 2014). In the CKD population, micro-MRI has been used to study bone microarchitecture in patients on dialysis and those pre- and post-kidney transplantation (Wehrli et al., 2004; Sharma et al., 2018; Rajapakse et al., 2012; Link et al., 2002; Leonard et al., 2019; Rajapakse et al., 2017). In a group of 17 patients on haemodialysis (HD), micro-MRI was used to quantify trabecular and cortical structural parameters, which are traditionally evaluated with bone biopsy (Wehrli et al., 2004), compared to an age- and sex-matched control group and results were consistent with increased bone fragility in HD patients compared to the control group.

To date, micro-MRI to evaluate bone microarchitecture associated with severe SHPT in patients prior to parathyroidectomy has not been reported. A previous study using DXA in a cohort of patients following parathyroidectomy, demonstrated increased BMD post-surgery (Fang et al., 2018), however this was based on retrospective data over a four-year period. HR-pQCT has been evaluated in patients with primary hyperparathyroidism undergoing a parathyroidectomy with studies reporting significant improvement in total, cortical, and trabecular volumetric bone density as early as 6 months post parathyroidectomy, as well as improved FEA stiffness and failure load (Hansen et al., 2012). Micro-MRI in patients with SHPT undergoing surgery may provide more detailed information regarding bone microarchitecture and bone strength compared to other current imaging techniques. This imaging technology has the capacity to distinguish patients who have evidence of bone disease related to high PTH and those who may not have significant structural bone disease despite high PTH. This information could be valuable to determine who may benefit from parathyroidectomy or who may not, or even who might potentially suffer adverse consequences from parathyroidectomy with risk of developing adynamic bone disease. Static MRI parameters, unlike bone biopsy, provide a representation of the cumulative impact on bone structure due to changes in bone turnover over time.

The landscape of management for SHPT in Australia significantly changed in August 2015 following withdrawal of government reimbursement for the calcimimetic agent cinacalcet. Over the past five years there has been an increase in the number of patients with severe and progressive SHPT requiring surgical management with a parathyroidectomy, as medical treatment with cinacalcet was not as readily available and other therapies for SHPT, such as calcitriol, are not always effective at suppressing PTH levels. At our institution, the number of parathyroidectomies performed doubled from 2015 to 2019 following withdrawal of funding for cinacalcet. Establishing a non-invasive method to evaluate and monitor underlying bone pathology in SHPT is essential and will assist in future management of this challenging problem, particularly in comparing the effectiveness of medical *versus* surgical SHPT treatment. The aim of this study was to determine micro-MRI parameters of bone microarchitecture in patients with SHPT undergoing parathyroidectomy and correlate MRI findings of bone disease with biochemical markers of SHPT at the time of surgery.

2. Methods

2.1. Study participants

Patients with CKD undergoing planned surgical parathyroidectomy for SHPT at The Royal Melbourne Hospital between January 2019 and February 2020 were approached to participate in this single-centre, cross-sectional study. Patients over the age of 18 and able to provide informed consent with no contraindication to MRI were included in the study. A detailed medical history and MRI safety questionnaire was recorded at pre-admission clinic prior to MRI scanning. The study was approved by the Melbourne Health Human Research Ethics Committee (#HREC2019.029) and was conducted in accordance with the Declaration of Helsinki.

2.2. MRI

Micro-MRI of the distal tibia was performed within two weeks of parathyroidectomy with a commercial 3.0-Tesla whole-body imager (Siemens Trio, Erlangen, Germany). Participants were imaged in a feet-first prone position. MRI acquisitions were performed at the distal tibial metaphysis using a 3D-turbo spin echo pulse sequence (Flip angle 180, repetition time/echo time 53/16 ms, field-of-view 70 mm × 70 mm, voxel size 0.273 × 0.273 × 0.6 mm^3, 12 signal averages, and echo train length of two). This sequence is commercially available on clinical MRI systems with a scan time of 12 min. The MRI scan was undertaken utilising a commercially available 15 channel transmit receive knee coil (Siemens, Erlangen, Germany). The centre of the coil was positioned so that images were obtained 1 cm proximal to the midpoint of the medial malleolus.

Raw MRI data was pre-processed *via* bone volume fraction (BVF) mapping and segmentation of the bone into trabecular and cortical compartments for structural measurements and topological analysis using published algorithms (Rajapakse et al., 2012; Wehrli, 2007). Data analysis was performed by two study investigators (CR, AO) offsite at the University of Pennsylvania, PA, USA. Segmentation was determined by delineating the periosteal and endosteal boundaries of the bone with an operator-guided semi-automatic algorithm using custom-built

Table 1
Description of MRI parameters evaluated in this study.

MRI parameter	Description
Surface to curve ratio (S/C)	Indicates the plate (surface) to rod (curve) ratio of the trabeculae. A higher ratio is a marker of greater trabecular network integrity (Rajapakse et al., 2014).
Erosion index (EI)	Represents topological parameters expected to increase *versus* those expected to decrease, with erosion cause by osteoclastic resorption. A higher index is consistent with greater trabecular deterioration (Rajapakse et al., 2014).
Bone volume/total volume (BV/TV) (%)	Represents the ratio of bone volume to total volume in the region of interest.
Trabecular thickness (TbTh) (mm)	Represents mean thickness of trabeculae
Trabecular number (TbN) (1/mm)	Represents mean number of trabecular per unit length
Trabecular separation (TbS) (mm)	Represents the bone marrow space between trabeculae
Cortical thickness (CTh) (mm)	Represents mean cortical bone thickness
Elastic modulus (GPa)	Main component of FEA and a marker of bone mechanical competence

Table 2
Demographics and clinical characteristics.

Demographics	Participants (n = 20)
Age, years	48 ± 12
Gender, male	15
Stage of CKD and/or dialysis modality	
- CKD stage 5 (non-dialysis)	1
- HD (satellite)	9
- HD (home)	2
- PD	5
- Kidney transplant recipient	3
Time on dialysis, years (n = 16)	2.9 [1–5]
Aetiology of CKD	
- Diabetic nephropathy	2
- Glomerulonephritis	9
- Reflux nephropathy	4
- Other	5
Co-morbidities	
- Previous parathyroidectomy	1
- Previous fracture	3
- Cinacalcet use in past 6 months	0
- Diabetes mellitus	4
- Cardiovascular disease	5
- Hypertension	13
- History of osteoporosis	1
- Failed transplant	6
- Smoker/ex-smoker	6

Data presented as number, mean ± standard deviation or median [interquartile range].
Abbreviations: CKD, chronic kidney disease; HD, haemodialysis; PD, peritoneal dialysis;

software (Rajapakse et al., 2012) and standard microarchitecture parameters were derived for trabecular and cortical bone. Table 1 outlines MRI parameters evaluated in this study.

2.3. FEA

FEA allows for *in vivo* estimation of bone mechanical properties. Three-dimensional micro-finite element models generated from acquired images of the distal tibia were subjected to stimulated loading in inferior-superior direction, to compute the elastic modulus of the entire cross-section of the bone (Chang et al., 2017; Rajapakse et al., 2009; Rajapakse et al., 2014; Rajapakse et al., 2010). This data can be interpreted as bone mechanical competence or strength – the higher the elastic modulus, the greater the strength of the bone.

2.4. Biochemical measurements

Serum was collected in all patients prior to parathyroidectomy (within two weeks of surgery). Samples were collected to measure the following parameters: PTH, calcium, phosphate, alkaline phosphatase (ALP), C-reactive protein (CRP), and albumin. Serum calcium level was adjusted as follows if serum albumin was < 40 g/L: corrected serum calcium (mmol/L) = measured serum calcium (mmol/L) + 0.02 (40 - serum albumin (g/L)).

2.5. Statistical analysis

Results are presented as mean (± standard deviation) for normally distributed variables and as median (and interquartile range) for variables with non-parametric distribution. Relationships were studied using Pearson or Spearman correlation, depending on the distribution of variables. Two-tailed p values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0 for Macintosh (SPSS, Chicago, IL). Graphics were created with GraphPad Prism 8 for Macintosh (La Jolla, CA, USA).

3. Results

3.1. Demographics and biochemical outcomes

Twenty-three patients underwent surgical parathyroidectomy at The Royal Melbourne Hospital from January 2019 to February 2020. All patients were approached to participate in the study. Three patients were excluded due to MRI being contraindicated (claustrophobia, n = 2; foreign material on orbital X-ray, n = 1). Twenty patients were enrolled in the study, with micro-MRI performed within two weeks of surgery (2.5 [1.3–4] days). Sixteen patients were on dialysis, three patients had a functioning kidney transplant (mean time post-transplant 14 ± 5 months) and one patient was pre-dialysis with CKD stage 5. Mean serum creatinine for patients with a kidney transplant was 90 ± 35 μmol/L and the patient with CKD stage 5 had a serum creatinine of 560 μmol/L. All patients had biochemical evidence of SHPT and were therefore included in the study to maximise study sample size. Participant demographics are outlined in Table 2 and biochemistry outlined in Table 3.

3.2. MRI findings

MRI of participants showed trabecular bone volume (BV/TV) of 10.8 ± 2.9% and surface-to-curve ratio (S/C) of 5.4 ± 2.3.

Table 3
Biochemistry at time of parathyroidectomy.

Biochemistry	Participants (n = 20)	Normal range
PTH, pmol/L	138.5 [39.6–186.7]	1.7–10.0
Adjusted calcium, mmol/L	2.5 ± 0.2	2.1–2.6
Phosphate, mmol/L	1.7 ± 0.6	0.75–1.50
ALP, IU/L	176 [103–274]	30–120
CRP mg/L	2 [1–5]	< 3
Albumin g/L	31 ± 1.5	32–45

Data presented as mean ± standard deviation or median [interquartile range].
Abbreviations: ALP, alkaline phosphatase; CRP, C-reactive protein; PTH, parathyroid hormone.

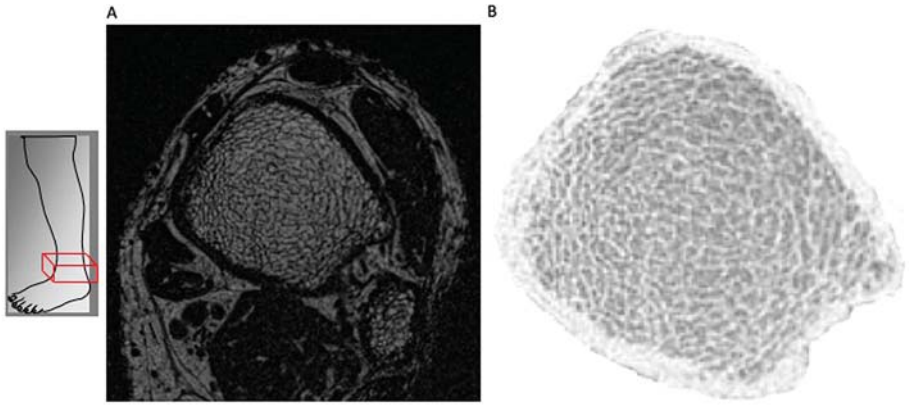

Fig. 1. A: Anatomic site of MRI image of distal tibia and fibula. B: High-resolution MRI image through distal tibia showing trabecular and cortical microarchitecture.

Table 4
MRI parameters in patients with SHPT at time of parathyroidectomy.

MRI parameter	All patients (n = 20)	CKD stage 5 patients (n = 17; dialysis n = 16, non-dialysis, n = 1)	Renal transplant patients (n = 3)	p value
S/C	5.4 ± 2.3	5.3 ± 2.4	5.2 ± 1.5	0.96
EI	1.01 ± 0.3	1.02 ± 0.3	0.98 ± 0.27	0.93
BV/TV (%)	10.8 ± 2.9	10.9 ± 3	10.4 ± 2.7	0.88
TbTh (mm)	0.13 ± 0.007	0.128 ± 0.007	0.127 ± 0.006	0.86
TbN (1/mm)	0.84 ± 0.17	0.84 ± 0.16	0.81 ± 0.17	0.89
TbS (mm)	1.11 ± 0.22	1.09 ± 0.2	1.16 ± 0.3	0.89
CTh (mm)	2.7 ± 0.63	2.8 + 0.63	2.3 ± 0.5	0.46

Data presented as mean ± standard deviation.
Abbreviations: S/C, surface to curve ratio; EI, erosion index; BV/TV (%), trabecular bone volume; TbTh (mm), trabecular thickness; TbN (1/mm), trabecular number; TbS (mm), trabecular separation; CTh (mm), cortical thickness.

Table 5
MRI parameters in patients with SHPT at time of parathyroidectomy based on gender.

MRI parameter	Male (n = 15)	Female (n = 5)	p value
S/C	5.8 ± 2.4	3.9 ± 0.77	0.06
EI	0.9 ± 0.3	1.2 ± 0.26	0.14
BV/TV (%)	11.5 ± 3	9 ± 21.1	**0.04**
TbTh (mm)	0.13 ± 0.008	0.12 ± 0.002	**0.01**
TbN (1/mm)	0.88 ± 0.17	0.72 ± 0.08	0.08
TbS (mm)	1.05 ± 0.2	1.3 ± 0.2	0.08
CTh (mm)	2.8 ± 0.66	2.3 ± 0.3	0.17
Elastic modulus (GPa)	2.04 ± 0.43	2.12 ± 0.47	0.8

Data presented as mean ± standard deviation.
Abbreviations: S/C, surface to curve ratio; EI, erosion index; BV/TV (%), trabecular bone volume; TbTh (mm), trabecular thickness; TbN (1/mm), trabecular number; TbS (mm), trabecular separation; CTh (mm), cortical thickness. Bold data indicate significance at p value < 0.05.

Representative MRI images illustrating trabecular and cortical compartments of a study participant are shown in Fig. 1. Mean values of trabecular and cortical structural parameters assessed at the distal tibia by MRI in the whole cohort, as well as divided into combined dialysis patients and pre-dialysis CKD stage 5 patient (n = 17) compared with patients with a functioning kidney transplant (n = 3), and by gender (15 male, 5 female), are presented in Tables 4 and 5 respectively. There were no statistically significant differences in trabecular or cortical parameters between CKD stage 5 patients (dialysis and non-dialysis) compared to patients with a kidney transplant. Female patients with SHPT had lower trabecular bone volume (BV/TV, 9 ± 21.1% *versus* 11.5 ± 3%, p = 0.04) and trabecular thickness (TbTh, 0.12 ± 0.002 mm *versus* 0.13 ± 0.008 mm, p = 0.01) compared to male patients, there was no difference in cortical thickness between genders (p = 0.17). Table 6 outlines MRI parameters at the distal tibia in previously published cohorts compared to our current study cohort. Micro-MRI remains an emerging imaging modality therefore no normal reference ranges for the distal tibia exist at present. As a result, micro-MRI parameters from two publications with non-CKD patients (Wehrli et al., 2004; Benito et al., 2003) are used as predicted normal ranges.

3.3. Correlation between biochemical variables and structural MRI parameters

There was a weak correlation between ALP and trabecular parameters specifically BV/TV (r = 0.5, p = 0.025) and TbTh (r = 0.5, p = 0.026) (Fig. 2). Unlike previous published literature by Sharma et al. (2018), which showed that PTH correlated with both S/C and erosion index (EI), in our study there was only a correlation between PTH and cortical thickness (r = 0.46, p = 0.04) (Fig. 2). Spearman correlations are outlined in Table 7.

3.4. FEA results

Elastic modulus in the study cohort ranged between 1.31 and 2.80 (mean 2.07 ± 0.44). Two patients with a history of calcaneal and neck of femur fractures had low elastic modulus of 1.31 and 1.65 respectively. There was a negative correlation between age and elastic modulus (r = −0.48, p = 0.03), and there was no correlation between dialysis vintage or gender and elastic modulus.

4. Discussion

This study examines bone microarchitecture of the distal tibia in patients with CKD and SHPT at the time of parathyroidectomy using the novel imaging technique of micro-MRI. Key findings include significant trabecular topological abnormalities with low S/C ratio, BV/TV and elevated EI consistent with greater trabecular deterioration and lower

Table 6
Comparison of demographic and distal tibial MRI parameters to published studies.

Parameter	Study cohort (n = 20)	Benito et al. (2003) (n = 10)	Benito et al. (2003) (n = 10)	Sharma et al. (2018) (n = 14)	Wehrli et al. (2004) (n = 17)	Wehrli et al. (2004) (n = 17)
Population	Dialysis patients with SHPT	Eugonadal men	Hypogonadal men	CKD pre kidney transplant	HD patients	Control group
Age	48 ± 12	53.7 ± 13.2	53.1 ± 13.4	46 ± 11.3	40.3 ± 6.4	40.2 ± 6.7
Gender, male	15 (75%)	10 (100%)	10 (100%)	9 (62.5%)	9 (53%)	9 (53%)
CKD stage	CKD5 on dialysis	No CKD	No CKD	CKD pre transplant	CKD5 on dialysis	No CKD
S/C	5.4 ± 2.3	10.8 ± 2.4	6.9 ± 1.8	5.51 ± 1.36	5.6 ± 1.5	6.4 ± 0.6
EI	1.01 ± 0.3	0.89 ± 0.13	1.21 ± 0.24	0.91 ± 0.25	1.17 ± 0.45	0.97 ± 0.21
BV/TV (%)	10.8 ± 2.9	14.3 ± 1.1	12 ± 1.6	10.4 ± 2.2	12 ± 2.2	13.3 ± 2
TbTh (mm)	0.128 ± 0.007			0.126 ± 0.005	0.151 ± 0.019	0.156 ± 0.013
TbN (1/mm)	0.84 ± 0.17			0.82 ± 0.14		
TbS (mm)	1.107 ± 0.219			1.120 ± 0.023		
CTh (mm)	2.698 ± 0.630			2.632 ± 0.545		

Abbreviations: S/C, surface to curve ratio; EI, erosion index; BV/TV (%), trabecular bone volume; TbTh (mm), trabecular thickness; TbN (1/mm), trabecular number; TbS (mm), trabecular separation; CTh (mm), cortical thickness; CKD, chronic kidney disease.

trabecular integrity and bone volume in patients with SHPT compared to published cohorts of individuals with no kidney disease. There was also evidence of reduced cortical thickness and low elastic modulus in keeping with poor bone mechanical competence. Although there were statistically significant correlations with ALP, PTH and some trabecular and cortical microarchitecture parameters, these associations were weak and highlight the imprecision of using bone turnover markers alone to identify and monitor renal bone disease.

Renal osteodystrophy is commonly seen in patients with CKD stage 5 on HD (Trombetti et al., 2013), with bone abnormalities contributing to increased fracture risk (Yenchek et al., 2012) as well as an associated increased risk of vascular calcification (Moldovan et al., 2011). The majority of studies evaluating BMD and fracture in patients with CKD have been performed using DXA or HR-pQCT. West et al. demonstrated in pre-dialysis CKD patients that hip BMD, calculated by either DXA or HR-pQCT, was significantly lower in those with a history of incident fractures compared to those without (Yenchek et al., 2012). A major concern with DXA in the CKD population is the inability to accurately analyse bone microstructure. Differentiation between cortical and trabecular bone is very challenging and can be influenced by artefactual vascular calcification projections particularly at the lumbar spine.

Alterations of bone microarchitecture contribute to skeletal fragility, independent of areal BMD, and are equally important to evaluate fracture risk (Boutroy et al., 2005; Sornay-Rendu et al., 2007). Negri et al. imaged the distal radius and tibia with HR-pQCT in patients on HD and showed that significantly deceased cortical and trabecular parameters correlated with severity of SHPT in women (Negri et al., 2012). Of note, the medium serum intact PTH was 63 pmol/L [4.3–295.5] in this group, which is much lower than the median serum PTH in our current study participants (138.5 pmol/L [39.6–186.7]). Elevated PTH has previously been associated with reduced cortical volumetric BMD, increased cortical porosity and reduced cortical thickness (Osima et al., 2018). Our study found a weak positive correlation between PTH and cortical thickness (r = 0.46, p = 0.04). This was a surprising finding as we would have expected to see a negative association between PTH and cortical thickness, and larger studies using micro-MRI are required to explore this association further. Although PTH is routinely used to monitor SHPT in patients with CKD, it is not a maker of bone turnover *per se*, as it does not accurately reflect osteoblastic or osteoclastic action in the bone. Additionally, not all circulating PTH in patients with advanced CKD is biologically active, with a proportion of PTH undergoing oxidation thereby losing biological activity (Hocher et al., 2013), and circulating PTH has an extremely short half-life (minutes), therefore PTH levels fluctuate significantly and a trend rather than a single value is more useful to monitor SHPT progression. PTH alone is also insufficient to differentiate the type of renal bone disease present, as suggested by the weak correlations in the study cohort.

Similar findings to our study were identified in a study of 74 patients on HD (mean PTH 355 pg/mL), with particularly female dialysis

Table 7
Spearman correlations (r) between trabecular and cortical microarchitecture parameters (determined by MRI) and bone turnover markers.

MRI parameter	ALP	PTH
S/C	0.34	0.23
EI	−0.32	−0.12
BV/TV (%)	0.5*	0.27
TbTh (mm)	0.5*	0.32
TbN (1/mm)	0.44	0.22
TbS (mm)	−0.44	−0.22
CTh (mm)	0.1	0.46*

Abbreviations: S/C, surface to curve ratio; EI, erosion index; BV/TV (%), trabecular bone volume; TbTh (mm), trabecular thickness; TbN (1/mm), trabecular number; TbS (mm), trabecular separation; CTh (mm), cortical thickness.
* $p \leq 0.05$.

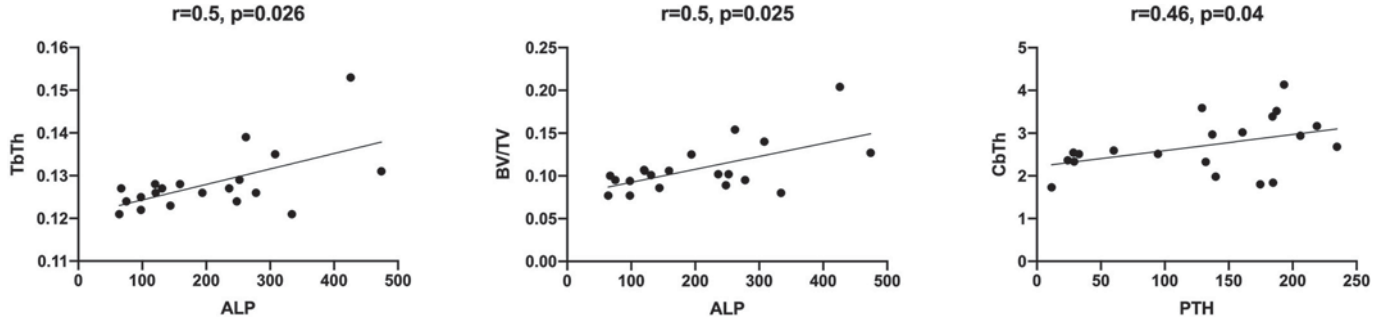

Fig. 2. Spearman correlations (r) between trabecular and cortical topological parameters derived by MRI and bone turnover markers (PTH and ALP).

patients having markedly impaired bone microarchitecture when assessed by HR-pQCT compared to a gender- and race-matched healthy control group (Cejka et al., 2011). Addition of FEA to bone microarchitecture analysis of the distal radius and tibia by HR-pQCT was evaluated by Trombetti et al. (2013) in patients on HD and findings were consistent with previous studies. Addition of FEA, unsurprisingly, showed that stiffness and predicted load failure were lower at both the distal radius and tibia in women with CKD but not in men, compared with age-matched controls.

Few studies have assessed the utility of micro-MRI in evaluating bone microarchitecture in CKD patients. Sharma et al. (2018) compared bone microarchitecture assessment using DXA, pQCT and micro-MRI with 'gold standard' bone biopsy in 14 patients with CKD prior to undergoing surgery (13 kidney transplantation, one parathyroidectomy). Micro-MRI changes in this study correlated well with bone histomorphometry and DXA, with significant correlations observed between histomorphometric mineralisation and turnover indices and various MRI parameters. MRI-derived trabecular parameters were also significantly related to femoral neck BMD. Importantly, micro-MRI parameters were comparable to results in our study and corroborate findings of severe renal osteodystrophy in the current cohort, both in dialysis patients and a small sample of kidney transplant recipients, with bone loss and deranged microarchitecture in relatively young and mostly male patients. Another study of high-resolution MRI in patients with CKD was performed by Link et al. (2002), investigating structural measures of the calcaneus compared to BMD in the lumbar spine using DXA in 60 kidney transplant recipients, pre- and post-transplantation. This study reported significantly lower BV/TV, TbN, TbTh and a higher TbS, together with significantly lower BMD of the lumbar spine in patients with prevalent fractures compared to those without.

Micro-MRI has emerged as a novel tool to accurately and safely assess both trabecular and cortical bone without risk of radiation exposure. The ability to incorporate FEA provides useful information regarding bone strength which could be used to monitor treatment effect. Micro-MRI was used in a cohort of hypogonadal and eugonadal males to assess trabecular architecture of the distal tibia (Benito et al., 2003). Benito et al. showed that the S/C ratio was 36% lower ($p = 0.004$) and the EI was 36% higher ($p = 0.003$) in hypogonadal men, suggesting that male hypogonadism was associated with marked deterioration of trabecular architecture. When comparing these results to male patients in the current study, our patients with severe SHPT have lower S/C ratio and BV/TV than both hypogonadal and eugonadal males with normal kidney function. Testosterone levels were not measured in the study population, although it is likely that a significant proportion of male patients would have hypogonadism given the prevalence of this endocrinological issue in kidney disease is reported to be greater than 50% (Albaaj et al., 2006; Gungor et al., 2010).

Peak BMD or BV/TV is a genetic predisposition influenced by multiple environmental factors and women typically have a lower peak BV/TV compared to males. In an exclusively female population, micro-MRI was used to assess trabecular structural bone parameters of the distal radius in a postmenopausal osteopaenic cohort pre- and post-treatment with bisphosphonates (Folkesson et al., 2011; Greenspan et al., 2010). To date, however, there is no published literature of bone microarchitecture parameters in premenopausal women with normal kidney function. In our study there were statistically significant reductions in both BV/TV and TbTh in females compared to males with SHPT, despite no significant differences in age or dialysis vintage.

Similar to the study by Benito et al., FEA analysis of the distal tibia with micro-MRI was performed in another cohort of eugonadal and hypogonadal males to assess the effects of testosterone treatment. Zhang et al. (2008) showed improved elastic moduli of the tibial trabecular bone by increased trabecular plate thickness following testosterone therapy in hypogonadal males. These studies highlight the usefulness of FEA derived from micro-MRI for monitoring treatment response. FEA using specialised MRI algorithms have been validated for the distal tibia (Rajapakse et al., 2018) and more recently for the femur (Rajapakse and Chang, 2018; Rajapakse et al., 2020). FEA of the femur is especially useful for evaluating fracture risk in the hip as it can simulate forces experienced during a sideways fall, which accounts for the main orientation in which hip fractures occur (Keyak et al., 2011). Using FEA derived from micro-MRI images in patients with SHPT could enable targeted therapies to be comprehensively evaluated with the effect on bone microarchitecture, and ultimately risk of fracture, established.

There are some limitations to our study including the small sample size and cross-sectional nature of the study which does not allow assessment of bone changes over time and evaluation as to whether surgical treatment may lead to improvement in bone microarchitecture. A 12-month follow up study is planned for our cohort of study patients to assess this question however, where each patient can be used as their own control. Another limitation is the lack of a matched dialysis population without severe SHPT. There was also no concurrent DXA scan evaluation performed, although the predictive validity of fracture of this imaging modality in dialysis patients has previously been described in the literature (Iimori et al., 2011). One strength of our study is the use of a commercially available, routinely used RF coil and sequence acquisition for MRI scanning, which will hopefully allow this imaging modality to be more widely accessible. Also, the use of FEA in this patient cohort is novel and likely to become a tool for monitoring treatment in the future.

5. Conclusion

In conclusion, using a novel, non-invasive and ionising-radiation free imaging modality, patients with severe SHPT prior to parathyroidectomy showed evidence of significant bone microarchitecture changes with trabecular deterioration, reduced trabecular bone integrity, low trabecular and cortical bone volume, and reduced mechanical competence of bone as identified on FEA. Commonly used bone turnover markers in CKD, including PTH and ALP, correlated poorly with trabecular and cortical topological parameters and confirm the need for high quality imaging to be used as a diagnostic tool in renal bone disease. The use of micro-MRI in this population has the potential to guide treatment strategies in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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