The efficacy of routine cholecalciferol supplementation in haemodialysis patients: an investigation into the effects of improved serum 25(OH)D on mineral bone markers, anaemia, and health-related quality of life

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D) in Health Science

by

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To my husband Jack and our daughters Philippa and Harper

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I never planned to undertake a doctorate. I became a health professional in order to make a difference to people's lives. Eighteen months into my dietetic career I became intrigued by an evidence gap and found myself writing a research proposal. The eighteen months that followed, involved tireless grant applications, many knock backs, and a complete study redesign. The end of 2013 I decided to bite the bullet and I applied to The University of Warwick; I planned to start my Ph.D 1st April 2014 without any funding secured but a determination to succeed. However, in January 2014 my break came, a research grant from the British Renal Society and British Kidney Patient Association (BRS and BKPA) provided me with funding for one day a week, for two years, proving persistence pays off. The Ph.D has been life-changing, it has provided training and discipline, as well as a wealth of transferable skills.

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"I was taught that the way of progress was neither swift nor easy" — Marie Curie

Declaration

I am aware of The University of Warwick's regulations governing plagiarism and I declare that this thesis represents my own work except where I have stated otherwise.

I declare that this thesis, or part of it, has not been submitted for any degree at another university.

All sources have been acknowledged by means of references.

Sharon A Huish

Abstract

Low 1,25$(OH)_2$D in end stage renal disease (ESRD) is considered a consequence of reduced 1α-hydroxylase rather than 25(OH)D deficiency. University Hospital Coventry and Warwickshire introduced cholecalciferol supplementation for all 350 haemodialysis patients in 2014. This project aimed to determine if cholecalciferol safely and effectively improves serum 25(OH)D, and investigate effects on bone markers, anaemia and health-related quality of life (HRQOL).

Routine biochemical data (serum corrected calcium, phosphate, PTH, ferritin, and haemoglobin) and erythropoietin dose, was collected for 12 months pre, and 15 months post, introduction of cholecalciferol. A 3-month repletion phase was assumed. Two 12-month observation periods were compared, T-12 to T-1 and T4-T15. Two sub-studies were undertaken; blood samples were taken at baseline (T0) and 12 months (T12) for the measurement of vitamin D metabolites and hepcidin (n=81), SF36 and EQ-5D questionnaires were completed at baseline, 4, 8 and 12 months (n=141).

Mean serum 25(OH)D increased from 27.4±25.3nmol/L (T0) to 120.6±27.1nmol/L (T15) (P<0.001), and 1,25$(OH)_2D_3$ increased from 45.3±25.7pmol/L (T0) to 106.6±44.0pmol/L (T12), (P<0.001). Mean corrected calcium and phosphate increased from 2.29±0.13mmol/L and 1.49±0.36mmol/L pre, to 2.35±0.13mmol/L (P<0.001) and 1.54±0.41 (P=0.045) post, respectively. Mean PTH was 41.2±38.7pmol/L pre, and 37.2±35.3pmol/L post (P=0.12). Mean monthly EPO dose was 141.30±127.16µg pre and 139.34±139.58µg, post (P=0.03). Mean ferritin and haemoglobin were 380.7±148.8µg/L and 106.2±9.7g/L pre and 399.2±156.2µg/L (P = 0.35) and 105.0±10.2g/L (P = 0.004) post, respectively. Mean serum hepcidin was 110.7±71.0ng/mL (T0) and 109.8±61.0 ng/mL (T12) (P = 0.73). SF-36 physical and mental component summary scores were 33.1 (27.6-39.9) and 48.6 (38.8-55.1) (T0) and 33.0 (27.5-40.2), (P=0.70) and 48.0 (40.4-56.6), (P=0.84) (T12), respectively. EQ-5D value index scores were 0.64 (0.39-0.75) (T0) and 0.64 (0.38-0.77) (T12), (P=0.91).

In conclusion, 1,25$(OH)_2D_3$ deficiency in ESRD is partly a consequence of 25(OH)D deficiency. Routine cholecalciferol is safe and effective, and may offer benefits above active analogue monotherapy.

List of Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
$1,24,25(OH)_3D$	1,24,25-trihydroxyvitamin D
24,25(OH)D	24,25-hydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
ACR	Albumin:creatinine ratio
BMI	Body mass index
CaSR	Calcium sensing receptor
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CKD-MBD	Chronic kidney disease - mineral bone disorder
DBP	Vitamin D binding protein
eGFR	Estimated glomerular filtration rate
EPO	Erythropoietin
ESA	Erythropoiesis stimulating agent
ESRD	End stage renal disease
FGF23	Fibroblast growth factor 23
GFR	Glomerular filtration rate
Hb	Haemoglobin
HD	Haemodialysis
HHD	Home haemodialysis
HRQOL	Health-related quality of life
IIH	Idiopathic infantile hypercalcaemia
IQR	Interquartile range
MCS	Mental component summary
MDRD	Modification of Diet in Renal Disease
NHS	National Health Service
OBLs	Osteoblasts
OCLs	Osteoclasts
OPG	Osteoprotegerin
PCS	Physical component summary
PD	Peritoneal dialysis
PTH	Parathyroid hormone
PTHR	Parathyroid hormone receptor
RANK	Receptor activator of nuclear factor–kappa B
RANKL	Receptor activator of nuclear factor–kappa B ligand
RBC	Red blood cell
RCT	Randomised controlled trial
RRT	Renal replacement therapy
SD	Standard deviation
SHPT	Secondary hyperparathyroidism
TGFβ	Transforming growth factor β
VDR	Vitamin D receptor
VMR	Vitamin D metabolite ratio

Chapter 1 Introduction

1.1 Research Aim

The aim of this thesis is to determine if routine cholecalciferol supplementation in haemodialysis patients, safely and effectively improves serum 25(OH)D, and investigate effects on markers of mineral bone disease, anaemia, and health-related quality of life.

This chapter explores the background to chronic kidney disease, vitamin D, and the role of vitamin D supplementation within end stage renal disease, specifically for people receiving haemodialysis.

1.2 Chronic kidney disease

1.2.1 Physiology of the kidneys

The primary role of the kidneys is to maintain internal equilibrium through selective retention and removal of water, electrolytes, amino acids, ions, glucose and waste products from the blood (Shirley & Unwin, 2010). To achieve this, the kidneys carry out the following three processes: filtration, reabsorption, and secretion. These processes occur in the nephron, the functional part of the kidney. Each kidney contains approximately one million nephrons (Shirley & Unwin, 2010). The structure and functions of the nephron are described in Figure 1.1.

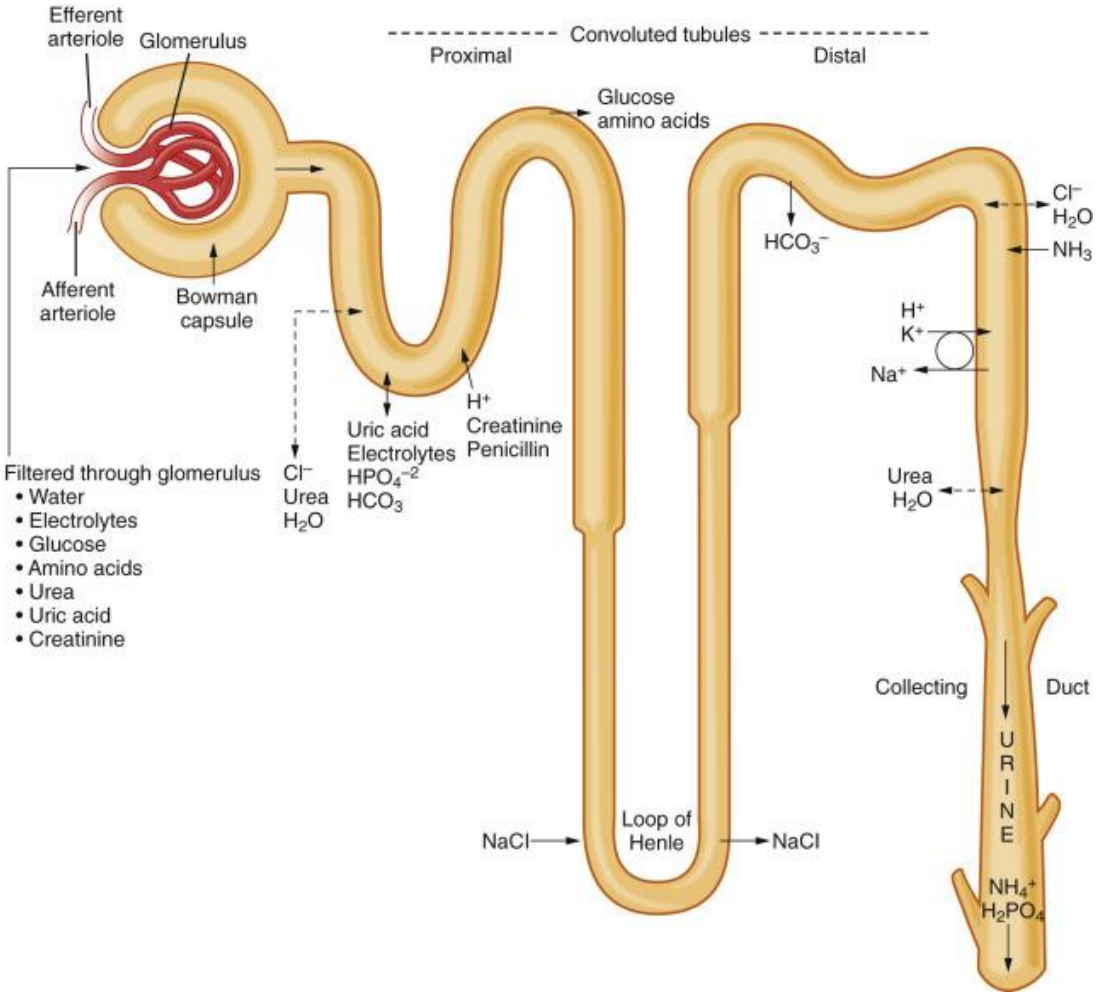

Figure 1.1 The structure and function of the nephron.
Blood enters the glomerulus via the afferent arteriole and exits via the efferent arteriole. The initial filtration step occurs in the glomerular capillaries within the Bowman capsule. The proximal convoluted tubule is the major resorptive segment of the nephron, reabsorbing glucose, amino acids, water, sodium, chloride, and bicarbonate. The loop of Henle acts as a countercurrent multiplier to create an osmotic medullary gradient in the outer medullary tissue. This facilitates the selective reabsorption of water and solutes, whilst concentrating the urine. The distal convoluted tubule plays a role in potassium, sodium, and divalent cation homeostasis, and the secretion of urea and ammonia into the collecting duct. The collecting ducts regulate water absorption and the final concentration of urine (Meltzer, 2019)

1.2.2 Measurement of kidney function

Glomerular filtration rate (GFR) is the total rate at which fluid is filtered into all the nephrons. GFR is measured in millilitres per minute (mL/min) and represents the amount of filtrate that is produced per minute. GFR is used to assess kidney function, it is expressed per body surface area in square metres ($mL/min/m^2$), as surface area reflects kidney size. The healthy normal range for GFR is wide

ranging, but is typically 120mL/min/1.73m^2 (Shirley & Unwin, 2010). GFR can be measured but it is usually estimated for convenience.

GFR measurement is based on the renal clearance of a substance that is freely filtered, not reabsorbed or secreted (Yaqoob & Ashman, 2021). Historically GFR was measured by urinary inulin clearance over a 24 hour period (Cameron & Greggor, 1998). Measuring inulin clearance is laborious and invasive, and as such is not practical in routine clinical practice. Instead, GFR is routinely estimated from serum creatinine, using an equation that incorporates age, gender, and race. A number of equations exist, but the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation is used currently (Levey *et al.*, 2009; NICE, 2014). Estimated GFR is referred to as eGFR.

Creatinine is a non-protein nitrogenous waste product derived from the dehydration of creatine (Kumar & Gill, 2018; Price & Finney, 2000). Creatine is synthesised in the liver, pancreas, and kidneys from the transamination of three amino acids - arginine, glycine, and methionine (Kumar & Gill, 2018). Creatine circulates in the blood and is phosphorylated in skeletal muscle and the brain, to phosphocreatine, a high-energy compound (Kumar & Gill, 2018; Price & Finney, 2000). The majority of the creatinine is produced in muscle; serum levels are influenced by skeletal muscle mass, age, gender, variable absorption, and renal tubular secretion (Florkowski & Chew-Harris, 2011). Therefore, creatinine based estimations of GFR have limitations. A correction factor is recommended for people of African-Caribbean or African family origin, and caution is advised for interpretation in people with extremes of muscle mass; for example, amputees or patients with muscle hypertrophy or cachexia (NICE, 2014). Alternative estimates, such as Modification of Diet in Renal Disease (MDRD), or Cockcroft-Gault, exist but these are also limited by the assumptions based on serum creatinine (Jelliffe, 1973; Levey *et al.*, 1999). If clinically indicated, direct measurement of GFR may be preferred (NICE, 2014).

Chronic kidney disease (CKD) is an umbrella term used to describe a wide-ranging number of disorders that affect kidney structure and function. It is defined as “abnormalities of kidney function or structure for more than 3 months, with implications for health” (NICE, 2014). This includes eGFR <60 mL/min/1.73m^2 on at least 2 occasions separated by a period of at least 90 days (NICE, 2014). CKD is classified into 5 stages according to eGFR and urinary albumin:creatinine ratio

(ACR). CKD stages represent normal renal function with other evidence of kidney damage (stage 1) through to kidney failure respectively (stage 5) (Figure 1.2) (NICE, 2014). CKD is a progressive condition affected by both modifiable and non-modifiable risk factors (Taal & Brenner, 2008).

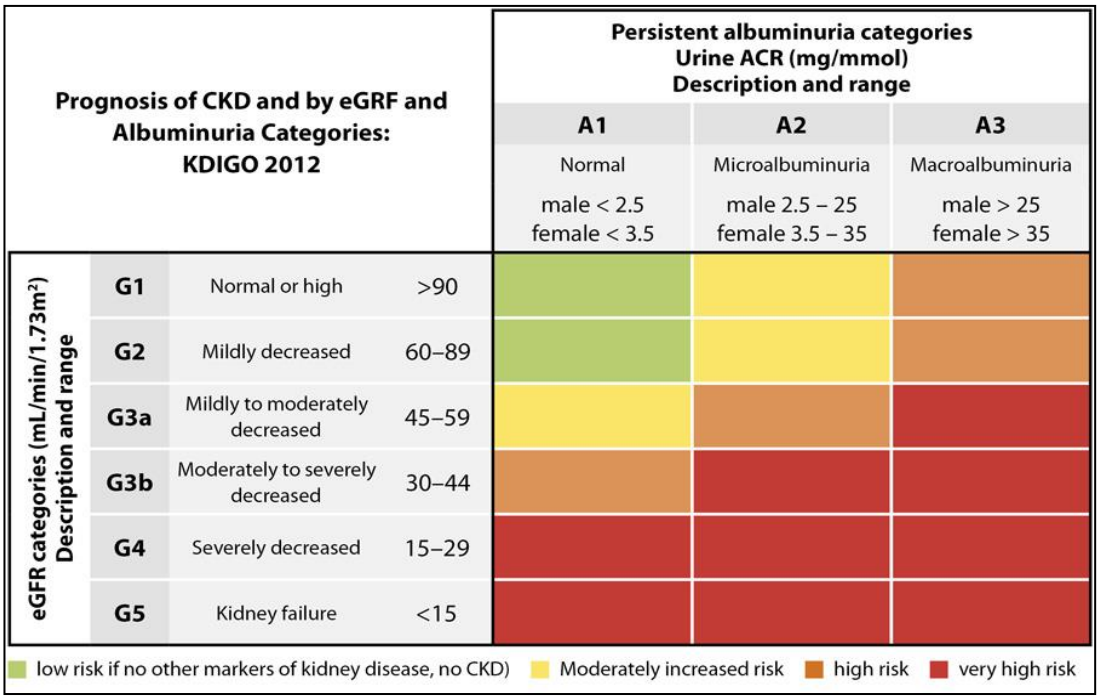

Figure 1.2 Stages of CKD and associated risk of adverse outcomes as defined by eGFR (estimated glomerular filtration rate) and ACR (albumin:creatinine ratio).

An increased ACR or decreased eGFR are independently associated with increased risk of adverse outcomes and in combination multiply the risk of adverse outcomes. The colours, and their associated risk, reflect the ranking of the adjusted relative risk (RR) for future complications including mortality, cardiovascular disease, and kidney failure. If eGFR is <60ml/min/1.73m^2, even if the lowest value group for albuminuria, there is a significant increased RR for all outcomes (NICE, 2014; Weinstein & Anderson, 2010).

1.2.3 Chronic kidney disease – prevalence and mortality

More than 1.8 million people have been diagnosed with CKD in the UK, representing approximately 3% of the population (NHS England, 2017; ONS, 2017). The prevalence of CKD stages 1-5 as a whole is difficult to ascertain due to the asymptomatic nature of the early stages (Bello *et al.*, 2010). Rates of 14% in men and 13% in women have been reported in England (Roderick *et al.*, 2011). Globally CKD prevalence is reported to be between 11 and 13% (Hill *et al.*, 2016). The actual prevalence is thought to be much greater again, affecting as many as 35% of

adults ≥35 years of age in England (NHS Health Survey for England, 2016). In terms of national health service (NHS) costs in England, CKD is estimated to cost £1.44 - £1.45 billion per year (Health Survey for England, 2016; Kerr *et al.*, 2012). Over 50% of this money is believed to be spent on renal replacement therapy (RRT) for end stage renal disease (ESRD) (Kerr *et al.*, 2012).

Clinically, the importance of this is demonstrated by the increased risk of mortality that can be seen from the early stages of the disease. Using eGFR (≥60mls/min/1.73m^2 to <15mls/min/1.73m^2), to represent stage of CKD, a large community-based population study in the USA documented an independent and graded association between risk of all-course mortality and CKD (Figure 1.3A), and risk of hospitalisation and CKD (Figure 1.3B). The risk of death and hospitalisation were evident at an eGFR of ≥60mls/min/1.73m^2 (rate 0.76 and 13.54 per 100 persons a year respectively) and increased along with progression of the disease (Go *et al.*, 2005).

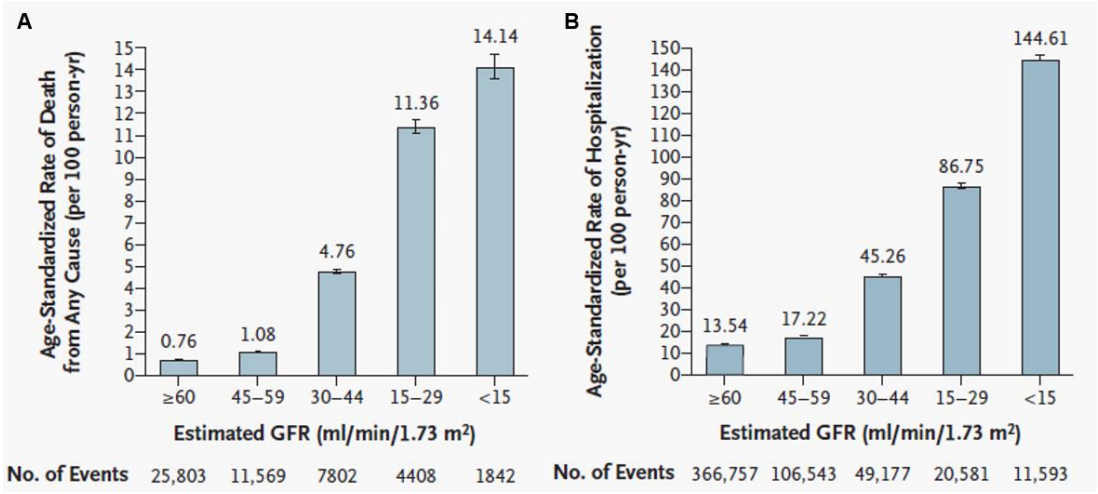

Figure 1.3 Age-standardized rates of death from any cause (A), and Hospitalization (B), according to estimated GFR.
Using estimated glomerular filtration rate (eGFR), ≥60mls/min/1.73m^2 to <15mls/min/1.73m^2, to represent stage of CKD, a large USA community-based population study of 1,120,295 adult outpatients with CKD demonstrated an independent and graded association between: risk of all-course mortality and CKD (A), and risk of hospitalisation and CKD (B). The risk of death and hospitalisation were evident at an eGFR of 60mls/min/1.73m^2 and increased along with progression of the disease. Error bars represent 95% confidence intervals. The rate of events is listed above each bar (Go *et al.*, 2005).

ESRD refers to when the kidneys are unable to support the body's requirements for the removal of waste products, and maintenance of acid-base, salt, and fluid balance. There are 64,887 adult patients having RRT in the UK with numbers increasing at a rate of 3% annually (UKRR, 2019). RRT exists in four main modalities: renal transplantation, haemodialysis (HD), peritoneal dialysis (PD) and home haemodialysis (HHD). Transplantation is the most common treatment modality in the UK (55.2%), followed by in-centre HD (37.3%), PD (5.4%) and HHD (2%) (UKRR, 2019).

People requiring RRT are offered a treatment modality choice tailored to their individual circumstances and delivered close to, or in, the home (NHS England, 2015). The benefits of self-care dialysis include those relating to patient empowerment, convenience, flexibility, and the ability to do more frequent and/or longer dialysis sessions, in order to optimise health outcomes. The limitations of the standard in-centre HD frequency have been recognised in recent years with research showing an increased risk of hospitalisation or death after the 'two-day break' (Foley *et al.*, 2011). However, a survival benefit in people having more frequent dialysis when compared to the standard three times a week in-centre HD prescription has not been shown (Rocco *et al.*, 2011). Frequent dialysis treatment has been associated with reduced cardiovascular risks; however, a downside to more frequent HD is increased vascular access interventions (FHN Trial Group, 2010). Not everyone has the option to, or wishes to, carry out dialysis themselves at home. Those wishing to dialyse at home need to be motivated to learn how to safely carry out the process, and also require functioning dialysis access.

1.2.4 Complications associated with chronic kidney disease

CKD is associated with significant morbidity and mortality. As described in section 1.2.1, the kidneys play an important role in maintaining homeostasis, as a result, CKD can affect almost every body system. Except for hypertension, there are few complications associated with the early stages of CKD (GFR ≥60ml/min). Most complications tend to develop gradually as GFR decreases below 60ml/min, and are seen increasingly in CKD stages 4 and 5 (GFR <30ml/min) (Wheeler, 2010) (Figure 1.4). Such clinical manifestations include cardiovascular disease, hypertension, dyslipidaemia, anaemia, altered bone and mineral metabolism, metabolic acidosis, malnutrition, sodium and water retention, hyperkalaemia, endocrine abnormalities, immunosuppression, and psychological problems

(Wheeler, 2010). Certain adverse outcomes, such as cardiovascular disease, can be prevented or delayed by early detection of CKD and subsequent management (Remuzzi *et al.*, 2002). However, under-diagnosis of CKD hinders such intervention (Coresh *et al.*, 2001; McClellan *et al.*, 1997; Obrador *et al.*, 1999). Clinical guidelines encourage the early identification of CKD in primary care and timely referral to a Nephrologist, to allow optimal treatment of the disease and associated complications.(Levey *et al.*, 2003; NICE, 2014). For patients, CKD is both a health and lifestyle burden; research to help improve treatments and quality of life for those living with kidney disease is one of Kidney Research UK's key research strategies (Davies, 2015).

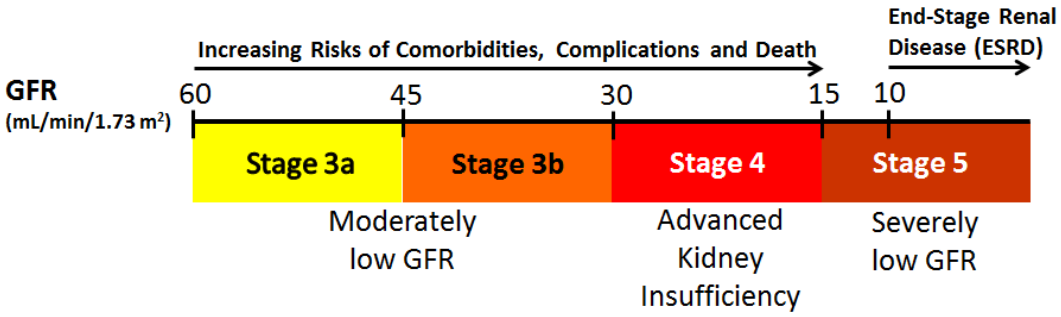

Figure 1.4 Chronic kidney disease (CKD) and its stages.
CKD represents a progressive loss of kidney function over time. As the GFR decreases, the CKD stage increases along with the relative risk of comorbidities, cardiovascular disease, all-cause mortality, and kidney failure itself (PKT, 2020). A GFR <60ml/min/1.73m^2 has a relative risk significant for all outcomes (Weinstein & Anderson, 2010).

The Ph.D will focus on chronic kidney disease mineral bone disorders (CKD-MBD) and anaemia, in relation to vitamin D. An overview of vitamin D will be given, followed by the problems relating to vitamin D deficiency and metabolism in CKD.

1.3 Vitamin D synthesis and signalling

Vitamin D occurs in 2 forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_3 is synthesised in the skin when it is exposed to ultraviolet (UV) radiation (Holick, 1981; Tian *et al.*, 1993), or obtained from diet and supplements. Vitamin D_2 is obtained from supplements, fortified foods, and some plant based foods. Up to 95% of the vitamin D we require can be provided by sunlight, meaning the classification of vitamin D as a vitamin (something required in the diet) is considered incorrect (Holick, 2007; Holick, 2010).

UV radiation, from sunlight (maximal effective wavelength 295nm) causes photolysis of 7-dehydrocholesterol to previtamin D_3 in the epidermis of the skin; this is then converted to vitamin D_3 by the keratinocytes (Holick, 1981; Webb, 2006) (Figure 1.5). Melanin, in the epidermis, can reduce the effectiveness of sunlight in making vitamin D_3 by absorbing UV radiation in competition with 7-dehydrocholesterol (Holick *et al.*, 1980). Skin pigmentation, season and latitude all influence cutaneous photochemical synthesis of vitamin D_3. Maximum vitamin D_3 production occurs in summer months and depending on latitude, little or no vitamin D_3 is made in the winter (Stamp & Round, 1974; Webb *et al.*, 1988). In the UK, sufficient vitamin D_3 synthesis can occur, on sunny days, between the hours of 10:00 and 15:00, April to September (Wacker & Holick, 2013). It is suggested that 10-20 minutes of daily skin-sun exposure during these times may result in a serum 25(OH)D increase of 5-10nmol/L (Wacker & Holick, 2013).

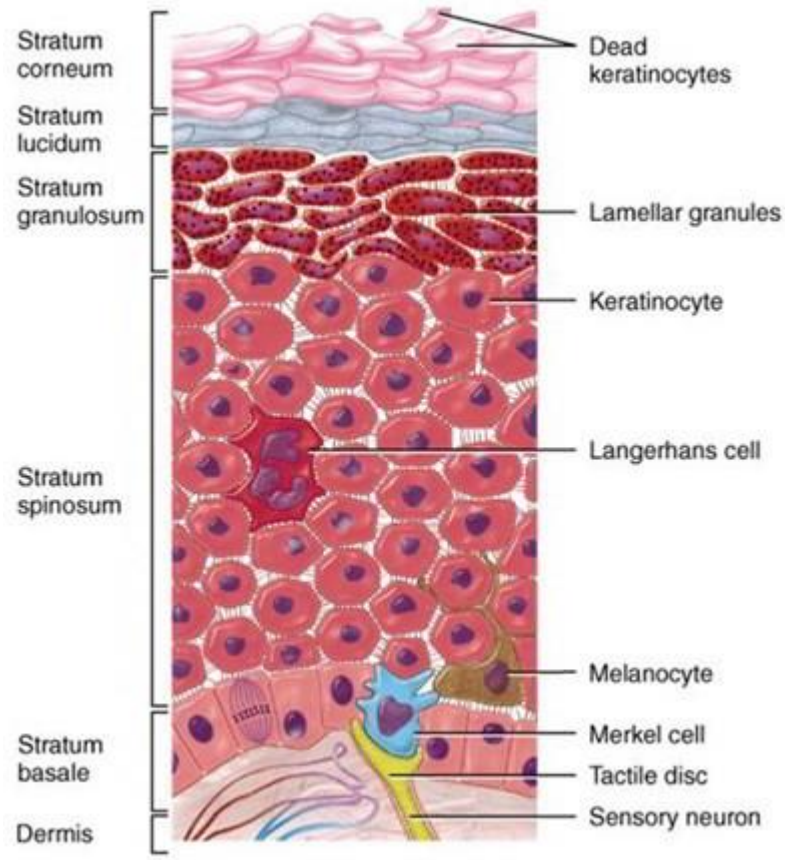

Figure 1.5 Structure of the epidermis
The epidermis is the major source of vitamin D. It is made up of 5 layers; the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum (the most superficial layer). UV radiation, from sunlight (maximal effective wavelength 295nm) causes photolysis of 7-dehydrocholesterol to previtamin D_3 in the epidermis; this is converted to vitamin D_3 by the keratinocytes (Holick, 1981; Webb, 2006). Keratinocytes also possess the enzymes (CYP27A1, 25-hydroxylase, and CYP27B1, 1α-hydroxylase) to metabolise vitamin D_3 to 25(OH)D and then 1,25$(OH)_2$D. Like most other cells, keratinocytes express the vitamin D receptor (VDR) so can respond to endogenous 1,25$(OH)_2$D (Bikle & Pillai, 1993).

Vitamin D circulates primarily bound to the vitamin D binding protein (DBP) (Chen *et al.*, 2010). Vitamin D undergoes activation via a series of hydroxylation steps. The first hydroxylation step, in the liver, is catalysed by either a low capacity, high specificity microsomal 25-hydroxylase (CYP2R1), or a high capacity, low affinity mitochondrial 25-hydroxylase (CYP27A1) into 25(OH)D (calcidiol) (Cheng *et al.*, 2004; Cheng *et al.*, 2003). CYP2R1 exhibits the highest affinity for vitamin D and as such is considered the most important of the 25-hydroxylases (Al Mutair *et al.*, 2012). However, CYP2R1 knock out mice are still able to produce 25(OH)D

confirming the involvement of other 25-hydroxylases, in particular CYP27A1 (Zhu *et al.*, 2013).

25(OH)D is the primary circulating form. The second hydroxylation step of vitamin D primarily occurs in the kidney, where mitochondrial CYP27B1 catalyses the conversion of 25(OH)D to 1,25$(OH)_2$D (Holick *et al.*, 1971; Takeyama *et al.*, 1997). 1,25$(OH)_2$D acts by binding to the VDR. The VDR is expressed in many cell types but is notable in the bone, intestine and kidney (Wang *et al.*, 2012). Here 1,25$(OH)_2$D acts primarily to maintain calcium and bone mineral homeostasis through enhanced calcium and phosphate absorption in the gastrointestinal tract, increased bone resorption, suppression of PTH secretion, and upregulation of FGF23 (discussed in detail in section 1.6.2). 1α-hydroxylase is also expressed in many extra-renal tissues including the parathyroid glands, testes, and skin (Zehnder *et al.*, 2001). Extra-renal production of 1,25$(OH)_2$D is not shown to contribute significantly to circulating 1,25$(OH)_2$D levels or calcium homeostasis. Instead, it is associated with autocrine/paracrine actions and believed to be important for both immune and inflammatory responses (Hewison *et al.*, 2007). In recent years there has been an increased recognition for the role, and many potential benefits, of extra-renal 1,25$(OH)_2$D synthesis (Jones, 2011).

1,25$(OH)_2$D is further metabolised by 24-hydroxylase (CYP24A1) to 1,24,25$(OH)_3$D. 24-hydroxylase also acts on 25(OH)D to produce 24,25$(OH)_2$D (Jones, 2011). The production of these metabolites is considered degradation; expression of 24-hydroxylase and 1α-hydroxylase is reciprocal in order to control 1,25$(OH)_2$D levels (Jones, 2011). Figure 1.6 presents an overview of the hydroxylation steps.

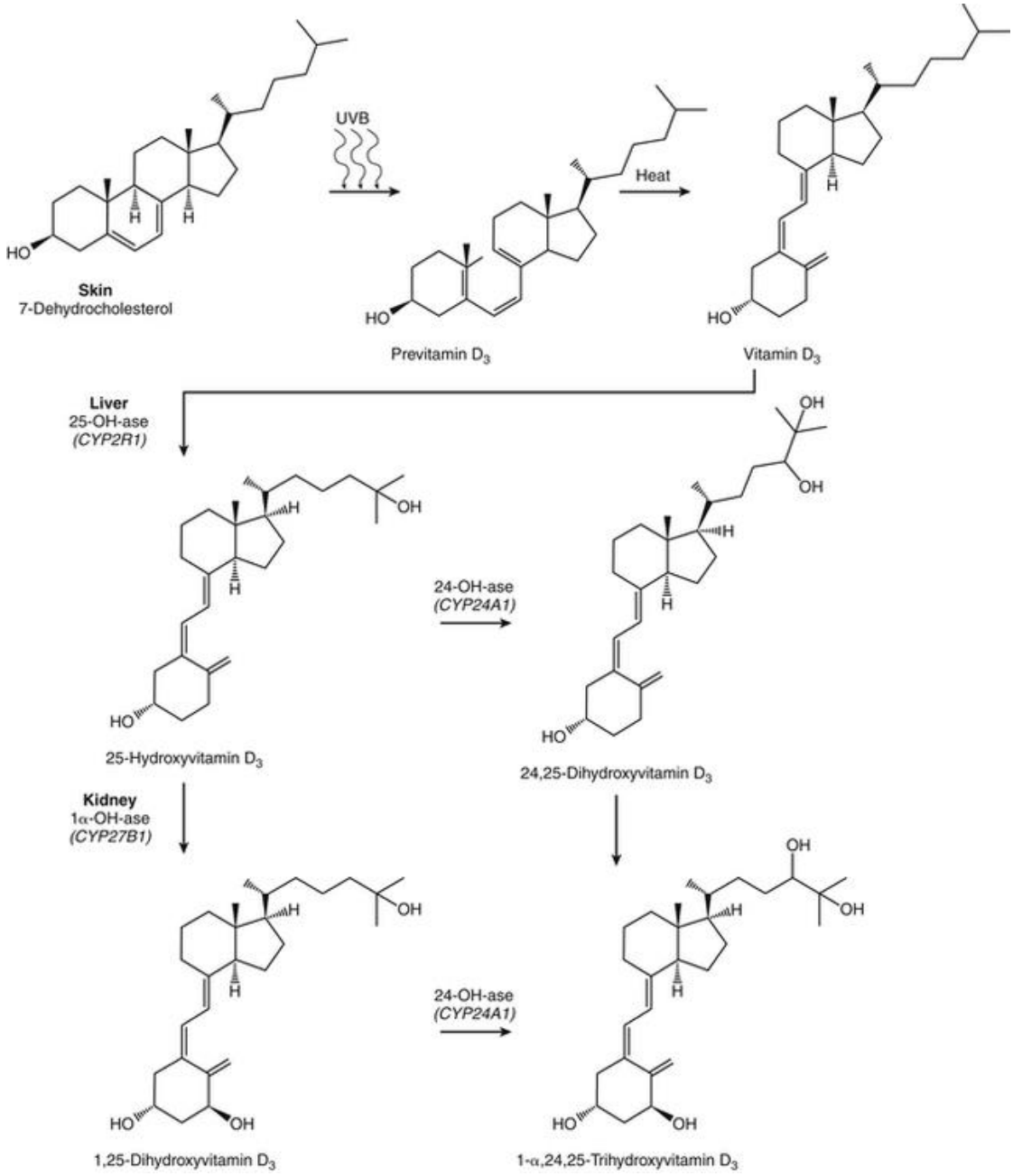

Figure 1.6 Vitamin D metabolism - the chemical structures and hydroxylation steps

25-hydroxylase (CYP2R1) converts vitamin D to 25(OH)D in the liver. 1α-hydroxylase (CYP27B1) converts 25(OH)D to $1,25(OH)_2D$ in the kidney. Other tissues contain these enzymes, but the liver is the main source of 25-hydroxylase, and the kidney the main source for 1α-hydroxylase. $1,25(OH)_2D$ is further metabolised by 24-hydroxylase (CYP24A1) to $1,24,25(OH)_3D$. 24-hydroxylase also acts on 25(OH)D to produce $24,25(OH)_2D$. The production of these metabolites is considered degradation; expression of 24-hydroxylase and 1α-hydroxylase is reciprocal in order to control $1,25(OH)_2D$ levels (Clinton, 2021; Jones, 2011).

1.4 Vitamin Assessment

Defining vitamin D deficiency, insufficiency and sufficiency is challenging due to a lack of consensus (Heaney, 2013; Rosen & Taylor, 2013). As such the prevalence of hypovitaminosis D is confounded by the level of 25(OH)D used to define adequacy. There exists several position statements and clinical practice guidelines that define optimal vitamin D status along with associated health outcomes. However, three of the most authoritative reports, those from The UK's Scientific Advisory Committee on Nutrition (SACN), The American Institute of Medicine's (IOM) and The US Endocrine Society (ES), all differ substantially. The ES report a 25(OH)D level <50 nmol/l as deficient, a level of <75nmol/L as insufficient, and a level of ≥75nmol/L as optimal (Holick *et al.*, 2011). The IOM on the other hand define levels of >50nmol/L as adequate for good bone health in almost all individuals (97.5% of the population) and suggest a level of 40nmol/L would meet the needs of 50% of the population (Ross *et al.*, 2011). SACN recommend maintaining 25(OH)D level of >25nmol/L in order to protect musculoskeletal health (SACN, 2016). This has led to confusion for clinicians, researchers, and the public, and added to the challenge of managing vitamin D deficiency. The NHS has adopted SACN recommendations for the general population however clinical laboratories may differ locally in terms of what is defined as an ideal reference range. At UHCW, and across Coventry and Warwickshire, vitamin D deficiency is defined as <30nmol/L, this target was based on the National Osteoporosis Society guidelines (APC, 2017; Aspray *et al.*, 2014).

The variation is likely representative of limitations in the existing literature. This includes difficulty distinguishing the sole effect of vitamin D as many intervention trials also include co-therapy with calcium. Studies do not account for sunlight exposure, seasonal variation, or dietary intake, and are either not adequately powered, or do not have long enough follow up periods to assess the longer-term effects (Holick et al., 2012). Many studies also include low dose vitamin D in the placebo arm (Holick *et al.*, 2012). In addition to this, the data is also dependent on the reliability of the serum 25(OH)D measurement, which as discussed in section 1.4.3, has its own shortcomings.

1.4.1 Assessment of vitamin D status

25(OH)D is the metabolite of vitamin D that is usually measured clinically to assess vitamin D status (Holick *et al.*, 2011; SACN, 2016). The 25-hydroxylation step in the liver is dependent on supply of vitamin D, so serum 25(OH)D reflects serum vitamin D levels. Serum 25(OH)D is a combination of both $25(OH)D_2$ and $25(OH)D_3$; (Holick, 2009).

$1,25(OH)_2D$ is not a reliable marker of vitamin D status for many reasons. These include: a short half-life, 4-15 hours vs. 21-30 days of 25(OH)D, and the fact serum $1,25(OH)_2D$ levels are maintained at the expense of 25(OH)D thus giving the false perception of sufficiency (Lips, 2007). In addition, unlike circulating 25(OH)D, the level of circulating $1,25(OH)_2D$ remains unchanged with exposure to UV radiation; it is instead, tightly controlled by the endocrine system in response to changes in serum calcium, phosphate and PTH (Webb, 2006).

Despite serum total 25(OH)D being the current clinical marker of vitamin D status controversy exists around how reliable it truly is.

1.4.2 Limitations in 25(OH)D as a marker of vitamin D status

Questions about the reliability of serum 25(OH)D as a marker of vitamin D status are related to issues surrounding its binding to DBP and albumin (approximately 85% is bound to DBP and 15% is albumin bound), less than 0.1% is unbound or 'free' (Chun *et al.*, 2014). In healthy individuals, free vitamin D and 25(OH)D are shown to strongly correlate, implying total 25(OH)D is predictive of free 25(OH)D (Aloia *et al.*, 2015; Bikle *et al.*, 1986; Nielson *et al.*, 2016b). Whether conditions that impact on DBP affect this correlation, meaning free 25(OH)D is more clinically useful than total 25(OH)D for certain populations, has been considered (Chun & Nielson, 2018). Free 25(OH)D does not appear to correlate better with serum bone markers than total 25(OH)D meaning it may not offer additional insight in the management of mineral bone disorders (Bikle *et al.*, 2017a; Chun & Nielson, 2018; Jemielita *et al.*, 2016).

Interest in $24,25(OH)_2D$ has also grown in recent years. Findings have shown that FGF23 stimulates 24-hydroxylase, and CYP24A1 mutations have been discovered

in cases of idiopathic infantile hypercalcaemia (IIH) (Quarles, 2012). CYP24A1 variation affects 24-hydroxylase activity (catabolism of serum 25(OH)D) therefore 25(OH)D concentration may reflect a state of fast or slow metabolism with corresponding high or low serum $24,25(OH)_2D$ levels (Petkovich & Jones, 2011; Wang *et al.*, 2010). Measurement of $24,25(OH)_2D$, and the ratio of this metabolite to 25(OH)D, may offer further insight and in turn support clinical management of disorders relating to vitamin D deficiency, hypercalcaemia, and hypophosphataemia (Kaufmann *et al.*, 2014). Further research, along with standardised measurement, is needed to gain more understanding in the clinical relevance of alternative vitamin D markers (Chun & Nielson, 2018).

1.4.3 Limitations in measuring total 25(OH)D

Various methods exist for the measurement of 25(OH)D including immunoassays (both radioimmunoassay and enzyme linked immunoassays), high performance liquid chromatography and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Holick, 2009). LC–MS/MS measures both $25(OH)D_2$ and $25(OH)D_3$, can distinguish between vitamin D metabolites, and is currently considered the gold standard method (Jones & Kaufmann, 2016). Unlike LC-MS/MS, immunoassays cannot quantify $25(OH)D_2$ and $25(OH)D_3$ separately and are unable to differentiate between other vitamin D metabolites (Wallace *et al.*, 2010). However, due to cost, and practicalities, the immunoassay remains the current method of choice for clinical laboratories (Jones & Kaufmann, 2016).

The vitamin D metabolite $24,25(OH)_2D$ is not differentiated by immunoassays, particularly those involving no chromatographic steps, this can result in a falsely higher 25(OH)D value (up to 15% overestimate) (Romagnoli *et al.*, 2013). Some commercial kits provide corrections for $24,25(OH)_2D$, but, accuracy is reduced if values are low or high (Romagnoli *et al.*, 2013). LC-MS/MS methods facilitate rapid analysis of many vitamin D metabolites, including $24,25(OH)_2D$ (Jones & Kaufmann, 2016).

Whilst immunoassay methods have shown bias and increased variability when compared to LC-MS/MS, LC-MS/MS is known to have faults of its own (Jukic *et al.*, 2017). In addition to hydroxylation, C3-epimerisation of the main vitamin D metabolites (25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$ and $1,24,25(OH)_3D$) is possible (Kamao *et al.*, 2004). The 3-epi-25(OH)D epimer (3-epi) may interfere with the

results obtained by LC-MS/MS. These compounds have the same molecular weight as vitamin D metabolites and thus form the same mass to charge parent and product ion pairs upon ionization (Romagnoli *et al.*, 2013). A selective column or increased chromatographic separation needs to be used in order to avoid the epimer from being included as part of the total 25(OH)D concentration (Jukic *et al.*, 2017). LC-MS/MS also requires more expertise and costly equipment (Jukic *et al.*, 2017). The steps involved in LC-MS/MS are outlined in Figure 1.7

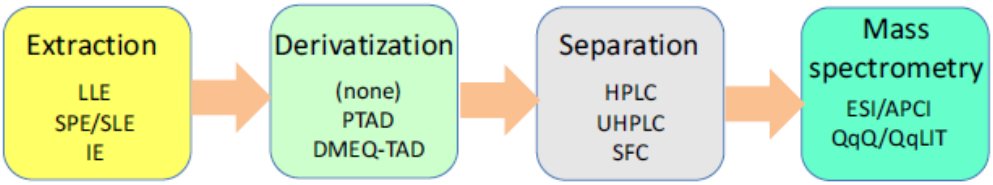

Figure 1.7 Steps involved in Liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology for quantification of vitamin D metabolites.
APCI, atmospheric pressure chemical ionization; DMEQ-TAD, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione; ESI, electrospray ionization; HPLC, high performance chromatography; IE, Immunoextraction; LLE, liquid-liquid extraction; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; QqLIT, quadrupole linear ion trap; QqQ, triple quadrupole; SFC, supercritical fluid chromatography; SLE supported liquid extraction; SPE, solid-phase extraction; UHPLC, ultra-high performance chromatography (Kaufmann *et al.*, 2018).

It is important to consider the assay used when interpreting results, both in the clinical setting itself, as well as those in clinical studies. Chapter 8 compares serum 25(OH)D results from both immunoassay and LC-MS/MS methods, and further explores whether 25(OH)D is the most appropriate marker of vitamin D status.

1.5 Vitamin D and Chronic Kidney Disease – the problem

Vitamin D deficiency and insufficiency (defined in this instance as serum 25(OH)D <37.5nmol/L and 37.5-75nmol/L respectively) is widespread amongst the general population and linked with morbidities including cardiovascular disease, diabetes, cancer and osteoporosis as well as increased mortality risk (Holick, 2004). Patients with chronic kidney disease (CKD) frequently have low circulating serum levels of 25(OH)D. The prevalence of hypovitaminosis D is shown to increase with falling

eGFR. Approximately 50% of people with CKD stage 3 are believed to be vitamin D insufficient or deficient and this increases to up to 95% in people with end stage renal disease (ESRD) on haemodialysis (Saab *et al.*, 2007; Tokmak *et al.*, 2008; Wolf *et al.*, 2007). A preliminary study at UHCW in 2014 compared serum 25(OH)D level in patients with CKD stage 5 and a group of healthy, medication controlled, hypertensive patients. 100% of the CKD patients had serum 25(OH)D levels <75nmol/L of whom 43% were deficient (<30nmol/L), whereas in the hypertensive patients 29% were 25(OH)D replete (≥75nmol/L) and 71% insufficient, of which only 21% were deficient Figure 1.8 (Huish *et al.*, 2014). The high prevalence of deficiency seen in ESRD is likely the result of reduced sunlight exposure, an ethnically diverse and ageing population (both of which have implications on skin synthesis of vitamin D), poor diet due to dietary restrictions and/or uraemic symptoms, and the uraemic state itself which hinders hydroxylation of vitamin D in the liver (Michaud *et al.*, 2010).

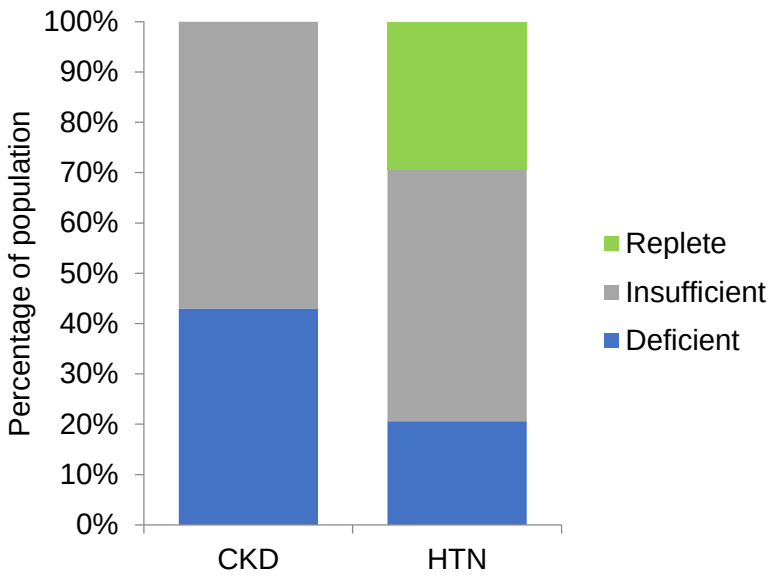

Figure 1.8 Low serum 25(OH)D is prevalent in CKD patients
Serum 25(OH)D was assessed in CKD stage 5 patients (CKD: eGFR ≤15) and healthy medication-controlled hypertensive subjects (HTN: eGFR >60). 25(OH)D was measured using the Elecsys Vitamin D Total Assay (Roche). Vitamin D thresholds were defined as <30nmol/L, deficient: 30-74nmol/L, insufficient: ≥75nmol/L, replete. 100% of CKD patients had levels <75nmol/L and 43.0% were deficient (<30nmol/L). In contrast in the HTN group 29% were replete and only 21% were deficient.

The impact of CKD on 25(OH)D and 1,25$(OH)_2$D catabolism may contribute to vitamin D deficiency. In CKD 24-hydroxylase activity is likely to be affected by a combination of low 25(OH)D and 1,25$(OH)_2$D, reduced VDR activation, and high PTH levels (Jones, 2011). In addition, 24-hydroxylase can be induced by metabolic acidosis and increased FGF23 which occur in CKD. It is also believed that use of active vitamin D analogues can induce 24-hydroxylase resulting in catabolism of 25(OH)D, 1,25$(OH)_2$D, and of the administered active vitamin D metabolites themselves (Bosworth & de Boer, 2013). This further exacerbates, and complicates management of vitamin D deficiency and CKD-MBD (Bosworth & de Boer, 2013).

Classically vitamin D is known for its role in calcium homeostasis and bone health, which is largely attributed to the active form 1,25$(OH)_2$D. One of the pathophysiological consequences of CKD is the reduced renal capacity to synthesise 1,25$(OH)_2$D. 1,25$(OH)_2$D concentration is shown to decrease along with progression of CKD (Levin *et al.*, 2007), and nephrectomy significantly reduces circulating 1,25$(OH)_2$D (Weisman *et al.*, 1978). This results in reduced VDR activation, decreased mineral uptake from diet, and subsequent reduction in serum calcium. This is well recognised and appreciated in ESRD where the therapeutic use of 1,25$(OH)_2$D (calcitriol), or a synthetic analogue (alfacalcidol), to reduce PTH and manage renal bone disease is routine (KDIGO, 2009; KDIGO, 2017; NICE, 2007; Steddon & Sharples, 2015). Alfacalcidol is a precursor of calcitriol (lacking 25-OH group); it is already hydroxylated in position 1 and therefore does not require activation in the kidney (Takahashi *et al.*, 2014) (Figure 1.9).

A case-control study of 1000 incident haemodialysis patients in the USA reported a significant increased risk of all-cause death in patients with vitamin D deficiency (using 25(OH)D <25nmol/L to define deficiency) (Wolf *et al.*, 2007). Patients with ESRD receiving active vitamin D treatment (type not specified) appear to have a 26% reduction in mortality, independent of other cardiovascular risks, parathyroid hormone (PTH) and bone mineral markers (Wolf *et al.*, 2007).

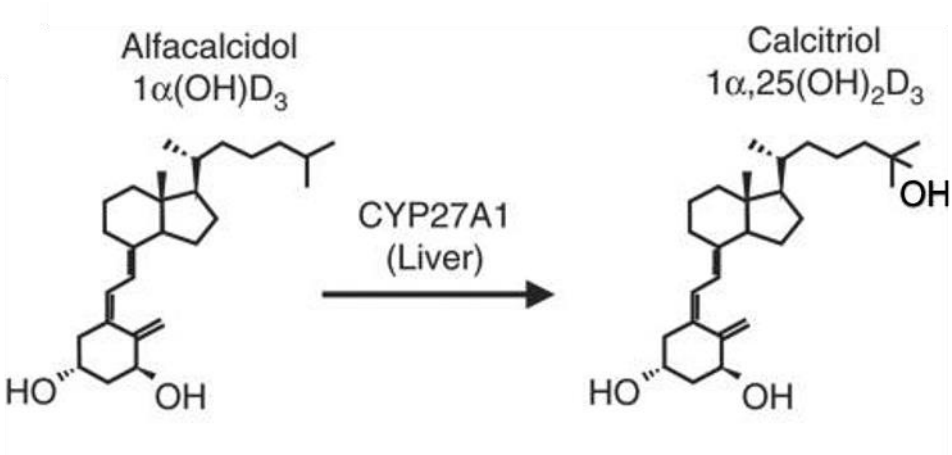

Figure 1.9 Structure of alfacalcidol
Alfacalcidol is metabolised to $1,25(OH)_2D_3$ in the liver by CYP2YA1.

Whilst supplementation with an active form of vitamin D alone effectively suppresses secretion of PTH, it does not provide adequate replacement in the management of vitamin D deficiency and insufficiency because it does not replace the substrate, 25(OH)D. There is a growing body of evidence to suggest benefits to improving plasma 25(OH)D levels using supplementation with ergocalciferol or cholecalciferol. There is much debate about the combined use of both active and non-active vitamin D treatments. UK Renal Association guidelines state that there is insufficient evidence for measurement and/or repletion of 25(OH)D in patients who are already receiving an active vitamin D analogue (Steddon & Sharples, 2015). This is due to the focus being on ensuring adequate $1,25(OH)_2D$ for CKD-MBD management. KDIGO 2017 CKD-MBD guidelines suggest 25(OH)D status may be assessed, and if indicated, supplementation given. Yet there are no current guidelines that emphasize the need to ensure adequate serum 25(OH)D in ESRD patients, or how to achieve it. This stems from the reduced ability for renal conversion of 25(OH)D into $1,25(OH)_2D$. However, the extrarenal activity of 1α-hydroxylase and the subsequent synthesis of $1,25(OH)_2D$ by many tissues; for example, the adrenal medulla, brain, pancreas, skin, colon, prostate, macrophages, and other organs is intact in ESRD fproduction are not well understood but the mechanism of intracellular $1,25(OH)_2D$ production is dependent upon sufficient circulating substrate (25(OH)D). Consequently, the potential benefits of non-renal synthesis of $1,25(OH)_2D$ cannot be achieved through the sole therapeutic use of active vitamin D analogues.

Cholecalciferol treatment to optimise serum 25(OH)D levels may improve mineral bone markers. Jean and colleagues (2009) gave cholecalciferol supplementation (100,000IU per month) to 107 haemodialysis patients for 15 months; 25(OH)D repletion (≥75nmol/L) was achieved in 91% of patients, and median PTH reduced from 295pg/mL (range 190-450) at baseline to 190pg/mL (range 110-273) at 15 months, $P < 0.05$. A surprising finding for the authors was a significant increase in serum $1,25(OH)_2D$, from 14±14pmol/L at baseline to 45±13pmol/L at 15 months, $P < 0.001$; demonstrating that even in the presence of ESRD, $1,25(OH)_2D$ production not only occurs, but it is sufficient to increase circulating serum concentrations. The same authors also reported new cinacalcet prescriptions decreased from 45 to 3 during three 12 month periods following introduction of vitamin D (cholecalciferol or 25(OH)D (calcifediol)) supplementation into routine care (Jean *et al.,* 2010). In addition, none of the patients requiring cinacalcet or parathyroidectomy had been treated with vitamin D (cholecalciferol or 25(OH)D (calcifediol)) prior to the diagnosis of SHPT (Jean *et al.,* 2010). Improving 25(OH)D levels may improve PTH by increasing serum $1,25(OH)_2D$ (increasing calcium), but also through local production and secretion of $1,25(OH)_2D$ in parathyroid cells themselves (Segersten *et al.*, 2002).

Whilst people with kidney disease are at greater risk of bone disease, they also have the non-renal related health requirements of the general population. Vitamin D deficiency has been associated with reduced muscle strength, anaemia, increased inflammation and infection, and reduced health-related quality of life (Boudville *et al.*, 2010; Kiss *et al.*, 2011; Kumar *et al.*, 2011; Lac *et al.*, 2010; Matias *et al.*, 2010; Srisakul *et al.*, 2011; Stenvinkel *et al.*, 1999). The non-classical (non-bone related) effects of vitamin D may actually present the greatest deficiency risks, and largest potential supplementation benefits, in CKD patients; this is explored in section 1.8 and 1.9.

1.6 Chronic Kidney Disease - Mineral Bone Disorder (CKD-MBD)

1.6.1 Normal bone homeostasis

The skeleton constantly undergoes bone remodelling, a process that occurs in the foetus and continues throughout a person's lifetime (Clarke, 2008). This continuous renewal of bone is designed to maintain bone integrity as well as mineral homeostasis. The bone acts as a buffer through absorption of excess acids, calcium and phosphate, and the release of minerals (calcium, phosphate, and magnesium) into circulation. Remodelling involves removal of old bone and replacement with newly formed and subsequently mineralised bone (Clarke, 2008). The bone remodelling cycle involves a complex series of highly regulated sequential steps coupling both resorption and formation. These roles are mediated by cytokines, including interleukins 1, 6, and 11, colony-stimulating factors, and calciotropic hormones including parathyroid hormone (PTH), 1,25-dihydroxy vitamin D ($1,25(OH)_2D$), calcitonin, and oestrogen (Rucci, 2008). The receptor activator of nuclear factor–kappa B (RANK) and its ligand, RANKL, are involved in bone resorption and formation. RANKL is released by osteoblasts (OBLs) and binds to RANK on immature and mature osteoclasts (OCLs), to initiate bone resorption. Osteoclasts resorb bone (resorption phase), facilitating the release of cytokines and proteins from the bone matrix (bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and transforming growth factor β (TGFβ). These recruit osteoblasts which produce osteoid, and promote its mineralisation (formation phase), completing the bone remodelling process (Figure 1.10). Osteoprotegerin (OPG; a decoy receptor) is secreted by osteoblasts and protects bone from excessive resorption by binding to RANKL and preventing it from binding to RANK (Boyce & Xing, 2008).

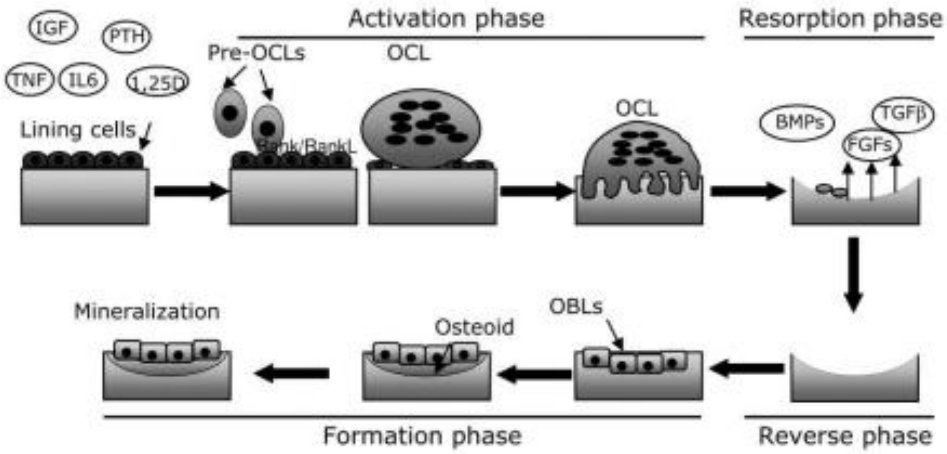

Figure 1.10 The bone remodelling process.
Bone remodelling is regulated by various cytokines, including interleukins 1, 6, and 11, colony-stimulating factors, and calciotropic hormones including parathyroid hormone (PTH), 1,25-dihydroxy vitamin D ($1,25(OH)_2D$), calcitonin, and oestrogen. The receptor activator of nuclear factor–kappa B (RANK) and its ligand, RANKL, are involved in bone resorption and formation. RANKL is released by bone-forming cells known as osteoblasts (OBLs) and stimulates RANK to form osteoclasts (OCLs), which mediate bone resorption. The activation of lining cells leads to increased surface expression of RANKL. RANKL interacts with its receptor RANK thus triggering osteoclast differentiation (Activation phase). Osteoclasts resorb bone (Resorption phase), facilitating the release of proteins from the bone matrix (bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and transforming growth factor β (TGFβ). These recruit osteoblasts which produce the new bone matrix, and promote its mineralisation (Formation phase), completing the bone remodelling process (Rucci, 2008).

Cortisol, an adrenal gland hormone, has complex effects on the skeleton; small amounts are necessary for normal bone development, but large amounts inhibit bone formation. Glucocorticoids, synthetic forms of cortisol, are used in the treatment of many medical conditions including asthma and arthritis. These can cause bone loss due to decreased bone formation and increased bone breakdown, leading to increased fracture risk (Kanis *et al.*, 2004). Oestrogen is a key hormone in bone remodelling (Khosla *et al.*, 2012). Deficiency, observed in postmenopausal women, and conditions affecting the oestrogen sensing receptor in men, leads to an increase in bone remodelling in which resorption surpasses formation causing decreased bone mass (Clarke, 2008; Faustini-Fustini *et al.*, 1999). Bone remodelling is largely controlled by the calcium-regulating hormones PTH and $1,25(OH)_2D$ (Raisz, 1999). PTH synthesis and secretion tends to increase with age, leading to an increase in bone turnover and subsequent reduction in bone mass (Burr, 1997; Jiang *et al.*, 2020). $1,25(OH)_2D$ increases calcium and phosphate

absorption in the gastrointestinal tract as well as having direct effects on bone resorption and formation (Li *et al.*, 1998). Other systemic hormones which influence skeletal growth include calcitonin, growth hormones, testosterone, and thyroid hormones (Raisz, 1999). Calcitonin is synthesised and released by the thyroid gland in response to increased serum calcium. It can block bone resorption through inactivating osteoclasts; however, the influences of calcitonin on bone health are thought to be relatively transient (Holt & Wysolmerski, 2011). The growth hormone system stimulates bone resorption and bone formation with the dominant effect being formation, resulting in an increase in bone mass (Ohlsson *et al.*, 1998). Testosterone directly and indirectly effects skeletal growth through its effects on bone, and through the stimulation of muscle growth which increases pressure on bone, increasing bone formation. Testosterone is also converted into oestrogen in fat cells, which as discussed above has a key role in bone remodelling (Mohamad *et al.*, 2016). Thyroid hormones increase the rate of bone formation and resorption. Deficiency can impair growth, yet increased amounts can cause excessive bone breakdown, weakening the skeleton (Vestergaard & Mosekilde, 2002)

1.6.2 Calcium balance

Calcium plays a crucial role in bone mineralisation as well as being vital for a number of physiological mechanisms. Calcium homeostasis is largely regulated by PTH, $1,25(OH)_2D$ and calcium, and their receptors (PTHR, VDR and CaSR) (Peacock, 2010). Together these regulate calcium transport in the gastrointestinal tract, kidney, and bone (Figure 1.11).

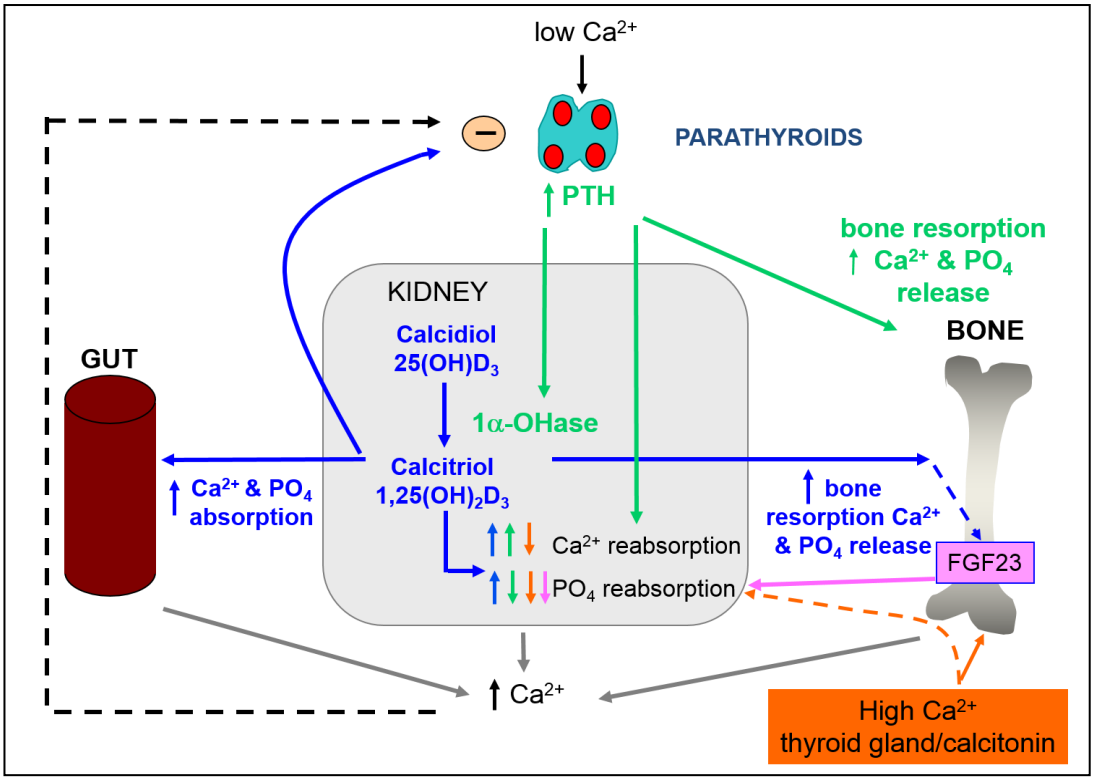

Figure 1.11 Guide to the control of calcium homeostasis in healthy individuals Changes in serum calcium concentration are identified by the CaSR on the parathyroid glands.
PTH is released in response to low serum calcium levels and increases calcium resorption from the bone, as well as calcium reabsorption and phosphate excretion in the kidney. PTH also increases 1α-hydroxylase (1α-OHase) which catalyses the conversion of 25(OH)D to $1,25(OH)_2D$. $1,25(OH)_2D$ activates the VDR to increase intestinal calcium and phosphate absorption, inhibit further PTH secretion, and increase bone resorption. In the presence of hypercalcaemia, these actions are reversed in order to decrease serum calcium. FGF23 is released from bone cells in response to a rise in serum phosphate and works to normalise phosphate levels through increased urinary phosphate excretion and reduced synthesis of $1,25(OH)_2D$ (Bland, 2010).

The CaSR is a plasma membrane glycosylated protein, expressed on tissues regulating calcium homeostasis, including the parathyroid glands, intestine and kidney, and other tissues (Zhang *et al.*, 2015). A reduction in circulating serum calcium results in deactivation of the CaSR on the parathyroid glands stimulating the secretion of PTH (Figure 1.12) which activates the PTHR to increase calcium resorption from the bone, as well as calcium reabsorption and phosphate excretion in the kidney (Hendy *et al.*, 2013). Figure 1.13 presents an overview of the CaSR signalling pathways.

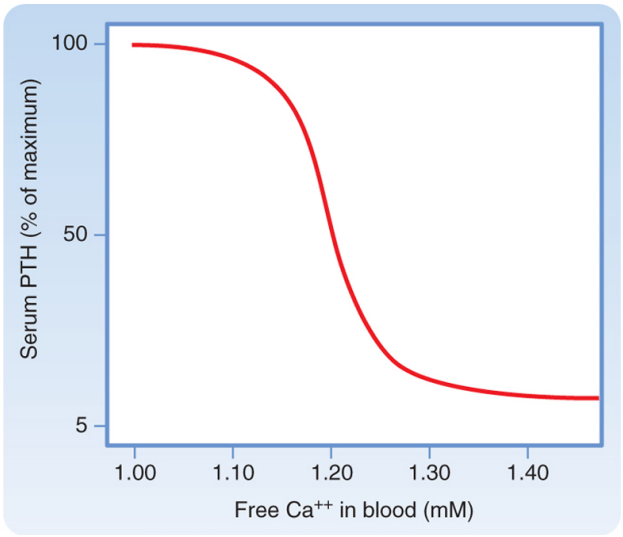

Figure 1.12 Parathyroid hormone secretion in response to calcium concentration.
Parathyroid hormone (PTH) secretion maximises as calcium drops toward 1.00mM (plasma calcium concentration of 2.0mmol/L) and is supressed by >50% as calcium increases to 1.20mM (Koeppon & Stanton, 2008).

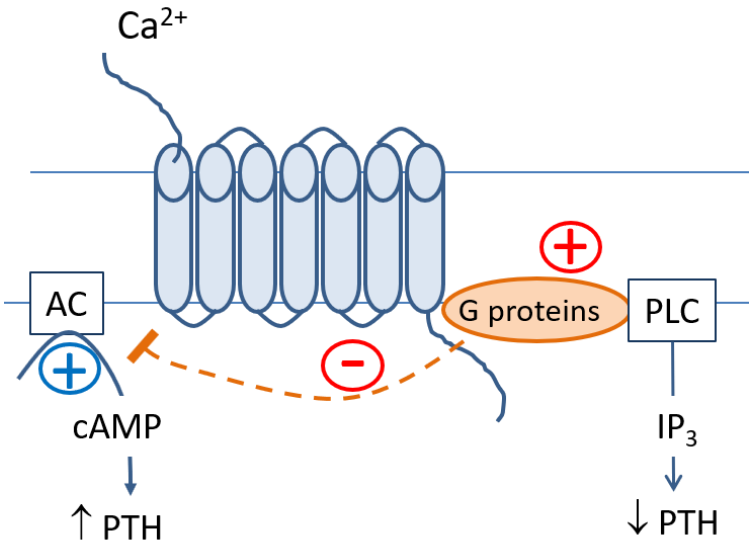

Figure 1.13 The extracellular calcium-sensing receptor signalling.
The extracellular calcium-sensing receptor (CaSR) is a seven transmembrane glycosylated protein that interacts with G proteins. The interaction between calcium (Ca^{2}+) and the CaSR results in activation of phosphatidylinositol-specific phospholipase C (PLC) which stimulates the release of diacylglycerol and produces inositol-1,4,5-trisphosphate (IP_3). IP_3 triggers the release of calcium from the endoplasmic reticulum and causes suppression of PTH. The CaSR also activates inhibitory G protein signalling, and inhibits adenylate cyclase (AC), reducing cyclic adenosine monophosphate (cAMP) formation and suppression of PTH. Decreases om Ca^{2+} decrease CaSR signalling, leading to increased cAMP and increased production and secretion of PTH (DiMeglio & Imel, 2014).

PTH increases the enzyme 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1; 1α-hydroxylase), which catalyses the conversion of 25-hydroxvitamin D (25(OH)D) to $1,25(OH)_2D$ (Brenza & DeLuca, 2000). $1,25(OH)_2D$ activates the vitamin D receptor (VDR) to increase intestinal calcium and phosphate absorption, inhibit further PTH secretion, and increase bone resorption. In the presence of hypercalcaemia, these actions are reversed to decrease serum calcium. This integrated hormonal system is tightly regulated and works effectively to maintain normocalcaemia in healthy individuals (Bland, 2010). Fibroblast growth factor 23 (FGF23) is released from osteocytes and osteoblasts in response to a rise in serum phosphate and $1,25(OH)_2D$; it works to normalise phosphate levels through increased urinary phosphate excretion and reduced intestinal absorption. FGF23 downregulates the luminal expression of sodium-phosphate cotransporters (NPT2a and 2c) in the proximal tubule and NPT2b in the intestine (Shimada *et al.*, 2004b). It also inhibits 1α-hydroxylase and stimulates 24-hydroxylase to suppress the production and increase removal of $1,25(OH)_2D$ which in turn reduces uptake of calcium and phosphate in the gut (Shimada *et al.*, 2004a).

1.6.3 Chronic kidney disease and secondary hyperparathyroidism

The kidney is the major site for expression and activity of 1α-hydroxylase. Expression has been shown in the renal proximal tubule, distal convoluted tubules, collecting ducts, thick ascending loop of Henle and papillary epithelium (Brunette *et al.*, 1978; Zehnder *et al.*, 1999). Whilst the kidney has a central role in $1,25(OH)_2D$ production, 1α-hydroxylase activity has been found in several other cell types throughout the body including the parathyroid glands, testes, skin, placenta, decidua, and macrophages (Campbell & Spector, 2012; Delvin *et al.*, 1985; Weisman *et al.*, 1979; Zehnder *et al.*, 2001; Zehnder *et al.*, 2002).

One of the consequences of CKD is reduced renal capacity to synthesise $1,25(OH)_2D$. Early animal studies demonstrated nephrectomy significantly reduced circulating $1,25(OH)_2D$ (Weisman *et al.*, 1978). Human studies have since confirmed $1,25(OH)_2D$ concentration decreases as CKD progresses (Craver *et al.*, 2007; Levin *et al.*, 2007). Reduced $1,25(OH)_2D$ results in reduced VDR activation, decreased mineral uptake from diet, and subsequent reduction in serum calcium. In normal physiology, $1,25(OH)_2D$ suppresses PTH secretion by the parathyroid glands (Dusso, 2011); in the presence of CKD this mechanism is dysregulated. The resulting increased levels of serum PTH fail to effectively increase renal 1α-

hydroxylase activity or tubular calcium reabsorption in the kidney and therefore the normalisation of serum calcium is increasingly dependent on the resorption of bone and release of calcium from the skeleton (Brown *et al.*, 1982). Urinary phosphate excretion is also reduced by CKD. As a result, FGF23 and PTH are both increased to in a bid to prevent hyperphosphataemia. Consequently, PTH secretion becomes excessive as the defective feedback mechanism strives to maintain normal levels of both calcium and phosphate, resulting in secondary hyperparathyroidism (SHPT). The continuous production of PTH results in parathyroid hyperplasia which reduces sensitivity to calcium and $1,25(OH)_2D$ by the downregulation of VDR and CaSR. A vicious cycle develops as a result (Brown *et al.*, 1982; Cunningham *et al.*, 2011; Slatopolsky *et al.*, 1999) (Figure 1.14).

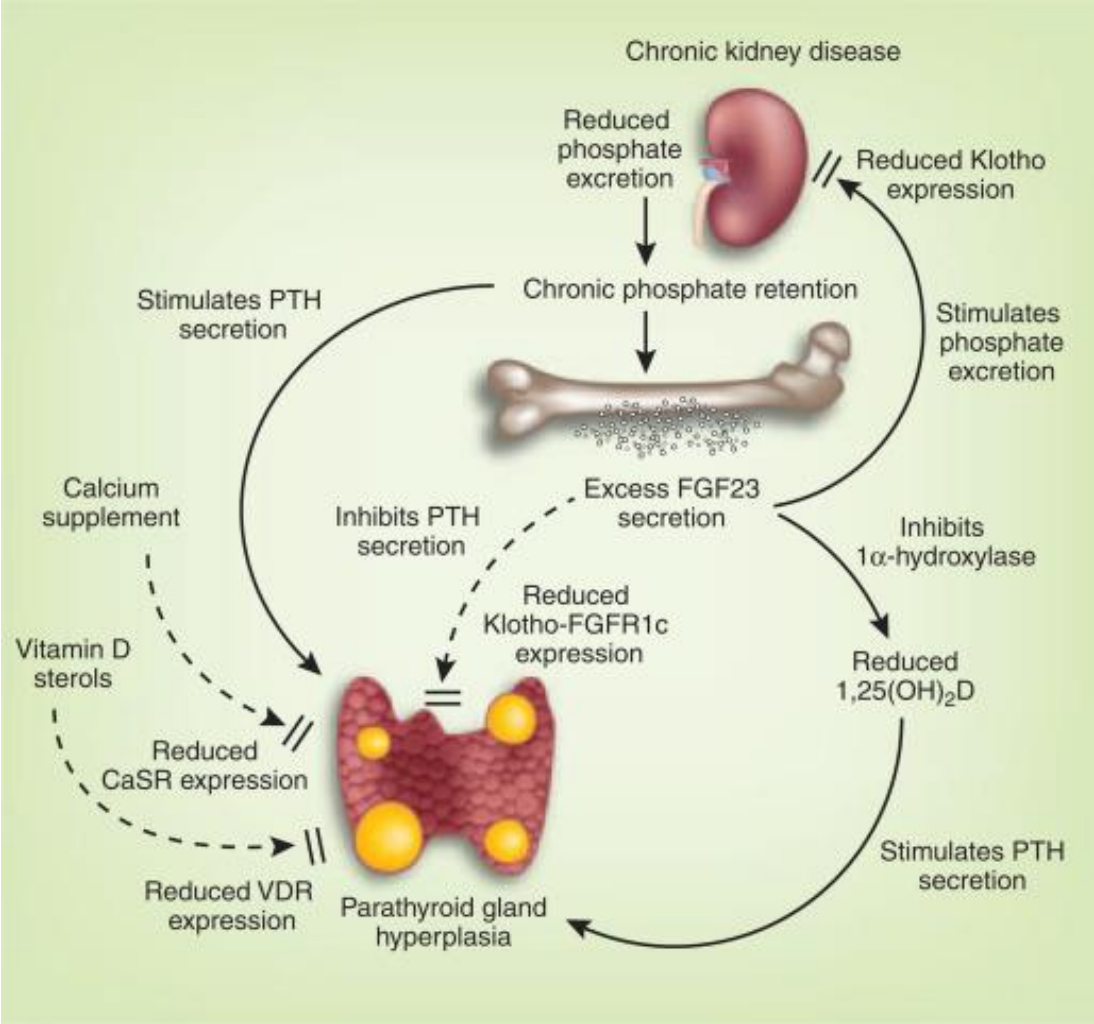

Figure 1.14 Secondary hyperparathyroidism
FGF23 is secreted by bone to maintain neutral phosphate balance. FGF23 inhibits 1α-hydroxylase, reducing $1,25(OH)_2D$ and causing secondary hyperparathyroidism (SHPT) to develop. Hyperphosphataemia and hypocalcaemia occur as CKD progresses; further stimulating PTH synthesis and secretion. PTH secretion becomes excessive as the defective feedback mechanism strives to maintain normal levels of both calcium and phosphate. FGF23 should suppress PTH synthesis and secretion, but in CKD there is resistance of the parathyroid due to down regulation of the FGF23 receptor: klotho complex. Continuous production of PTH results in parathyroid hyperplasia which reduces sensitivity to calcium and $1,25(OH)_2D$ by the downregulation of VDR and CaSR (Komaba & Fukagawa, 2009; Silver & Naveh-Many, 2009).

SHPT prevalence increases along with decreasing GFR and is present in most patients with ESRD (Vikrant & Parashar, 2016). A cross sectional prospective multicentre study explored the relationships between 25(OH)D, 1,25(OH)2D, calcium, phosphate and PTH in CKD patients that were not prescribed vitamin D (n = 1814) (Levin *et al.,* 2007). Median serum calcium and phosphate were stable across eGFR ranges, with serum phosphate only increasing once eGFR reached

<20mls/min/1.73m^2. Median PTH increased above normal range (65pg/mL or 6.8pmol/L) much earlier in CKD progression (eGFR of 45mls/min/1.73m^2) (Levin *et al.,* 2007). As discussed previously, increases in PTH occur to maintain serum calcium and phosphate within the normal range; prevalence of SHPT (PTH >65pg/mL) is reported to be ~12% at an eGFR >80mls/min/1.73m^2 and increases to 56% once eGFR reduces to <40mls/min/1.73m^2 (Levin *et al.,* 2007). This increasing prevalence of SHPT coexists with increasing prevalence of both 1,25(OH)$_2$D and 25(OH)D deficiency (Levin *et al.* 2007) (Figure 1.15).

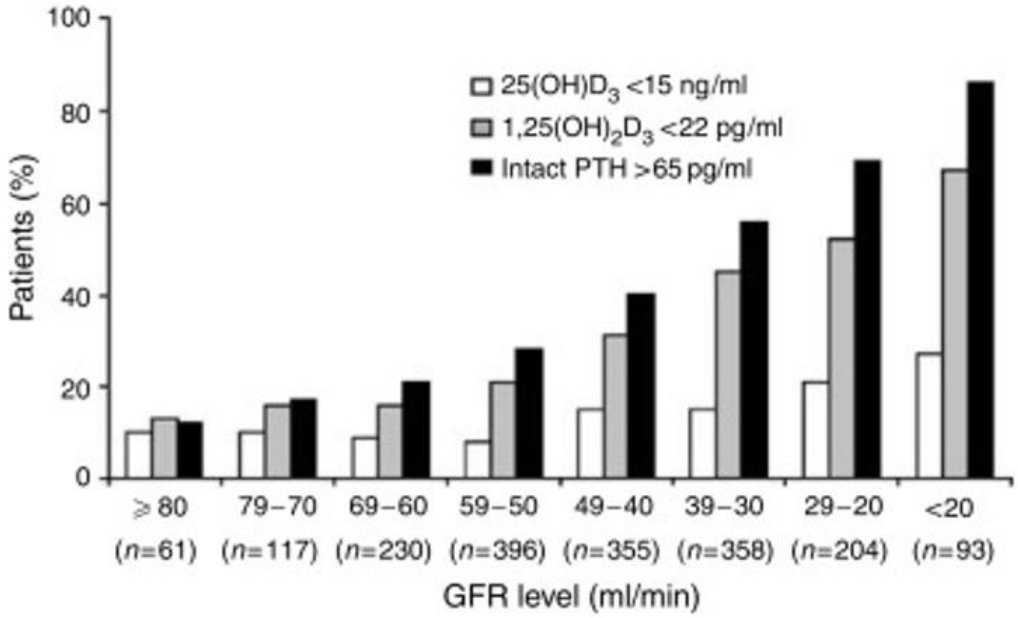

Figure 1.15 Prevalence of 25(OH)D and 1,25(OH)$_2$D deficiency, and secondary hyperparathyroidism, in relation to GFR
Prevalence of secondary hyperparathyroidism (PTH >65pg/mL) was ~12% at an eGFR >80mls/min/1.73m^2 and this increased to ~56% once eGFR reduced to <40mls/min/1.73m^2. There was a decline in both 1,25(OH)$_2$D and 25(OH)D concentrations as eGFR reduced; 1,25(OH)$_2$D deficiency was more prevalent, and prevalence increased at a greater rate, than 25(OH)D deficiency (Levin *et al.,* 2007).

SHPT is associated with clinical manifestations including bone pain, deformity and/or fracture, as well as vascular calcification, major contributors to morbidity and mortality (Block *et al.*, 2004; Jean *et al.*, 2012). The prevalence of vascular calcification has been seen in up to 100% of haemodialysis patients and is considered to be linked with cardiovascular disease, the number one cause of death in this population (Fox *et al.*, 2012; Kraus *et al.*, 2015; Mahmoodi *et al.*, 2012; UKRR, 2019). Hyperphosphatemia, which causes osteogenic transdifferentiation of

smooth muscle cells, appears to be the major contributor to vascular calcification (Giachelli, 2009; Jono *et al.*, 2000; Steitz *et al.*, 2001).

1.6.4 Calciphylaxis

A severe, yet rare, complication of SHPT is calciphylaxis, also referred to as calcific uremic arteriolopathy (CUA). This can occur in the skin as a result of arteriolar calcification of subcutaneous fat and dermis. Calciphylaxis has been associated with mortality rates of approximately 50% within a year of diagnosis (McCarthy *et al.*, 2016; Nigwekar *et al.*, 2016).

With the exception of one study of 242 prevalent HD patients in Hawaii, reporting a prevalence of 4.1% (Angelis *et al.*, 1997), calciphylaxis prevalence data has historically been absent from international literature (Brandenburg *et al.*, 2012). This is beginning to change. A recent study of >250,000 HD patients in North America identified 1030 new calciphylaxis cases during the 5 year follow up and concluded an incidence rate of 3.49 per 1000 patient-years (Nigwekar *et al.*, 2016). Other reported annual incidence rates include 0.04% seen during an 8 year study period (2008-2015) by the German Calciphylaxis Registry (Brandenburg et al., 2017), and 0.03% reported in Japan (Hayashi *et al.*, 2012). Limitations in these data are believed to exist due to misdiagnosis and underreporting (Nigwekar, 2017). The UK Calciphylaxis Study (UKCS) which commenced in 2012 and plans to conclude the end of 2021 aims to identify calciphylaxis risk factors, underlying disease processes, and which treatment strategies confer a favourable outcome. Preliminary data from the UKCS demonstrate calciphylaxis is more prevalent in females (58%), those of Caucasian ethnicity (95%) and people who have diabetes (63%). 59.3% of the UKCS cohort died during the follow up period (median follow-up was 6.5 months) (Chinnadurai *et al*., 2019). Length of time on dialysis (dialysis vintage), higher serum calcium, phosphate and PTH, and younger age have been associated with a risk of calciphylaxis (Angelis *et al.,* 1997; Weenig *et al.*, 2007). Calciphylaxis treatment strategies include a multifaceted approach of wound care and surgery, increased dialysis frequency, and medical management including calcimimetics, bisphosphonates, sodium thiosulphate and possible discontinuation of warfarin and calcium containing phosphate binders (Seethapathy *et al.,* 2019). Where improvement in severe SHPT cannot be achieved medically, surgical parathyroidectomy is considered (Seethapathy *et al.,* 2019).

Whilst most common in those receiving RRT, calciphylaxis is also seen in those with CKD in the pre-dialysis stage, including people with an eGFR >60mls/min/1.73m^2 (McCarthy *et al.*, 2016). Cases have been seen in people with normal kidney function, and warfarin use has been identified as a risk factor (Bajaj *et al.*, 2018; Nazarian *et al.*, 2009; Saifan *et al.*, 2013).

1.7 Chronic kidney disease and anaemia

1.7.1 Red blood cell production

Erythropoietin (EPO), a glycoprotein hormone mainly produced by the kidney, plays an essential role in the growth, and survival, of erythroid progenitor cells (Eckardt & Kurtz, 2005). 90% of EPO is made in the kidneys and the remainder produced by various organs including the liver (Adamson, 1996). EPO production is regulated by hypoxia-inducible transcription factors (HIF) and increased in response to the stress of anaemia or hypoxemia (Adamson, 1996; Suzuki & Yamamoto, 2016; Zivot *et al.*, 2018). A deficiency of either EPO or iron causes a lack of red blood cells (RBCs), and subsequent low levels of Hb (microcytic anaemia); the symptoms of which include fatigue, shortness of breath, dizziness, pallor, cramp, and anorexia (Elstrott *et al.*, 2020).

Circulating EPO binds to EPO receptors on the erythroid progenitor cells, erythroid burst-forming units, and erythroid colony-forming units in the bone marrow (Adamson, 1994). EPO receptors are glycoproteins belonging to the type I superfamily of single-transmembrane cytokine receptors (D'Andrea & Zon, 1990). The binding of EPO to its receptors rescues progenitor cells and the earliest erythroblasts from apoptosis thereby facilitating cell division and subsequent maturation into RBCs (Jelkmann, 2011). EPO maintains the number of RBCs in circulation through stimulating cell proliferation and differentiation of erythrocyte progenitors in the marrow (Kato, 2016) (Figure 1.16). Mature RBCs are released into the blood stream where they survive for approximately 115 days (Franco, 2012). Iron is also essential for RBC production, specifically for synthesis of haemoglobin (Hb). Iron provides the site for oxygen binding in Hb and as such facilitates the transport of oxygen to tissues (Eckardt & Kurtz, 2005).

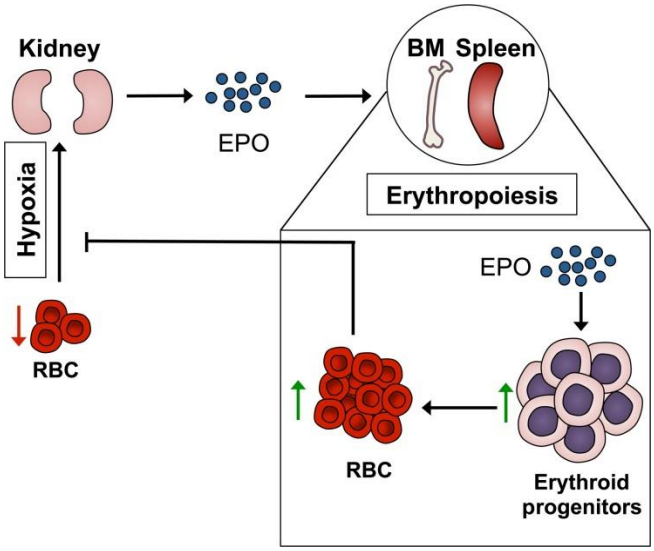

Figure 1.16 Systemic regulation of erythropoiesis through Erythropoietin (EPO).
The kidney produces EPO in response to hypoxia. EPO is carried in the blood to the bone marrow (BM) and spleen where it stimulates proliferation and survival of erythroid progenitors, including burst-forming unit-erythroid and colony-forming unit-erythroid cells. Expansion of the erythroid-committed progenitor pool increases the number of RBC, correcting hypoxemia and returning EPO production back to normal levels (Liang & Ghaffari, 2016).

Hepcidin, a liver derived peptide, is responsible for maintaining systemic iron homeostasis and has been shown to inhibit the production of RBCs by preventing the release of stored iron from macrophages and hepatocytes (Tsuchiya & Nitta, 2013; Young & Zaritsky, 2009). Hepcidin binds its receptor, ferroportin, with high affinity causing its internalisation and degradation (Nemeth *et al.*, 2004). Ferroportin is a cellular iron transporter; the binding of hepcidin blocks iron export to transferrin, and transport to the tissues (Nemeth *et al.*, 2004). Hepcidin expression is regulated mainly at a transcriptional level by various stimuli, including iron concentration, hypoxia, erythropoiesis, and inflammation (Arosio, 2014).

1.7.2 Anaemia and chronic kidney disease

In ESRD, the renal endocrine system fails to produce adequate amounts of EPO required for RBC production. EPO production and secretion decline along with GFR as CKD advances. Deficiency has been identified in CKD stages 1 and 2, with a deficit of up to 64% shown in CKD stage 5 (Mercadal *et al.*, 2012). For this reason, the prevalence of anaemia progresses together with the disease; the exact prevalence is not well reported, and varies according to definition, but anaemia is

believed to affect nearly all patients by the time they reach ESRD (KDOQI, 2006). Anaemia in CKD is associated with reduced health-related quality of life (HRQOL), and increased cardiovascular disease (CVD), hospital admissions, and mortality (Farag *et al.*, 2011; KDOQI, 2006; Macdougall & Eckardt, 2010). Renal anaemia is typically normochromic and normocytic however both the life span and production of RBCs is reduced (Macdougall & Eckardt, 2010). Normal bone marrow would usually have capacity to increase erythropoiesis to compensate for reduction in RBC life; this response is EPO induced and thus impaired in CKD (Macdougall & Eckardt, 2010). Therapeutic intravenous EPO has improved renal anaemia by reducing the need for repeated blood transfusions (KDIGO, 2012). However, the required doses of EPO are variable with some patients requiring high doses despite adequate iron supplementation, and a proportion of patients experiencing EPO resistance (Danielson & Y. Beguin, 1995; Kalantar-Zadeh *et al.*, 2003; Tong & Nissenson, 2001).

Typically poor responders end up on higher doses of EPO which are expensive and associated with an increased risk of hypertension (Krapf & Hulter, 2009). The average weekly EPO cost per HD patient at UHCW is £19.38 (£1,007.76 per patient per annum); assuming these costs are nationally representative, this equates to £25,698,475.60 per year for all UK HD patients. In addition to the financial burden, EPO resistance, which occurs in 10-20% of patients, is independently associated with an increased morbidity and mortality (KDOQI, 2006; Regidor *et al.*, 2006; Zhang *et al.*, 2004). As a consequence, the National Kidney Foundation (NKF) Kidney Disease Outcomes and Quality Initiative (KDOQI) updated its recommended Hb target to 110-120g/L and advised for levels not to exceed 130g/L (KDOQI, 2007; Phrommintikul *et al.*, 2007; Szczech *et al.*, 2008; Vaziri, 2008). Previously, the target had been 110g/L or greater. The UK Renal Association currently recommend a target Hb of 100-120g/L for CKD patients receiving ESA (erythropoiesis stimulating agent) therapy (Renal Association, 2017).

CKD patients require increased amounts of iron in order for EPO to work effectively; therefore iron replacement is routinely given (KDIGO, 2012). Maintaining serum ferritin above 100 microgram/L, regardless of ESA use, is currently recommended; it is recognised that dialysis patients will require intravenous iron in order to achieve this (KDOQI, 2007; Renal Association, 2017). This compares with a recommended lower limit of 15 microgram/L for the general population (NICE, 2018). An optimal ferritin target for HD patients is yet to be proven. DeVita and colleagues carried out

a small randomised controlled trial (RCT), n = 36, that demonstrated targeting higher ferritin levels (400microgram/L) resulted in a 28% reduction in EPO compared with a 200microgram/L ferritin target (DeVita *et al.*, 2003). Similar results were seen by another similar sized RCT, n = 32, which demonstrated a 40% reduction in EPO use with a mean ferritin of 730microgram/L compared with 297microgram/L (Besarab *et al.*, 2000). IV iron has been associated with increased survival, compared with no IV iron when doses of up to 400mg per month are used; whereas doses of >400mg/month have been linked with increased mortality (Kalantar-Zadeh *et al.*, 2005). Another, larger (n = 10,899) study, observational in design, demonstrated no association between any dose of IV iron and risk of mortality (Miskulin *et al.*, 2014). Existing literature is limited by small study size, or uncontrolled study design. For this reason, the risks and benefits of IV iron treatment are not completely understood; large RCT studies are needed (Fishbane *et al.*, 2014).

The increased financial expense, as well as increased mortality risk, suggest a need to investigate ways of optimising the benefits of EPO at low doses (Kumar *et al.*, 2011). Renal anaemia is considered multifactorial and may actually crossover with the anaemia of chronic disease resulting from a combination of reduced EPO production and efficacy, as well as reduced iron availability induced by increased levels of inflammation (Babitt & Lin, 2012). Hepcidin, which inhibits the release of stored iron, is inversely correlated with GFR; circulating levels increase along with the increased inflammation and reduced renal excretion seen in ESRD (Young & Zaritsky, 2009).

1.7.3 Anaemia, inflammation, and chronic kidney disease

CKD is considered to be an inflammatory condition. Serological markers such as C-reactive protein (CRP), fibrinogen, and tumor-necrosis factor-alpha (TNFα), which demonstrates an activated inflammatory response, have been shown in 30% of pre-dialysis CKD patients and 50% of dialysis patients (Stenvinkel *et al.*, 1999; Yeun & Kaysen, 1997; Zimmermann *et al.*, 1999). Contributory factors include increased synthesis, and reduced renal clearance, of inflammatory cytokines such as interleukin 1 (IL-1), IL-2 and IL-6 and TNFα, infections related to dialysis access, existing co-morbidities, and the dialysis process itself (Farag *et al.*, 2011). Chronic inflammation is associated with EPO resistance, reduced HRQOL, and shown to directly impact on clinical outcomes, including CVD and mortality (Zimmermann *et*

al., 1999). Low serum albumin levels are strongly associated with systemic inflammation, and are well recognised as an outcome predictor in CKD; further implicating the role of inflammation (Avram *et al.*, 1996). A low GFR is associated with an abnormally low serum albumin; approximately 10% prevalence is seen in those with an eGFR of 55mls/min/1.73m^2 compared with almost 30% once the eGFR reaches 10mls/min/1.73m^2 (Kopple *et al.*, 2000).

1.7.4 Anaemia, health-related quality of life, and chronic kidney disease

Health-related quality of life (HRQOL) refers to the extent in which a disease limits the capacity to lead a normal life, and has grown in recognition in recent years for its importance as an outcome measure in all areas of clinical research (Mapes, 2004). As well as a measure of a patients perception of their own health, it is an independent predictor of mortality and hospitalisation (Mapes, 2004). HRQOL has been shown to correlate with CKD, reducing with progression of disease (Finkelstein *et al.*, 2009). The compromised HRQOL in haemodialysis patients is well documented with convincing evidence suggesting that HRQOL predicts outcomes in this population (Mapes, 2004). Improving HRQOL on dialysis was one of the top research priorities identified in a patient survey carried out in 2013 (Davies, 2015; KRUK, 2013).

1.8 Vitamin D, anaemia, and inflammation in CKD

One reported benefit of 25(OH)D supplementation is the improvement in anaemia in CKD. Low levels of serum 25(OH)D have been linked with increased EPO requirements suggesting that vitamin D supplementation may improve EPO response (Kiss *et al.*, 2011; Srisakul *et al.*, 2011; Stenvinkel, 2001). A retrospective study of 142 maintenance haemodialysis patients in Hungary found serum 25(OH)D levels significantly correlated with Hb level and ESA dose even after controlling for confounding factors (see Figure 1.17) (Kiss *et al.*, 2011).

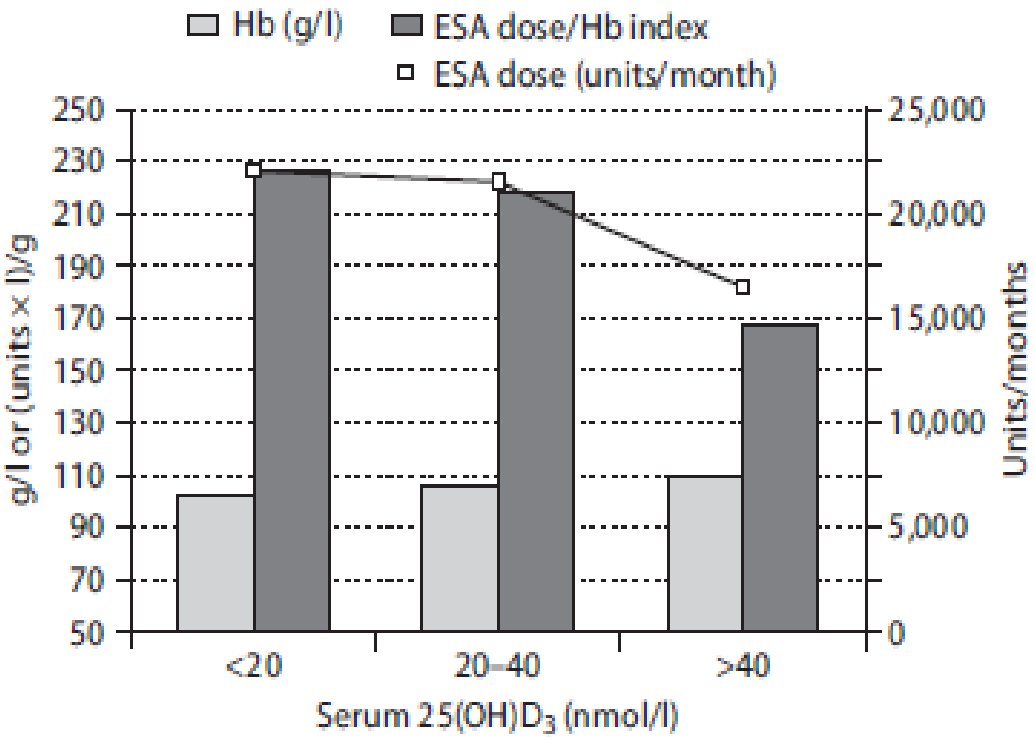

Figure 1.17 Serum 25(OH)D is independently associated with erythropoietin dose
Data from 142 patients was included for analysis and serum 25(OH)D levels were found to significantly correlate with Haemoglobin (Hb) level and erythropoietin stimulating agent (ESA) dose even after controlling for confounding factors. Associations between serum 25(OH)D_3 concentrations vs. haemoglobin (Hb) level (light grey bars), monthly ESA dose (line) and ESA dose/Hb index (dark grey bars). $P \leq 0.05$ for trend in all three associations (Kiss *et al.*, 2011).

Similar results were demonstrated in retrospective cohort study of 153 patients looking at the correlation between 25(OH)D levels and ESA usage in CKD patients (Lac *et al.*, 2010). Patients were grouped according to their change in 25(OH)D levels, and a 24% reduction (P =0.025) in ESA dose was seen in the patients going from a low-to-normal serum vitamin D level. In addition, a trend towards increased ESA dosage was seen in patients who became vitamin D deficient from a previously sufficient state; overall there appeared to be an ESA sparing effect with 25(OH)D repletion (Lac *et al.*, 2010). The same was found by a pilot study of 81 vitamin D-deficient HD patients which demonstrated that ergocalciferol supplementation may reduce EPO doses (Kumar *et al.*, 2011). Mean 25(OH)D levels after 4 months of ergocalciferol supplementation had increased to 71.1±21.5nmol/L compared to 38.2±17.7 nmol/L at baseline. The median dose of EPO dropped to 18,400 units per month from 21,933 units per month at the beginning of the study (P = 0.17). Overall, 57% of study participants required less EPO after ergocalciferol was

administered, however a higher EPO dose was required by 43% and the differences seen were not significant. Furthermore only 44% of study subjects achieved the optimal 25(OH)D level (≥75nmol/L), further limiting findings. A similar study of 119 HD patients showed 94% of patients were able to achieve vitamin D levels of ≥75nmol/l after 300,000 IU ergocalciferol was administered during a 6 month period from May to October (Saab *et al.*, 2007). 91% of study subjects had vitamin D deficiency or insufficiency (<75nmol/l) at baseline. Vitamin D deficiency resolved in all patients and only 6% of patients remained vitamin D insufficient: mean levels increased to 133.8±40.7nmol/L from 42.2±21.2nmol/L at baseline. The authors reported a significant reduction in median weekly EPO dose (P = 0.001). The weekly EPO dose reduced in 64% of patients, increased in 28% of patients and remained stable in 8% of patients. This benefit was mostly seen in patients with baseline 25(OH)D levels less than 50nmol/L, suggesting that patients who were most deficient in vitamin D exhibit the greatest benefit with regard to EPO sensitivity. All four studies lacked a control arm.

Randomised Control Trials (RCTs) of vitamin D supplementation in HD patients have generally been small and do not provide high-level evidence for any outcome. Two prior double blinded RCTs, both based in the USA, have reported on ESA dose. The first was small (n=50), and randomised patients to receive cholecalciferol, or placebo, for 6 months (Mose *et al.*, 2014). The second was a comparatively larger study (n= 276), that randomised patients to receive ergocalciferol, or placebo, for 6 months (Miskulin *et al.*, 2016). Mean serum 25(OH)D levels increased from 30nmol/L to 80nmol/L, and 40nmol/L at baseline to 98nmol/L, in the intervention arms, respectively, but neither study saw a change in EPO dose over the 6 months in the intervention or control groups (Miskulin *et al.*, 2016; Mose *et al.*, 2014).

In vitro and *in vivo* studies have demonstrated increased erythropoiesis when supplementing vitamin D to a previously vitamin D deficient state (Aucella *et al.*, 2003; Icardi *et al.*, 2013). The mechanism suggested for this effect is via the role of 25(OH)D in the suppression of pro inflammatory cytokines inhibitory to erythroid progenitors (Icardi *et al.*, 2013). Serum 25(OH)D may affect iron homeostasis by lowering serum levels of the iron-regulatory protein hepcidin (Babitt & Lin, 2012; Tsuchiya & Nitta, 2013). Cholecalciferol supplementation has also been shown to reduce IL-6, a pro-inflammatory cytokine known to be the primary mediator in hepcidin production, by 30% (Andrews, 2004; Tsuchiya & Nitta, 2013) (Figure 1.18).

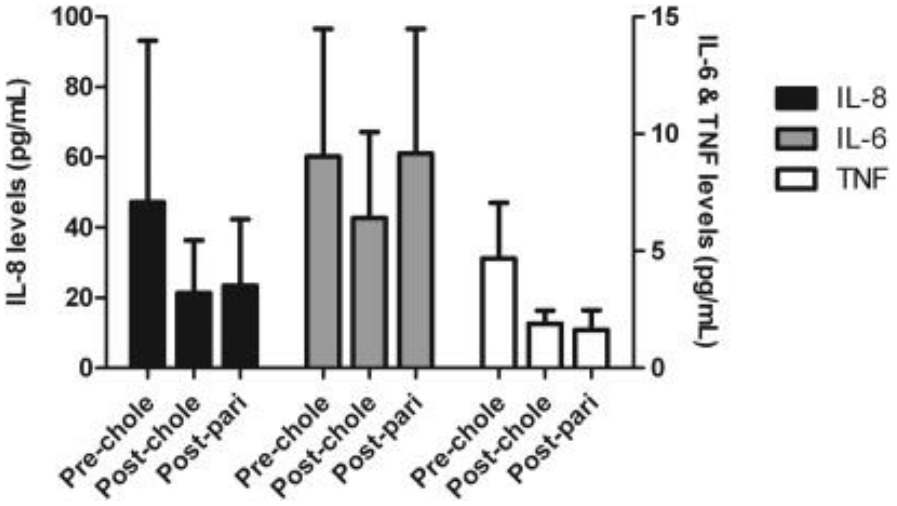

Figure 1.18 Change in serum IL-8, IL-6 and TNF-α levels after cholecalciferol and paricalcitol therapy.
Stubbs and colleagues (2010) investigated the effects of cholecalciferol and paricalcitol therapies on the inflammatory phenotype. A 55% decrease in serum IL-8 levels (P = 0.13), 30% decrease in IL-6 levels (P = 0.05), and a 60% decrease in TNF-α (P = 0.05) was seen in response to cholecalciferol therapy. Serum IL-8 and TNF-α levels remained lower after discontinuation of cholecalciferol and restarting of paricalcitol yet IL-6 levels returned to near baseline (n = 7).

Given the potential risks, and costs, associated with EPO use (these are discussed in section 1.7.2), reducing EPO dose is desirable, particularly if it can be achieved safely and inexpensively. Promoting erythropoiesis through improving serum 25(OH)D may have a role in the treatment of renal anaemia. In addition to its anti-inflammatory effects, adequate serum 25(OH)D enables local tissue synthesis of active $1,25(OH)_2D$ in non-renal tissues (Johal & Levin, 2009; Zehnder *et al.*, 2001). The production of local $1,25(OH)_2D$ in haematopoietic tissue improves erythropoiesis not only through its anti-inflammatory actions but also because it works synergistically with EPO on erythroid burst forming units, thus having an erythropoietin-sparing effect (Aucella *et al.*, 2003). Extra-renal $1,25(OH)_2D$ synthesis is also associated with the induction of antibacterial proteins including cathelicidin (Liu *et al.*, 2006). This has been associated with the abrogation of inflammatory immune responses suggesting increased serum 25(OH)D may also result in better immune responses and thus reduced infection risk (Adams & Hewison, 2008). These benefits relate to locally synthesised extra-renal $1,25(OH)_2D$ and are dependent upon sufficient serum 25(OH)D.

Whilst studies have linked improved serum 25(OH)D with a reduced requirement for EPO, current evidence is weakened by the uncontrolled study design seen in the majority of reported research and also failure to successfully replete serum 25(OH)D levels in all patients. Baseline 25(OH)D levels have varied considerably between studies and it is suggested that higher doses of vitamin D are required in patients with empty vitamin D stores (Tokmak *et al.*, 2008). Anaemia guidelines have suggested that the role of non-active vitamin D supplementation in EPO responsiveness requires further investigation (KDIGO, 2012). There is presently an absence of UK studies looking at 25(OH)D supplementation and EPO use, and more research is needed in order to influence practice.

Most haemodialysis patients require EPO therapy; they also have reduced iron availability (Mercadal *et al.*, 2012; Nemeth *et al.*, 2004). Hepcidin is modulated by inflammation, and increased hepcidin blocks the release of stored iron. Vitamin D supplementation may reduce inflammation resulting in lower serum hepcidin which will improve iron availability and RBC production. In addition, improved serum 25(OH)D may result in the synthesis of $1,25(OH)_2D$ in hematopoietic tissues which may also aid red blood cell production. Both mechanisms would result in a reduced requirement for EPO; Figure 1.19 outlines the proposed mechanisms.

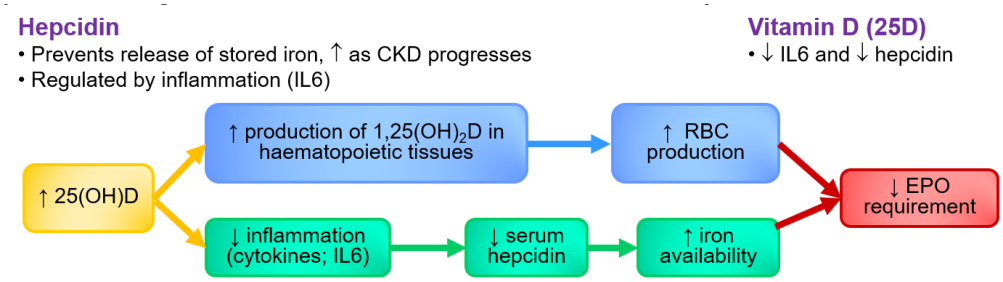

Figure 1.19 The proposed mechanism by which cholecalciferol supplementation reduces erythropoietin (EPO) requirement, inflammation, and improves iron availability.
Nearly all haemodialysis patients require EPO therapy however they also have reduced iron availability. Hepcidin is modulated by inflammation and increased hepcidin blocks the release of stored iron. Vitamin D supplementation may reduce inflammation resulting in lower serum hepcidin which will improve iron availability and red blood cell (RBC) production. In addition, improved serum 25(OH)D may result in the synthesis of $1,25(OH)_2D$ in hematopoietic tissues which may also aid RBC production. Both mechanisms would in turn result in a reduced requirement for EPO.

1.9 Vitamin D and health-related quality of life in CKD

Health-related quality of life (HRQOL) reflects the impact of disease on an individuals' sense of well-being, and as such is useful in assessing the effectiveness of therapeutic interventions (Soni *et al.*, 2010). Benefits on inflammation and EPO may result in improved Health-related quality of life (HRQOL) scores. Evidence also suggests vitamin D supplementation may directly impact on HRQOL; however, the mechanism for this is not yet understood.

HRQOL has been shown to correlate with CKD, reducing along with progression of disease, Figure 1.20 (Mujais *et al.*, 2009). Compromised quality of life in haemodialysis patients is well documented with convincing evidence suggesting that HRQOL predicts outcomes in this population (Anand *et al.*, 2011; Kušleikaitė *et al.*, 2010; Mapes, 2004). Factors affecting HRQOL for haemodialysis patients include: dialysis burden, body image issues related to having a fistula, impaired mental wellbeing, anxiety, and a lack of autonomy related to dependence on dialysis for survival (Moreiras-Plaza *et al.*, 2011). Recognition for the importance of improved HRQOL has grown in recent years, yet the prevalence of mental-health problems, including anxiety and depression, within the ESRD population remain understudied (Kimmel *et al.*, 2008; Pei *et al.*, 2019). Reported rates for anxiety, and depression, in ESRD are 21.1 - 51.6% and 23.3 - 65.3%, respectively (Alavi *et al.*, 2009; Turkistani *et al.*, 2014; Yoong *et al.*, 2017).

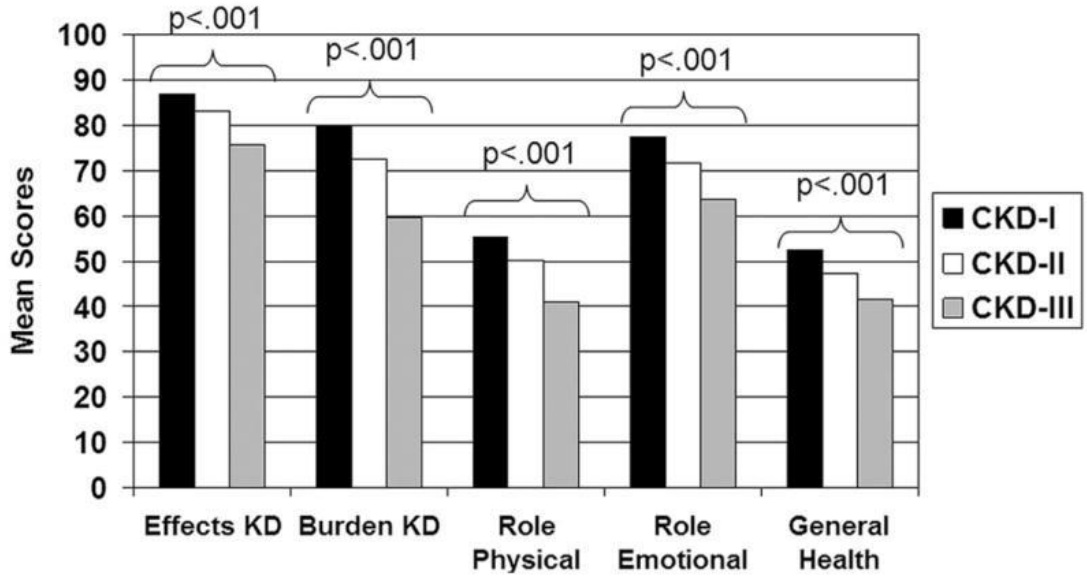

Figure 1.20 Health-related quality of life (HRQOL) declines progressively along with chronic kidney disease (CKD) stage.
HRQOL domain scores show progressive decline as CKD advances. CKD-I = CKD stage 3, CKD-II = CKD stage 4 and CKD-III = CKD stage 5. KD = kidney disease. P values represent the trend across the CKD stages (Mujais *et al.,* 2009).

Improving serum 25(OH)D levels through vitamin D supplementation may reduce inflammation and improve anaemia management which in turn may positively impact on clinical outcomes including mortality and HRQOL (Kiss *et al.*, 2011; Lac *et al.*, 2010; Srisakul *et al.*, 2011; Stenvinkel, 2001). Evidence also suggests that vitamin D supplementation may improve HRQOL through increased muscle strength and physical function, as well as its anti-inflammatory role (Anand *et al.*, 2011; Jones, 2011). This is further supported by the association of low serum 25(OH)D levels with muscle atrophy, increased falls, musculoskeletal pain, and poorer physical function, all of which impact on HRQOL (Boudville *et al.*, 2010).

1.10 Vitamin D Supplementation in End Stage Renal Disease

In 2003 the US National Kidney Foundation KDOQI guidelines recommended ergocalciferol supplementation for CKD stages 3 and 4; this included a serum 25(OH)D target of 75nmol/L and a clear supplementation strategy to achieve it (K/DOQI, 2003). However, these recommendations were superseded in 2009, and then 2017, by global CKD-MBD guidelines which do not specify a serum 25(OH)D target or vitamin D supplementation strategy (KDIGO, 2017). Recommendations for

ESRD have remained focused on active vitamin D analogues throughout this time (KDIGO, 2017; KDOQI, 2003).

Until recently the high prevalence of 25(OH)D deficiency and the associated bone and non-bone related problems were not well appreciated in ESRD; treatment strategies almost exclusively focused on calcitriol, alfacalcidol and paricalcitol. This is largely attributed to a continued lack of understanding for the role and benefits of the different vitamin D forms, and of extra-renal $1,25(OH)_2D$ synthesis, in addition to the engrained concept that only $1,25(OH)_2D$ (the fully active vitamin D form) is required for bone mineral homeostasis. Existing evidence clearly demonstrates that the notion of vitamin D deficiency being purely a bone related problem is outdated. In response to a growing body of evidence, recognition of 25(OH)D deficiency and the appreciation for the benefits of supplementation is beginning to improve. The focus, at least in CKD patients (pre-dialysis), has reverted to 25(OH)D repletion, rather than active analogue use, as first line treatment for SHPT. UK National guidelines for CKD changed in 2014 and now suggest treatment with vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol) in those with an eGFR <30mls/min/1.73m^2 and increased PTH (NICE, 2014). Global renal guidelines also suggest vitamin D levels may be checked and supplementation given, if required, as per the general population, in all stages of CKD (KDIGO, 2017).

As discussed in section 1.4, supplementation guidelines for the general population vary and the UK NHS has adopted SACN (2016) recommendations (400IU per day, to achieve 25(OH)D ≥25nmol/L). SACN recommendations do not consider the influence of chronic disease including i) the effects of CKD on vitamin D metabolism, or ii) the optimal 25(OH)D threshold for CKD. 400IU has been shown to increase 25(OH)D concentration in people with CKD stages 3 and 4 from 17±9nmol/L to 31±15nmol/L; SACNs 25nmol/L target was not achieved by all (Sekkarie, 2006). A need to distinguish a serum 25(OH)D target in relation to its protective effects on each chronic disease has been proposed (Muscogiuri, 2018). However, even with a disease-specific target, given other factors affect dose response (BMI for example), defining a single recommended daily dose may not be possible.

Management of vitamin D deficiency is complicated further by a push from primary care to get patients to purchase their own over the counter (OTC) preparations (APC, 2017). In Coventry and Warwickshire, those with an eGFR

<30mls/min/1.73m^2, should, theoretically, be exempt from this. However, fiscal pressures on the NHS no doubt complicate management of vitamin D deficiency. OTC vitamin products have limitations beyond the expense of their purchase; they are classed as food supplements and do not require the same licensing standards as prescribed preparations, meaning the actual dose may differ from the amount stated on the packet. The British Pharmacopeia specification for licensed medicines is 90-125% of the labelled claim - this compares with 80-150% for food supplements. 11 OTC vitamin D supplements were tested by a London research group and results for vitamin D content ranged from 41.2±10.6% to 165.3±17.8% of labelled claim; 8 of the 11 products failed to meet the food supplement standards (Wan *et al.*, 2020). The same group tested 2 licensed products; these met British Pharmacopeia specification with 90.9±0.7% and 90.5±3.9% of labelled claim (Wan *et al.*, 2020). A previous US study demonstrated similar issues with OTC vitamin D preparations, with a variance of 9% to 146% of labelled claim (LeBlanc *et al.*, 2013).

Currently there are no renal specific guidelines for vitamin D supplementation (ergocalciferol or cholecalciferol) in relation to recommended dosage and the serum 25(OH)D level to aim for. This, together with the limitations discussed above, means recommendations have not widely translated into practice. Leading guidelines recommend treating renal patients the same as the general population (KDIGO, 2017). However, these do not consider the implications of CKD on vitamin D metabolism and deficiency risk. Given the endocrine functions of the kidney, it could be argued that the ES guidelines are more applicable than the IOM and SACN reports, which are aimed at the general population.

1.10.1 Vitamin D Toxicity

Cholecalciferol supplementation is considered safe because it increases the inactive substrate 25(OH)D rather than directly increasing 1,25$(OH)_2$D. As discussed in section 1.6.2, conversion of 25(OH)D into 1,25$(OH)_2$D is tightly regulated in response to serum calcium and PTH. Safe serum 25(OH)D level has been defined as <200nmol/l (Vieth, 1999) and review of the available literature demonstrates that all haemodialysis patients supplemented with vitamin D (even those on very high doses) remained within this safe range (further discussed in section 2.1.6). The safe upper limit for vitamin D_3 supplementation in the general population varies between guidelines from 4,000IU-10,000IU per day (Efsa Panel on Dietetic Products, 2012; Ross *et al.*, 2011). Cholecalciferol has been shown to

be more effective than ergocalciferol at improving circulating 25(OH)D levels (Armas *et al.,* 2004, Tripkovic *et al.,* 2012). Vitamin D supplementation therapy is inexpensive, considered safe (side effects being hypercalcemia and hyperphosphatemia), and there is continued emerging evidence to suggest many benefits.

The next chapter reports the results of a systematic review undertaken to determine the dose of cholecalciferol required to replete haemodialysis patients' serum 25(OH)D levels to ≥75nmol/L; to inform the development of clinical guidelines for the treatment of these patients at UHCW.

Chapter 2 Systematic review and cholecalciferol supplementation guideline development

There is an absence of guidance on vitamin D supplementation in ESRD, specifically in relation to how much vitamin D is required to achieve 25(OH)D repletion. Therefore, a systematic review of the current literature, looking at the safety and efficacy of cholecalciferol supplementation in haemodialysis patients was justified. The aim of systematic review was to inform the development of a new guideline, to introduce cholecalciferol supplementation as part of standard care to UHCW HD patients.

Whilst the laboratory reporting system at University Hospitals Coventry and Warwickshire (UHCW) defines a serum 25(OH)D level of <50nmol/L as insufficient, this does not take into account individual risk factors, or the health benefits of vitamin D in CKD, which have largely been associated with serum 25(OH)D levels ≥75nmol/L. There is no established 'ideal' serum 25(OH)D cut off for patients receiving haemodialysis (HD). Therefore the optimal serum 25(OH)D level of 75nmol/L was chosen based on the ES guidelines, the rationale for this is discussed in section 1.10 (Holick *et al.*, 2011). This level was used to inform the systematic review.

2.1 Systematic review

The title for the systematic review was developed based on the PICO (Population, Intervention, Comparison, Outcome) system (section 2.1.1).

Title

What dose of cholecalciferol is required to replete haemodialysis patients' serum 25(OH)D levels to ≥75nmol/L?

Aim

To identify the appropriate dose of cholecalciferol to safely and effectively, increase and maintain serum 25(OH)D to ≥75nmol/L.

Objectives

- To systematically identify all relevant research studies that have been reported to date looking at cholecalciferol supplementation in haemodialysis patients.
- To systematically select appropriate studies according to predetermined inclusion and exclusion criteria.
- To obtain the full text of all the finally selected papers and critically appraise the studies.
- To summarise the findings and literature review.

2.1.1 Systematic review methods

The PROSPERO systematic review register was reviewed to check the proposed research question was original.

Study eligibility Criteria

A Cochrane review was not possible due to the lack of randomised controlled trials in this field. Therefore, a review of relevant intervention studies that met pre-set criteria was undertaken. Studies needed to be available in English.

Developing the PICO (Population, Intervention, Comparison, Outcome)

- **P**opulation (or Patient or Problem): Adults (≥ 18 years of age) with ESRD receiving haemodialysis
- **I**ntervention: Cholecalciferol
- **C**omparison: N/A
- **O**utcomes: Serum 25(OH)D measured and reported and dose and frequency of cholecalciferol reported

Information sources

Following meetings with librarians at UHCW and The University of Warwick a list of search terms was drawn up; this was based on all known words that could be used to cover the criteria set out in the PICO. The choice of databases to include was also decided at these meetings. As a means of cross checking the integrity of the

search, papers familiar to the researchers were identified and the search results checked to ensure these papers were included. A search of the following databases was carried out between February and June 2014; Embase, Medline, Web of Science, Cochrane, Cinahl and Proquest.

Full electronic search strategy

All database searches were based on the following Medline search which was carried out 7th February 2014 (Table 2.1).

1. Dietary Supplements/
2. supplement*.mp.
3. (dosage or dose).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
4. drug therapy.mp. or exp Drug Therapy/
5. 1 or 2 or 3 or 4
6. exp Vitamin D/ or exp Vitamin D Deficiency/
7. vitamin d.mp.
8. cholecalciferol.mp. or exp Cholecalciferol/
9. colecalciferol.mp.
10. 6 or 7 or 8 or 9
11. 5 and 10
12. hemodialysis.mp. or exp Renal Dialysis/
13. exp Kidney Failure, Chronic/ or haemodialysis.mp.
14. esrd.mp.
15. end stage renal disease.mp.
16. end stage renal failure.mp.
17. chronic kidney disease.mp. or exp Renal Insufficiency, Chronic/
18. 12 or 13 or 14 or 15 or 16 or 17
19. 11 and 18
20. 12 or 13 or 14 or 15 or 16
21. 11 and 20
22. limit 21 to english language

Table 2.1 Medline search criteria

The list of search terms was based on all known words that could be used to cover the criteria set out in the PICO: **P**opulation: adults (≥ 18 years of age) with ESRD receiving haemodialysis, **I**ntervention: Cholecalciferol, **C**omparison: N/A, **O**utcomes: serum 25(OH)D measured and reported and dose and frequency of cholecalciferol reported.

Criteria for journal paper selection

Search results were screened according to preset inclusion and exclusion criteria (Table 2.2 and Table 2.3). The initial title screen was carried out by myself, and the

abstract and full text screening, was undertaken by 2 reviewers (Ph.D supervisor Dr Rosemary Bland and myself).

Subjects	Chronic Kidney Disease CKD Dialysis End Stage Renal Disease ESRD Haemodialysis Hemodialysis Kidney Failure	
Intervention	Cholecalciferol Colecalciferol Oral Supplementation Supplement Supplements Vitamin D Vitamin D3	
Exclude	1,25 dihydroxyvitamin D 1,25(OH)D 1-alfa 1-alfacalcidol 22-oxacalcitol active vitamin D alfacalcidol alphacalcidol Animals Dogs bone disease prevention calcification Calcimimetics calcium carbonate supplementation studies calcium kinetics cellular studies Children Cincalcet CKD stage 1 - 4 Conservative cystinosis Denosumab Early CKD Ergocalciferol Home dialysis hypophosphatemic	Intravenous IV Lanthanum low calcium dialysate Maxacalcitol myeloma patients non-dialysis non-dialyzed non-renal studies Omega 3 Paricalcitol Peritoneal Dialysis Prediabetes Pre-dialysis Rickets Sevelamer Transplant Velcalcetide Vitamin D2 Calcium Oxalate 24,25(OH)D3 PTH assay Fistula Sarcoidosis Phosphate binder

Table 2.2 Inclusion and exclusion criteria according to paper title.
The criteria for inclusion and exclusion according to paper title for the systematic review.

Subjects	Haemodialysis Hemodialysis
Intervention	Cholecalciferol Colecalciferol Oral Vitamin D3
Exclude	1,25(OH)D 1-alfa 1-alfacalcidol active vitamin D alfacalcidol alphacalcidol CKD stage 1 - 4 Conservative Early CKD Ergocalciferol Home dialysis Intravenous IV non-dialysis non-dialyzed Paricalcitol Peritoneal Dialysis Transplant Calcifediol/calcidiol/25(OH)D supplement Non-intervention studies Cinacalcet Combined therapy studies (eg. Cholecalciferol plus omega 3) Vitamin D2

Table 2.3 Inclusion and exclusion criteria according to paper abstract
The criteria referred to for inclusion and exclusion according to paper abstract for the systematic review.

The criteria for selecting a paper for full text assessment of a paper was predefined and is set out in Table 2.2 and Table 2.3. A form was created for the full paper screening process (Figure 2.1).

Reviewer: SH RB Other	Date:		
Reference:			
Study ID: (first author, year)	**Endnote (EN) No:**		
	(circle as appropriate)		
1) Full article available	**Yes**	**No**	**Unsure**
2) Participants – adults (≥18 years) on haemodialysis *If studies include both peritoneal dialysis and haemodialysis patients then only include if results for haemodialysis patients can be identified separately.*	**Yes**	**No**	**Unsure**
3) Intervention - Vitamin D3 (Cholecalciferol) *This includes only oral supplements and excludes those where vitamin D is taken as part of a multivitamin/mineral preparation or in combination with another supplement/therapy*	**Yes**	**No**	**Unsure**
4) Blood vitamin D level measured (serum 25(OH)D)	**Yes**	**No**	**Unsure**
5) Dose and frequency of vitamin D3 reported	**Yes**	**No**	**Unsure**
If all yes, include. If not all yes, exclude.	**Include**	**Exclude**	**Unsure**
Any comments:			

Figure 2.1 Inclusion and exclusion criteria for full text screening
The above form was completed to assess inclusion or exclusion of full papers for the systematic review. Two reviewers (Sharon Huish and Dr Rosemary Bland) completed this form after reading each full text paper.

2.1.2 Study selection

The combined search of Embase, Medline, Web of Science, Cochrane, Cinahl and Proquest identified 3592 citations. After 745 duplicates were removed this provided 2847 results for screening. A further 2500 were excluded following screening of title. 316 papers were excluded after abstract review which left 31 articles requiring full text screening; we identified 3 further papers from a search of the reference lists of the 31 full-text articles, meaning 34 full articles were assessed. The final assessment stage identified 17 papers for further evaluation (Figure 2.2).

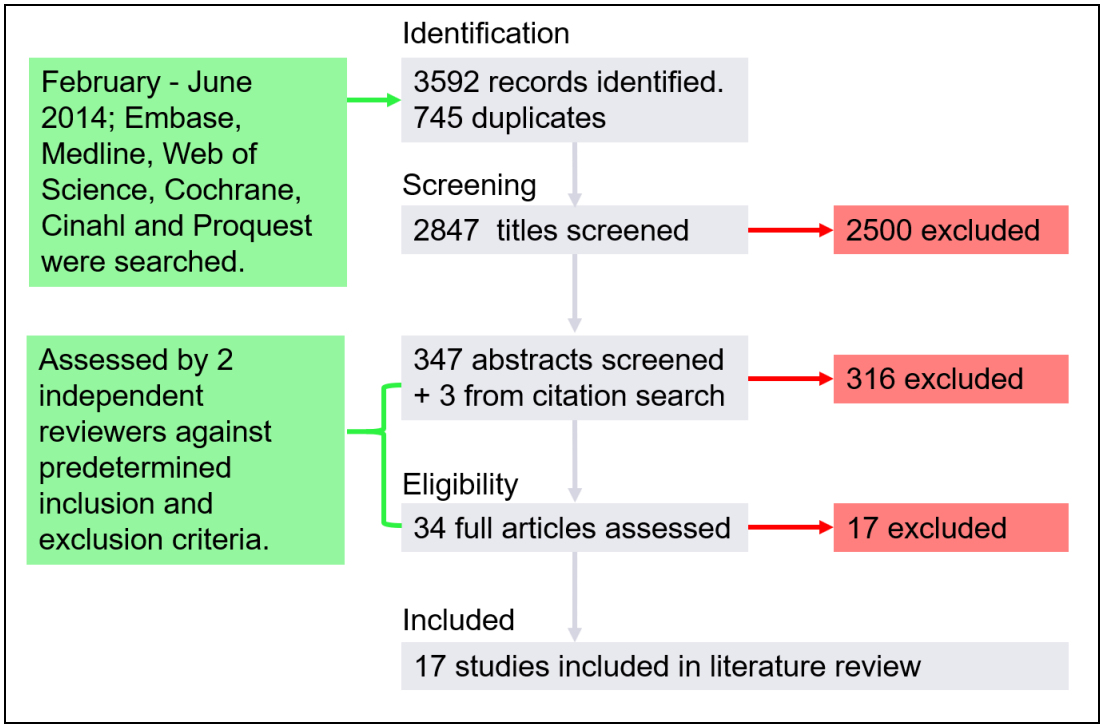

Figure 2.2 Systematic review study selection
The combined search of Embase, Medline, Web of Science, Cochrane, Cinahl and Proquest identified 3592 citations. After 745 duplicates were removed this provided 2847 results for screening. 2500 were excluded after title review and a further 316 after abstract review. Full text assessment of 34 articles identified 17 papers which were reviewed.

2.1.3 Study characteristics

Although search parameters were not limited by year, final included papers were published between 2008 and 2014 and involved a total of 855 HD patients, of which 595 received cholecalciferol as the intervention. Studies were all small in terms of study size but still varied in participant numbers; the mean study size was 37, median 24 (range 7-158). 8 of the 17 studies were randomised controlled trials (RCTs) and 9 were prospective cohort studies. Participant age was reported as mean by some studies and median by others; for the 10 studies reporting age as a mean, the overall mean was 62.2±14.1 years. 7 studies reported participant age as median; the median age varied from 53.6-69.0 (range 20 – 87). 59% and 41% of the collective population were male and female, respectively. 2 of the studies did not report participant age and 1 of these did not report gender either (Tokmak *et al.*, 2008, Stubbs *et al.*, 2010). 15 of the studies used immunoassays for analysis of serum 25(OH)D, 1 used liquid chromatography–tandem mass spectrometry (LC–MS/MS) and 1 high-performance liquid chromatography. None of the studies were

UK based, the majority were carried out in European countries (n = 10), 1 in Turkey, 4 in the USA, 1 in Australia and 1 in Brazil. Table 2.4 represents the characteristics of the included studies including the assay used to measure 25(OH)D, which, as discussed in section 1.4.3, is an important consideration.

Study Design	Source	Included in analysis (n)	Age median (IQR) or mean ±SD	%male	25(OH)D assay	Country
Randomised Controlled Trials	Armas L *et al.*, 2012	42	57.6 (47.2-64.2)	70	Immunoassay (Nichols Advantage)	USA
	Delanaye P *et al.*, 2013	30	75±9	70	Immunoassay (DiaSorin LIAISON)	Belgium
	Hewitt *et al.*, 2013	45	60 (53-71)	48	Immunoassay (DiaSorin LIAISON)	Australia
	Marckmann *et al.*, 2012	52	71 (62-78)	75	LC-MS/MS (Applied Biosystems, Dionex)	Denmark
	Massart *et al.*, 2014	53	62 ±12.3	64	Immunoassay (DiaSorin LIAISON)	Belgium
	Seibert *et al.*, 2013	33	66.9 ±10.8	55	Immunoassay (DiaSorin LIAISON)	Germany
	Tokmak *et al.*, 2008*	64	Not reported	59	Immunoassay (Nichols Advantage)	Germany
	Wasse H *et al.*, 2012	52	49±13	62	Immunoassay (DiaSorin LIAISON)	USA
Intervention Studies (no control arm)	Armas L *et al.*, 2013	30	53.6 (45.8-65.4)	57	Immunoassay (DiaSorin LIAISON)	USA
	Bucharles *et al.*, 2012	30	59±15	47	Immunoassay (DiaSorin LIAISON)	Brazil
	Daroux M *et al.*, 2012	37	66.4±18.6	70	Immunoassay (DiaSorin LIAISON)	France
	Hryszko T *et al.*, 2013	22	69 (20-84)	59	Immunoassay (Elecsys, Roche)	Poland
	Jakopin E *et al.*, 2014	65	63.3 (26 - 87)	52	Immunoassay (Elecsys, Roche)	Slovenia
	Jean G *et al.*, 2009	107	66.4±15	56	Immunoassay (DiaSorin LIAISON)	France
	Matias P *et al.*, 2010	158	62.8±14.8	47	Radioimmunoassay (IDS)	Portugal
	Ozkurt S *et al.*, 2013	28	52±18	54	HPLC (ImmuChrom)	Turkey
	Stubbs JR *et al.*, 2010	7	Not reported	-	Immunoassay (DiaSorin LIAISON)	USA

Table 2.4 Characteristics of included studies.

8 of the 17 studies were randomised controlled trials (RCTs) and 9 were prospective cohort studies. Collectively the studies analysed data from 855 participants; the mean study size 37, median 24 (range 7-158). Participant age was reported as mean by some studies and median by others; for the 10 studies reporting age as a mean, the overall mean was 62.2 ±14.1 years. 7 studies reported participant age as median; the median age varied from 53.6 - 69.0 years (range 20 – 87). 59% and 41% of the collective population were male and female, respectively. 2 of the studies did not report participant age and 1 of these did not report gender either.

2.1.4 Baseline serum 25(OH)D levels, study length, and cholecalciferol dose

Most studies reported mean baseline serum 25(OH)D (n = 13). Mean baseline 25(OH)D level was <50nmol/L in all but one study where it was 55.4nmol/L (Matias *et al.*, 2010), overall mean 35.5±14.8mmol/L. 4 studies reported median baseline 25(OH)D; this ranged from 20.7-35.4nmol/L (Table 2.5). The study intervention period varied considerably between all the included studies; mean 31.4 weeks (range 3-104, IQR 12-39). Only two studies were carried out over 102 weeks (24 months), however adequate repletion was only achieved in 9.2% and 57% of patients implying the supplementation strategy was inadequate and suboptimal, respectively (Jakopin *et al.*, 2011; Tokmak *et al.*, 2008). The average cholecalciferol dose used was 53,940IU with interventions varying from 2,700 – 200,000IU, and the frequency of dosing differed from thrice weekly to once monthly. The most common dose used was 20,000IU weekly, yet this was only given in 5 studies (Armas *et al.*, 2013; Bucharles *et al.*, 2012; Ozkurt & Musmul, 2013; Seibert *et al.*, 2013; Tokmak *et al.*, 2008). The number of patients receiving cholecalciferol as an intervention, the length of intervention, baseline serum 25(OH)D levels and equivalent weekly dose are all presented in Table 2.5.

Source	No. HD subjects receiving cholecalciferol	Study Intervention duration months (weeks)	Baseline 25(OH)D median (IQR) or mean±SD nmol/L	Equivalent weekly cholecalciferol dose (IU)
Armas L *et al.*, 2012	20	3.5 (15)	33.2 (27.7-40.4)	10,333
Delanaye P *et al.*, 2013	16	12 (52)	30±12.5	12,500
Hewitt *et al.*, 2013	21	6 (26)	44.9±12.5	12,500 - 50,000
Marckmann *et al.*, 2012	12	2 (8)	20.7 (16.3-28.9)	40,000
Massart *et al.*, 2014	26	9 (39)	42.7±16.0	6,250 - 25,000
Seibert *et al.*, 2013	15	3 (12)	29.4±11.2	5,000 - 40,000, average 21,166 ±8,333
Tokmak *et al.*, 2008*	64 then 23	24 (104)	64 pts at T0 16.7±9.6, 23 pts at T9 84.0±31.7	20,000 for 9 months reduced to 5,000 for last 15 months
Wasse H *et al.*, 2012	25	3 weeks	35.7±14.2	200,000
Armas L *et al.*, 2013	30	3 (12)	35.4 (28.7-46.2)	20,000
Bucharles S *et al.*, 2012	30	6 (24)	45.2±16.5	50,000 for 12 weeks then 20,000
Daroux M *et al.*, 2012	13	3 (12)	27.5±15.0	50,000
Hryszko T *et al.*, 2013	22	3 (12)	39.6±15.5	10,000 or 50,000
Jakopin E *et al.*, 2014	65	24 (104)	28.6±16.7	10,000
Jean G *et al.*, 2009	107	15 (64)	32 (7-56)	25,000
Matias P *et al.*, 2010	158	6 (26)	55.4±30.0	8,100 - 50,000
Ozkurt S *et al.*, 2013	28	3 (12)	31.2±17.7	20,000
Stubbs JR *et al.*, 2010	7	2 (8)	34.7±5.5	100,000

Table 2.5 Baseline serum 25(OH)D levels and study intervention dosage and duration.

Mean baseline 25(OH)D level was <50nmol/L in all but one study; overall mean 35.5±14.8mmol/L. 4 studies reported median baseline 25(OH)D; this ranged from 20.7-35.4nmol/L. The study intervention periods varied considerably between the included studies; mean 31.4 weeks (range 3-104, IQR 12-39). Only two studies were carried out over 102 weeks (24 months). The most common dose used was 20,000IU weekly, yet this was only given within 5 studies. To compare dosing regimens between studies, the data was converted into equivalent weekly dose; mean 32,581IU, range 5,000 – 200,000IU.

2.1.5 Repletion of serum 25(OH)D

14 studies reported average repletion of 25(OH)D to ≥75nmol/L, however, repletion of ≥90% of the population was only achieved in 7 of these (Armas *et al.*, 2013; Bucharles *et al.*, 2012; Jean *et al.*, 2009; Marckmann *et al.*, 2012; Ozkurt & Musmul, 2013; Stubbs *et al.*, 2010; Wasse *et al.*, 2012). Table 2.6 presents the study design and size, intervention duration, serum 25(OH)D levels at baseline and study end, number of reported incidences of hypercalcaemia, percentage of participants that achieved repletion and the limitations of these 7 studies. All studies were small; the number of subjects receiving cholecalciferol ranged from 7 – 107; total for all 7 studies = 239. Study intervention periods ranged from 8 – 64 weeks, however, 5 of the 7 studies were for ≤12 weeks (Armas *et al.*, 2013; Marckmann *et al.*, 2012; Ozkurt & Musmul, 2013; Stubbs *et al.*, 2010; Wasse *et al.*, 2012). Cholecalciferol dose varied from 20,000 – 200,000 IU per week; the highest dose was only given for 3 weeks (Wasse *et al.*, 2012).

The longest intervention periods that successfully achieved serum 25(OH)D repletion in ≥90% of the population were 6 and 15 months in duration (Bucharles *et al.* 2012; Jean *et al.* 2009). Longer study duration provides insight into maintenance of serum 25(OH)D and therefore the findings of these 2 studies were of particular interest. Bucharles and colleagues (2012) gave subjects 50,000IU of cholecalciferol for 12 weeks followed by a maintenance dose of 20,000IU weekly for a further 12 weeks. Subjects were highly selected from a single centre haemodialysis population based in Brazil, exclusion criteria were severe hyperparathyroidism (PTH >300 pg/mL), prescribed a vitamin D analogue, history of CVD, known inflammatory disorder, malignancy, chronic infections, autoimmune diseases. Cholecalciferol supplements were given out by dialysis nursing staff during the subject's routine dialysis visits. Serum 25(OH)D was measured at baseline, 3 months and 6 months and levels were 45±16nmol/L, 115±36nmol/L and 101±26nmol/L, respectively (n = 30). Serum 25(OH)D was successfully repleted and maintained in all patients. Serum 25(OH)D levels did not increase further between 3 and 6 months suggesting the maintenance dose given was appropriate. The study was carried out in Brazil, in their autumn and winter months; the seasonal timing would likely have affected the results and therefore a longer follow up period would have been informative with regards to the impact of seasonal change on serum 25(OH)D levels and supplementation strategy. Jean and colleagues (2009) gave subjects 100,000IU of cholecalciferol for 15 months. All 250 HD patients in their clinic at the time were

screened against the following exclusion criteria: 25(OH)D ≥75 nmol/L, actual or recent (≤ 3 months) treatment with vitamin D derivatives (including active), prescription of cinacalcet and bisphosphonates, uncontrolled hypercalcaemia (>2.55mmol/L), hyperphosphataemia (>2.0mmol/L) and severe hyperparathyroidism (SHPT: PTH >600pg/mL). 161 consented and enrolled and results from 107 were recorded at study end. Cholecalciferol was provided and administered by nursing staff at the first dialysis session of each month. Serum 25(OH)D was recorded at the following time points: -3, 0, 1, 3, 9, and 15 months, bloods were taken immediately prior to the monthly cholecalciferol administration. Repletion (serum 25(OH)D ≥ 75nmol/L) was achieved in 82% of subjects by month 3, 88% by month 9 and 91% by month 15; results are presented in Table 2.7. The majority of patients were 25(OH)D replete by month 3, however some patients took until month 15 to achieve repletion and others (9%), did not achieve repletion. This suggests the supplementation strategy is affective for the majority but not all. The study was carried out in France and the length of follow-up covered all seasons, demonstrating that serum 25(OH)D levels do not increase too high despite ongoing supplementation during the summer months. The selection criteria, which excluded all patients receiving an active vitamin D analogue, limits the translation of results in the 'real-life' setting where the use of cholecalciferol supplementation, as a concurrent therapy, would likely be sought.

Study Type	Author	HD subjects receiving D3 (n)	Study Duration months (weeks)	D3 Dose (IU)	Frequency	Baseline 25(OH)D median (range) or mean±SD	Study end 25(OH)D median (range) or mean (±SD)	Incidences of hypercalcaemia	% of patients repleted to ≥75nmol/L	Limiting Factors
RCT	Marckmann *et al.*, 2012	12	2 (8)	40,000	weekly	20.7 (16.3-28.9)	114.9 (82.5-153.0)	2	100	Very small study and short intervention period
	Wasse H *et al.*, 2012	25	0 (3)	200,000	weekly	35.7±14.2	130.8±44.9	0	91	Short intervention period
Prospective cohort	Armas L *et al.*, 2013	30	3 (12)	20,000	weekly	35.4 (28.7-46.2)	123.1 (105.6-145.0)	0	93	Short intervention period
	Bucharles *et al.*, 2012	30	6 (24)	50,000 for 12 weeks then 20,000	weekly	45.2±16.5	100.8±25.6	3	100	Highly specified inclusion
	Jean *et al.*, 2009	107	15 (64)	100,000	monthly	32 (7-56)	105.8 (52-192)	0	91	Highly specified inclusion. Excluded active analogues use
	Ozkurt *et al.*, 2013	28	3 (12)	20,000	weekly	31.2±17.7	149.8±38.7	0	100	Short study period
	Stubbs *et al.*, 2010	7	2 (8)	50,000	twice a week	34.7±5.5	134.5±8.2	0	100	Very small study and short intervention period

Table 2.6 Overview of the 7 studies that achieved 25(OH)D repletion (≥75nmol/L) in ≥90% of subjects.

Months	-3	0	1	3	9	15
25(OH)D nmol/L (range)	31±11 (3 - 55)	32±13 (7-56)	68.3±19 (30-130)	97.7±28 (45-198)	105.7±28 (49-190)	105.8±27 (52-192)
% 25(OH)D >75nmol/L	0	0	46	82	88	91

Table 2.7 Serum 25(OH)D changes in response to cholecalciferol supplementation in the longest intervention study.
Jean and colleagues (2009) gave haemodialysis patients (n=107) 100,000IU cholecalciferol monthly for 15 months; supplementation was provided and administered by nursing staff at the first dialysis session of each month. 25(OH)D was recorded at the following time points: -3, 0, 1, 3, 9, and 15 months, bloods were taken immediately prior to the monthly cholecalciferol administration. Repletion was achieved by in 82% of subjects by month 3, 88% by month 9 and 91% by month 15.

2.1.6 Safety of cholecalciferol supplementation

Hypercalcaemia is the identified risk of vitamin D toxicity (Vieth, 2007); the safety of cholecalciferol supplementation is assessed based on serum calcium. Of the total combined study population receiving cholecalciferol (n=595) and throughout the duration of the studies, only 14 incidences of hypercalcaemia were reported in patients receiving cholecalciferol; there were no cases of hypercalcaemia in 10 of the 17 studies (Armas *et al.*, 2012; Armas *et al.*, 2013; Daroux *et al.*, 2013; Delanaye *et al.*, 2013; Hryszko *et al.*, 2013; Jean *et al.*, 2009; Matias *et al.*, 2010; Ozkurt & Musmul, 2013; Stubbs *et al.*, 2010; Wasse *et al.*, 2012).

Of the 7 studies that reported incidences of hypercalcaemia; two studies reported no differences in hypercalcaemic incidence between intervention vs. placebo arms (Hewitt *et al.*, 2013; Massart *et al.*, 2014). In another study, which identified 2 cases of hypercalcemia, both patients were receiving calcium-based phosphate binders and active vitamin D analogues (Marckmann *et al.*, 2012). Isolated events occurring in a single patient, within other studies, occurred in patients also receiving active vitamin D analogue treatment (Jakopin *et al.*, 2011; Seibert *et al.*, 2013). Tokmak and colleagues (2008) reported 3 cases of hypercalcaemia, all of which normalised following a brief suspension of the active vitamin D analogue, and the calcium levels of these patients remained within target range following the active vitamin D analogue being restarted at a lower dose. Bucharles and colleagues (2012) reported 3 incidences of hypercalcaemia which were not well explained in the paper, yet the authors concluded that cholecalciferol was safe. Authors did not

link hypercalcaemia directly to cholecalciferol in any of the studies (Bucharles *et al.*, 2012; Hewitt *et al.*, 2013; Jakopin *et al.*, 2011; Marckmann *et al.*, 2012; Massart *et al.*, 2014; Seibert *et al.*, 2013; Tokmak *et al.*, 2008). Cholecalciferol supplementation was considered safe by all included studies.

2.1.7 Summary

In summary the systematic review confirmed that hypovitaminosis D is common in haemodialysis patients. Study duration was a limiting factor; most study intervention periods were less than one year. Repletion of ≥90% of the population was only achieved in 7 studies; in 6 of these studies the minimum weekly dose of cholecalciferol was 20,000IU for ≥8 weeks, and in the remaining study, patients were given 200,000IU a week for 3 weeks. No cases of hypercalcaemia were directly linked with cholecalciferol supplementation and where used, concurrent use of cholecalciferol, with active vitamin D analogues, was deemed safe (Armas *et al.*, 2013; Bucharles *et al.,* 2012; Jean *et al.*, 2009; Marckmann *et al.*, 2012; Ozkurt & Musmul, 2013; Stubbs *et al.*, 2010; Wasse *et al.*, 2012). Based on the review data, and the varying repletion success, it was evident that a specific guideline for cholecalciferol supplementation in the haemodialysis population was needed.

2.2 Clinical Guideline development

The cholecalciferol supplementation guidelines were developed as part of my clinical renal dietitian role, alongside other renal multidisciplinary team members, consisting of a consultant nephrologist and a specialist renal pharmacist from UHCW. A summary of the information from the systematic review was discussed with two vitamin D experts: Dr Rosemary Bland and Professor Martin Hewison. The guideline aimed to ensure that all haemodialysis patients at UHCW Trust were provided with standardised and effective management of hypovitaminosis D (hypovitaminosis D was defined as a serum 25(OH)D of less <75nmol/L). All patients undergoing hospital haemodialysis should have had their vitamin D status checked and monitored as per the guideline (Table 2.8). The intention was to maintain 25(OH)D levels at ≥75nmol/L. The clinical guideline, and subsequent routine screening of serum 25(OH)D in all patients having in-centre haemodialysis at UHCW NHS Trust, was introduced in November 2014.

Taking account of the systematic review findings which demonstrated 20,000IU of cholecalciferol weekly was sufficient, along with consideration of local clinical guidelines for the general population (Appendix A), which incorporate a 5 week high dose repletion phase using 60,000IU, a supplementation guideline for haemodialysis patients was developed (section 2.2.2 summarises The cholecalciferol supplementation guideline).

2.2.1 Clinical approval

UHCW NHS Trust has a clinical guideline approval committee in which all newly developed guidelines and amendments to existing guidelines go to. The committee is multidisciplinary, and the purpose is to scrutinise guidance to ensure the following: i) clinical need ii) safety iii) ability to be implemented within the existing workforce iv) minimal risk of misinterpretation v) plan for audit and review. The committee requested the following amendment to the summary table within the guideline to help minimise risk of misinterpretation. 1. For the dosage to be written as ‘units’ rather than the abbreviation IU (international units). The guideline was approved in November 2014.

Duties and responsibilities

The clinical guideline approval committee agreed the following: it is the responsibility of all healthcare professionals involved in the care of haemodialysis patients to be familiar with this guideline and its contents. This includes, but is not limited to; renal physicians, pharmacists, dietitians, and nurses working in haemodialysis care.

2.2.2 The cholecalciferol supplementation guideline

Cholecalciferol supplementation should be included as part of standard haemodialysis prescriptions. The first version of the cholecalciferol supplementation guideline recommended a repletion dose of 40,000 units weekly followed by a maintenance dose of 20,000 units weekly. There was not a UK licensed high dose cholecalciferol preparation available for use in November 2014. The preparation used was Dekristol® (a preparation licensed for use in Germany). The maintenance dose was revised following preliminary data analysis which demonstrated serum 25(OH)D concentrations were increasing above the upper target of 150nmol/L. This is discussed further with presentation of results, in section 4.3.1. The revised guideline is outlined in Table 2.8. A UK licensed high dose cholecalciferol preparation (Fultium D3®) was available at the time of the guideline revision and this replaced Dekristol®.

25(OH)D concentration	Cholecalciferol Dose
< 50nmol/L	40,000 units = two 20,000 unit capsules once weekly
50 - 75nmol/L	20,000 units = one 20,000 unit capsule every other week
76-150nmol/L	If not already taking cholecalciferol - no indication to start. If taking cholecalciferol already - maintain levels on maintenance dose of 20,000 units = one 20,000 unit capsule every other week
> 150nmol/L	STOP cholecalciferol, recheck level in 3 months and provided <150 nmol/L restart maintenance dose of 20,000 units = one 20,000 unit capsule every other week

Table 2.8 Clinical guideline for cholecalciferol supplementation in haemodialysis patients at UHCW (final version).

2.2.3 Detailed description of guideline

Serum 25(OH)D: maintain between 76-150 nmol/L

- Serum 25(OH)D levels should be checked in all haemodialysis patients prior to commencing cholecalciferol.
- Serum 25(OH)D levels should be repeated every 3 months until the vitamin D level is stable within target range and the prescribed cholecalciferol dose is stable.

  - A stable cholecalciferol dose is defined as no changes made to the patient's cholecalciferol dose over the past 6 months (based on the previous 2 serum vitamin D results).
- In the absence of a clinical indication to recheck a patient's vitamin D level, for example concerns of toxicity, there should be no need to check a patient's vitamin D level within 3 months of the last vitamin D result.
- Once stable, vitamin D levels should be monitored annually.

Serum calcium: maintain between 2.08-2.58mmol/L (based on the local laboratory reference range and corrected for serum albumin)

- In the absence of hypercalcaemic disease such as granulomatous disease, cholecalciferol should not cause hypercalcaemia.
- If hypercalcaemia occurs the following calcium therapies should be reviewed; calcium supplements, calcium based phosphate binders, alfacalcidol, calcitriol and paricalcitol. Where required calcium therapies should be discontinued.
- Cholecalciferol should not be discontinued unless hypercalcaemia remains after the above calcium therapies are stopped.
- If the event that cholecalciferol needs to be discontinued the patients' serum 25(OH)D level should be checked in order to assess for toxicity

Phosphate: maintain serum phosphate between 1.1 and 1.7mmol/L

- Hyperphosphataemia should continue to be managed with dietary advice and/or phosphate binders as per current guidelines (NICE, 2013)

Parathyroid Hormone: maintain serum PTH between 8 – 38 pmol/L (2-9 times the upper laboratory target)

- Cholecalciferol should be given concurrently with current treatment regimens for hyperparathyroidism; as per by current UHCW and NICE guidance.
- Improvement of vitamin D status using cholecalciferol supplementation may help manage hyperparathyroidism. This may lead to a reduction in the required dose of active vitamin D analogues; alfacalcidol, calcitriol and paricalcitol.

Dissemination and Implementation

The guidelines were disseminated and implemented through the monthly renal multidisciplinary and bi-monthly renal governance meetings and made available on UHCW Trusts' e-library so that all members of the renal multidisciplinary had access to them.

Training

All new clinical renal staff complete a local introduction and training programme facilitated by the renal department. Included within this, is training on the implementation of all relevant the clinical guidelines.

Cholecalciferol administration

If required, cholecalciferol should be prescribed by the patient's renal consultant within their dialysis prescription book (Appendix B) and administration was overseen by nursing staff during the patient's dialysis sessions.

2.2.4 Quality control and safety

To ensure the guideline was implemented correctly I oversaw the initial prescribing and cross-checked prescription books against serum 25(OH)D results. Following the initial high-dose repletion phase and the repeat testing of serum 25(OH)D at 3 months; the cholecalciferol dose was reviewed by the patient's renal consultant. Cross-checks were carried out in 20 patients from each of the dialysis units within the first two months and again between months 4 and 6. These cross-checks, along the preliminary data (serum 25(OH)D and corrected calcium) from the initial 6 months after introduction cholecalciferol supplementation were used to review the safety and effectiveness of the guideline (see section 4.3).

In-centre haemodialysis patients have routine monthly bloods tests as part of their clinical care. These blood results are reviewed by their renal consultant. The clinical guidelines for cholecalciferol supplementation included the following guidance for the consultants to follow to ensure safety.

Serum corrected calcium and phosphate is measured monthly in all in-centre haemodialysis patients. Serum 25(OH)D was measured every 3 months. As part of the clinical guideline the advice provided for the renal consultants to ensure safety was:

- Serum corrected calcium >2.65 mmol/l
    - Suspend active vitamin D analogue and recheck corrected calcium level within two weeks
    - If the patient is not prescribed active vitamin D or if the corrected calcium remains high after two weeks: suspend cholecalciferol
- Serum phosphate >2.0mmol/l
    - Dietitian review of compliance with phosphate binders and/or diet and recheck phosphate level next month
    - Check dialysis adequacy
    - If phosphate remains high (above usual trend for patient) despite good compliance with phosphate binders and/or diet and dialysis adequacy is sufficient suspend cholecalciferol
- Serum 25(OH)D
    - >75nmol/L reduce cholecalciferol to 20,000IU alternate weeks
    - >150nmol/L suspend cholecalciferol

If cholecalciferol is withdrawn based on 25(OH)D level (>150nmol/L) alone (in the absence of hypercalcaemia and hyperphosphataemia), if at the next 3 monthly blood test the 25(OH)D level has returned to within target range (75nmol/L – 150nmol/L), cholecalciferol should be recommenced at the maintenance dose.

2.3 Study rationale

Preliminary data from the first 100 UHCW haemodialysis patients screened for vitamin D deficiency was assessed (59% male, median age 67 years (range 26-92). Virtually all patients (96%) had suboptimal levels suggesting 336 of the 350 haemodialysis patients would require cholecalciferol supplementation. The local

guideline to ensure deficiency is identified and appropriately treated in our patients (section 2.2.2) provided a unique opportunity, not only to collect repletion data, but also to collect biochemical and qualitative data in order to investigate classical and non-classical effects of vitamin D. This is something that can only be studied in the ESRD population, due to the reduced renal synthesis of $1,25(OH)_2D$. Currently an optimal serum 25(OH)D level remains controversial; it is anticipated that the current and future studies together with other emerging evidence will help better determine a target serum 25(OH)D in relation to health outcomes in the haemodialysis population. As well as exploring both bone and non-bone related actions of 25(OH)D, it was hoped this study would provide the data needed to support the implementation of vitamin D supplementation to the 24,202 UK haemodialysis patients (UKRR, 2019). Whilst vitamin D is currently a hot research topic, the precise research question the Ph.D is looking to answer, and investigation of non-routine vitamin D metabolites, is unique.

A randomised placebo controlled trial would generally be considered to be more favourable to the before and after study undertaken here. However, it has been considered unethical to withhold treatment in a cohort of patients who are profoundly vitamin D-deficient (Frame *et al.*, 2018). RCTs investigating vitamin D supplementation present many difficulties including lack of guidance on appropriate intervention period, how to use a placebo (in order to overcome the ethical issues studies are required to use a low dose vitamin D in the control arm which has implications for the results), and also vitamin D can be purchased over the counter which offers up its own research management dilemmas (Frame, 2020; Lappe & Heaney, 2012). The research design is fair and ethically favourable; moreover, supplementing all patients with vitamin D in an organised, and well planned out manner, allowed the collection of real-life meaningful data. The following chapter outlines the study design and methods.

Chapter 3 Study design & methods

The previous chapter reviewed the literature and discussed the development of UHCW cholecalciferol supplementation guidelines for haemodialysis patients. In order to see the impact of these guidelines a longitudinal study was carried out to compare clinical data, before and after, the introduction of supplementation. The study design and methods are described in this chapter.

3.1 Aim

The overarching aim of this thesis is to investigate the effects of optimising serum 25(OH)D on mineral bone disease, anaemia, health-related quality of life and vitamin D metabolism in haemodialysis patients.

Research objectives

- To undertake a systematic literature review looking at the efficacy of cholecalciferol in haemodialysis patients
- To assess the efficacy of cholecalciferol supplementation in replenishing and maintaining serum 25(OH)D levels to ≥75nmol/L in haemodialysis patients
- To assess classical benefits of optimising serum 25(OH)D (measured by routine markers of mineral bone disease: corrected calcium, phosphate, and parathyroid hormone)
- To assess non-classical benefits of optimising serum 25(OH)D (anaemia measured using serum haemoglobin, hepcidin, and EPO dose requirements; and extra-renal 1α-hydroxylase activity measured through serum $1,25(OH)_2D_3$)
- To assess the impact of vitamin D supplementation on HRQOL measures, assessed using questionnaires.

- To explore the integrity of the 25(OH)D immunoassay, and to assess the role of other vitamin D metabolites ($1,25(OH)_2D_3$, $24,25(OH)_2D_3$, Epi-$25(OH)D_3$) and vitamin D metabolite ratios (25(OH)D:$24,25(OH)_2D_3$, $1,25(OH)_2D_3$:$24,25(OH)_2D_3$, and 25(OH)D:$1,25(OH)_2D_3$) as indicators of vitamin D status

3.2 Study design

The research study was separated into 5 parts; three aspects are addressed within the main longitudinal study and two aspects as sub-studies (Figure 3.1).

The main study

1. Vitamin D status: prevalence of deficiency and effectiveness of supplementation

2. The impact of cholecalciferol supplementation on routinely collected data: markers of mineral bone disease; serum corrected calcium, phosphate and PTH

3. The impact of improved serum 25(OH)D on erythropoietin (EPO) requirements

The sub-studies

4. Sub-study one: The impact of serum 25(OH)D on iron metabolism (hepcidin), vitamin D metabolites and vitamin D metabolite ratios ($1,25(OH)_2D_3$, $24,25(OH)_2D_3$, Epi-$25OHD_3$, 25(OH)D:$24,25(OH)_2D_3$, $1,25(OH)_2D_3$:$24,25(OH)_2D_3$, and 25(OH)D:$1,25(OH)_2D_3$).

5. Sub-study two: The impact factor: the effect of serum 25(OH)D on health-related quality of life (HRQOL)

The whole study (main study and sub-studies)

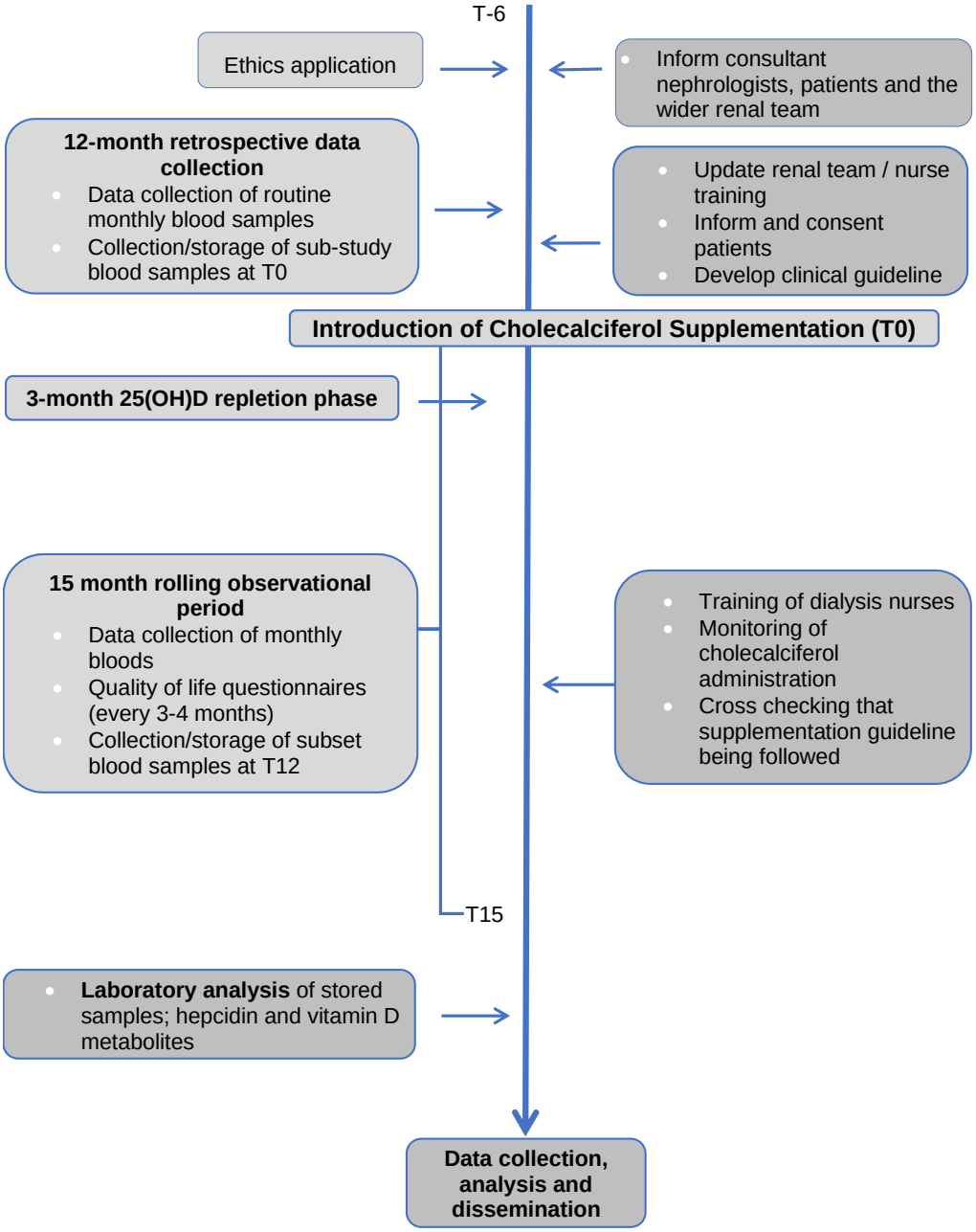

Figure 3.1 Diagram of the whole study design including the main study and sub-studies).
The study was designed around a change in standard care: the introduction of routine cholecalciferol supplementation for all 350 haemodialysis patients at UHCW. The whole study investigated the effects on routinely collected data, sub-study one investigated the effects on vitamin D metabolites, and sub-study two, the effects on HRQOL. The tasks relating to the development of the clinical guideline for cholecalciferol supplementation including its implementation and monitoring are included on the right hand side of the diagram. T-6 and T15 = 6 months prior to, and 15 months post, the introduction of cholecalciferol supplementation. T0 = baseline.

3.3 Study methods

All 350 patients receiving in-centre haemodialysis at UHCW received cholecalciferol as required based on their serum 25(OH)D levels. Based on a pre-study audit (see section 2.3) 96% of patients were expected to require cholecalciferol supplementation (336 of 350 haemodialysis patients). Repletion data: serum 25(OH)D, along with routinely collected dialysis data (corrected calcium, phosphate, ferritin, PTH, Hb, and EPO dose), was extracted from the renal database PROTON (section 3.3.3) and analysed for all patients as part of a clinical audit of new treatment, as such it did not require individual patient consent. Consent was obtained for the completion of HRQOL questionnaires and for the collection of additional, non-routine, blood samples for investigation of other vitamin D metabolites and hepcidin (sub-studies one and two). Ethical approval was obtained for both the main study and the sub-studies.

Section 2.2 outlines the overall management of cholecalciferol supplementation. The overall structure, integration of the study aspects, and timeline are illustrated in Figure 3.1.

3.3.1 Ethical Approval

NHS Research Ethics Committee Ethical Approval was applied for using the online Integrated Research Application System (IRAS). A separate application was submitted for the sub-study. Following minor amendments to the protocol, ethical approval was received on 1st July 2014 (main-study) and 28th August 2014 (sub-study). REC reference numbers: 14/NS/1012 and 14/EE/10, respectively. As a requirement of UHCW NHS Trust local R&D approval was also received (Appendix C). The patient information sheets, and consent forms can be found in the appendices (Appendix D and E)

3.3.2 Informed written consent

Patients were approached during their haemodialysis visits and information sheets containing a comprehensive description of both the purpose of the study and the study protocol were provided to all potential participants (Appendix D). At least 48 hours was given between the patients being given the information leaflet and them

being approached regarding enrolment. Opportunity for questions and answers was given following which informed consent was obtained (the consent forms can be found in Appendix E). Enrolment to the study began in November 2014. Once consented the HRQOL questionnaires were handed out to patients, and non-routine blood samples taken, when they attended for dialysis; participation did not involve additional hospital visits.

3.3.3 Recording and storage of data

During the set-up of the study the services provided by the information, communication, and technology centre were utilised to set up a customised Microsoft Access database. Microsoft Access was chosen as a database over the sole use of Excel because it facilitates the option to generate data queries and reports, and allows the storage of additional information, for example medications within multiple tables (Microsoft, 2020). The database was designed to allow the data to be easily extracted to facilitate analysis. Each patient was assigned a study number in which their information was stored anonymously, on an encrypted USB drive, in full compliance with Medical Research Council (MRC) guidelines (MRC, 2012) and the Data Protection Act (DPA, 1998, 2018). Data was extracted from the Trusts clinical systems, collated, cross-referenced, and input into the research database for analysis. Data extraction was carried out by myself with the assistance of the Trusts' Information Technology department. Once extracted, I personally checked through the data to ensure the results were reflective of the 'pre-dialysis' monthly blood samples (not post dialysis results). All additional, duplicate, or non-relevant blood results were deleted to provide one set of blood results per subject per month. If a patient had more than one set of bloods results for any given month, then the results on, or nearest to, the date their monthly bloods were due to be taken were used. All anomalous results were cross referenced to check validity. This data processing, whilst laborious, was critical to ensure reliable end data.

3.3.4 Statistical analysis

This study was designed around standard care and as such all 350 haemodialysis patients under the care of UHCW NHS Trust were included in the primary outcome measure analysis. Based on UHCW haemodialysis numbers in recent years, an annual loss to follow-up rate of up to 20% is be expected in this patient cohort due

to change in dialysis modality, transplant, ill health, or death. A worst case drop-out rate was assumed during the study set-up which gave us a sample size of 280 patients. All 350 patients were invited to participate in the HRQOL sub-study, and the plan was to recruit at least 80 patients for the assessment of hepcidin and vitamin D metabolites to provide full data on at least 50 patients (81 were recruited). This allowed a generous dropout rate over the 12-month follow up to adequately provide for exploration of estimates of change between observational periods (Johanson & Brooks, 2009).

Statistical analyses was conducted using SPSS, Stata and XLSTAT. Shapiro-Wilk normality tests were carried out in SPSS initially to test the distribution of data; the majority of the data are not normally distributed, in these instances nonparametric tests were used for the statistical analysis. Where the data are normally distributed, parametric tests were carried out. The Chi Squared test was used to test for differences in results between groups where data was not continuous (for example ethnicity and gender). Whole group, and grouped analysis were carried out using a related samples Wilcoxon signed rank test, or paired t-test, to compare data, pre and post cholecalciferol supplementation. Data are summarised using proportions, means and standard deviations, or medians and ranges where appropriate. Change in serum 25(OH)D, EPO dose, serum Hb, ferritin, corrected calcium, phosphate, PTH, hepcidin, and vitamin D metabolites (3-epi-25(OH)D_3, 1,25$(OH)_2D_3$, 24,25$(OH)_2D_3$), prior to and post cholecalciferol supplementation were investigated. Relationships were explored using Spearman's rank correlation coefficients where there was a non-normal distribution of data, and Pearson's rank correlation coefficient for normally distributed data. Data were stratified to further investigate relationships; where this was carried out the related statistics are discussed in the relevant results sections. The HRQOL was scored according to the SF36 and EQ5D algorithms, and relevant value index data sets, and analysed using the Kruskal-Wallis (non-parametric test for analysis of variance). Passing-Bablok regression, and Bland-Altman bias plot analysis was carried out to test for difference between the serum 25(OH)D results from 2 assay methods (Immunoassay and LC-MS/MS).

3.3.5 Roll out of cholecalciferol supplementation

UHCW NHS Trust has 5 dialysis units; the main unit which is based at the University Hospital in Coventry and 4 satellite units; Nuneaton, Rugby, Whitnash

(Leamington Spa), and Stratford upon Avon. The introduction of routine serum 25(OH)D testing and subsequent cholecalciferol supplementation was rolled out across a period of 5 months from November 2014 through to April 2015 (Table 3.1). This roll of the programme was to provide for awareness and education around the new clinical guideline, and to minimise impact on the clinical laboratories.

Dialysis Unit	Implementation Month	Number of patients
Nuneaton	November/December 2014	59
Stratford upon Avon	January 2015	54
Whitnash	February 2015	31
University Hospital	March/April 2015	130
Rugby	April 2015	76

Table 3.1 The roll out of 25(OH)D testing and cholecalciferol supplementation across UHCW dialysis centres
UHCW NHS Trust has 5 dialysis units. The main unit which is based at the University Hospital in Coventry and 4 satellite units, Nuneaton, Rugby, Whitnash (Leamington Spa), and Stratford upon Avon. The introduction of routine serum 25(OH)D testing and subsequent cholecalciferol supplementation was rolled out across a period of 5 months from November 2014 through to April 2015.

3.3.6 Cholecalciferol administration

If required, based on serum 25(OH)D level, cholecalciferol was prescribed by the patient's renal consultant, within their dialysis prescription book (Appendix B) and administration was carried out by nursing staff during the patient's dialysis sessions. Cholecalciferol was given as part of standard care and therefore not the responsibility of this study. Nevertheless, the supplementation strategy did impact on the success of this research and therefore monitoring of the roll out, and administration, was overseen, as part of my clinical role. This involved visits to all the satellite units and checking patients' serum 25(OH)D levels and cholecalciferol prescription.

3.4 Main study - prevalence of 25(OH)D deficiency, effectiveness of supplementation and the impact on mineral bone markers

3.4.1 Main Study Inclusion

The 350 patient cohort included all patients having in-centre dialysis under UHCW NHS Trust. Patients having home haemodialysis were not included in the study because routine blood tests are less frequent in these patients.

There were no exclusion criteria for in-centre dialysis patients; all patients, regardless of acute illness or co-morbidities, were included in the main study. However, hypercalcaemia was a contraindicator to cholecalciferol supplementation and therefore not all patients were guaranteed to be given cholecalciferol.

3.4.2 Routinely collected data

All UHCW in-centre haemodialysis patients have the following information collected as part of routine clinical care:

- Monthly blood tests - ferritin, Hb, corrected calcium, phosphate and 3 monthly 25(OH)D and PTH. The data was stored on the renal database system PROTON.

- EPO - doses are prescribed within the patients' dialysis prescription books and administration is recorded on an electronic database. EPO therapy continued to be adjusted by clinicians as standard practice to achieve target haemoglobin between 10-12 g/L, according to UHCW renal anaemia guidelines.

The above data was monitored monthly, for the 12 months prior to, and for the 15 months post, the introduction of cholecalciferol supplementation.

Serum 25(OH)D levels were measured by the clinical laboratories at UHCW using Elecsys Vitamin D Total Assay (Roche). The percentage coefficient of variation varied according to mean serum level; it was 13.6% for 10.2nmol/L, 9.1% for 33.5nmol/L and 6.3% for 73.8nmol/L (manufacturer information from UHCW biochemists). The assay is unable to detect levels below 7.5nmol/L or above 175nmol/L (Ong *et al.*, 2012); UHCW laboratories reported any results <10nmol/L

as '<10nmol/L' and results >175nmol were reported as '>175nmol/L'. For analysis purposes all results reported as <10nmol/L were given a value of 0nmol/L and all results reported as >175nmol/L were given a value of 175 nmol/L. This was decided following discussions with Ph.D supervisors Professor Hewison and Associate Professor Dr Bland. The options of using 0, 5 or 10nmol/L were all discussed as options for levels less than 10nmol/L. A value had to be determined for analysis purposes and the consensus was 0nmol/L. In terms of serum 25(OH)D categories for determining degree of deficiency; levels of 0, 5 or 10nmol/L all fall into the same category and therefore the decision to use the value 0nmol/L for patients who had a level of <10nmol/L did not affect the reporting of prevalence of serum 25(OH)D deficiency (vitamin D status) (Prevalence of 25(OH)D deficiency is presented in section 4.2.

The safety of cholecalciferol supplementation was assessed by monitoring patients' serum corrected calcium levels. Hypercalcaemia is an identified risk associated with cholecalciferol (BNF, 2019). Studies have suggested that serum 25(OH)D levels of 300nmol/L are safe, with concentrations needing to be >375nmol/L to cause hypercalcaemia (El-Hajj Fuleihan *et al.,* 2006; Holick, 2007; Kimball *et al.,* 2017). The upper limit of 250nmol/L, as recommended by the ES, is therefore considered conservative (Holick *et al.,* 2011).

Translating serum 25(OH)D and corrected calcium levels into dosing recommendations has resulted in safe upper limits for cholecalciferol supplementation (in the general population) that vary between from 4,000IU-10,000IU per day (Efsa Panel on Dietetic Products, 2012; Ross *et al.*, 2011). The doses used as part of the UHCW clinical guideline (40,000IU weekly for repletion and 20,000IU weekly for maintenance) are well within these safety limits. Due to the reduced synthesis of $1,25(OH)_2D$ in ESRD, and the routine monthly monitoring of serum corrected calcium, the risk in this population group may be reduced compared with the general population. The systematic review (reported in section 2.1) considered the safety of cholecalciferol supplementation specifically in haemodialysis patients, and all patients supplemented with cholecalciferol (even those on very high doses) remained within a safe serum 25(OH)D range according to the ES guidelines (Holick *et al.,* 2011). As discussed in Chapter 1 (sections 1.3 and 1.6.2), $1,25(OH)_2D_3$, not 25(OH)D, is responsible for maintaining calcium homeostasis through enhanced calcium absorption in the gastrointestinal tract, increased bone resorption, suppression of PTH secretion, and upregulation of

FGF23. Cholecalciferol was given concurrently with active vitamin D analogues, known hypercalcaemic agents, therefore the careful monitoring of serum corrected calcium was a priority of both the clinical team, as well as the study.

3.5 Sub-study design

3.5.1 Sub-study one: The impact of serum 25(OH)D on iron metabolism, vitamin D metabolites and vitamin D metabolite ratios

An annual loss to follow-up rate of up to 20% can be expected in UHCW HD patients due to change in dialysis modality, transplant, ill health, or death. This is before actual study withdrawal for other reasons, such as a patient having a change of mind, are taken into consideration. The sub-study was an exploratory study and as such complete data on 50 patients was sought (section 3.3.4 discusses sample size). With this in mind, the study protocol aimed to consent approximately 80 patients in order to allow for a generous loss to follow-up of up to 38%. This was achieved; eighty-one patients were recruited for additional studies into iron metabolism and vitamin D metabolites. All patients that met the following criteria were approached for inclusion:

- Established on haemodialysis for ≥1 month
- Prescribed EPO
- No hospital admissions in the 4 weeks prior to inclusion.
- No known active malignancy
- Able to self-consent to providing additional blood samples for research purposes
- Able to enrol for duration of study (for example if a patient had a planned transplant they would be excluded)

Patients were recruited during their haemodialysis visits. Patient information sheets were given out 48 hours prior to consent being obtained (section 3.3.2).

Two additional, non-routine, blood samples (to provide five 2ml serum samples) were taken from each consented patient prior to, and again 12 months post, the introduction of cholecalciferol supplementation. The additional blood samples were collected by nurses when patients attended their dialysis sessions; participation did not involve additional hospital visits. Samples were labelled with the following details: patient initials, study ID, time point (T0 = baseline, T12 = 12 month follow up). Blood samples were taken by clinical renal nurses and stored on ice for up to 1 hour until processing by the clinical research nurses and the serum stored in at -80^{o}C in the Trusts' research tissue bank. UHCW research tissue bank is regulated by the Human Tissue Authority; study sample storage was overseen by the tissue

bank manager in line with the regulations set out by the Human Tissue Act (HTA, 2004). Stored serum samples were later analysed for serum hepcidin, 25(OH)$D_{2,}$ 25(OH)D_3, 25(OH)D_2, Epi-25OH$D_{3,}$ 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$. These are not routinely measured in UHCWs hospital laboratory and samples were transferred on dry ice, according to local tissue bank procedures, to Professor Hewison's research laboratory at The University of Birmingham for LC-MS/MS analysis. According to Trust procedures and HTA regulations a tissue sample transfer forms were completed.

3.5.2 Sub-study two: The effect of cholecalciferol supplementation on health-related quality of life (HRQOL)

141 patients consented to investigation into HRQOL and completed at baseline (T0) 4 months (T4), 8 months (T8), and 12 months (T12). Questionnaire results were compared across time points in order to investigate the effect of serum 25(OH)D repletion (Chapter 7). The 12 months follow up period was chosen to ensure adequate time to achieve serum 25(OH)D repletion, in order to reliably test for effects. This timeframe covered all seasons, and therefore minimised the seasonal influence on both vitamin D status and general well-being. The following 2 questionnaires are widely cited and validated for patients with ESRD (Gómez-Besteiro *et al.*, 2004; Johansen *et al.*, 2001; Merkus *et al.*, 1997; Terada & Hyde, 2002; Yang *et al.*, 2014)

1. Short-Form Health Survey 36 (SF-36): The SF-36 questionnaire is a multipurpose, 36-item survey that measures eight domains of health: physical functioning, role limitations due to physical health, bodily pain, general health perceptions, vitality, social functioning, role limitations due to emotional problems, and mental health (SF, 2020; Ware Jr & Sherbourne, 1992)

2. The European Quality of Life (EQ-D5) questionnaire is a simple to use generic measure of health status that measures 5 domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression (EQ-5D, 2020).

Paper questionnaires were completed by patients and stored in locked cabinets within the research offices at UHCW. Questionnaires were anonymous and only

included the patients study number and the date of completion. Once all questionnaires were completed the data was collected into an excel spreadsheet for analysis. This was stored on an encrypted USB stick (data collection information in discussed in section 3.3.3). HRQOL was scored according to the SF36 and EQ5D algorithms and differences between timepoints investigated. Results are presented in Chapter 7.

The following chapter (Chapter 4) is the first of the result chapters; the prevalence of vitamin D deficiency is presented, as well as data representing the effectiveness and safety of cholecalciferol supplementation.

Chapter 4 Prevalence of 25(OH)D deficiency, repletion, and safety of cholecalciferol supplementation

From November 2014 - April 2015, 350 haemodialysis patients under the care of UHCW were initiated on regular cholecalciferol supplementation, as indicated, based on their serum 25(OH)D levels. Supplementation was given as part of standard routine care to increase and maintain serum 25(OH)D to 75-150nmol/L (supplementation details are outlined in section 2.2.2). Patients were followed up for 15 months; the prevalence of hypovitaminosis D, and the effectiveness, and safety, of cholecalciferol supplementation are presented in this chapter.

4.1 Population characteristics

The demographics and characteristics of the whole study population are summarised in Table 4.1. Coventry has a higher percentage of people from black and minority ethnic (BAME) groups compared to the national average (Census, 2011a).. However despite this the population remains predominantly of White British ethnicity (66.6%) (Census, 2011a). The UHCW haemodialysis population has a higher majority of individuals of White British ethnicity than Coventry city average at 71.4% vs. 66.6% respectively. This is likely to be related to UHCW renal centre catchment, which also covers Warwickshire; White British people account for 88.5% of Warwickshire's population (Census, 2011b). The majority of study patients were male, and this is reflective of the overall UK dialysis population as seen in Table 4.2 (Byrne C, 2018). Primary renal diagnosis (PRD) is not thought to affect the outcomes of this study; it is reported because this it is linked with patient outcomes and therefore a matter of clinical interest (Byrne C, 2018).

The age, gender and ethnicity of the study population appears reflective of the UK haemodialysis (HD) population as a whole (Table 4.2). The median age of UHCW haemodialysis patients was 69.0 years compared with 67.2 nationally. The majority of study patients were white and male, 74.3% and 60.6%, this compared with 76.4% and 61.9% of the total UK haemodialysis population, respectively. The primary renal diagnosis (PRD) was 'unknown' in a greater proportion of the UHCW population than the UK, yet a higher percentage of the UK HD population reported PRD as

'other'. This may be partly related to inconsistencies in reporting between centres (Byrne C, 2018).

Variables	Haemodialysis Patients (n=350)
Age, years, median (range, IQR)	69.0 (24-95, 54.5-78.3)
Male, n (%)	212 (60.6)
Ethnicity, n (%)	
White	260 (74.3)
South Asian	55 (15.7)
Black	23 (6.6)
Chinese	1 (0.3)
Other	11 (3.1)
Dialysis Vintage, years, median (range, IQR)	2.4 (0-17.5, 0.8-5.1)
Diabetes n (%)	
Type 1	11 (3.1)
Type 2	91 (26)
Previous RRT, n (%)	
Peritoneal Dialysis	96 (27)
Transplant	44 (13)
Primary Renal Diagnosis, n (%)	
Unknown	70 (20)
Diabetic Nephropathy	65 (19)
Renal Vascular Disease	29 (8)
Hypertension	34 (10)
Glomerulonephritis	51 (15)
Pyelonephritis	31 (9)
Polycystic	27 (8)
Other	42 (12)
Missing	1 (0)

Table 4.1 Demographics and clinical characteristics of 350 UHCW haemodialysis patients (the main study population).
Most patients were male (60.6%) and of White British ethnicity (74.3%).

Variables	UHCW HD population	UK HD population
Age, median (years)	69.0	67.2
Male (%)	60.6	61.9
Ethnicity (%)		
White	74.3	76.4
South Asian	15.7	12.2
Black	6.6	7.8
Chinese	0.3	0.7
Other	3.1	2.9
Dialysis Vintage, years, median	2.4	3.2
Diabetes (%)	29	58
Primary Renal Diagnosis, (%)		
Unknown	20.0	15.1
Diabetic Nephropathy	18.6	16.9
Renal Vascular Disease	8.3	2.9
Hypertension	9.7	6.1
Glomerulonephritis	14.6	19.1
Pyelonephritis	8.9	10.3
Polycystic	7.7	10.0
Other	12.0	16.5
Missing	0.3	3.2

Table 4.2 Demographics and characteristics of the study population compared with the UK in-centre haemodialysis population.

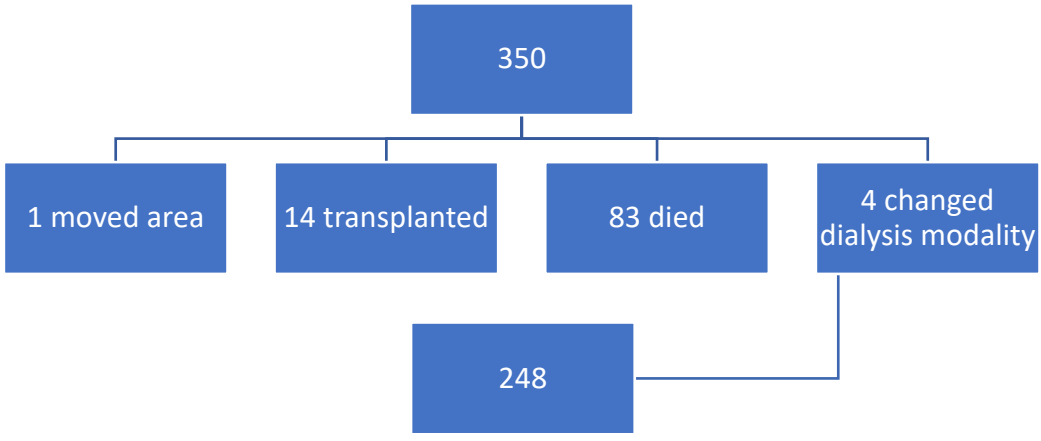

Figure 4.1 Number included at the start of the study, reasons for drop-out and number of patients at study end.
Study participant numbers for the main study cohort at baseline and study end, together with reasons for drop-out. 350 haemodialysis patients were included in the study. During the 15-month follow up phase the total withdrawal was 102 (29%). This was due to; relocation (n=1), renal transplantation (n = 14), death (n=83) and change in dialysis modality to peritoneal dialysis or home haemodialysis (n=5).

Study participant numbers for the main study cohort at baseline and study end, together with reasons for drop-out, are presented in Figure 4.1. 350 haemodialysis patients were included in the study; during the 15-month follow up phase the total drop out was 102 patients (29%). Reasons for this were relocation (n=1), renal transplantation (n=14), death (n=83) and change in dialysis modality to peritoneal dialysis or home haemodialysis (n=5). This left 248 patients at study end. Patients' data was included up to the point of study drop-out. Therefore, providing there was sufficient data, patients were still included in the analysis. New HD starters had vitamin D levels tested, and if indicated received cholecalciferol supplementation, but were not included in the study.

4.2 Prevalence of serum 25(OH)D deficiency at baseline

Serum 25(OH)D results were available for 328 patients at baseline (T0). The results from 22 patients were missing; this occurred due to these patients not being at dialysis the day monthly bloods were taken; reasons for this included holidays, illness, non-concordance, change in dialysis days, and hospital appointments. 25(OH)D status was categorised according to ES guidelines (Holick *et al.*, 2011). Virtually all patients (94.2%, 309 of 328) had insufficient serum 25(OH)D levels (<75nmol/L). Only 19 patients (5.8%) had optimal levels (≥75nmol/L) as seen in Figure 4.2. Median baseline serum 25(OH)D was 22nmol/L with patients' levels ranging from 0-140nmol/L as presented in Figure 4.3. The deficient group (n = 209) had a median of 15.0nmol/L (range 0-29nmol/L), the insufficient group (n = 100) had a median of 39.5nmol/L (range 30-71nmol/L) and the replete group (n = 19) had a median of 97nmol/L (range 75-140nmol/L) as shown in Figure 4.4.

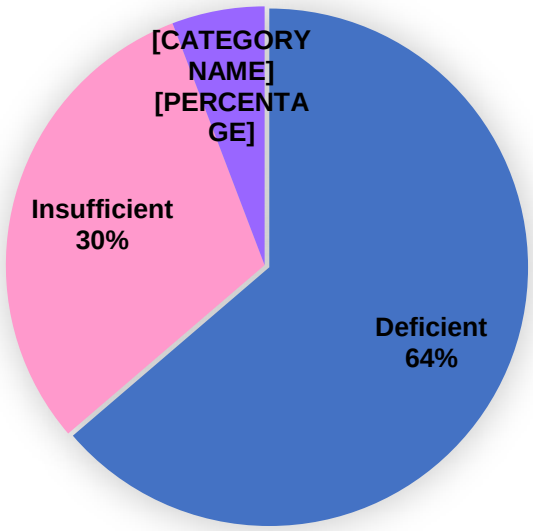

Figure 4.2 Prevalence of hypovitaminosis D in UHCW haemodialysis patients.
Vitamin D status of 328 haemodialysis patients screened at University Hospitals of Coventry and Warwickshire (UK). 94% (309 of 328) had serum 25(OH)D <75nmol/L (64% (210 of 328) deficient, <30nmol/L; 30% (98 of 328) insufficient, 30-74nmol/L). Only 19 patients (6%) had optimal levels (≥75nmol/L). Data represents individual patient values grouped into deficient (<30nmol/L), insufficient (30-74nmol/L) and optimal (≥75nmol/L).

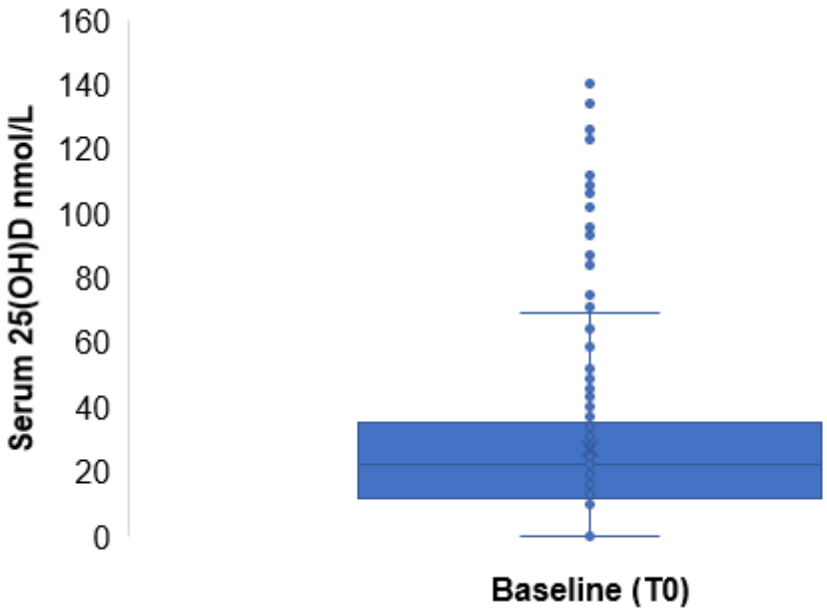

Figure 4.3 Baseline (T0) serum 25(OH)D concentrations of 328 haemodialysis patients at UHCW.
Data representing the spread of individual patient values; median (22nmol/L) and range (0-140nmol/L).

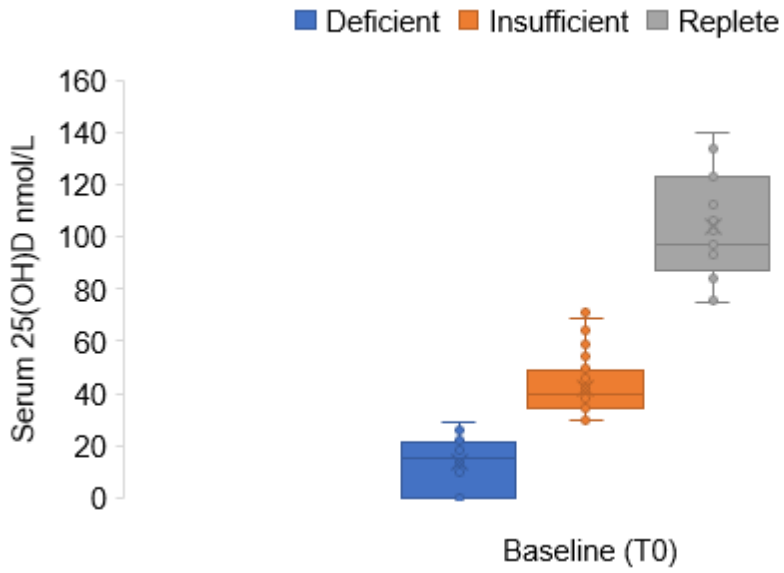

Figure 4.4 Box and whisker plots illustrating the baseline (T0) serum 25(OH)D concentrations of haemodialysis patients at UHCW as vitamin D status categories.
Data represents individual patient values grouped into deficient (<30nmol/L), insufficient (30-74nmol/L) and replete (≥75nmol/L) according to ES guidelines (Holick *et al.*, 2011). The deficient group (n=209) had a median of 15.0nmol/L (range 0-29nmol/L), the insufficient group (n=100) had a median of 39.5nmol/L (range 30-71nmol/L) and the replete group (n=19) had a median of 97nmol/L (range 75-140mmol/L) (n=328).

In the general population, age, sex, and ethnicity have been linked with vitamin D status; lower 25(OH)D levels are associated with increased age and female sex, and higher 25(OH)D levels are associated with white ethnicity (Mitchell *et al.*, 2012; Mithal *et al.*, 2009; Verdoia *et al.*, 2015). These factors did not appear to affect prevalence, or degree, of serum 25(OH)D deficiency in UHCW haemodialysis patients (Figure 4.5 and Figure 4.6 and Table 4.3)

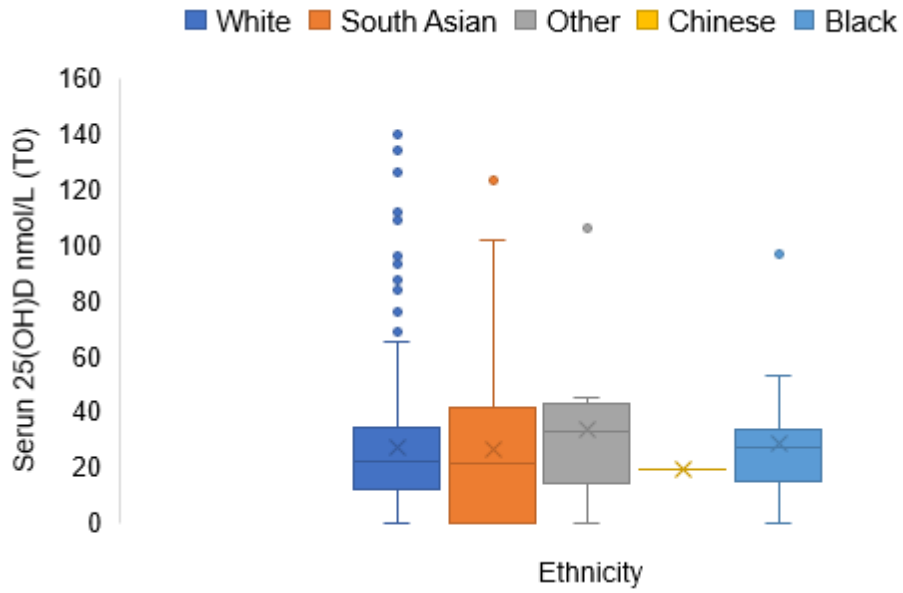

Figure 4.5 Baseline (T0) serum 25(OH)D concentrations of haemodialysis patients in Coventry according to ethnicity.
Baseline serum 25(OH)D concentrations were 22nmol/L (0-140, 13-34) (White, n=248), 21nmol/L (0-123, 0-41) (South Asian, n=48), 33nmol/L (0-106, 20-42) (Other, n=10), 19nmol/L (19, 19) (Chinese, n=1), 27nmol/L (0-97, 16-33) (Black, n=21). There was no difference between baseline 25(OH)D according to ethnicity (Kruskal–Wallis P = 0.768). Values represent median (range, IQR).

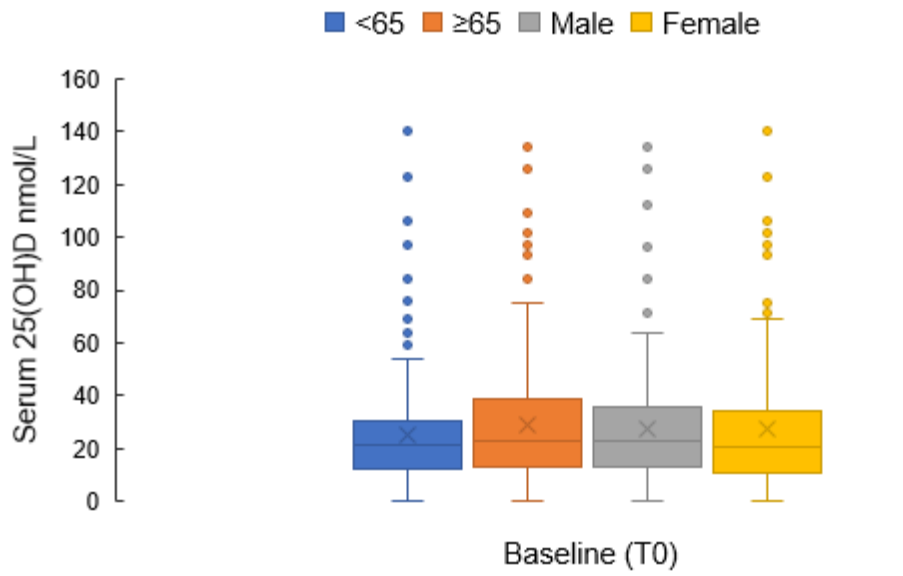

Figure 4.6 Baseline (T0) serum 25(OH)D concentrations of haemodialysis patients in Coventry according to sex (male and female) and age (<65 years and ≥65 years).
There was no difference between 25(OH)D concentrations between males and females, 21nmol/L (0-140nmol/L) vs. 26nmol/L (0-135nmol/L), respectively, P = 0.295. Likewise, there was no difference between age groups; 21nmol/L (0-140nmol/L) and 23nmol/L (0-135nmol/L) in the <65 and ≥65 years of age groups, respectively, P = 0.186 (n=328) (Independent samples Mann Whitney U test). Data represent median (range).

Variable	25(OH)D deficient n = 209	25(OH)D insufficient n = 100	25(OH)D replete n = 19
Age, years, median (range, IQR)	67.9 (53.9-77.4)	69.8 (56.6-80.3)	72.7 (54.0-80.2)
Male, n (%)	122 (58.4)	67 (67.0)	9 (47.4)
Ethnicity n (%)			
White	163 (78.0)	71 (71.0)	14 (73.7)
South Asian	29 (13.9)	16 (16.0)	3 (15.8)
Black	12 (5.7)	8 (8.0)	1 (5.3)
Chinese	1 (0.5)	0 (0.0)	0 (0.0)
Other	4 (1.9)	5 (5.0)	1 (5.3)
Dialysis Vintage, years, median (range, IQR)	2.2 (0-17.5, 0.75-4.69)	2.6 (0-14.0, 1.27-5.30)	3.7 (0-13.2, 1.08-8.21)
Diabetes			
Type 1, n (%)	5 (2.4)	5 (5)	1 (5.3)
Type 2, n (%)	63 (30.0)	19 (19)	4 (21.1)

Table 4.3 Baseline characteristics of study population according to serum 25(OH)D status (n = 328).

Study population characteristics were explored in relation to their baseline serum 25(OH)D status. There were no differences between the groups in terms of age, P = 0.1708, gender, P = 0.171, or ethnicity P = 0.833 (Kruskal–Wallis and Chi Squared).

4.3 Repletion of 25(OH)D

As per the clinical guideline (Table 4.4), cholecalciferol supplementation was commenced at 40,000 units or 20,000 units weekly according to each patients' serum 25(OH)D level. The supplementation dose and clinical guideline was due to be reviewed at 6 months (discussed in quality control and safety, section 2.2.4).

25(OH)D concentration	Cholecalciferol Dose
< 50nmol/L	40,000 units = two 20,000 unit capsules once weekly
50 - 75nmol/L	20,000 units = one 20,000 unit capsule once weekly
76-150nmol/L	If not already taking cholecalciferol - no indication to start. If taking cholecalciferol already - maintain levels on maintenance dose of 20,000 units = one 20,000 unit capsule once weekly
> 150nmol/L	STOP cholecalciferol, recheck level in 3 months and provided <150 nmol/L restart maintenance dose of 20,000 units = one 20,000 unit capsule once weekly

Table 4.4 Clinical guideline summary for cholecalciferol supplementation in haemodialysis patients at UHCW (version 1).
Cholecalciferol supplementation was given based on serum 25(OH)D concentration. This was measured every 3 months initially; once stable, in the absence of a clinical indication such as hypercalcaemia, serum 25(OH)D was monitored annually.

Serum 25(OH)D was measured prior to commencing cholecalciferol supplementation and then every 3 months. According to the clinical guideline, once stable, in the absence of a clinical indication such as hypercalcaemia, serum 25(OH)D should be monitored annually (2.2.2). The number of patients 25(OH)D results available for analysis at each time point are outlined in Table 4.5, this reduced over the 15-month follow up period from 328 at T0 to 113 at T15. The largest reduction in the number of reported results occurred from T12 (n = 230) to T15 (n = 113). This was attributed to the stable serum 25(OH)D levels seen from T9 to T12.

Time point (months)	Number of patients 25(OH)D results included in analysis
T0	328
T3	285
T6	264
T9	237
T12	230
T15	113

Table 4.5 The number included in the analysis of serum 25(OH)D at each time point during the study.

Serum 25(OH)D was measured prior to commencing supplementation and then every 3 months according to the clinical guideline; this was done as part of standard routine care and therefore not the responsibility of the study. The number of 25(OH)D results available for analysis reduced over the 15-month prospective follow up period. The largest drop was from T12 to T15, due to serum 25(OH)D being stable so, in accordance with the guideline, testing frequency was reduced.

4.3.1 Review of clinical guideline

As discussed in section 2.2.4, preliminary data during the initial 6 months following the introduction of cholecalciferol supplementation, were reviewed to assess the safety and effectiveness of the guideline.

The initial high dose repletion phase from baseline to 3 months (T0-T3) resulted in 76.5% (218 of 285) of patients achieving adequate repletion (serum 25(OH)D ≥75nmol/L). 12.3% of patients (35 of 285) had serum 25(OH)D levels greater than 150nmol/L (the clinical guideline upper limit) at T3. In the second 3 month period (T3 – T6), this trend continued, and at T6 24.6% (65 of 264) had serum 25(OH)D levels >150nmol/L (Figure 4.7).

Once this preliminary data was obtained, the clinical guideline was revised, and the maintenance dose reduced from 20,000 units per week to 20,000 units every other week (Table 4.6). The amended guideline went through the approval processed as discussed in section 2.2.1. The reduction in maintenance cholecalciferol dose led to a reduction in the number of patients overshooting the upper serum 25(OH)D target from 65 of 264 (24.6%) at 6 months to 26 of 237 (11.0%) at 9 months ($P < 0.001$) and did not change between 9 and 12 months (P for difference = 0.728). As previously discussed, according to the guideline, after the first year, providing 25(OH)D stable, testing could be reduced to annually; therefore, less serum 25(OH)D results were known at T15. The percentage of patients achieving and

maintaining target serum 25(OH)D concentration (75-150nmol/L) was not affected by the reduction in maintenance dose (Figure 4.7).

25(OH)D concentration	Cholecalciferol Dose
< 50nmol/L	40,000 units = two 20,000 unit capsules once weekly
50 - 75nmol/L	20,000 units = one 20,000 unit capsule every other week
76-150nmol/L	If not already taking cholecalciferol - no indication to start. If taking cholecalciferol already - maintain levels on maintenance dose of 20,000 units = one 20,000 unit capsule every other week
> 150nmol/L	STOP cholecalciferol, recheck level in 3 months and provided <150 nmol/L restart maintenance dose of 20,000 units = one 20,000 unit capsule every other week

Table 4.6 Revised clinical guideline for cholecalciferol supplementation in haemodialysis patients at UHCW.
The Maintenance cholecalciferol dose was reduced from 20,000 units once weekly to 20,000units every other week.

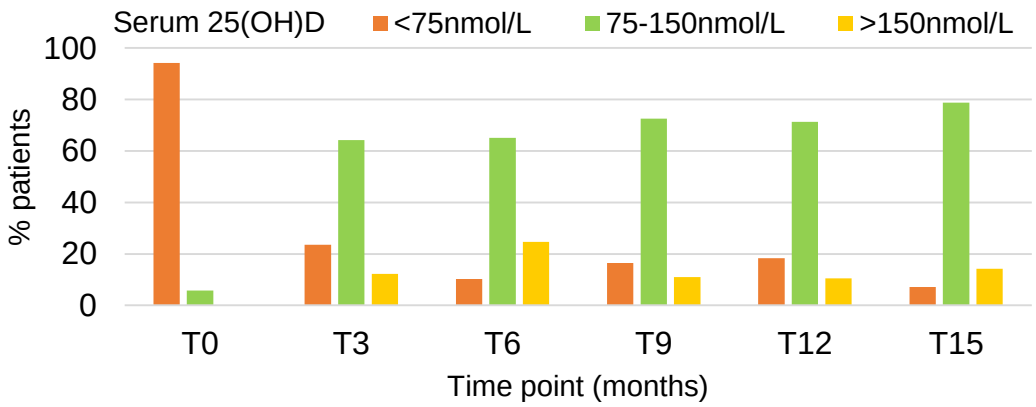

Figure 4.7 Serum 25(OH)D concentration of UHCW haemodialysis patients according to clinical guideline target ranges throughout the 15-month prospective observational period.
94.2% (309 of 328) had low serum 25(OH)D levels, and only 19 patients (5.8%) had optimal levels (≥75nmol/L) at baseline. Supplementation was effective; optimal serum 25(OH)D was achieved in 64.2% (183 of 285) of patients at 3 months (T3). 24.6% (65 of 264) of patients reached serum 25(OH)D concentrations above the upper target (150nmol/L) at T6. Following review of the clinical guideline and reduction in maintenance cholecalciferol dose from 20,000IU weekly to 20,000IU every other week, this reduced to 11% (26 of 237) at T9 and plateaued.

4.3.2 Effectiveness of supplementation guideline

Cholecalciferol supplementation effectively increased serum 25(OH)D levels to ≥75nmol/L in 76% of patients by T3. This increased further and was maintained in 82 to 93% of patients throughout the remaining study duration (T6-T15), as presented in Figure 4.8. Using the UHCW guideline serum 25(OH)D target range of 75nmol/L-150nmol/L, 64.2 – 78.8% of patients had an optimal, within target, serum 25(OH)D concentration from T6 – T15 (Figure 4.9).

39 patients (13.7%) had insufficient levels of serum 25(OH)D (30-74nmol/L) at T3. Whilst a proportion of patients had insufficient 25(OH)D throughout the study, only 9 (7.1%) were insufficient at study end (T15) (Figure 4.8).

1 - 2% of patients remained 25(OH)D deficient throughout T6-T12; This was attributed to cholecalciferol either not being prescribed, the patients not receiving it, or choosing not to take it (Figure 4.8).

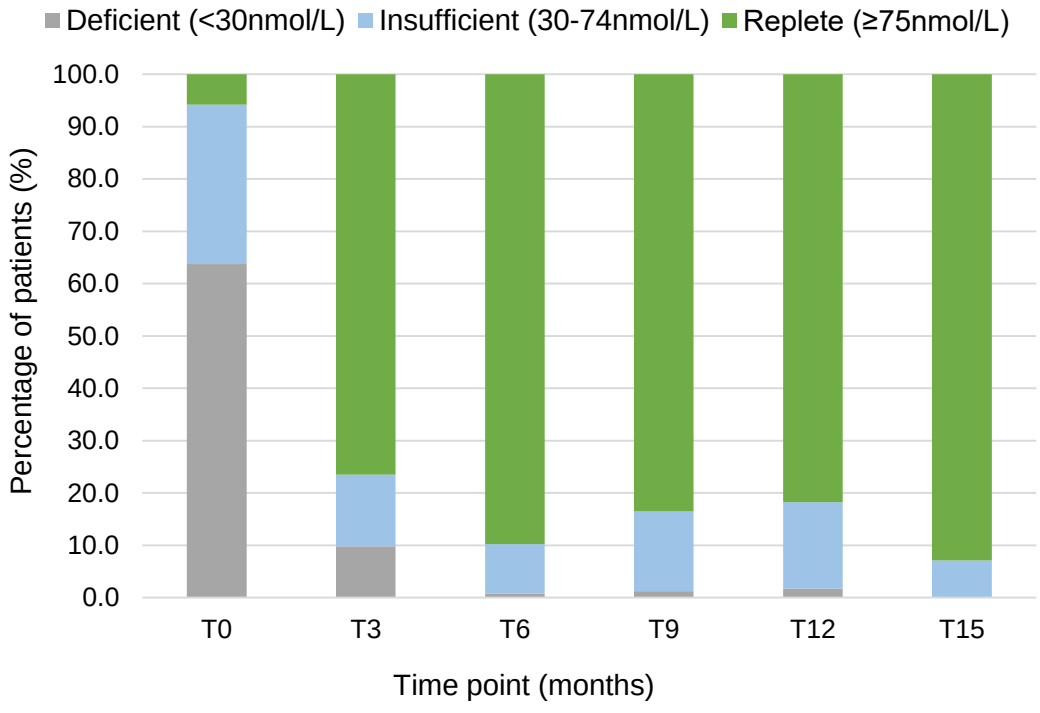

Figure 4.8 Serum 25(OH)D status in UHCW haemodialysis patients at each time point.
Cholecalciferol supplementation was effective. 76% (217 of 285) achieved repletion to ≥75nmol/L by T3 (T0 – T3 P <0.001), and 82% (216 of 264) by T6 (T0 – T6 P <0.001), this was maintained in 82% (216 of 264) to 93% (105 of 113) of patients throughout the remaining study duration (T6 - T15 P =0.776). 1 - 2% of patients remained 25(OH)D deficient throughout T6-T12. Wilcoxon signed rank test.

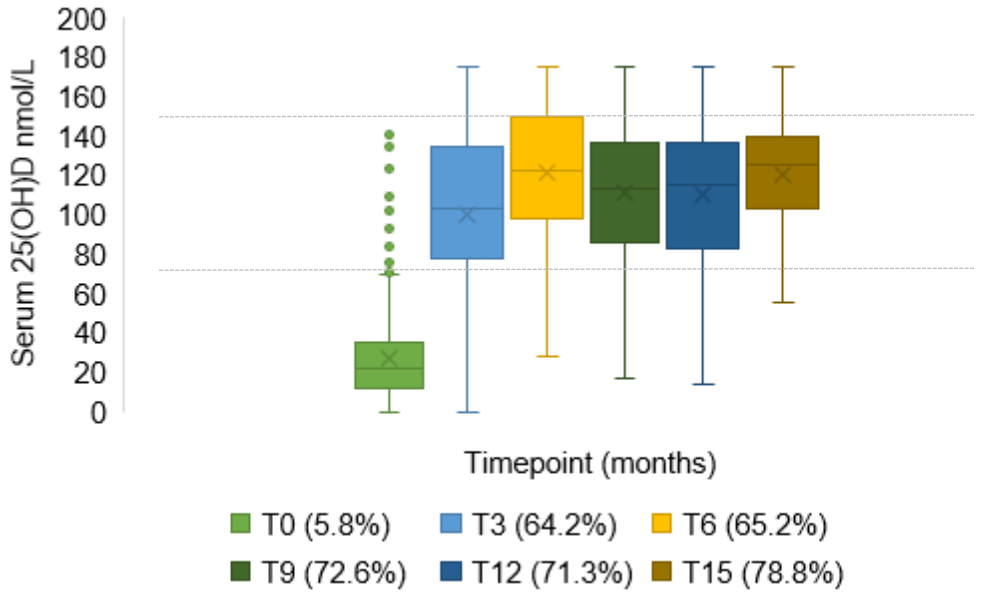

Figure 4.9 Serum 25(OH)D levels in relation to the clinical guideline targets at each time point throughout the 15-month prospective observational period. Once serum 25(OH)D repletion was obtained (T3), the majority of patients maintained serum 25(OH)D levels within the target range (75-150nmol/L) from T3 through to study end (T15). This demonstrates that the cholecalciferol supplementation guideline was effective at increasing and maintaining serum 25(OH)D levels. Data represent individual patient values at each time point; the lines across the graph area represent the lower and upper serum 25(OH)D targets (75nmol/L and 150nmol/L).

4.4 Assessment of safety

The safety of cholecalciferol supplementation was assessed based on serum corrected calcium. Prior to cholecalciferol supplementation (T-12 to T-1) the whole cohort mean corrected calcium was 2.29±0.13mmol/L (median 2.29, range 1.56-3.10), n = 290. In the 12 months following the initial repletion phase (T4 to T15) this increased to 2.35±0.13mmol/L (median 2.34, range 1.60-3.18), $P < 0.001$, but stayed well within the 2.08-2.58mmol/L target (Table 4.7). Data was stratified to include only patients that achieved repletion (mean serum 25(OH)D ≥75nmol/L from T3-T15), and the same analysis was repeated, n=238. Overall, the results reflected whole cohort analysis (Table 4.7). The range in corrected calcium was variable across the whole 28-month observation period with high and low corrected calcium levels being identified both pre and post cholecalciferol supplementation as illustrated in Figure 4.10.

Corrected Calcium (mmol/L)	No. of patients' results	T -12 to T-1	T4 to T15	Wilcoxon signed rank test
All patients	290	2.29 ± 0.13	2.35 ± 0.13	P <0.001
25(OH)D ≥75nmol/L T3-T15	238	2.29 ± 0.13	2.35 ± 0.13	P <0.001

Table 4.7 Mean serum corrected calcium T-12 to T-1 and T4 to T15.
Prior to 25(OH)D repletion (T-12 to T-1) the whole cohort mean corrected calcium was 2.29±0.13mmol/L (n = 290); in the 12 months following vitamin D repletion (T4 to T15) this increased to 2.35±0.13mmol/L, P <0.001, but stayed well within target range (2.08-2.58mmol/L). Data was stratified to include only patients that achieved a mean serum 25(OH)D ≥75nmol/L from T3-T15, and the same analysis was repeated, the outcome remained unchanged. Data represent mean±SD.

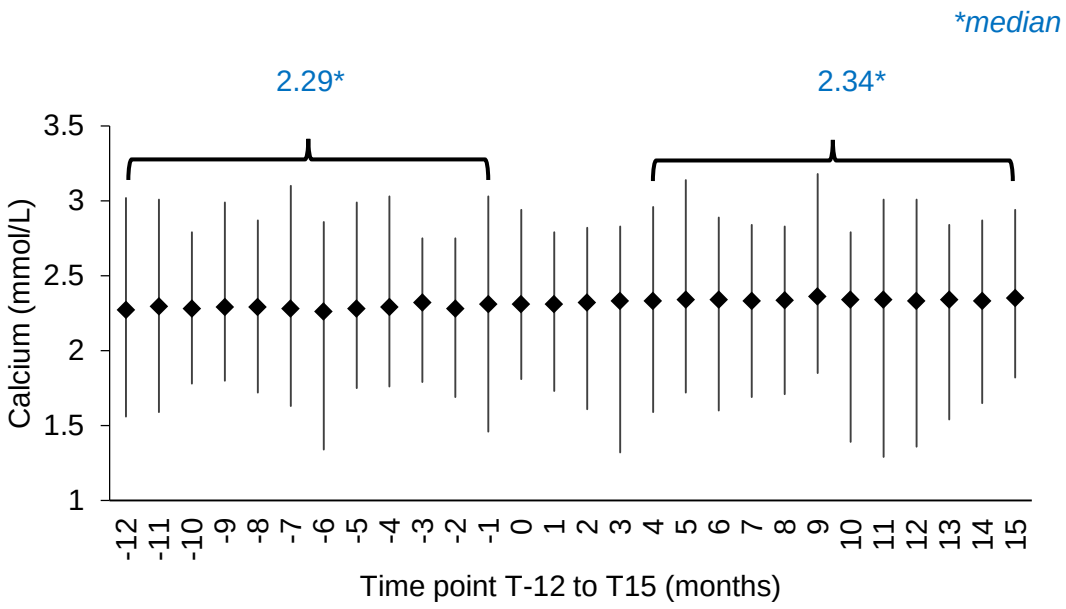

Figure 4.10 Variation in serum corrected calcium across the study.
The overall median in the 12 months pre introduction of cholecalciferol was 2.29mmol/L (1.56–3.10mmol/L), this compared with 2.34mmol/L (1.60–3.18mmol/L), in the 12-month post supplementation follow up period, P< 0.001. The range was variable across the whole 28-month observation period with high and low corrected calcium levels being identified both pre and post cholecalciferol supplementation. Data represent median serum corrected calcium at each timepoint together with the range. Wilcoxon signed rank test.

4.4.1 Correlation between corrected calcium and 25(OH)D

As discussed in 1.6.2, serum calcium levels rise in response to increased calcium absorption in the gut; this is related to, and facilitated by, the actions of $1,25(OH)_2D$ not 25(OH)D. In the presence of adequate substrate (25(OH)D), serum $1,25(OH)_2D$ is produced, as required, in response to serum calcium and parathyroid hormone levels. Calcium is therefore not directly associated with 25(OH)D. To demonstrate this, and further investigate the relationship in terms of safety, the correlation between the two biomarkers was investigated. There was no correlation seen between serum corrected calcium and 25(OH)D at any timepoint (Table 4.8); this is presented further in section 5.3.1.

Time point	T0	T3	T6	T9	T12	T15
Correlation between corrected calcium and 25(OH)D (ρ)	0.092 P=0.098 n = 322	0.027 P=0.663 n = 273	0.049 P=0.437 n = 256	0.051 P=0.440 n = 231	0.106 P=0.113 n = 223	-0.089 P=0.352 n = 111

Table 4.8 The relationship between serum corrected calcium and serum 25(OH)D during the prospective follow up period.
Cholecalciferol supplementation was initiated at T0. Serum 25(OH)D levels were tested at 3 monthly intervals (T0-T15). There was no correlation between serum 25(OH)D and corrected calcium at any timepoint. Results were only included if both a serum corrected calcium and 25(OH)D were available for the patient at the specified time point (Spearman's ρ).

4.4.2 Cholecalciferol supplementation and serum $1,25(OH)_2D$

Serum $1,25(OH)_2D_3$ levels were measured in the sub-study one subjects (n = 81). The characteristics of the sub-study subjects are presented in Table 4.9. Complete data, baseline (T0) and 12 months (T12), was obtained for 55 participants (Figure 4.11).

Variables	Sub-study one Haemodialysis Patients (n=81)	UHCW Haemodialysis Patients (n=350)
Age, years, median (range)	69 (27-76)	69 (24-95)
Male, n (%)	50 (61.7)	212 (60.6)
Ethnicity, n (%)		
White	75 (92.6)	260 (74.3)
South Asian	4 (4.9)	55 (15.7)
Black	1 (1.2)	23 (6.6)
Other	1 (1.2)	12 (3.4)
Dialysis Vintage, years, median (range)	2.8 (0.1-17.5)	2.4 (0-17.5)
Diabetes n (%)		
Type 1	2 (2.5)	11 (3.1)
Type 2	19 (23.5)	91 (26.0)

Table 4.9 Characteristics of UHCW haemodialysis patients recruited into sub-study one.
Patients recruited into sub-study one were representative of UHCW haemodialysis population for all characteristics except for ethnicity. Minority ethnic groups were under-represented, a recognised problem in research and something that requires addressing in future studies (Redwood & Gill, 2013).

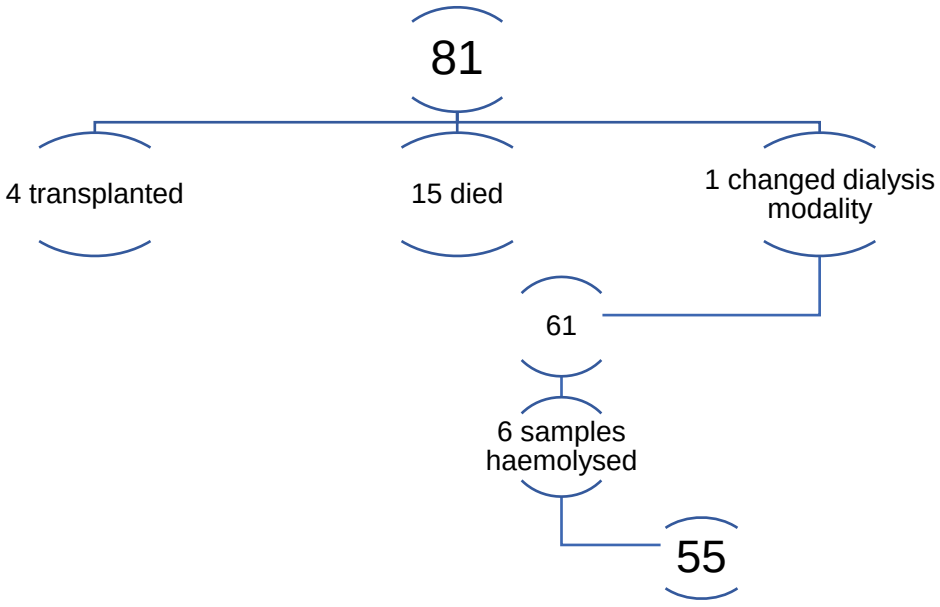

Figure 4.11 Sub-study one withdrawal.
81 haemodialysis patients were recruited for participation in sub-study one. During the 15-month follow up phase the total withdrawal was 25% (n = 20). The results (T0 and T12) from 55 patients were included in the data analysis.

Despite 82% (66 of 81) of the sub-study patients being prescribed an active vitamin D analogue, serum $1,25(OH)_2D_3$ levels were low at baseline, 45.3±25.7pmol/L. Cholecalciferol supplementation resulted serum $1,25(OH)_2D_3$ repletion in 96% of patients (53 of 55) at 12 months, 106.6±44.0pmol/L, P <0.001 (Figure 4.12). The laboratory reference range for normal $1,25(OH)_2D_3$ concentration is 60-108pmol/L (Bouillon, 2017; Feldman, 2018).

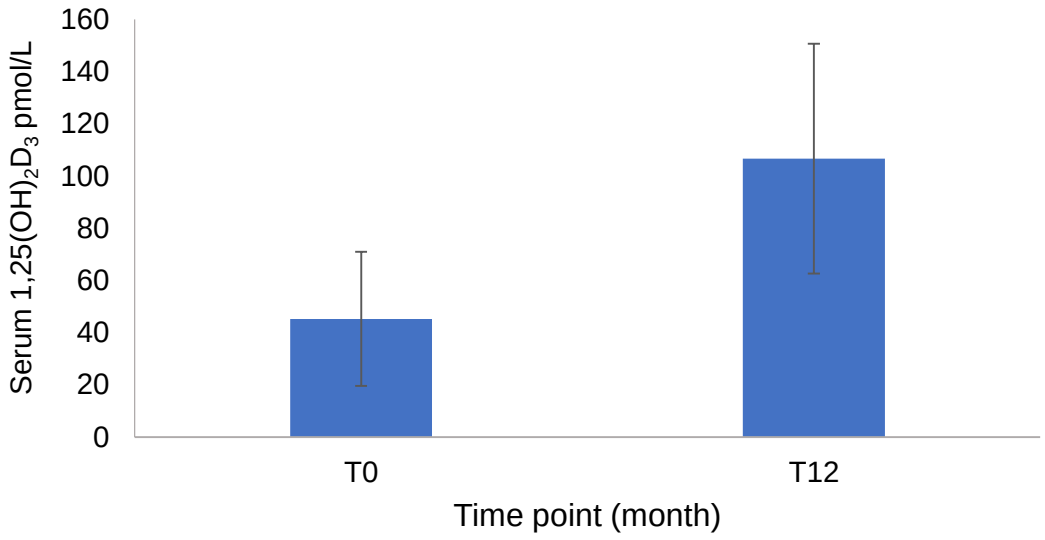

Figure 4.12 Mean serum $1,25(OH)_2D_3$ level in sub-study one participants at baseline (T0) and 12 months post introduction of cholecalciferol supplementation (T12).
Serum $1,25(OH)_2D_3$ increased following cholecalciferol supplementation from 45.3±25.7pmol/L (T0) to 106.6±44.0pmol/L (T12), P <0.001. The normal reference range for serum $1,25(OH)_2D_3$ is 60-108pmol/L (Bouillon, 2017; Feldman, 2018) therefore $1,25(OH)_2D_3$ repletion was achieved. n = 55. Data represent mean±SD. Wilcoxon signed rank test.

Data was stratified according to whether patients were prescribed an active vitamin D analogue at baseline (45 of 55) or not (10 of 55). Both groups had low baseline levels; mean serum $1,25(OH)_2D_3$ was only slightly lower (ns) in patients not prescribed an active vitamin D analogue at T0, 41.8±28.7pmol/L vs.

46.1±25.3pmol/L, P = 0.536 (Figure 4.13). Serum $1,25(OH)_2D_3$ was higher at T12 in those prescribed an active vitamin D analogue but this did not reach significance, 111.3±45.4pmol/L compared with 85.7±28.9 in those not prescribed an active vitamin D analogue (P = 0.269). The lack of significance may have related to small numbers (one group on had 10 subjects). The mean values for both groups achieved $1,25(OH)_2D_3$ normalisation at T12.

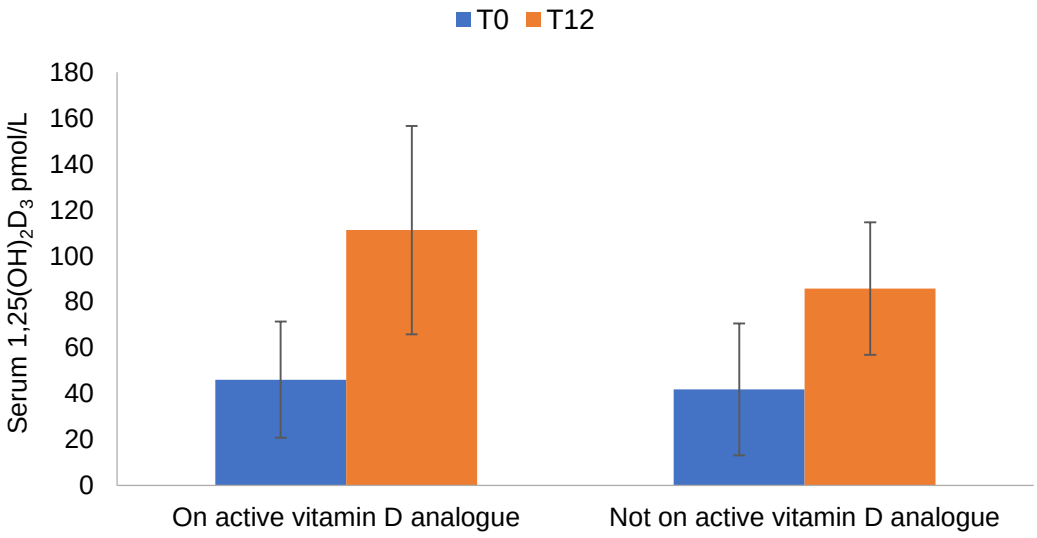

Figure 4.13 Mean serum $1,25(OH)_2D_3$ in sub-study participants according to active analogue use, at baseline (T0) and 12 months post introduction of cholecalciferol supplementation (T12).
Data was grouped according to whether the patients were prescribed an active vitamin D analogue at baseline (n = 45) or not (n = 10). Mean serum $1,25(OH)_2D_3$ was lower (*ns*) in the patients not prescribed an active vitamin D analogue at T0 (41.8±28.7pmol/L vs. 46.1±25.3pmol/L, P = 0.536). Both groups had low baseline $1,25(OH)_2D_3$ and normalisation of $1,25(OH)_2D_3$ was achieved at T12 (P <0.001). Serum $1,25(OH)_2D_3$ was higher at T12 in those prescribed an active vitamin D analogue (*ns*) (111.3±45.4pmol/L compared with 85.7±28.9, P = 0.269). Data represent mean ±SD. Wilcoxon signed rank test.

$1,25(OH)_2D_3$ levels failed to increase in 2 patients despite their serum 25(OH)D concentrations optimising from 40.2nmol/L and 23.6nmol/L at baseline to 144nmol/L and 136.4nmol/L at 12 months, respectively. The results for these 2 patients are presented in Table 4.10. Serum corrected calcium reduced in both patients reinforcing the link with $1,25(OH)_2D_3$ and not 25(OH)D, this reduction in calcium did not cause an increase in PTH.

Parameter	Patient 1		Patient 2	
	Pre	Post	Pre	Post
25(OH)D	40.2nmol/L (T0)	144nmol/L (T12)	23.6nmol/L (T0)	136.4nmol/L (T12)
$1,25(OH)_2D_3$	64.1pmol/L (T0)	55.0pmol/L (T12)	68.4pmol/L (T0)	64.3pmol/L (T12)
Corrected calcium	2.42mmol/L (Mean T-12 to T-1)	2.36mmol/L (Mean T4 to T-15)	2.54mmol/L (Mean T-12 to T-1)	2.34mmol/L (Mean T4 to T-15)
PTH	15.5pmol/L (Mean T-12 to T-1)	15.2pmol/L (T12)	58.5pmol/L (Mean T-12 to T-1)	48.3pmol/L (Mean T4 to T-15)

Table 4.10 Pre and post vitamin D repletion results for serum 25(OH)D, $1,25(OH)_2D_3$, corrected calcium and PTH.
Results from the 2 patients in whom there was no improvement in serum $1,25(OH)_2D_3$ from T0 to T12 despite achieving optimal 25(OH)D concentrations. Patient 1 was not prescribed an active vitamin D but patient 2 was. Serum corrected calcium reduced in both patients reinforcing the link with $1,25(OH)_2D_3$ and not 25(OH)D, this reduction in calcium did not cause an increase in PTH.

Serum $1,25(OH)_2D_3$ was compared to serum 25(OH)D levels (Figure 4.14). No correlation was seen between serum 25(OH)D and serum $1,25(OH)_2D_3$ at baseline or 12 months. This absence of correlation was expected because the production of $1,25(OH)2D_3$ is tightly regulated, occurring in response to serum calcium and parathyroid hormone levels rather than a change in serum 25(OH)D. Therefore $1,25(OH)2D_3$ is not expected to correlate with 25(OH)D.

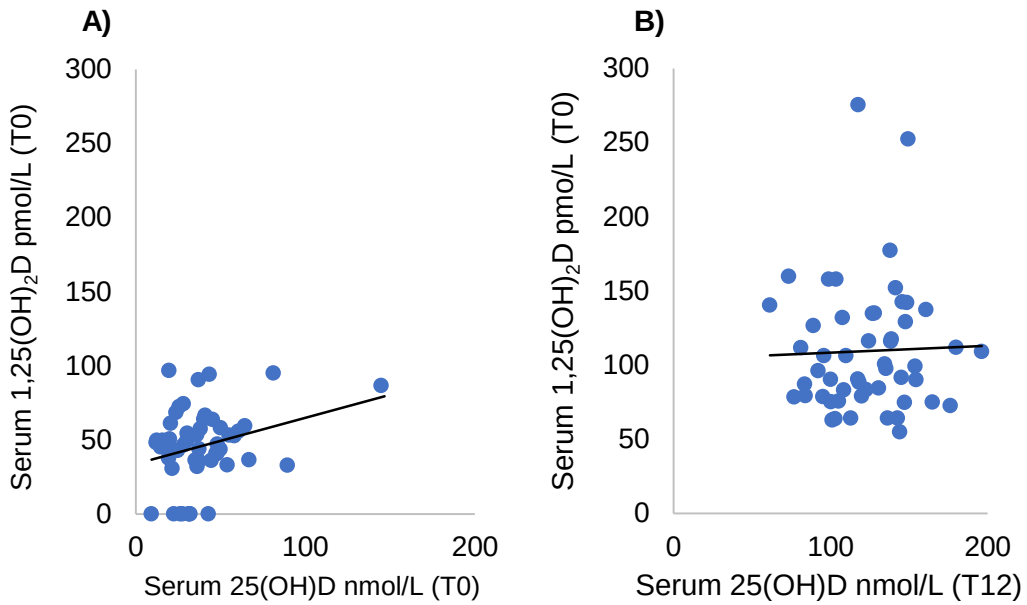

Figure 4.14 The relationship between serum 25(OH)D and 1,25(OH)$_2$D$_3$ at baseline (T0) and 12 months (T12).
Scatter plots exploring the relationship between 25(OH)D and 1,25(OH)$_2$D$_3$ in haemodialysis patients, prior to commencing routine cholecalciferol supplementation (T0) and at 12 months (T12 There was no correlation between serum 1,25(OH)$_2$D$_3$ and 25(OH)D at baseline **(A)** or 12 months **(B)**, ρ 0.165, P = 0.230, and ρ 0.180, P = 0.188, respectively, n = 55 (Spearman's ρ).

The relationship between serum corrected calcium and 1,25(OH)$_2$D$_3$ was explored pre and post introduction of cholecalciferol supplementation (Figure 4.15). Whilst no correlation was seen between corrected calcium and 1,25(OH)$_2$D$_3$ prior to initiation of cholecalciferol (when serum 25(OH)D and 1,25(OH)$_2$D$_3$ levels were both low), a correlation was seen at 12 months (Figure 4.15).

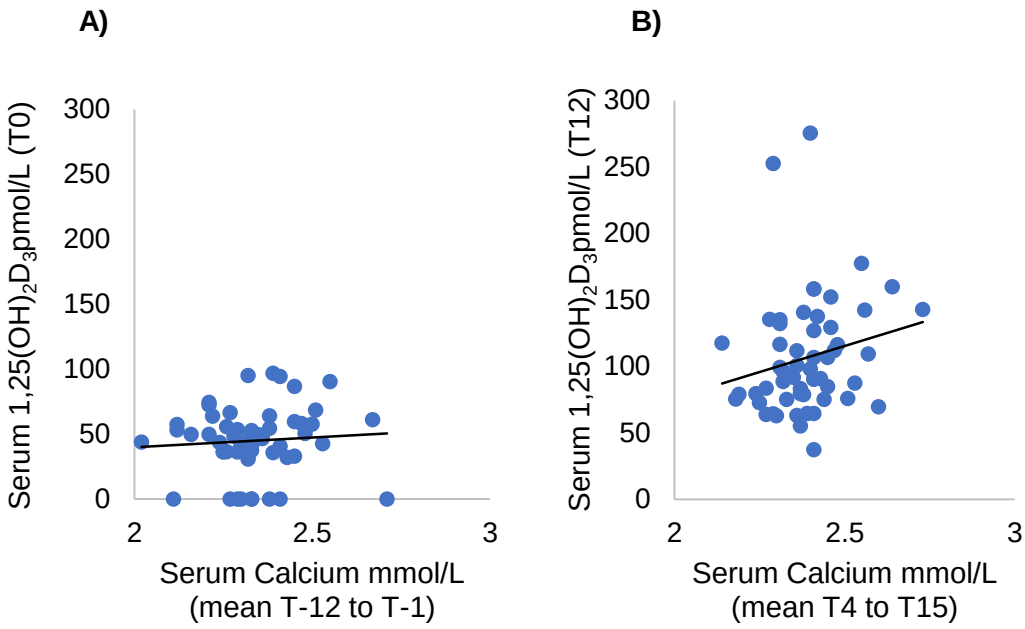

Figure 4.15 The relationship between serum $1,25(OH)_2D_3$ and corrected calcium in haemodialysis patients pre, and post, cholecalciferol supplementation.
There was no correlation seen between serum corrected calcium and $1,25(OH)_2D_3$ at baseline **(A)**, ρ 0.094, P = 0.50. There was a positive correlation following serum 25(OH)D repletion **(B)**, ρ 0.288, P = 0.033, n = 55 (Spearman's ρ).

4.4.3 Limitations of cholecalciferol supplementation

Based on data from the systematic review (Chapter 2), it was anticipated that 25(OH)D repletion would be seen in most patients after 3 months of cholecalciferol supplementation. This was the case, with 76% (216 of 285) achieving optimal 25(OH)D concentrations by T3 and 90% (238 of 264) by T6. However, a number of patients did not achieve repletion, 24% (68 of 285) at T3 and 10% (26 of 264) at T6).

The supplementation guideline was dependent on a clinician checking patients' serum 25(OH)D results and prescribing cholecalciferol accordingly. Implementing new guidelines, and changing practice, in the pressured clinical setting, is not without challenges (Grimshaw & Russell, 1994). Cholecalciferol supplementation was introduced as part of standard care, meaning it did not have the same degree of oversight, and monitoring, as a clinical trial. These limitations are discussed here.

However, the monitoring, and investigation, into these issues, was not within the scope of the study.

One potential problem is that blood tests may have been missed. This could occur if a patient missed dialysis on the day monthly bloods were due (for example due to being on holiday or in hospital) or because 25(OH)D was not added to the blood request, due to staff oversight. The supplementation guideline specified that cholecalciferol should be suspended if serum 25(OH)D >150nmol/L and then restarted 3 months later providing the serum 25(OH)D reduced to ≤150nmol/L; this relied on testing and prescribing practices. If a patient missed a blood test, or if cholecalciferol did not get restarted due to clinician oversight, their serum 25(OH)D level may have fallen below optimal. This risk would have only applied to the few patients that achieved serum 25(OH)D levels >150nmol/L.

The guideline adopted a conservative upper serum 25(OH)D limit of 150nmol/L (Holick *et al.*, 2011). A higher upper limit of 175nmol/L for serum 25(OH)D may have provided greater scope for avoiding the stopping and starting of cholecalciferol; the number of patients with serum 25(OH)D 151-175nmol/L was 29 (T3), 47 (T6), 20 (T9) 23 (T12), and 14 (T15). Having an upper target of 175nmol/L would have resulted in a higher percentage of subjects maintaining a serum 25(OH)D within the target range (75-175nmol/L).

Another limiting factor on the success of repleting serum 25(OH)D is non-concordance, either through direct refusal to nursing staff during administration, or through acceptance at that point and then later disposal of the capsule. There were only two known cases, out of 350 patients, where this occurred.

Many factors are believed to affect serum 25(OH)D response to cholecalciferol supplementation (Mazahery & von Hurst, 2015). These include, but are not limited to, body mass index (BMI), fat stores, ethnicity, and uraemia (Aloia *et al.*, 2008; Camozzi *et al.*, 2016; Michaud *et al.*, 2010). Having a guideline that stipulates one rule for all does not take these known influences into consideration and is therefore not without limitations.

4.5 Discussion

Hypovitaminosis D was highly prevalent in UHCW HD patients. These findings are in keeping with those of others (Saab *et al.*, 2007; Tokmak *et al.*, 2008; Wolf *et al.*, 2007). Cholecalciferol, given as a 'one rule fits all' dose, during dialysis sessions, is effective at increasing and maintaining serum 25(OH)D at a population level. Whilst the current study did not have the controlled environment of a clinical trial, the cholecalciferol was prescribed by renal physicians and given by nursing staff which minimised the risk of non-adherence. Previous studies have shown a varying response to cholecalciferol in ESRD with 0-100% of the study populations achieving serum 25(OH)D repletion to ≥75nmol/L (Jakopin *et al.,* 2014; Armas *et al.,* 2012; Delanaye *et al.,* 2013; Massart *et al.,* 2014; Marckmann *et al.,* 2012). Reasons for this are likely to include varying doses, intervention periods, and compliance.

A statistically significant increase in serum corrected calcium was seen; the overall mean corrected calcium remained well within the target range, and positively, the incidences of hypocalcaemia (<2.08mmol/L) reduced. Most patients (96%, 278 of 290) maintained a mean serum corrected calcium level within the laboratory target range following cholecalciferol supplementation. There were no adverse incidences relating to calcium following the introduction of cholecalciferol supplementation and the range of serum corrected calcium levels seen were comparable in both the 12-month observation periods, 1.56-3.10mmol/L (pre) vs. 1.60-3.18mmol/L (post).

As expected, there was no correlation between 25(OH)D and corrected calcium, or 25(OH)D and $1,25(OH)_2D_3$. Serum corrected calcium did not correlate with $1,25(OH)_2D_3$ at baseline, but a correlation was seen at 12 months, once 25(OH)D repletion was achieved. Increased serum corrected calcium is facilitated by the actions of $1,25(OH)_2D$, not 25(OH)D. In the presence of adequate substrate (25(OH)D) serum $1,25(OH)_2D$ is produced (as required) in response to serum calcium and parathyroid hormone levels. Calcium is expected to correlate with $1,25(OH)_2D$; the absence of this correlation at baseline suggests the lack of substrate was limiting production of $1,25(OH)_2D$ (25(OH)D and $1,25(OH)_2D_3$ concentrations were low at baseline). Once 25(OH)D repletion was achieved, production and secretion of $1,25(OH)_2D$ was facilitated, serum $1,25(OH)_2D_3$ normalised, and a statistically significant correlation between serum corrected calcium and $1,25(OH)_2D$ was seen. The results demonstrate that calcium is not directly related to 25(OH)D, but rather indirectly through the actions of $1,25(OH)_2D$.

Production of $1,25(OH)_2D$ is tightly regulated meaning optimising 25(OH)D through cholecalciferol supplementation will not result in unlimited $1,25(OH)_2D$ and severe hypercalcaemia.

248 patients (71% of the whole study cohort) were prescribed an active vitamin D analogue (calcitriol or alfacalcidol) at baseline. Given the therapeutic nature of active vitamin D analogues is to increase serum calcium (BNF, 2019), this was an important safety consideration. An increase in serum calcium levels in response to cholecalciferol supplementation likely represents a persons' ability to synthesise $1,25(OH)_2D_3$ in the presence of adequate substrate (serum 25(OH)D). In this situation, patients may no longer require an active vitamin D analogue, or may at least require a reduction in dose. If the serum corrected calcium level increased beyond the upper target of 2.58mmol/L; the active analogue should have been discontinued as specified in the clinical guideline (section 2.2.2). For this reason, in most incidences, the high corrected calcium was an isolated result, however there were some situations where there were 2 or 3 concurrent high corrected calcium results; this may have been caused by a delay in the active analogue being reduced, or discontinued, or may have been down to the decision of the managing clinician. A slightly high corrected calcium may not have given rise to concern; whilst the clinical guideline was there as guidance, it could be overruled by clinician discretion. The results indicate that concurrent use of active analogues is safe for haemodialysis patients providing serum corrected calcium levels are monitored regularly. Whilst an increase in median serum corrected calcium was seen, almost all hypercalcaemia seen was considered mild according to the NICE definition (<3.00mmol/L) (NICE, 2019).

There is very little data reporting how much calcium increases with active vitamin D analogues in CKD or ESRD. The serum calcium response is affected by other factors including serum $24,25(OH)_2D_3$ level, calcium supplements, and calcium-based phosphate binders. One study demonstrated that in order to increase corrected calcium from ~2.35mmol/L to ~2.45mmol/L, and maintain corrected calcium at this level, patients needed between 0.25mcg and 1mcg daily; 0.25mcg being the most commonly required dose (Hamdy *et al.*, 1995).

The recording and reporting of prescription changes, and what percentage of patients were still prescribed active analogues at the study end, was beyond the

scope of this study. The low incidence of hypercalcaemia seen, meant there was no indication to stop active vitamin D analogue use, in most patients.

Despite most patients being prescribed an active vitamin D analogue, mean $1,25(OH)_2D_3$ levels were low at baseline. Results suggest that adequate serum $1,25(OH)_2D_3$ can only be achieved through 25(OH)D repletion; relying solely on active analogue treatment is not sufficient. Prescribers have historically been led to believe that active vitamin D analogues are the key to addressing the lack of 1α-hydroxylase (and subsequent $1,25(OH)_2D$) in ESRD, yet these results demonstrate otherwise. By their very nature, active vitamin D analogues are hypercalcaemic; cholecalciferol is safer, and it may provide for management of both 25(OH)D and $1,25(OH)_2D$ deficiencies. If active analogues are required in addition to cholecalciferol, concurrent therapy is safe. The following chapter explores the impact of cholecalciferol supplementation, and 25(OH)D repletion on mineral bone markers.

Chapter 5 The impact of cholecalciferol supplementation on routine markers of mineral bone disease; serum corrected calcium, phosphate, and parathyroid hormone

Chapter 4 demonstrated the effective repletion of serum 25(OH)D in response to cholecalciferol supplementation and considered the effect on serum corrected calcium in relation to safety. This chapter considers the effects of cholecalciferol supplementation, and subsequent 25(OH)D repletion, on markers of CKD-MBD, namely corrected calcium, phosphate and PTH.

Serum parathyroid hormone (PTH) was measured every 3 months, and serum corrected calcium and phosphate measured monthly as part of standard care in all subjects (n = 350). Patients that had a parathyroidectomy prior to, or at any point during the study, were excluded from the analysis of PTH. Any patients that did not have adequate data to provide a mean for both 12-month time points (at least 2 results from both time periods) were excluded. Data were analysed both as a population whole and also stratified to exclude any patients that were already 25(OH)D replete at baseline, or that did not achieve repletion (serum 25(OH)D ≥75nmol/L). Stratification was undertaken to investigate whether effects could be seen in the whole group, thus representative of routine care (the 'one rule fits all' cholecalciferol guideline) or were dependent on 25(OH)D concentration. The number of patients included in the analysis for each parameter is shown in Table 5.1.

Parameter	Number included in analysis	Number included in analysis: vitamin D replete only
PTH	280	232
Corrected calcium	290	238
Phosphate	290	238

Table 5.1 The number of patients included in analysis.
Patients that had a parathyroidectomy prior to, or at any point during the study, were excluded from the analysis of PTH but not the analysis of serum corrected calcium and phosphate. Any patients that did not have adequate data in order to provide a mean at both the pre and post 12-month time points (at least 2 results from both time periods) were excluded. Data was looked at as a population whole,

and then stratified to exclude patients that were 25(OH)D replete at baseline or did not achieve 25(OH)D repletion.

5.1 The effect of cholecalciferol supplementation on serum PTH

Whole cohort data was looked at as a whole and then subsequently grouped based on mean PTH levels pre vitamin D supplementation (T-12 to T-1). The PTH cut offs for the groups were decided based on the renal association targets for end stage renal disease patients at the time the research was conducted, which was 2-9 times the upper-normal limit for the local laboratory reference (Steddon & Sharples, 2015). The PTH groups were as follows: over-suppressed (<8.4pmol/L), on target (8.4-37.8pmol/L), high (37.9-85pmol/L) and very high (>85pmol/L).

Prior to vitamin D supplementation, when 94.2% of patients (309 of 328) had insufficient serum 25(OH)D levels, the whole cohort mean PTH was 41.2±38.7pmol/L (T-12 to T-1); following the introduction of vitamin D supplementation, a numerical change was seen 37.2±35.3pmol/L (T4 to T15) (*ns*). The grouped analysis revealed no difference in PTH for the patients that already had a mean PTH within target range at baseline. A statistically significant decrease in PTH was seen in the high and very high PTH groups, 52.2±13.5pmol/L vs. 46.5±24.4pmol/L (P = 0.020), and 130.7±26.7pmol/L vs. 92.9±59.8pmol/L (P <0.001), respectively. A statistically significant increase in PTH was seen in the over-suppressed group, 5.8±1.8 vs. 14.5±8.5 (P <0.001) (Table 5.2A)

Once data was stratified, and those that did not adequately replete were excluded, results were reflective of the whole cohort with the exception of the reduction seen in the high PTH group, the statistical significance was lost (Table 5.2B). Data indicate that those with the highest serum PTH pre-supplementation are likely to have the most significant PTH reduction when given cholecalciferol supplementation.

(A)

PTH Grouping (pmol/L)	No. of results	T -12 to -1	T4 – T15	%Change	Wilcoxon signed rank test
All patients	280	41.2 ± 38.7	37.2 ± 35.3	-9.7%	P = 0.123
Over-suppressed <8.4	28	5.8 ± 1.8	14.5 ± 8.5	+150.0%	P <0.001
8.4-37.8 On target	147	22.0 ± 8.5	24.1 ± 15.5	+9.5%	P = 0.513
37.9-84.9 High	71	52.2 ± 13.5	46.5 ± 24.4	-10.9%	P = 0.020
≥85.0 Very high	34	130.7 ± 26.7	92.9 ± 59.8	-28.9%	P <0.001

(B)

PTH Grouping for patients that achieved repletion (pmol/L)	No. of results	T -12 to -1	T4 – T15	% Change	Wilcoxon signed rank test
All patients	232	41.2±40.0	38.4±36.8	-6.8%	P = 0.737
Over-suppressed <8.4	24	5.9 ± 1.7	15.4 ± 8.5	+161.0%	P <0.001
8.4-37.8 On target	122	21.5 ± 8.5	24.6 ± 16.5	+14.4%	P = 0.179
37.9-84.9 High	58	53.6 ± 14.5	48.5 ± 24.5	-9.5%	P = 0.068
≥85.0 Very high	28	131.7 ± 24.3	97.4 ± 62.2	-26.0%	P = 0.004

Table 5.2 Whole cohort and grouped analysis of mean PTH in the 12 months pre (T-12 to T-1) and post (T4 toT15) cholecalciferol supplementation.
(A) Grouped analysis was carried out based on mean PTH levels pre cholecalciferol supplementation (T-12 to T-1). Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum PTH levels were tested at 3 monthly intervals and the change in mean between the two 12-month study observation periods compared. No difference was found in the patients that already had a mean on target PTH but a statistically significant change in PTH was seen in the over-suppressed, high and very high PTH groups. **(B)** Once data was stratified, and those that did not adequately replete were excluded, results were reflective of the whole cohort, however, the significance was lost in the reduction seen in the high PTH group. Data are presented as mean±SD.

5.1.1 The correlation between serum PTH and serum 25(OH)D

Relationships between PTH and 25(OH)D were explored pre and post serum 25(OH)D repletion. All patients with serum 25(OH)D ≥75nmol/L at baseline were excluded from analysis, as well as all patients with a mean serum 25(OH)D <75nmol/L following cholecalciferol supplementation (T4-T15). Any patients with insufficient data and those that had a parathyroidectomy at any stage throughout the observation periods were also excluded from analysis. At baseline, in the presence of low serum 25(OH)D, no correlation was seen between serum PTH and serum 25(OH)D (Figure 5.1A) Once vitamin D repletion had been achieved a weak positive correlation was seen (Spearman's ρ = 0.180, P = 0.005); an increase in serum 25(OH)D is associated with an increase in serum PTH (Figure 5.1B).

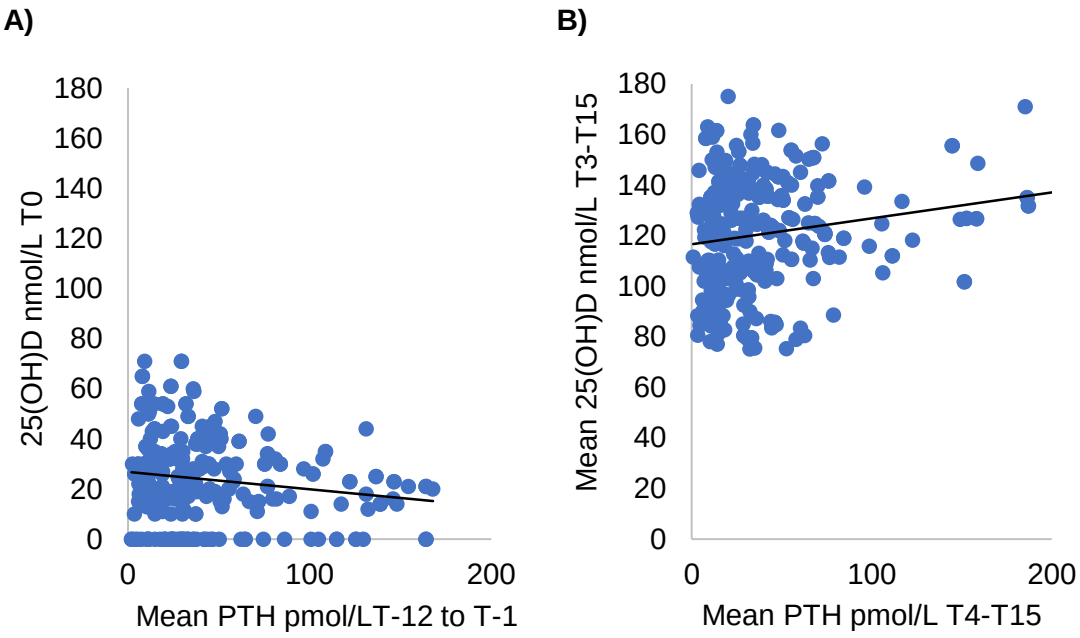

Figure 5.1 Relationship between baseline serum 25(OH)D and mean serum PTH prior to (A), and post (B) 25(OH)D repletion in haemodialysis patients. Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum 25(OH)D and PTH levels were tested every 3 months as part of standard routine care. **(A)** No correlation was seen between PTH and 25(OH)D at baseline, ρ = -0.084, P = 0.146, n = 302. **(B)** A weak correlation was seen between PTH and 25(OH)D in patients that achieved serum 25(OH)D repletion (≥75nmol/L), ρ = 0.180, P = 0.005, n = 245. Data represent mean values for both of the 12 month observation periods with the exception of baseline 25(OH)D. Spearman's ρ.

5.2 Cholecalciferol supplementation and serum phosphate

Prior to cholecalciferol supplementation (T-12 to T-1) the whole cohort mean phosphate was 1.49±0.36mmol/L; following the introduction of vitamin D supplementation (T4 to T15) this increased to 1.54±0.41mmol/L (P = 0.045).

Data were analysed for all and subsequently grouped based on mean phosphate levels pre vitamin D supplementation (T-12 to T-1). The phosphate cut offs for the groups were decided based on the renal association targets for end stage renal disease at the time the research was conducted (Steddon & Sharples, 2015). The phosphate groups were as follows, low (<1.1mmol/L), on target (1.1-1.7mmol/L), and high (>1.7mmol/L).

A statistically significant increase in phosphate was seen in the low and on target phosphate groups, 0.96±0.12mmol/L vs.1.14±0.35mmol/L (P = 0.004), and 1.40±0.17mmol/L vs. 1.47±0.34mmol/L (P = 0.003) respectively (Table 5.3A). A statistically significant reduction in phosphate was seen in patients with a high serum phosphate pre cholecalciferol supplementation, 1.99±0.26mmol/L vs. 1.82±0.41mmol/L (P = 0.012) (Table 5.3A).

Once data was stratified, and those that did not adequately replete were excluded, results remained reflective of the whole cohort analysis; phosphate increased overall from 1.49±0.35 to 1.52±0.38mmol/L (P = 0.023), and also increased in the ‘low’ and ‘on target’ phosphate groups, 0.94±0.13 to 1.15±0.32mmol/L (P = 0.002) and 1.40±0.16 to 1.46±0.30mmol/L (P = 0.002), respectively. Whereas a reduction in mean phosphate was seen in the ‘high’ phosphate group from 1.98±0.21 to 1.88±0.36mmol/L (P = 0.012). Results are presented in Table 5.3B.

The results indicate that cholecalciferol supplementation is likely to increase serum phosphate in patients with a low or on target serum phosphate at baseline. However, if the mean serum phosphate is high at baseline then cholecalciferol supplementation may reduce serum phosphate.

(A)

Phosphate Grouping (mmol/L)	No. of results	T -12 to -1	T4 – T15	% change	Wilcoxon signed rank test
All patients	290	1.49 ± 0.36	1.54 ± 0.41	+3.4%	P = 0.045
<1.1	28	0.96 ± 0.12	1.14 ± 0.35	+18.8	P = 0.004
1.1-1.7	191	1.40 ± 0.17	1.47 ± 0.34	+5%	P = 0.003
>1.7	71	1.99 ± 0.26	1.82 ± 0.41	-8.5%	P = 0.012

(B)

Phosphate Grouping for patients that achieved repletion (mmol/L)	No. of results	T -12 to -1	T4 – T15	% change	Wilcoxon signed rank test
All patients	238	1.49 ± 0.35	1.52 ± 0.38	+2.0%	P = 0.023
<1.1	23	0.94 ± 0.13	1.15 ± 0.32	+22.3%	P = 0.002
1.1-1.7	159	1.40 ± 0.16	1.46 ± 0.30	+4.3%	P = 0.002
>1.7	56	1.98 ± 0.21	1.88 ± 0.36	-5.1%	P = 0.028

Table 5.3 Whole cohort and grouped analysis of mean serum phosphate in the 12 months pre (T-12 to T-1) and post (T4 to T15) cholecalciferol supplementation in haemodialysis patients.
Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum phosphate levels were tested monthly as part of standard routine care. The change in mean phosphate between the two 12-month observation periods was compared. **(A)** A statistically significant increase in phosphate was seen in the low and on target phosphate groups, and a statistically significant reduction in phosphate was seen in patients with a high serum phosphate pre cholecalciferol supplementation. **(B)** Once data was stratified to exclude those that did not achieve 25(OH)D repletion, results remained reflective of the whole cohort analysis. Data are presented as mean±SD.

5.2.1 The correlation between serum phosphate and serum 25(OH)D

The relationship between serum 25(OH)D and phosphate was explored both pre and post cholecalciferol supplementation to see if serum 25(OH)D concentration made a difference to the relationship between the two parameters. Patients that were 25(OH)D replete at baseline were excluded from the pre supplementation

analysis; and patients that failed to achieve adequate repletion were excluded from the post supplementation (T3 to T15) analysis. No significant correlation was seen between phosphate and 25(OH)D pre, or post cholecalciferol supplementation (Figure 5.2 – A and B).

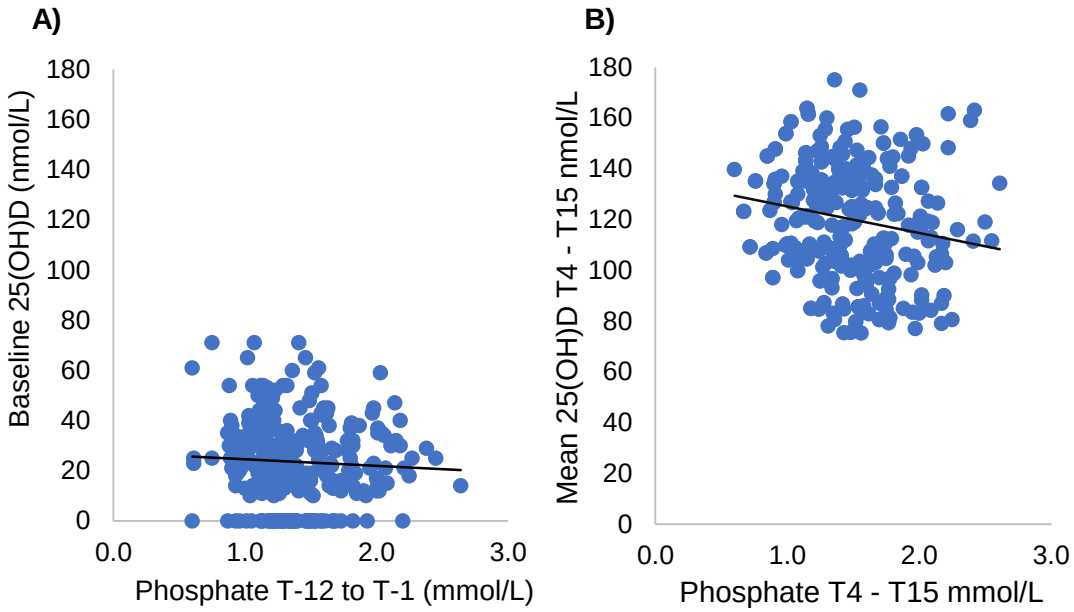

Figure 5.2 Relationship between baseline serum 25(OH)D and mean serum phosphate pre, and post, 25(OH)D repletion, in haemodialysis patients. Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum phosphate levels were tested monthly, and 25(OH)D every 3 months, as part of standard routine care. No correlation was seen between phosphate and serum 25(OH)D pre **(A)**, or post **(B),** repletion, ρ = -0.066, P = 0.251, n = 315, and ρ = -0.030, P = 0.644, n = 245, respectively. Patients with serum 25(OH)D ≥75nmol/L at baseline, those that did not adequately replete, and those with insufficient data, were excluded from analysis. Data represent mean values for both of the 12 month observation periods with the exception of baseline 25(OH)D. Spearman's ρ.

5.2.2 The correlation between serum phosphate and serum PTH

Given the involvement of vitamin D, notably 1,25$(OH)_2$D, and PTH, in the regulation of phosphate homeostasis (Bergwitz & Jüppner, 2010), the relationship between serum phosphate and PTH was explored both pre and post cholecalciferol supplementation to see if serum 25(OH)D concentration made a difference to the relationship between the two parameters. A weak correlation was seen between phosphate and PTH both pre, and post, serum 25(OH)D repletion, ρ = 0.215,

P <0.001, n = 302 and ρ = 0.233, P <0.001, n = 245, respectively, suggesting the higher a patients' phosphate, the higher their PTH (Figure 5.3 – A and B).

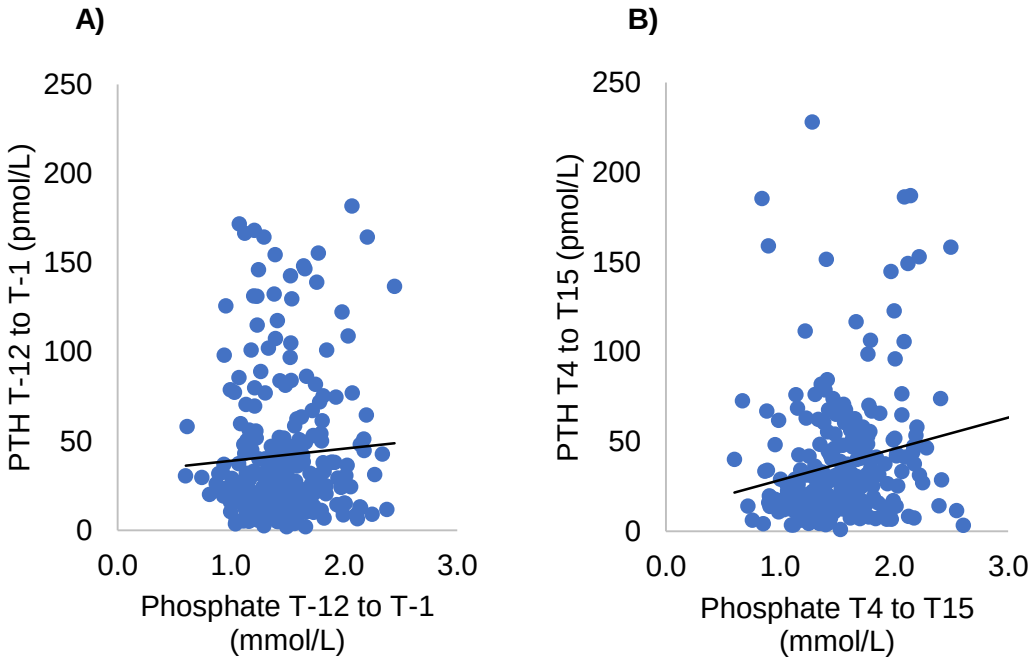

Figure 5.3 Relationship between mean serum PTH and mean serum phosphate pre, and post, 25(OH)D repletion, in haemodialysis patients. Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum phosphate levels were tested monthly, and PTH every 3 months, as part of standard routine care. A weak correlation was seen between serum PTH and serum phosphate both pre **(A)**, and post **(B)** serum 25(OH)D repletion ρ = 0.215, P < 0.001, n = 302 and ρ = 0.233, P < 0.001, n = 245, respectively. Patients with serum 25(OH)D ≥75nmol/L at baseline, those with insufficient data, those that did not achieve repletion, and those that had a parathyroidectomy at any stage throughout the observation periods were excluded from analysis. Data represent mean values for both of the 12 month observation periods. Spearman's ρ.

5.3 Cholecalciferol supplementation and serum corrected calcium

Serum corrected calcium data pre, and post, cholecalciferol supplementation were compared as a whole group, grouped according to mean corrected calcium levels pre cholecalciferol supplementation (T-12 to T-1), and finally, stratified to exclude those who did not adequately replete. For the grouped analysis, calcium based groupings were decided based on the renal association targets for end stage renal disease patients at the time the research was conducted (normal reference range for the local laboratory, at UHCW this was 2.08-2.58mmol/L) (Steddon & Sharples, 2015). The calcium groups were, low (<2.08mmol/L), on target (2.08-2.58mmol/L), and high (>2.58mmol/L).

Prior to vitamin D supplementation (T-12 to T-1) the whole cohort mean corrected calcium was 2.29±0.13mmol/L; following the introduction of vitamin D supplementation (T4 to T15) this increased to 2.35±0.13mmol/L (P <0.001). A statistically significant increase in corrected calcium was also seen in the low and on target calcium groups: 1.99±0.06mmol/L vs. 2.19±0.83mmol/L (P = 0.012), and 2.30±0.11mmol/L vs. 2.35±0.13mmol/L (P <0.001), respectively. However, a non-statistically significant reduction in corrected calcium was seen in patients with a high corrected calcium pre cholecalciferol supplementation, 2.67±0.07mmol/L vs. 2.44±0.19mmol/L (P = 0.068) (Table 5.4A), the lack of significance may reflect that there were only 4 patients in this group. The data indicate that cholecalciferol supplementation results in an increase to corrected calcium in patients with a low, or on target, corrected calcium at baseline. However, if the corrected calcium at baseline is already high then cholecalciferol does not further increase serum corrected calcium and may reduce it. Whilst overall a statistically significant increase in mean serum corrected calcium was seen, the mean value remained well within the target range at 2.35mmol/L (target 2.08-2.58mmol/L). When data was stratified for a mean serum 25(OH)D ≥75nmol/L the results reflected whole cohort grouped analysis (Table 5.4B).

(A)

Calcium Grouping (mmol/L)	No. of results	T -12 to -1	T4 – T15	% Change	Wilcoxon signed rank test
All patients	290	2.29 ± 0.13	2.35 ± 0.13	+2.6%	P <0.001
<2.08	9	1.99 ± 0.06	2.19 ± 0.83	+10.1%	P = 0.012
2.08-2.58	277	2.30 ± 0.11	2.35 ± 0.13	+2.2%	P <0.001
>2.58	4	2.67 ± 0.07	2.44 ± 0.18	-8.6%	P = 0.068

(B)

Calcium Grouping for patients that achieved repletion (mmol/L)	No. of results	T -12 to -1	T4 – T15	%Change	Wilcoxon signed rank test
All patients	238	2.29 ± 0.13	2.35 ± 0.13	+2.6%	P <0.001
<2.08	8	2.00 ± 0.06	2.20 ± 0.87	+10.0%	P = 0.012
2.08-2.58	227	2.30 ± 0.12	2.35 ± 0.13	+2.2%	P <0.001
>2.58	3	2.70 ± 0.03	2.42 ± 0.21	-10.4%	P = 0.109

Table 5.4 Comparison of mean serum corrected calcium in the 12 months pre, and post, cholecalciferol supplementation in haemodialysis patients.
Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase assumed. Serum corrected calcium was tested monthly and the change in mean serum corrected calcium between the two 12-month observation periods was compared for the whole cohort **(A)**, and data were stratified to exclude patients with serum 25(OH)D ≥75nmol/L at baseline and those that did not achieve repletion **(B)**. Data stratification did not affect results; a statistically significant increase in corrected calcium was seen overall, and in the low and on target calcium groups. A non-significant reduction in corrected calcium was seen in patients with a high serum corrected calcium pre cholecalciferol supplementation. Data are presented as mean±SD.

5.3.1 The correlation between serum corrected calcium and 25(OH)D

The relationship between calcium and 25(OH)D, with regards to safety of cholecalciferol supplementation, has already been discussed in 4.4.1. This current section investigates the relationship between the parameters depending on serum 25(OH)D status. There was no correlation between serum corrected calcium and

serum 25(OH)D in the presence of 25(OH)D deficiency or repletion (Figure 5.4 - A and B).

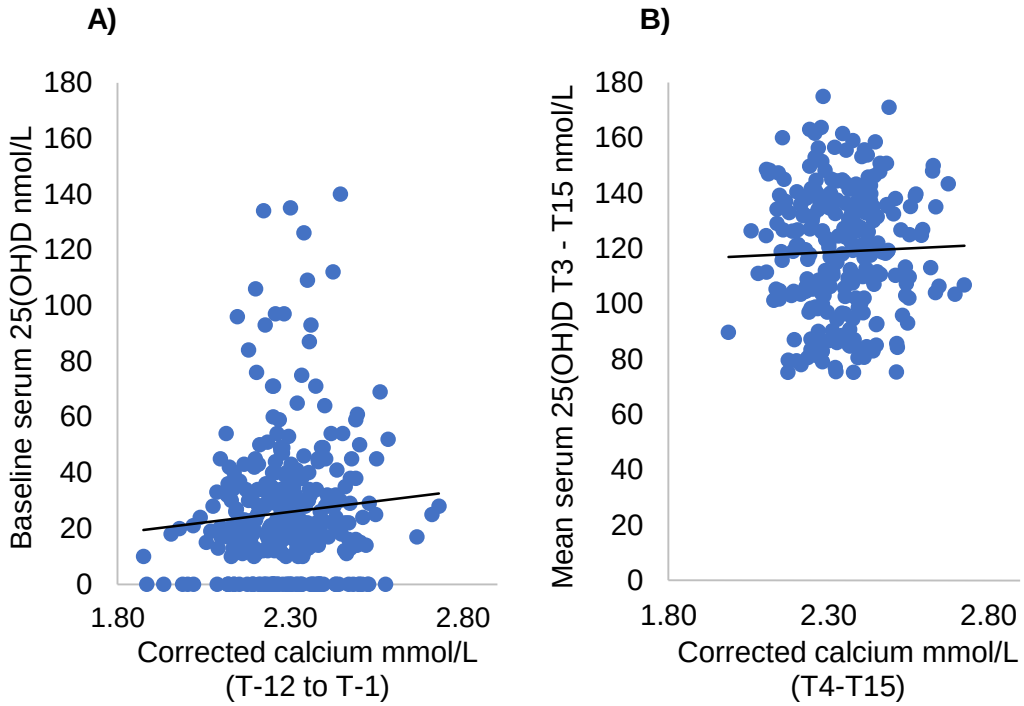

Figure 5.4 Relationship between baseline serum 25(OH)D and mean serum corrected calcium pre, and post, 25(OH)D repletion, in haemodialysis patients. Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum corrected calcium levels were tested monthly, and 25(OH)D every 3 months, as part of standard routine care. There was no correlation between serum corrected calcium and serum 25(OH)D pre **(A)**, or post **(B)**, 25(OH)D repletion, ρ = 0.061, P = 0.284, n = 315, and ρ = 0.039, P = 0.545, n = 245, respectively. Patients with insufficient data, and those with serum 25(OH)D ≥75nmol/L at baseline, and anyone that did not achieve repletion were excluded from analysis. Data represent mean values for both of the 12 month observation periods, and single, one off, measures for baseline 25(OH)D. Spearman's ρ.

5.3.2 The correlation between serum corrected calcium and serum phosphate

The data was further explored to investigate whether the relationships between calcium and the other bone miner markers was affected by serum 25(OH)D status. Correlations between serum corrected calcium and phosphate were carried out pre and post cholecalciferol supplementation. A weak correlation was seen between corrected calcium and phosphate pre 25(OH)D repletion, ρ = 0.119, P = 0.037, n = 308 (Figure 5.5A) Post repletion there was no correlation between phosphate and calcium, ρ = 0.084, P = 0.192, n = 245 (Figure 5.5B)

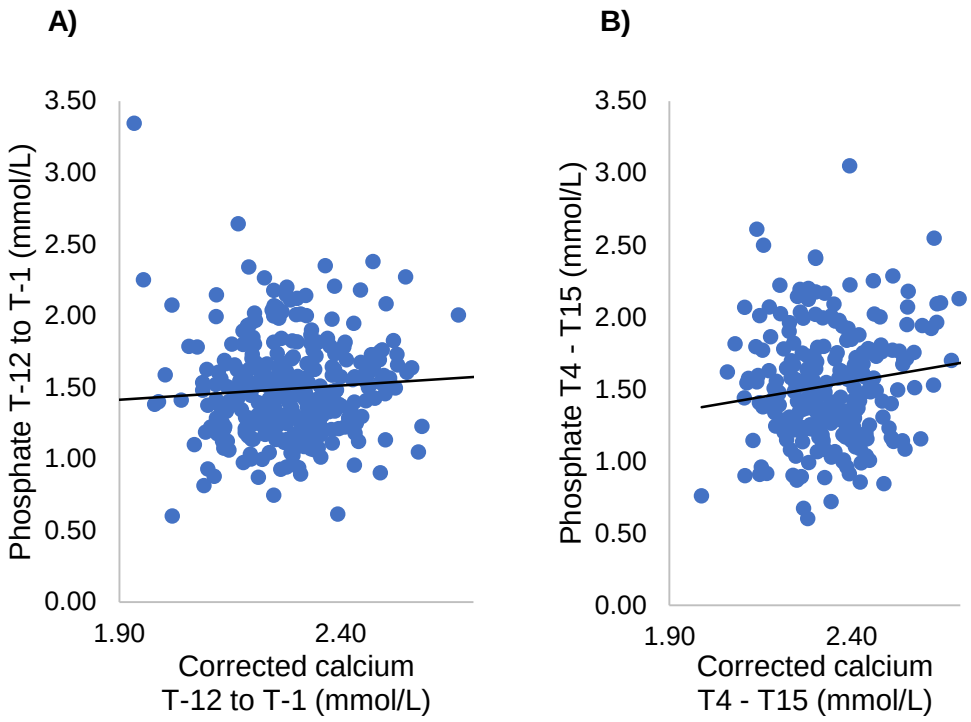

Figure 5.5 Relationship between mean serum corrected calcium and mean serum phosphate pre, and post, 25(OH)D repletion, in haemodialysis patients. Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum phosphate and corrected calcium were measured monthly, as part of standard routine care. **(A)** There was there was a weak correlation between serum calcium and phosphate pre repletion, ρ = 0.119, P = 0.037, n = 308. **(B)** Post repletion there was no correlation between phosphate and calcium, ρ = 0.084, P = 0.192, n = 245. Patients with insufficient data, and those with serum 25(OH)D ≥75nmol/L at baseline, and anyone that did not achieve repletion were excluded from analysis. Data represent mean values for both of the 12 month observation periods. Spearman's ρ.

5.3.3 The correlation between serum calcium and serum PTH

Increases in calcium inhibit PTH synthesis and secretion (Hendy *et al.*, 2013). Therefore, the relationship between the two was investigated pre and post cholecalciferol supplementation. A weak inverse correlation was seen between calcium and PTH pre and post 25(OH)D repletion, ρ -0.140, P = 0.015, n = 302 and ρ -0.113, P = 0.077, n = 245, respectively (Figure 5.6 – A and B).

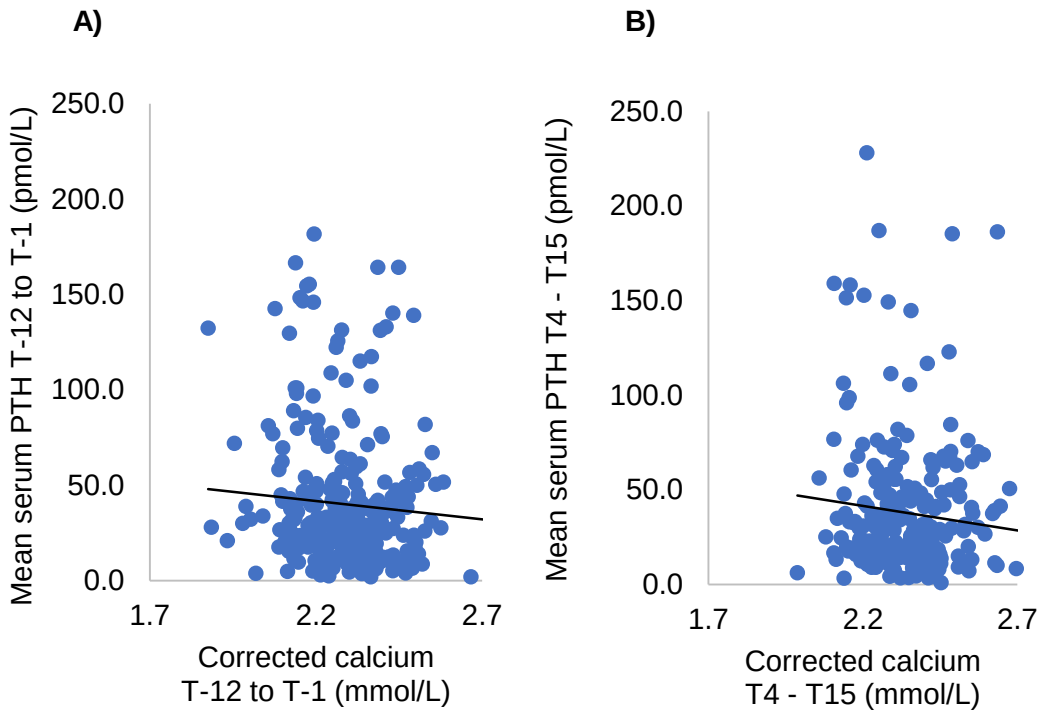

Figure 5.6 Relationship between mean serum corrected calcium and PTH pre, and post, 25(OH)D repletion, in haemodialysis patients.
Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Serum corrected calcium levels were tested monthly, and PTH every 3 months, as part of standard routine care. A weak inverse correlation between corrected calcium and PTH was seen both when serum 25(OH)D levels were low, ρ = -0.140, P = 0.015, n = 302 **(A)** and post 25(OH)D repletion, ρ = -0.113, P = 0.077, n = 245 **(B)**. Patients with insufficient data, and those with serum 25(OH)D ≥75nmol/L at baseline, and anyone that did not achieve repletion were excluded from analysis. Data represent mean values for both of the 12 month observation periods. Spearman's ρ.

5.4 Discussion

Due to the homeostatic mechanisms involved in calcium and phosphate management (section 1.6.1); serum calcium, phosphate and PTH response (to 25(OH)D repletion and subsequent 1,25$(OH)_2$D repletion) is expected to differ according to their concentration at baseline. For example, if serum calcium is low, the PTHR will be activated to trigger increased bone resorption and intestinal absorption of calcium; therefore, a statistically significant increase in serum calcium concentration, in response to 25(OH)D repletion, would be expected. As such, whole cohort analysis is confounded by the varying baseline concentrations, and varying response. In this study the data are stratified into groups based on pre-supplementation levels of calcium, phosphate and PTH, to facilitate investigation into the response to serum 25(OH)D repletion.

Cholecalciferol supplementation increased corrected calcium and phosphate, and reduced PTH in the whole cohort analysis, although the PTH reduction did not reach significance. Once patients were grouped according to baseline levels it became apparent that the response to cholecalciferol, for all 3 parameters, varied depending on pre supplementation levels (of serum calcium, phosphate and PTH). Corrected calcium, phosphate and PTH significantly reduced in patients with the highest levels of each parameter pre supplementation. Therefore, despite corrected calcium and phosphate increasing overall, if levels were already high, they did not increase further; instead, they reduced. This further supports the safety of cholecalciferol discussed in section 4.4. Results demonstrated a 26% reduction in PTH following 25(OH)D repletion in those with severe secondary hyperparathyroidism (PTH >85pmol/L). Interestingly, a statistically significant increase in PTH occurred in those with an over suppressed PTH concentration at baseline; suggesting cholecalciferol does not cause the same risk of over suppression and adynamic bone disease, as active vitamin D analogue use (Brandenburg & Floege, 2008; Goodman *et al.*, 1994).

The grouped analysis (based on baseline concentration) has not been carried out by others and therefore a comparison with existing evidence cannot be made. The effect of cholecalciferol supplementation on PTH in studies published to date has varied, from no significant change (Marckmann *et al.,* 2012; Armas *et al.,* 2012; Wasse *et al.,* 2012; Obi *et al.,* 2020), to a statistically significant reduction (Mose *et*

al., 2014; Tokmak *et al.,* 2008; Saab *et al.,* 2007; Matias *et al.,* 2010; Jean *et al.,* 2009).

Other studies have not seen a statistically significant change in serum calcium (Mose *et al.,* 2014; Delanaye *et al.,* 2013; Seibert *et al.,* 2012; Marckmann *et al.,* 2012; Armas *et al.,* 2012; Wasse *et al.,* 2012; Obi *et al.,* 2020) or serum phosphate (Seibert *et al.,* 2012; Jean *et al.,* 2009; Obi *et al.,* 2020).

Given the uncontrolled nature of the study, and no exclusion criteria, it is difficult to draw solid conclusions in terms of absolute benefits. The confirmation of safety is consistent, and thus the possibility of benefit to CKD-MBD markers, with no risk, supports the use of routine cholecalciferol in HD patients. Cholecalciferol supplementation improves PTH concentration further than sole active analogue use, with less risk of hypercalcaemia. The effects on CKD-MBD, together with appraisal of existing evidence are discussed further in Chapter 9.

Chapter 6 The impact of serum 25(OH)D on anaemia

The management of renal anaemia is challenging; partly due to varying individual response to EPO, and also because there are risks associated with high EPO doses (Danielson & Y. Beguin, 1995; Kalantar-Zadeh *et al.*, 2003; Tong & Nissenson, 2001). Therapies that improve efficacy of EPO are therefore warranted. Low levels of serum 25(OH)D have been linked with increased EPO requirements suggesting that vitamin D supplementation may improve the EPO response (Kiss *et al.*, 2011; Srisakul *et al.*, 2011; Stenvinkel, 2001). To investigate this, this chapter explores the relationship between serum 25(OH)D and markers of anaemia.

Serum ferritin and haemoglobin levels were measured monthly, and monthly EPO usage recorded, as part of standard care in all subjects (n = 350). For all patients receiving cholecalciferol, repletion data; (serum 25(OH)D levels), along with routine dialysis data (ferritin, Hb, and EPO dose), was collected monthly for 12 months prior to, and 15 months post, introduction of cholecalciferol supplementation. Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase was assumed. Any patients that did not have adequate data to provide a mean for both 12-month time points (at least 2 results from both time periods) were excluded. Data was analysed both as a population whole and stratified to exclude patients that did not adequately replete serum 25(OH)D. Serum hepcidin was measured in the sub-study one subjects once at baseline and once at 12 months (n = 81). Data analysis was carried out using a related samples Wilcoxon signed rank test to investigate change between the two 12 month observation periods. Spearman's rank correlation coefficient was applied to test for correlation between anaemia markers and serum 25(OH)D. All patients with serum 25(OH)D ≥75nmol/L at baseline, and those that did not adequately replete (repletion = serum 25(OH)D ≥75nmol/L) following cholecalciferol supplementation were excluded from the correlation analysis.

The number of patients included in the final analysis for each parameter is shown in Table 6.1.

Parameter	Number included in analysis (all patients)	Number included in analysis (patients that achieved 25(OH)D ≥75nmol/L)
Ferritin	293	244
Haemoglobin	294	244
Erythropoietin dose	264	220

Table 6.1 The number of patients included in the analysis of each anaemia marker.
EPO dose was only included where the patients' haemoglobin (Hb) was within target range (100-120g/L) that month. Any patients that did not have adequate data in order to provide a mean at both the pre and post 12-month observation periods (T-12 to T-1 and T4-T15) (at least 2 results from both time periods) were excluded.

6.1 The effect of cholecalciferol supplementation on serum ferritin

Whole group analysis, and grouped analysis based on mean ferritin levels (T-12 to T-1) as follows, low (<200µg/L), on target (200-499µg/L), and above target (>499µg/L), was carried out. The ferritin cut offs for the groups were based on local (UHCW) targets for HD patients which are informed by national and international guidelines (KDIGO, 2012; NICE, 2015). UHCW HD patients are given intravenous (IV) iron, as needed, to achieve the target ferritin. Administering IV iron to patients with a ferritin >500µg/L is considered to potentially increase the risk of iron toxicity without inferring benefit (KDIGO, 2012; NICE, 2015). Ferritin is an inflammatory marker; levels >500 µg/L are associated with increased inflammation in HD patients (Rambod *et al.*, 2008).

The whole group analysis found no difference between mean ferritin pre vs. post cholecalciferol supplementation. Prior to vitamin D supplementation (T-12 to T-1) mean ferritin was 380.7±148.8µg/L and following treatment with routine vitamin D supplementation (T4 to T15) mean ferritin was 399.2±156.2µg/L, P = 0.352. A statistically significant increase in mean ferritin was seen in patients that had low or on target ferritin concentrations pre cholecalciferol supplementation, 148.2±50.4 vs. 240.1±148.9µg/L (P = 0.003), and 359.0±81.1 vs. 396.3±140.9µg/L (P =0.004), respectively. A statistically significant decrease in ferritin occurred in those patients with a high ferritin pre cholecalciferol supplementation, 632.2±123.1 vs. 496.8±150.4µg/L, P <0.001 (Table 6.2A).

Data was stratified to exclude patients with serum ≥75nmol/L at baseline, as well as those that did not achieve repletion, and the same analysis repeated. Results replicated those seen in the whole cohort. Whole group analysis found no difference between mean ferritin, pre and post, cholecalciferol supplementation. T-12 to T-1 mean ferritin was 384.0±142.1µg/L and following 25(OH)D repletion (T4 to T15), mean ferritin was 398.5±143.8µ/L, P = 0.313. Statistically significant differences were again found in the grouped analysis. A significant increase in mean ferritin was seen in patients that had low or on target ferritin pre cholecalciferol supplementation, 149.0±52.2 vs. 233.5±144.0µ/L (P = 0.017), and 364.2±79.0 vs. 395.3±122.6µ/L (P = 0.006), respectively. A statistically significant decrease in ferritin occurred in those patients with a high ferritin pre supplementation, 619.3±126.2 vs. 500.3±144.5mmol/L (Table 6.2B).

Data indicate that cholecalciferol supplementation may have a positive impact on serum ferritin levels, increasing low levels, and reducing levels that are too high.

A)

Ferritin Grouping (µ/L)	No. of patients	T -12 to -1	T4 – T15	%Change	Wilcoxon signed rank test
All patients	293	380.7 ± 148.8	399.2 ± 156.2	+4.9%	P = 0.352
<200	25	148.2 ± 50.4	240.1 ± 148.9	+62.0%	P = 0.003
200-499	222	359.0 ± 81.1	396.3 ± 140.9	+10.4%	P = 0.004
>499	46	632.3 ± 123.1	496.8 ± 150.4	-21.4%	P <0.001

B)

Ferritin Grouping for patients that achieved repletion (µ/L)	No. of results	T -12 to -1	T4 – T15	%Change	Wilcoxon signed rank test
All patients	244	384.0 ± 142.1	398.5 ± 143.8	+3.8%	P = 0.313
<200	20	149.0 ± 52.2	233.5 ± 144.0	+56.7%	P = 0.017
200-499	188	364.2 ± 79.0	395.3 ± 122.6	+8.5%	P = 0.006
>499	36	619.3 ± 126.2	500.3 ± 144.5	-19.2%	P <0.001

Table 6.2 Mean ferritin in pre (T-12 to T-1) and post (T4 toT15) introduction of routine cholecalciferol supplementation in haemodialysis patients.
Cholecalciferol supplementation was initiated at T0 and a 3-month repletion phase assumed. Serum ferritin was tested monthly and the change in mean serum ferritin between the two 12-month observation periods was compared for the whole cohort **(A)**, and data were stratified to exclude patients with serum 25(OH)D ≥75nmol/L at baseline and those that did not achieve repletion **(B).** There was no difference between mean ferritin, pre, and post, cholecalciferol supplementation. The grouped analysis found a statistically significant increase in ferritin for patients in the low or on target ferritin groups. A statistically significant decrease in ferritin occurred in patients with a high mean ferritin pre supplementation. Data are presented as mean±SD.

6.1.1 The correlation between serum ferritin and serum 25(OH)D

Relationships between ferritin and 25(OH)D were explored pre and post serum 25(OH)D repletion. At baseline, in the presence of low serum 25(OH)D, there was a weak, non-significant, correlation between ferritin and serum 25(OH)D, ρ = 0.110, P = 0.063, n = 288 (Figure 6.1A). This remained unchanged in the presence of serum 25(OH)D repletion, ρ = 0.110, P = 0.062, n = 288 (Figure 6.1B).

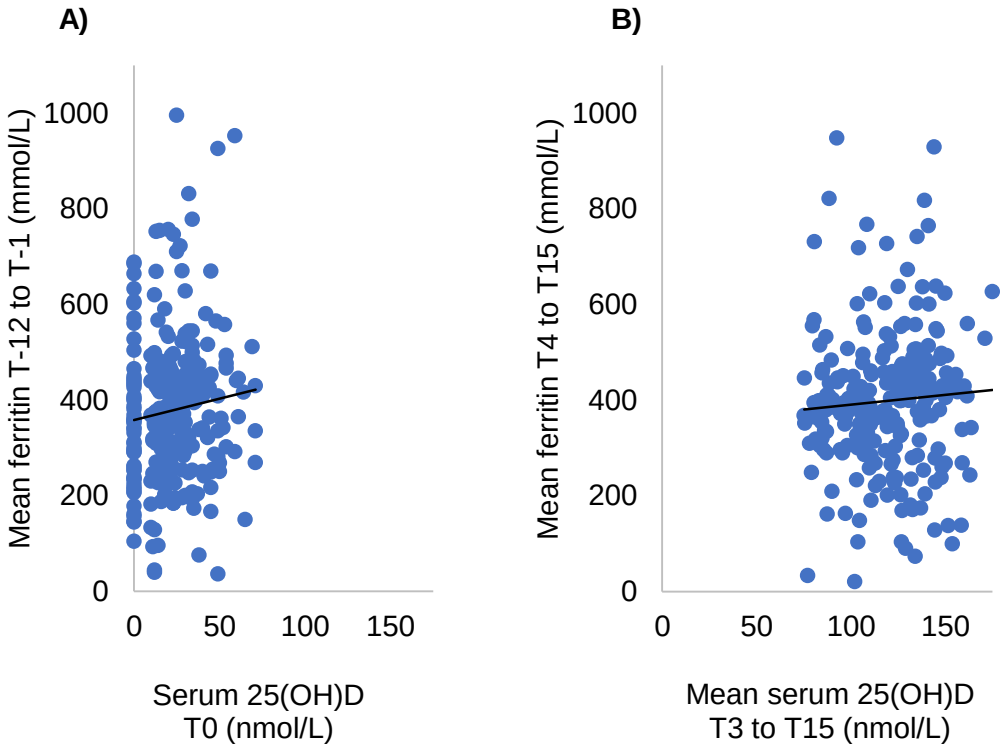

Figure 6.1 Relationship between mean serum ferritin and 25(OH)D pre, and post, serum 25(OH)D repletion, in haemodialysis patients.
There was a weak, non-significant, correlation between mean ferritin (T-12 to T-1) and serum 25(OH)D prior to **(A)**, and post **(B),** 25(OH)D repletion, ρ = 0.110, P = 0.062, n = 288 and ρ = 0.123, P = 0.052, n = 251, respectively. Patients with insufficient data, those with serum 25(OH)D ≥75nmol/L at baseline, and patients that did not adequately replete were excluded from analysis. Data represent mean values with the exception of baseline 25(OH)D. Spearman's ρ.

Baseline ferritin may affect the ferritin response to cholecalciferol supplementation; higher ferritin levels appear to reduce in response to cholecalciferol supplementation (Table 6.2 A and B). High ferritin (>500µg/L) is associated with increased inflammation in HD patients (Rambod *et al.*, 2008), and increased serum 25(OH)D has been linked with reduced inflammation (Alvarez *et al.*, 2013). With this in mind, data were stratified to explore the relationship between ferritin and serum 25(OH)D in only those patients that had a high mean ferritin T-12 to T-1 (>499µg/L); no correlation was seen pre repletion, ρ = -0.141, P = 0.346, n = 47, and moderate, non-significant, positive correlation seen post 25(OH)D repletion ρ = 0.57, P =0.735, n = 38 (Figure 6.2 A and B).

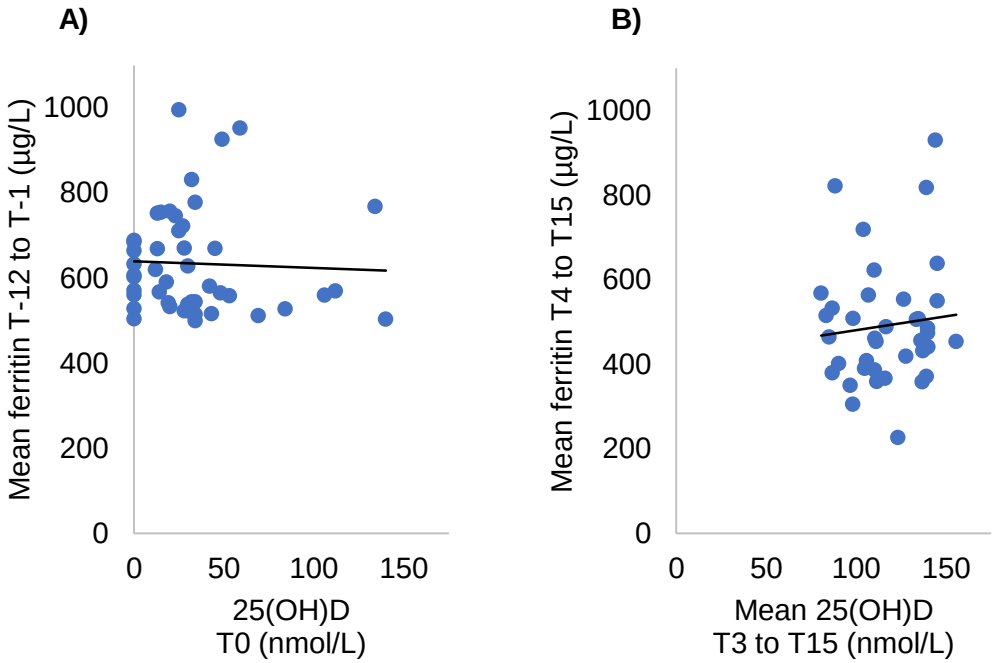

Figure 6.2 Relationship between baseline serum 25(OH)D and mean serum ferritin (T-12 to T-1) prior to, and post, 25(OH)D repletion in haemodialysis patients that had a high mean ferritin (>499mmol/L) (T-12 to T-1).
No correlation was seen pre repletion, ρ = -0.141, P = 0.346, n = 47 **(A)**, and moderate, non-significant, positive correlation seen post 25(OH)D repletion ρ = 0.57, P =0.735, n = 38 **(B).** Patients with insufficient data were excluded from analysis. Data represents mean patient values for ferritin and absolute values for 25(OH)D (T0) and mean values for both ferritin and 25(OH)D (T12). Spearman's ρ.

6.2 The effect of cholecalciferol supplementation on serum haemoglobin

Mean Hb levels pre (T-12 to T-1) and post (T4 to T15) cholecalciferol supplemetantion were compared for the whole cohort, and grouped according to mean Hb levels prior to baseline (T12 to T-1), low, (<100g/L), on target (100-120g/L), and high (>120g/L). The Hb cut offs for these groups were based on local (UHCW) targets for HD patients which are based on national and international renal guidelines (KDIGO, 2012; NICE, 2015).

Overall a statistically significant decrease in mean Hb was seen following routine cholecalciferol supplementation, 106.2±9.7 (T-12 to T-1) vs. 105.0±10.2g/L (T4 to T15), P = 0.004. The grouped analysis demonstrated a statistically significant decrease in the 'on-target' and 'high' Hb groups, 109.3±4.8 vs. 105.7±9.3g/L, P <0.001, and 125.3±4.7 vs. 119.5±12.2g/L, P = 0.023, respectively. The mean Hb in the 'low' Hb group increased from 92.4±6.5g/L (T-12 to T-1) to 99.6±9.7g/L (T4 to T15), P <0.001 (Table 6.3A).

The results did not change once the data was stratified to include only those that adequately repleted serum 25(OH)D; overall the mean Hb reduced, 107.1±9.1µg/L (T-12 to T-1) vs. 105.6±10.0µg/L (T4 toT15), P = 0.007. A decrease in mean Hb was also seen in the grouped analysis for the 'on target' and 'high' groups; 109.4±4.9 (T-12 to T-1) vs. 106.3±8.9µg/L (T4 to T15), P <0.001, and 125.3±4.7 (T-12 to T-1) vs. 119.5±12.2 (T4 to T15), P = .023, respectively. The mean Hb of the 'low' group increased from 93.5±5.5µg/L to 99.9±9.5g/L, P <0.001 (Table 6.3B).

A)

Haemoglobin Grouping (g/L)	No. of results	T -12 to -1	T4 – T15	% Change	Wilcoxon signed rank test
All patients	294	106.2 ± 9.7	105.0 ± 10.2	-1.1%	P = 0.004
<100 (low)	63	92.4 ± 6.5	99.6 ± 9.7	+7.8%	P < 0.001
100-120 (on target)	218	109.3 ± 4.8	105.7 ± 9.3	-3.3%	P < 0.001
>120 (high)	13	125.3 ± 4.7	119.5 ± 12.2	-4.6%	P = 0.023

B)

Haemoglobin Grouping for patients that achieved repletion (g/L)	No. of results	T -12 to -1	T4 – T15	%Change	Wilcoxon signed rank test
All patients	244	107.1 ± 9.1	105.6 ± 10.0	-1.4%	P = 0.007
<100 (low)	49	93.5 ± 5.5	99.9 ± 9.5	+6.8%	P < 0.001
100-120 (on target)	182	109.4 ± 4.9	106.3 ± 8.9	-2.8%	P < 0.001
>120 (high)	13	125.3 ± 4.7	119.5 ± 12.2	-4.6%	P = 0.023

Table 6.3 Mean Hb in the 12 months pre (T-12 to T-1) and post (T4 toT15) introduction of cholecalciferol supplementation.
Hb was measured monthly and the change in mean between the two 12-month observation periods compared. Data represent the whole cohort **(A)** and exclusion of patients that did not adequately replete, or already had a serum 25(OH)D ≥75nmol/L at baseline. A decrease in mean Hb was seen following cholecalciferol supplementation **(A).** The grouped analysis revealed an increase in mean Hb in the 'low' Hb group, and a decrease in mean Hb in the 'on target' and 'high' Hb groups **(A).** Results remained unchanged once stratified **(B).** Data are presented as mean±SD.

Overall, despite the decrease in Hb concentration seen, the whole cohort mean Hb stayed within target (100-120g/L). The grouped analysis saw the 'low' Hb groups' mean Hb increase to be almost within target and the 'high' Hb groups' mean Hb decreased to a concentration that was within the target level.

6.2.1 The correlation between serum haemoglobin and serum 25(OH)D

Relationships between Hb and 25(OH)D were explored pre and post serum 25(OH)D repletion. At baseline, in the presence of low serum 25(OH)D, there was a weak, yet statistically significant, positive correlation between Hb and serum 25(OH)D, ρ = 0.148, P = .012, n = 288 (Figure 6.3A). This remained the same post introduction of cholecalciferol supplementation, once vitamin D repletion had been achieved, ρ = 0.134, P = 0.034, n = 251 (Figure 6.3B).

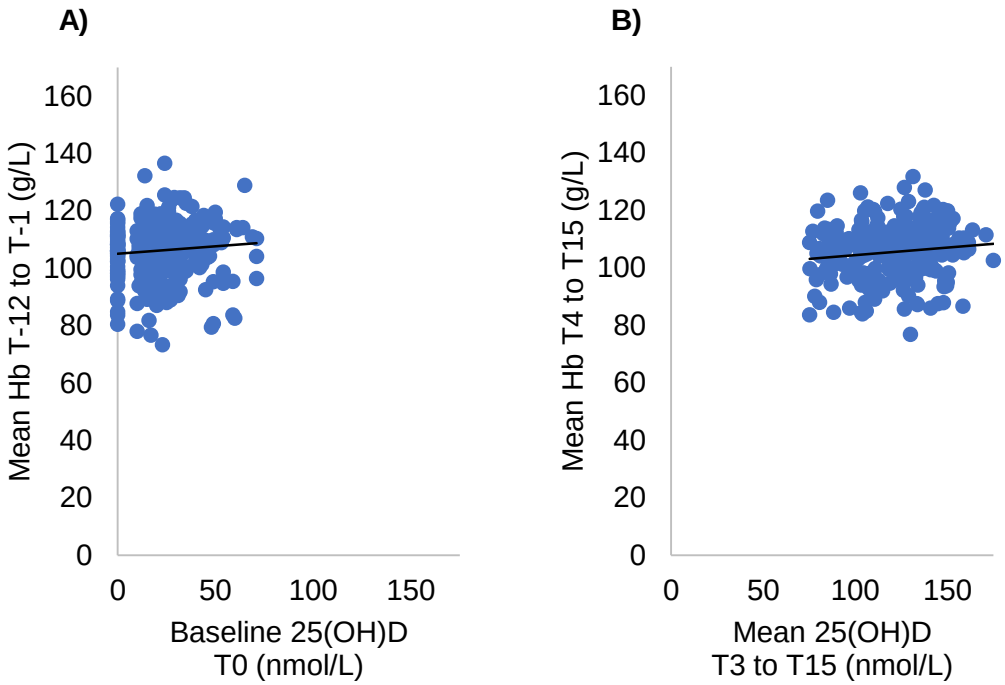

Figure 6.3 Relationship between Hb and 25(OH)D prior to, and post supplementation with cholecalciferol in haemodialysis patients.
(A) A weak, yet statistically significant, positive relationship between Hb and 25(OH)D is seen in patients with low serum 25(OH)D (<75nmol/l), ρ = 0.148, P = 0.012, n = 288. **(B)** Results were replicated following 25(OH)D repletion, ρ = 0.134, P = 0.034, n 251. Patients with insufficient data, those with serum 25(OH)D ≥75nmol/L at T0 and those that did not adequately replete were excluded from analysis. Spearman's ρ.

6.3 The effect of cholecalciferol supplementation on erythropoietin use

EPO dosage data was analysed for all patients at all monthly timepoints where their serum Hb concentration fell within the UHCW target, 100-120g/L; if the Hb was not within target the EPO dose that month was excluded from analysis (n=264). This

was to minimise the influence of EPO dose adjustments that occurred in relation to sudden changes in Hb. Mean total monthly EPO (µg) received during the 12 months prior to the introduction of vitamin D supplementation (T-12 to T-1) was compared to mean total monthly EPO (µg) received during the 12 months post vitamin D repletion (T4 to T15). Data showed that the total EPO use fell following vitamin D supplementation. Prior to cholecalciferol supplementation (T-12 to T-1) the mean EPO dose was 141.30±127.16µg (median 112.1, IQR 54.8-181.3), this reduced by 1.4% to 139.34±139.58µg (median 95.0, IQR 48.0-175.0) post cholecalciferol supplementation, P = 0.0258 (Figure 6.4).

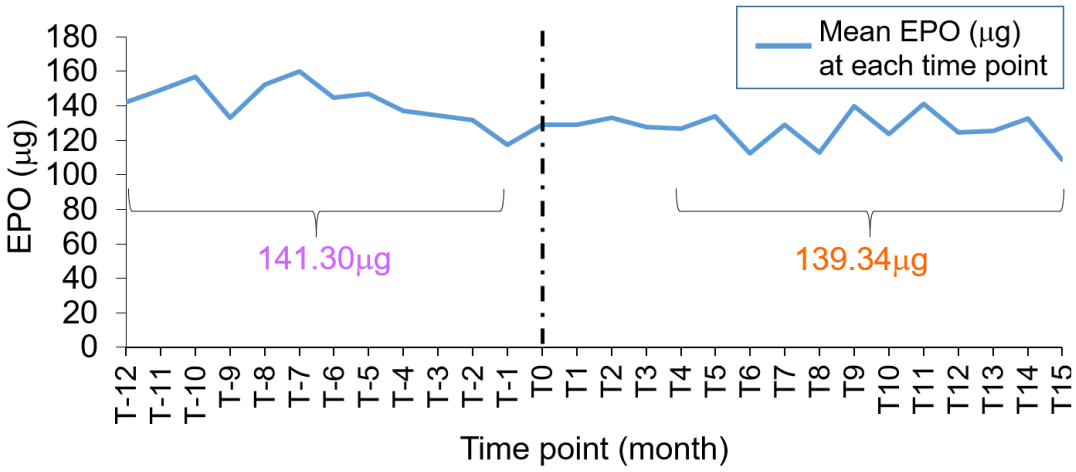

Figure 6.4 Mean monthly EPO dose across the retrospective (pre cholecalciferol supplementation) and prospective (post cholecalciferol supplementation) study periods.
Cholecalciferol supplementation was initiated at T0 and a 3 month repletion phase assumed. Two 12 month observation periods, pre (T-12 to T-1) and post (T4 to T15) introduction of cholecalciferol supplementation were compared to test whether cholecalciferol had an impact on EPO usage, 141.30±127.16µg T-12 to T-1 vs. 139.34±139.58µg T4 to T15, P = 0.0258, n = 264 (related-samples Wilcoxon signed rank test).

Data was stratified to exclude patients that did not adequately replete serum 25(OH)D, and the same analysis repeated. The outcome remained unchanged; EPO use significantly reduced following 25(OH)D repletion. In the presence of low serum 25(OH)D (T-12 to T-1) mean EPO dose was 141.4±129.4µg (median 112.2, IQR 54.3-180.0) and this reduced by 3.3% to 136.7±137.1µg (median 92.2, IQR 48.0-172.8) T4 to T15, P = 0.036, n = 220 (Figure 6.5).

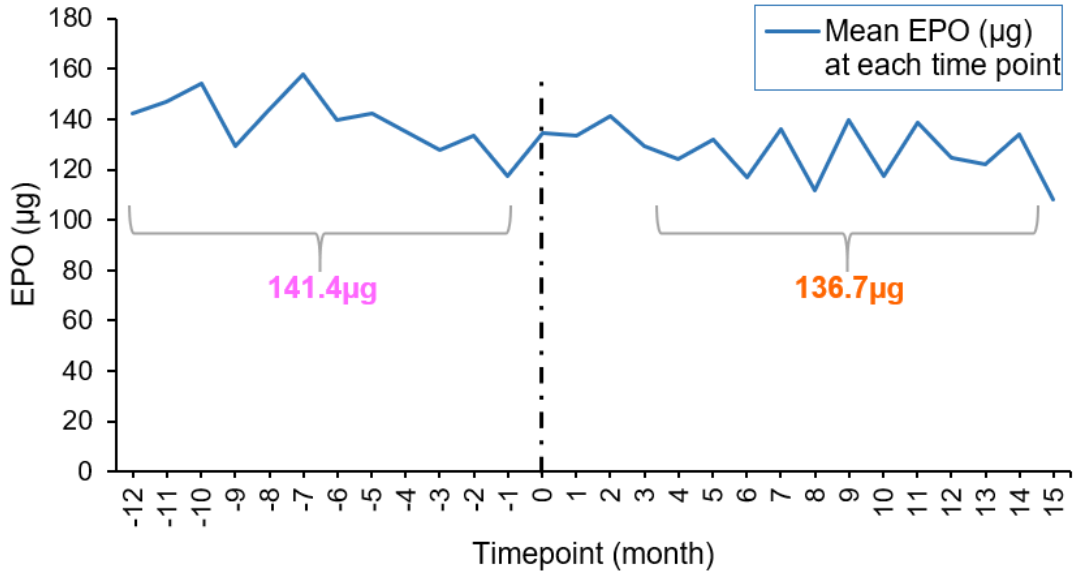

Figure 6.5 Mean monthly EPO dose pre, and post, serum 25(OH)D repletion. Cholecalciferol supplementation was initiated at T0 and a 3 month repletion phase assumed. Two 12 month observation periods, pre (T-12 to T-1) and post (T4 to T15) introduction of cholecalciferol supplementation were compared to test whether cholecalciferol had an impact on EPO usage, 141.4±129.4µg T-12 to T-1 vs. 136.7±137.1µg T4 to T15, P = 0.036, n = 220. Data excluded patients with serum 25(OH)D ≥75nmol/L at T0, and those that did not adequately replete. Wilcoxon signed rank test.

6.3.1 Correlation between EPO and serum 25(OH)D

Relationships between EPO usage and 25(OH)D were explored pre and post serum 25(OH)D repletion. At baseline, in the presence of low serum 25(OH)D, there was no correlation between mean EPO dose and serum 25(OH)D, ρ = -0.181, P = 0.162, n = 298 (Figure 6.6A). This remained unchanged post vitamin D repletion, ρ = -0.008, P = 0.895, n = 262 (Figure 6.6B).

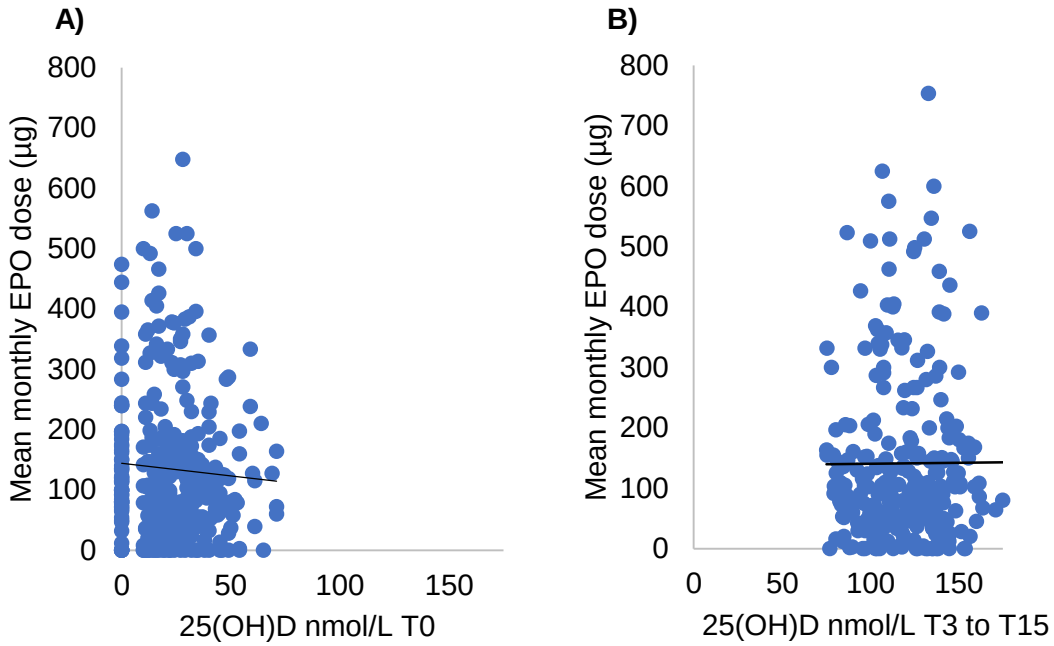

Figure 6.6 Relationship between mean monthly EPO dose and 25(OH)D pre, and post, 25(OH)D repletion, in haemodialysis patients.
There was no correlation between EPO dose and serum 25(OH)D in the presence of 25(OH)D deficiency **(A)**, or post repletion **(B)**, ρ = -0.081, P = 0.162, n = 298 and ρ = -0.008, P = 0.895, respectively. Patients with insufficient data, those with serum 25(OH)D ≥75nmol/L at baseline, and those that did not achieve repletion, were excluded from analysis. n = 262. Spearman's ρ.

6.4 The effect of cholecalciferol supplementation on serum hepcidin

Serum hepcidin levels were measured in the sub-study one subjects; complete serum hepcidin data, baseline (T0) and 12 months (T12), was available for 54 participants.

Whole group analysis, and grouped analysis based on baseline hepcidin levels (T0) as follows: lowest quartile (<67.5ng/mL), interquartile range (67.5-146ng/mL), and highest quartile (>146ng/mL), was carried out to investigate whether baseline levels affected response. Analysis of the 54 subjects was carried out in the first instance, then data was stratified to exclude patients with serum ≥75nmol/L at baseline, as well as those that did not achieve repletion, and the same analysis repeated (n = 49).

Prior to cholecalciferol supplementation (T0), mean hepcidin level was 110.7±71.0ng/mL and following cholecalciferol supplementation (T12) it was 109.8±61.0, P = 0.727, n = 54 (Table 6.4A). Stratification based on serum 25(OH)D

did not affect this outcome (Table 6.4B). The grouped analysis found a different response according to baseline hepcidin. Hepcidin concentration significantly increased from 44.4±12.3 to 73.2±40.9ng/mL in those with the lowest levels pre supplementation, P = 0.048; an increase was also seen once stratified according to 25(OH)D repletion; however, the significance was lost.

Patients with the highest baseline hepcidin saw a large but non-significant reduction in their serum hepcidin levels from 205.4±70.3 to 138.6±77.3 following supplementation with cholecalciferol; this reduction was also seen when stratified according to 25(OH)D repletion (Table 6.4 A and B). The lack of significance may reflect study numbers in the groups (n = 13).

(A)

Hepcidin (ng/mL)	No. of results	T0 (ng/mL)	T12 (ng/mL)	% Change	Wilcoxon signed rank test
All patients	54	110.7±71.0	109.8±61.0	-0.8%	P = 0.727
<67.5ng/mL	14	44.4±12.3	73.2±40.9	+64.9%	P = 0.048
67.5 – 146ng/mL	27	96.0±22.4	114.9±53.0	+19.7%	P = 0.159
>146ng/mL	13	205.4±70.3	138.6±77.3	-32.5%	P = 0.16

(B)

Hepcidin (ng/mL)	No. of results	T0 (ng/mL)	T12 (ng/mL)	% Change	Wilcoxon signed rank test
All patients	49	110.5±72.1	106.6±58.6	-3.5%	P = 0.886
<67.5ng/mL	13	45.4±12.2	71.9±42.3	+58.4%	P = 0.087
67.5 – 146ng/mL	23	94.3±21.5	107.5±43.2	+13.9%	P = 0.287
>146ng/mL	13	205.4±73.2	140.2±76.5	-31.7%	P = 0.16

Table 6.4 Baseline and 12 month serum hepcidin levels.
Hepcidin levels were measured in the sub-study one population at baseline (T0) and 12 months after the introduction of routine cholecalciferol supplementation (T12). The whole group analysis found no difference between hepcidin pre and post cholecalciferol supplementation (n = 54) **(A)**. This remained unchanged once data were stratified to exclude patients with serum 25(OH)D ≥75nmol/L at baseline and those that did not achieve repletion (n = 49) **(B)**. An increase in hepcidin concentration occurred for patients within the lowest quartile at baseline. No

difference was seen in patients with baseline hepcidin levels within the interquartile range, but a statistically significant reduction occurred for those with the highest hepcidin concentration. Mean±SD.

6.4.1 Correlation between serum hepcidin and serum 25(OH)D

Relationships between hepcidin and 25(OH)D were explored pre and post 25(OH)D repletion. At baseline (T0), in the presence of low serum 25(OH)D in the majority, there was no correlation between serum hepcidin and 25(OH)D, ρ = -0.13, P = 0.964, n = 49 (Figure 6.7A). This remained the unchanged 12 months post introduction of cholecalciferol supplementation (T12), ρ = -0.168, P = 0.255, n = 49 (Figure 6.7B).

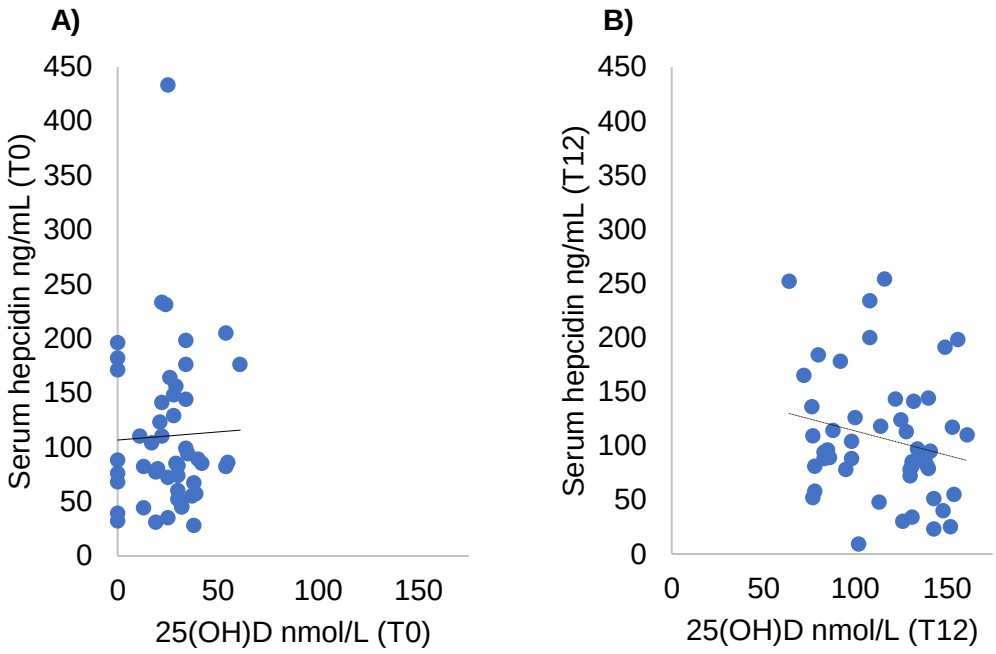

Figure 6.7 Relationship between serum hepcidin and serum 25(OH)D at baseline and 12 months.
Serum hepcidin and 25(OH)D were measured in a small cohort of haemodialysis patients that were recruited into the sub-study one. At baseline, in the presence of low serum 25(OH)D there was no correlation between serum hepcidin and 25(OH)D, ρ = -0.13, P = 0.964, n = 49 **(A)**. This remained unchanged once repletion was achieved, ρ = -0.168, P = 0.255, n = 49 **(B)**. Spearman's ρ.

6.4.2 The correlation between serum hepcidin and serum ferritin

Relationships between serum hepcidin and ferritin were explored pre and post cholecalciferol supplementation. At baseline (T0), in the presence of low serum 25(OH)D, there was a positive correlation between serum hepcidin and ferritin, ρ = 0.403, P = 0.003, n = 49 (Figure 6.8A). This strength of this correlation increased at

12 months (T12), once vitamin D repletion had been achieved, ρ = 0.588, P <0.001, n = 49 (Figure 6.8B).

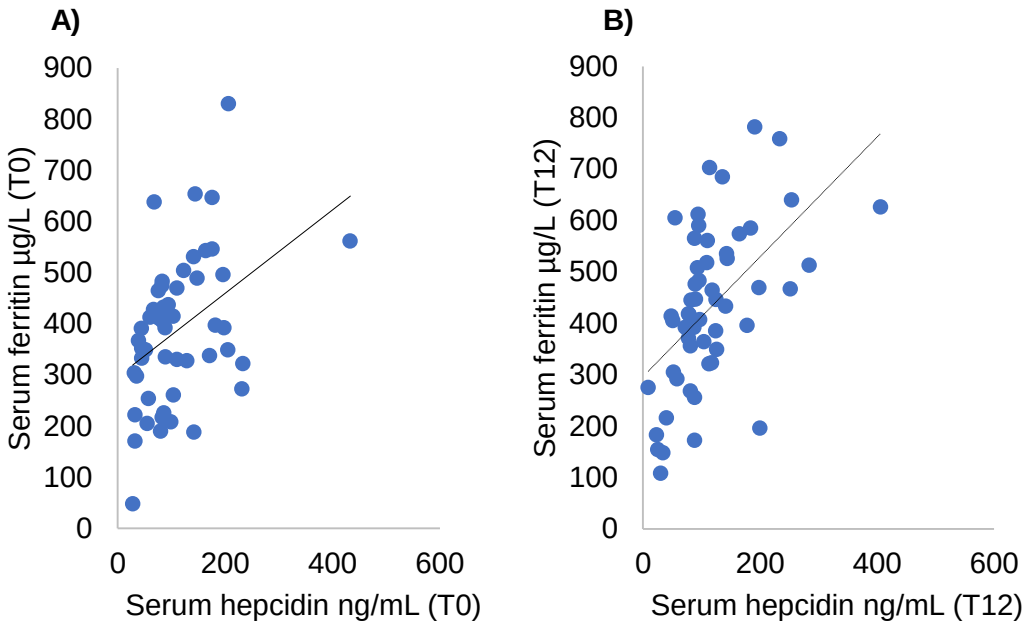

Figure 6.8 Relationship between serum hepcidin and serum ferritin pre (A), and 12 months post (B) 25(OH)D repletion
Serum hepcidin and ferritin were measured in a small cohort of haemodialysis patients that were recruited into the sub-study one. At baseline, in the presence of low 25(OH)D there was a positive correlation between serum hepcidin and ferritin, ρ = 0.403, P = 0.003, n = 49. This correlation increased following 25(OH)D repletion (T12), ρ = 0.588, P <0.001, n = 49. Spearman's ρ.

6.5 Discussion

A small but significant reduction in monthly EPO usage was seen. This is consistent with existing evidence which has linked increased 25(OH)D with reduced erythropoiesis stimulating agent (ESA) use (Saab *et al.,* 2007; Lac *et al.,* 2010; Kiss *et al.,*2011; Rianthavorn & Boonyapapong, 2013). In contrast, the findings of others, have shown no significant effect of improved 25(OH)D on the use of ESA (Matias *et al.,* 2010 and Obi *et al.,* 2020). Bhan and colleagues (2015) found ergocalciferol supplementation has no effect on EPO use, Hb, or ferritin; yet they did see an increase total iron binding capacity, P <0.05. Similar results showing no change in Hb, ferritin, or ESA resistance index, were also found in a recent RCT (Obi *et al.,* 2020).

There was no change in hepcidin overall, however levels increased in those with the lowest hepcidin levels at baseline, and reduced in those with the highest, implying the response is related to baseline hepcidin concentration. Despite seeing a 32.5% reduction (in the group with highest baseline concentration of hepcidin) the difference was not statistically significant; this may reflect the small study numbers once data were grouped (n = 13).

Other studies investigating the effect of cholecalciferol, or ergocalciferol, on hepcidin have resulted in differing outcomes; some have shown a reduction in hepcidin with improved 25(OH)D in healthy subjects (Bacchetta *et al.*, 2014; Smith *et al.*, 2017), and others have reported an increase in hepcidin in HD patients given cholecalciferol (Obi *et al.*, 2020). EPO use is considered to effect serum hepcidin concentration (Shoji *et al.*, 2013), it maybe this, or perhaps unrelated pathophysiological consequences of ESRD, which alter the hepcidin response to cholecalciferol. Additionally, dialysis is an inflammatory inducing process and high levels of inflammation in the patient cohort may mask response.

The complex nature of renal anaemia, and confounding variables, make it difficult to decipher the exact impact of improved serum 25(OH)D. This, in addition to the lack of control arm mean the findings, albeit thought provoking, are not clinical meaningful. The Ph.D findings, along with results from existing studies, are discussed further in Chapter 9.

Anaemia is associated with reduced health-related quality of life (HRQOL) in CKD patients (van Haalen *et al.*, 2020); the following chapter investigates the effect of cholecalciferol supplementation and improved serum 25(OH)D on HRQOL measures.

Chapter 7 The patient impact factor: health-related quality of life

Health-related quality of life (HRQOL) is used to measure the impact of an illness on a patient's physical, physiological, and psychological well-being (Ebrahim, 1995). Improved serum 25(OH)D levels may positively impact on HRQOL by reducing inflammation and improving anaemia (Kiss *et al.*, 2011; Lac *et al.*, 2010; Srisakul *et al.*, 2011; Stenvinkel, 2001). Vitamin D supplementation may improve HRQOL by improving muscle strength and physical function (Anand *et al.*, 2011; Jones, 2011). This chapter reports HRQOL measures from the Short-Form Health Survey 36 (SF-36) and European Quality of Life (EQ-5D) which are both widely cited and validated for patients with ESRD (Gómez-Besteiro *et al.*, 2004; Johansen *et al.*, 2001; Merkus *et al.*, 1997; Terada & Hyde, 2002; Yang *et al.*, 2014). The SF-36 measures health status, but does not inform cost effectiveness analysis, whereas the EQ-5D can be used for health economics (EuroQol Group, 1990). Whilst health economics was beyond the scope of this Ph.D, it was hoped using 2 questionnaires would help validate study findings and provide data for future analysis.

SF-36 and EQ-5D

1. Short-Form Health Survey 36 (SF-36). A 36-item survey that measures eight domains of health: physical functioning, role limitations due to physical health, bodily pain, general health perceptions, vitality, social functioning, role limitations due to emotional problems, and mental health (SF, 2020; Ware Jr & Sherbourne, 1992)

2. The European Quality of Life (EQ-5D). A questionnaire that measures 5 domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression (EQ-5D, 2020).

All 350 haemodialysis patients were invited participate in the study into the effects of cholecalciferol supplementation on HRQOL; 141 were consented and completed questionnaires were received from 122 people at baseline (timepoint 1), 74 at month 4 (timepoint 2), 50 at month 8 (timepoint 3), and 54 at month 12 (timepoint 4). Cholecalciferol supplementation was commenced, based on serum 25(OH)D, at

baseline and continued as indicated by serum 25(OH)D levels (according to the UHCW guideline outlined in Table 4.6, section 4.3.1). Data was analysed both as a population whole and stratified to exclude patients that were already vitamin D replete at baseline, and/or did not adequately replete during the study follow-up. Shapiro-Wilk's normality test revealed the data are skewed; statistical analysis was therefore carried out using the Kruskal-Wallis test (the non-parametric equivalent of analysis of variance (ANOVA)) and data are reported as median and IQR.

7.1 Population characteristics

The demographics and characteristics of the patients who completed HRQOL questionnaires are summarised in Table 7.1. Most patients were male (61.6%), and of White British ethnicity (87.7%) which is reflective of the Coventry HD population as a whole (section 4.1).

Variables	HRQOL study Haemodialysis Patients (n=122)	UHCW Haemodialysis Patients (n=350)
Age, years, median (IQR)	70 (54-78)	69 (24-95)
Male, n (%)	75 (61.6)	212 (60.6)
Ethnicity, n (%)		
White	107 (87.7)	260 (74.3)
South Asian	9 (7.4)	55 (15.7)
Black	4 (3.3)	23 (6.6)
Other	2 (1.6)	12 (3.4)
Dialysis Vintage, years, median (IQR)	2.5 (1.1-4.8)	2.4 (0-17.5)
Diabetes n (%)		
Type 1	3 (2.5)	11 (3.1)
Type 2	20 (16.4)	91 (26.0)

Table 7.1 Characteristics of UHCW haemodialysis patients who completed the HRQOL questionnaires.
Patients recruited into sub-study one were representative of UHCW haemodialysis population for all characteristics except for ethnicity. Minority ethnic groups were under-represented, a recognised problem in research and something that requires addressing in future studies (Redwood & Gill, 2013)

7.2 Short-Form Health Survey 36 questionnaire results

Results from the SF-36 questionnaire demonstrate that mental component summary (MCS) scores, and physical component summary (PCS) scores were below norm (50.0) at baseline and throughout the study (Table 7.2). HRQOL was not affected by cholecalciferol supplementation; there was no statistically significant difference between MCS scores, PCS scores, or any of the independent physical, or mental, health components across the 12 month study period, P = 0.8398 and P = 0.7030, respectively (Table 7.2A). Results were not altered by stratification according to vitamin D repletion (Table 7.2B).

(A)

Timepoint (n)	Baseline (114)	4m (74)	8m (50)	12m (54)	Kruskal Wallis
PCS	33.1 (27.6-39.9)	31.6 (26.2-39.2)	32.8 (27.8-40.3)	33.0 (27.5-40.2)	P = 0.703
*MCS	48.6 (38.8-55.1)	47.3 (36.5-56.1)	49.6 (34.1-58.0)	48.0 (40.4-56.6)	P = 0.840
Physical functioning	35.0 (15.0-65.0)	25.0 (10.0-45)	31.9 (10.0-60.0)	30.0 (6.0-55.0)	P = 0.232
Role physical	37.5 (18.8-56.3)	37.5 (18.8-56.3)	31.3 (18.8-56.3)	31.3 (12.5-31.4)	P = 0.988
Bodily pain	42.0 (31.0-74.0)	41.0 (22.0-74.0)	41.0 (22.0-74.0)	41.0 (31.0-74.0)	P = 0.861
General health	31.6 (17.0-47.0)	30.0 (20.0-50.0)	35.0 (25.0-52.0)	37.0 (25.0-55.0)	P = 0.257
*Vitality	37.5 (18.8-50.0)	31.3 (18.8-50.0)	37.5 (25.0-50.0)	34.8 (21.9-56.3)	P = 0.677
*Social functioning	50 (37.5-75.0)	50 (37.5-75.0)	62.5 (37.5-87.5)	62.5 (25.0-87.5)	P = 0.535
*Role emotional	75 (41.7-100.0)	66.7 (25.0-100.0)	58.3 (33.3-100.0)	75.0 (33.3-100.0)	P = 0.874
*Mental health	70.0 (55.0-85.0)	70.0 (50.0-85.0)	75.0 (55.0-85.0)	75.0 (60.0-90.0)	P = 0.600

(B)

Timepoint (n)	Baseline (96)	4m (62)	8m (44)	12m (47)	Kruskal Wallis
PCS	34.0 (28.2-41.2)	31.6 (27.3-41.0)	33.6 (28.6-40.6)	33.1 (27.9-40.2)	P = 0.655
*MCS	48.8 (38.9-54.8)	48.0 (37.1-56.1)	50.0 (34.1-57.7)	47.8 (37.5-56.5)	P = 0.977
Physical functioning	35.0 (15.0-67.5)	25.0 (10.0-55.0)	35.0 (15.0-65.0)	30.0 (6.0-55.0)	P = 0.284
Role physical	37.5 (21.9-56.3)	37.5 (18.8-56.3)	43.8 (25.0-62.5)	37.5 (12.5-56.3)	P = 0.948
Bodily pain	51.0 (31.0-74.0)	46.0 (22.0-84.0)	41.0 (22.0-84.0)	41.0 (31.0-84.0)	P = 0.946
General health	35.0 (20.0-47.0)	32.0 (20.6-47.0)	35.0 (25.0-52.0)	37.0 (25-52)	P = 0.425
*Vitality	35.4 (18.8-50)	34.4 (25-50)	37.5 (25-56.3)	37.5 (25-56.3)	P = 0.678
*Social functioning	50.0 (37.5-75)	50.0 (37.5-87.5)	62.5 (50.0-100.0)	62.5 (25.0-87.5)	P = 0.699
*Role emotional	75.0 (37.5-100.0)	66.7 (25.0-100.0)	66.7 (33.3-100.0)	70.8 (25-100.0)	P = 0.812
Mental health	70.0 (55.0-85.0)	70.0 (50.0-85.0)	75.0 (50.0-85.0)	75.0 (60.0-90.0)	P = 0.802

Table 7.2 SF-36 scores at each questionnaire time point.
Cholecalciferol supplementation was initiated at baseline and a 3-month repletion phase assumed. The SF-36 questionnaire was completed every 4 months for 1 year. Results were compared for all patients **(A)**, then data were stratified to exclude patients with serum 25(OH)D ≥75nmol/L at baseline and those that did not achieve repletion **(B).** There was no significant change in any component throughout the 12-month follow up period; results did not alter once stratified according to vitamin D repletion. PCS = Physical Component Summary, MCS = Mental Component Summary. *Mental health components. Data represent median (IQR).

7.3 The European Quality of Life questionnaire results

Results from the EQ-5D questionnaire are presented as value index scores and health scores. Value index scores are generated from the individuals' answers to 5 questions (covering 5 health domains); an index score of 1 reflects the value of full health (EQ-5D, 2020). The health scores have a scale of 0-100, where patients are asked to indicate their overall health on the day of questionnaire completion; a score of 100 means the best health they can imagine (EQ-5D, 2020).

The EQ-5D results reflected results from the SF-36 questionnaire, showing HRQOL was not affected by cholecalciferol supplementation. There was no difference between value index scores or EQ-5D health scores across the 12 month study period, P = 0.9143 and P = 0.4835, respectively (Table 7.3A). Results did not alter once data was stratified according to vitamin D repletion, P = 0.8028 and P = 0.6059 (Table 7.3B).

(A)

	Baseline	4 months	8 months	12 months	Kruskal-Wallis test
Number (n)	122	74	50	54	
Value index score	0.64 (0.39-0.75)	0.63 (0.33-0.75)	0.64 (0.45-0.81)	0.64 (0.38–0.77)	P = 0.9143
Health score	60.0 (50.0-75.0)	60.0 (50.0-75.0)	70.0 (50.0 -80.0)	60.0 (45.0-80.0)	P = 0.4835

(B)

	Baseline	4 months	8 months	12 months	Kruskal-Wallis test
Number (n)	96	64	44	47	
Value index score	0.64 (0.39-0.77)	0.64 (0.35-0.76)	0.67 (0.45-0.82)	0.65 (0.43–0.80)	P = 0.9709
Health score	63.0 (50.0–75.0)	60 (50.0-75.0)	70 (50.0-80.0)	60 (45.0–80.0)	P = 0.4728

Table 7.3 Summary of EQ-5D value index scores and health scores
Cholecalciferol supplementation was initiated at baseline and a 3-month repletion phase assumed. The EQ-5D questionnaire was completed every 4 months for 1 year. Results were compared for all patients regardless of serum 25(OH)D **(A)**, and then data were stratified to exclude patients with serum 25(OH)D ≥75nmol/L at baseline and those that did not achieve repletion **(B)**. There was no significant change in EQ-5D value index scores or health scores throughout the 12-month follow up period; results did not alter once stratified according to vitamin D repletion. Data represent median (IQR).

7.4 Discussion

SF-36 MCS and PCS scores were below norm at baseline and were not improved by increased serum 25(OH)D. SF-36 norm values are based on the general US population (Ware *et al.*, 2000); given the known co-morbidities (Wheeler, 2010), it could be anticipated that a cohort of patients with ESRD would fall below this. Using the SF-36 MCS score <38 as an indicator of depression (Matcham *et al.*, 2016), the percentage of the population at risk of depression (26%) was reflective of the prevalence rates reported by others (25-30% in ESRD) (Hedayati *et al.*, 2008; Wuerth *et al.*, 2003).

HRQOL is significantly reduced in ESRD and is associated with increased risk of hospital admission and mortality (Mapes, 2004). Despite the high prevalence of vitamin D deficiency, the effect of cholecalciferol supplementation on HRQOL, has yet to be investigated by clinical trials. Observational studies have shown an association between low serum 25(OH)D and reduced PCS and MCS scores in ESRD and CKD patients (Anand *et al.*, 2011; Kara *et al.*, 2019; Oh *et al.*, 2017). One small intervention study, gave ergocalciferol to 60 patients with ESRD, and found no effect on HRQOL following 6 months supplementation (Hewitt *et al.*, 2013). Mager and colleagues (2017) conducted a trial in patients with CKD stages 1-4, comparing daily vs. monthly cholecalciferol supplementation and investigating the effects on HRQOL. A statistically significant improvement in physical functioning was seen during the 6 month follow up but no significant change in any other measures of HRQOL, and there was no significant change in overall PCS or MCS scores (Mager *et al.*, 2017). The authors recommended further research, to include longer study follow up, and a control arm.

Without a control arm, it is unknown whether HRQOL would have deteriorated if the patients had not received vitamin D. The HRQOL in HD patients overtime is discussed further in the main discussion (Chapter 9). Study size is also a limiting factor; complete data for all 4 time points was only available for 50 patients. The systematic differences between those who completed the HRQOL study and those who dropped out was not explored. However, attrition bias (in addition to selection bias) is known to exist in studies; this limits the interpretation of study findings beyond those already presented by the small study size.(Nunan *et al.*, 2018) This is a recognised limitation of qualitative research whereby those who are less well are

more likely to drop out, potentially resulting in a false positive outcomes.(Kornbluh, 2015)

HRQOL is often a secondary end-point in vitamin D research, and as such studies are often not adequately powered to provide meaningful results (Hoffmann *et al.*, 2015). There is a large RCT currently underway in the UK, using cholecalciferol supplementation in ESRD, and investigating HRQOL as a secondary endpoint; this study aims to recruit 4200 participants and is the largest vitamin D supplementation study in ESRD to date (Hiemstra, 2015). The outcome of this study will hopefully provide greater insight; yet an adequately powered RCT with HRQOL as the primary end point is needed for clear conclusions to be drawn.

Chapter 8 Is 25(OH)D the correct vitamin D metabolite to measure?

The mechanisms for the assessment of vitamin D status are described in 1.4.1. This chapter explores the precision of the hospital 25(OH)D assay and the dynamic nature of vitamin D metabolism, through consideration of how vitamin D metabolites are formed and further metabolised.

The vitamin D multi-metabolite analysis was carried out by Professor Hewisons' team at The University of Birmingham using Liquid chromatography–tandem mass spectrometry (LC-MS/MS). The tissue transfer forms were completed by myself, as were all other research activities, including the statistical analysis, within this chapter.

Throughout this chapter, 25(OH)D, without a subscript indicates total 25(OH)D ($25(OH)D_2$ plus $25(OH)D_3$); subscripts will be used when a specific form of the vitamin is being referred to. Results from the Ph.D sub-study one will be presented, and existing evidence discussed, in order to address the following:

1. A comparison of 25(OH)D measurements
    - Ph.D sub-study results; Elecsys competitive immunoassay vs. Liquid chromatography–tandem mass spectrometry (LC-MS/MS).

2. The present, and future, assessment of vitamin D status
    - Total 25(OH)D, $25(OH)D_2$, and $25(OH)D_3$
        - Limitations of total 25(OH)D assessment
    - What should we be testing to assess vitamin D status?
        - 3-epimer forms of 25(OH)D and $1,25(OH)_2D$
        - $24,25(OH)_2D$ - a catabolic metabolite of vitamin D
        - Vitamin D Binding Protein (DBP) – serum carrier of vitamin D
        - The free fraction of 25(OH)D - fraction not bound to DBP or albumin

8.1 A comparison of 25(OH)D measurements

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) measures both 25(OH)D_2 and 25(OH)D_3, can distinguish between these and other vitamin D metabolites, and is currently considered the gold standard method for determination of serum vitamin D status (Jones & Kaufmann, 2016). Immunoassays cannot quantify 25(OH)D_2 and 25(OH)D_3 separately, or differentiate between all vitamin D metabolites (notably 25(OH)D and its 3-epi form), yet due to cost, and practicalities, immunoassays remain the current method of choice for clinical laboratories (Jones & Kaufmann, 2016).

All patients in sub-study one had their serum 25(OH)D_2 and 25(OH)D_3 levels measured at baseline (T0) and 12 months (T12) by LC-MS/MS, as well as having their total serum 25(OH)D measured as part of standard care by UHCW clinical laboratory, using the Elecsys competitive immunoassay. The LC-MS/MS 25(OH)D_2 and 25(OH)D_3 results were combined to give total 25(OH)D measurements. These were compared against those obtained by the immunoassay to investigate if there was a difference between the results from the two assay methods (complete data was available for 52 subjects). There was a statistically significant correlation between the results obtained by both assays at T0 and T12, Pearson's correlation coefficient [*r*] 0.788, P <0.001 and [*r*] 0.795, P <0.001, respectively (Figure 8.1).

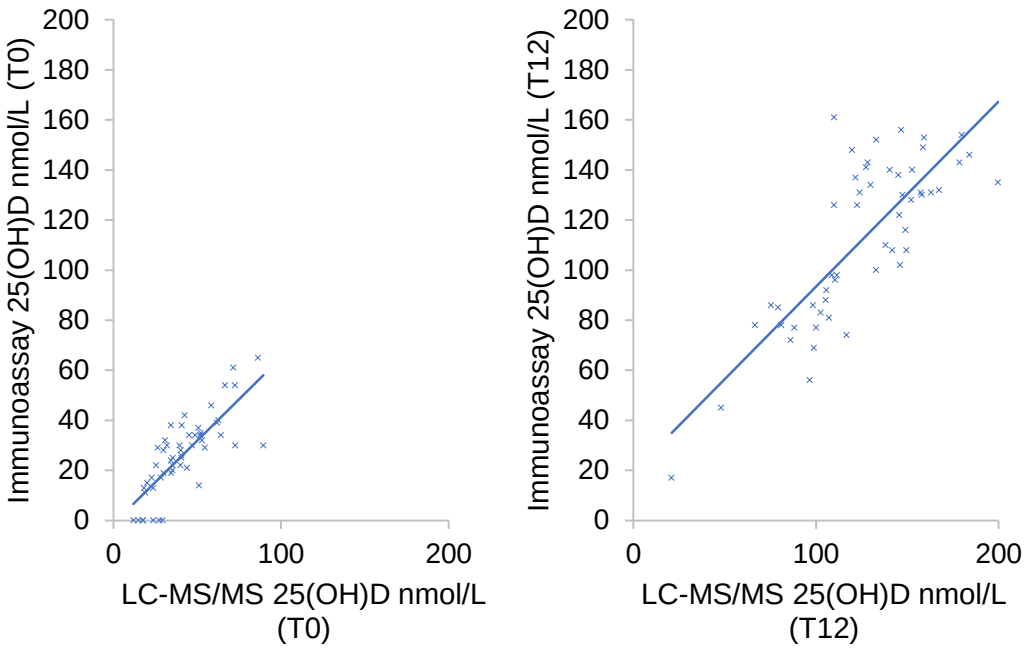

Figure 8.1 Comparison of total serum 25(OH)D results at baseline (T0) and 12 months (T12) using LC-MS/MS and Immunoassay.
All patients in the sub-study had their serum total 25(OH)D measured at baseline (T0), prior to cholecalciferol supplementation, and again at 12 months (T12) by LC-MS/MS, and by Elecsys competitive immunoassay, as part of routine clinical care. There was a statistically significant correlation between the results obtained by both assays at T0 and T12, Pearson's correlation coefficient [*r*] 0.788, P <0.001 and [*r*] 0.795, P <0.001, respectively (n=52).

Due to the limitations of correlation methods; they are not able to detect if there is a constant or proportional difference between two sets of data (Udovičić *et al.*, 2007), Passing-Bablok (PB) regression, and Bland-Altman bias plot analysis was carried out. PB regression analysis show the relationship between the LC-MS/MS and immunoassay 25(OH)D measurements is linear, intercept -8.5, slope core 0.9, P=0.303 at baseline and intercept -2.4, slope core 0.8, P=0.722 at 12 months (Figure 8.2). However, the Bland-Altman bias plot, together with paired t-test, show a significance difference; results from the Elecsys immunoassay were statistically significant lower at baseline (P <0.001) and 12 months (P <0.001) (Table 8.1 and Figure 8.3). The Bland-Altman analysis show the immunoassay had a negative bias compared with LC-MS/MS. A larger bias was present at baseline compared with 12 months -15.2 (95% CI -37.5 to 7.1, P<0.001) and -13.1 (95% CI -55.6 to 29.5, P<0.001). This confirms that results obtained from the Elecsys immunoassay are predicted to be lower than those obtained from LC-MS/MS.

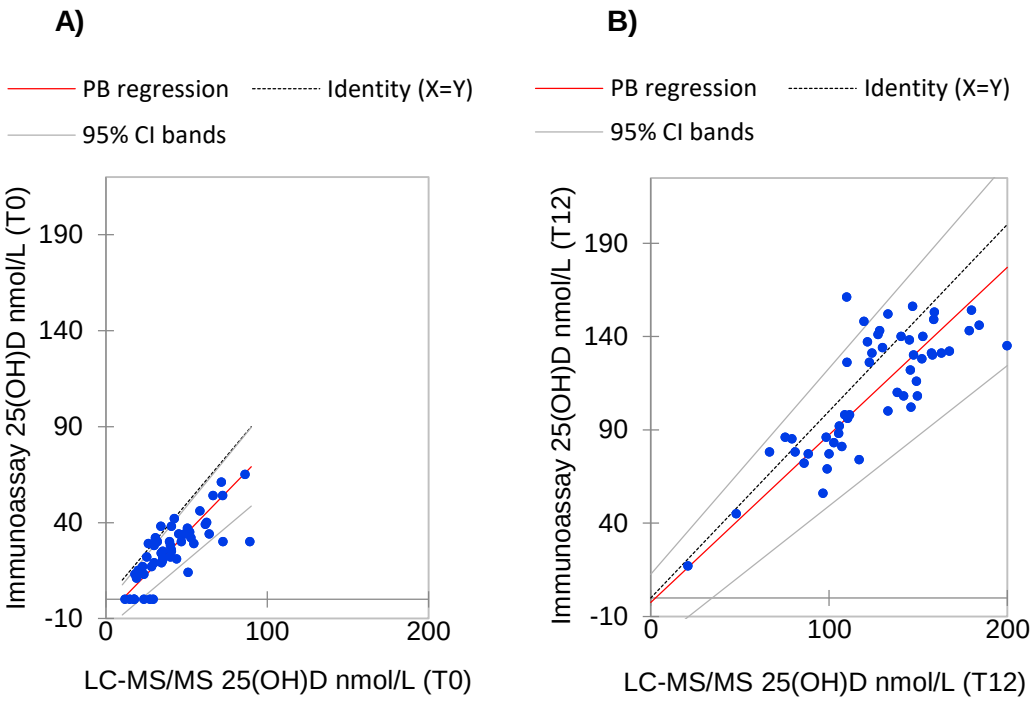

Figure 8.2 Passing-Bablok regression analysis of LC-MS/MS and Immunoassay at baseline (T0) and 12 months (T12).
All patients in the sub-study had their serum total 25(OH)D measured by both LC-MS/MS, and Elecsys competitive immunoassay, at baseline (A), prior to cholecalciferol supplementation, and again at 12 months (B) Passing-Bablok (PB) regression analysis show the relationship between the 25(OH)D measurements from the immunoassay and LC-MS/MS assay are linear: intercept -8.5, slope core 0.9, P = 0.303 at baseline, and intercept -2.4, slope core 0.8, P = 0.722 at 12 months (n=52).

25(OH)D (nmol/L)	Elecsys Immunoassay *Mean ± (SD)*	LC-MS/MS *Mean ± (SD)*	%Difference between the means (%)	95% CI	Paired t-test
Baseline	26.1 (15.5)	41.3 (18.4)	-15.2	-18.4 to -12.0	P <0.001
12 months	112.1 (32.5)	125.1 (35.0)	-13.1	-19.0 to -7.1	P <0.001

Table 8.1 Total serum 25(OH)D measures by LC-MS/MS and Elecsys Immunoassay.
All patients in the sub-study had their serum total 25(OH)D measured by both LC-MS/MS, and Elecsys competitive immunoassay at baseline, prior to cholecalciferol supplementation, and again at 12 months. The results from both assays were compared and a statistically significant difference was found at both time points. Bland-Altman bias (difference between the means) (n = 52).

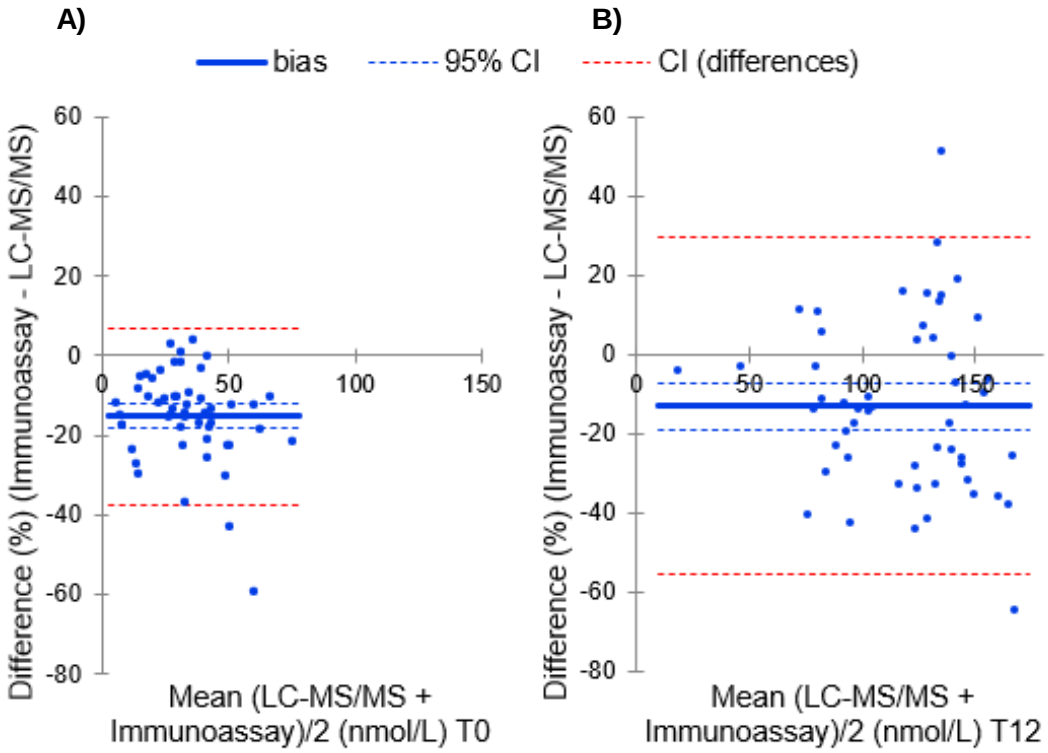

Figure 8.3 Bland-Altman bias plot analysis of LC-MS/MS and Immunoassay at baseline (T0) and 12 months (T12).
All patients in the sub-study had their serum total 25(OH)D measured at baseline (T0), prior to cholecalciferol supplementation, and again at 12 months (T12) by LC-MS/MS, and by Elecsys competitive immunoassay. A negative bias was found for the immunoassay at both time points, with a larger bias being present at T0 compared with T12: -15.2% (95% CI -37.5 to 7.1, P<0.001) and -13.1% (95% CI -55.6 to 29.5, P<0.001)) respectively (n = 52).

The results presented here, comparing the immunoassay against LC-MS/MS reveal a linear relationship yet negative bias and statistical difference. A negative bias in clinical terms offers more safety than a positive one given the risks associated with deficiency, coupled with modest risk of toxicity. LC-MS/MS is rarely used for 25(OH)D analysis in the clinical setting due to the greater resources, increased expertise, and time, required to carry it out (Jones & Kaufmann, 2016). However, imprecision of the immunoassay could cause misdiagnosis of vitamin D deficiency, risking clinical implications.

8.2 The present, and future, assessment of vitamin D status

8.2.1 Total 25(OH)D, 25(OH)D_2, and 25(OH)D_3

Serum 25(OH)D, the total of 25(OH)D_2 and 25(OH)D_3, is the measurement of vitamin D status currently (Association for Clinical Biochemistry, 2012; Ross *et al.*, 2011). Vitamin D_2 (ergocalciferol) becomes 25(OH)D_2 and vitamin D_3 (cholecalciferol) becomes 25(OH)D_3. Sources of vitamin D_2 include plant based, or fortified, foods, and supplements, and sources of vitamin D_3 include animal based foods, sunlight, and supplements (Holick, 2010). Measuring total 25(OH)D accounts for all vitamin D sources, whereas only measuring 25(OH)D_3, for example, could falsely diagnose vitamin D deficiency in someone taking ergocalciferol supplementation. Although not a focus of this chapter, a similar problem arises when considering 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ (Biancuzzo *et al.*, 2013).

Limitations of serum 25(OH)D

The prevalence of hypovitaminosis D within specific cohorts is confounded by variability in the level of 25(OH)D used to define adequacy (Bouillon, 2017). 25(OH)D assay variation has been a contributing factor to the lack of consensus defining vitamin D deficiency, insufficiency, and sufficiency; (Heaney, 2013; Rosen & Taylor, 2013) (Section 1.4.3).

Factors contributing to 25(OH)D immunoassay measurement variation include antibody affinity for 25(OH)D_2 and 25(OH)D_3, cross reactivity with 24,25$(OH)_2$D, and concentration of DBP (Carter, 2012). Some immunoassays are unable to detect 25(OH)D_2, due to low antibody affinity, this can cause falsely low 25(OH)D results; this is of particular relevance to populations where ergocalciferol supplementation is common (for example in the United States of America) (Shu *et al.*, 2013). Cross reactivity with 24,25$(OH)_2$D is another issue related to immunoassays, this can cause falsely high 25(OH)D results (Carter *et al.*, 2016; Cashman *et al.*, 2015). 24,25$(OH)_2$D is reported to cause up to 15% overestimation (Romagnoli *et al.*, 2013). In certain immunoassays, 25(OH)D is not well separated from DBP, meaning situations affecting DBP concentrations (such as pregnancy or liver disease) can impact on 25(OH)D results (Cavalier *et al.*, 2014; Heijboer *et al.*, 2012).

Whilst immunoassay methods have shown bias and increased variability when compared to LC-MS/MS; LC-MS/MS also has faults (Jukic *et al.*, 2017). 3-epi-

25(OH)D epimer (3-epi) does not interfere with immunoassays, but does cross react in a competitive binding protein assay; it is not always separated in high performance liquid chromatography and LC-MS/MS. Whilst 3-epi-25(OH)D has been found in people of all ages, an inverse relationship between 3-epi-25(OH)D and age has been shown, meaning this is more relevant in children (Singh *et al.*, 2006). 3-epi-25(OH)D circulates at ~5% the concentration of 25(OH)D; the impact of cross reactivity should therefore have minimal impact on 25(OH)D measurements, however much higher concentrations have been reported (Bailey *et al.*, 2013; Carter *et al.*, 2016; Cashman *et al.*, 2014; Chailurkit *et al.*, 2015; Jukic *et al.*, 2017; Lutsey *et al.*, 2015).

Efforts have been made to standardise 25(OH)D assays; this includes retrospective assay standardisation, to facilitate pooling of research data and, in turn, deliver an evidenced based definition for vitamin D status (Binkley *et al.*, 2017; Durazo-Arvizu *et al.*, 2017; NIH, 2017). Whilst standardisation of 25(OH)D measurement is vital, many other vitamin D metabolites exist, of which there is a growing body of research, suggesting physiological activity, and roles, within the diagnosis and management of disease (Herrmann *et al.*, 2017). Such vitamin D metabolites include 3-epi-25(OH)D_3, 3-epi-1,25(OH)$_2D_3$, 24,25(OH)$_2D_3$, and 1,24,25(OH)$_3D_3$, vitamin D binding protein (DBP), and free (bioavailable) 25(OH)D (discussed further in section 8.2.2). Figure 8.4 gives an overview of vitamin D metabolism.

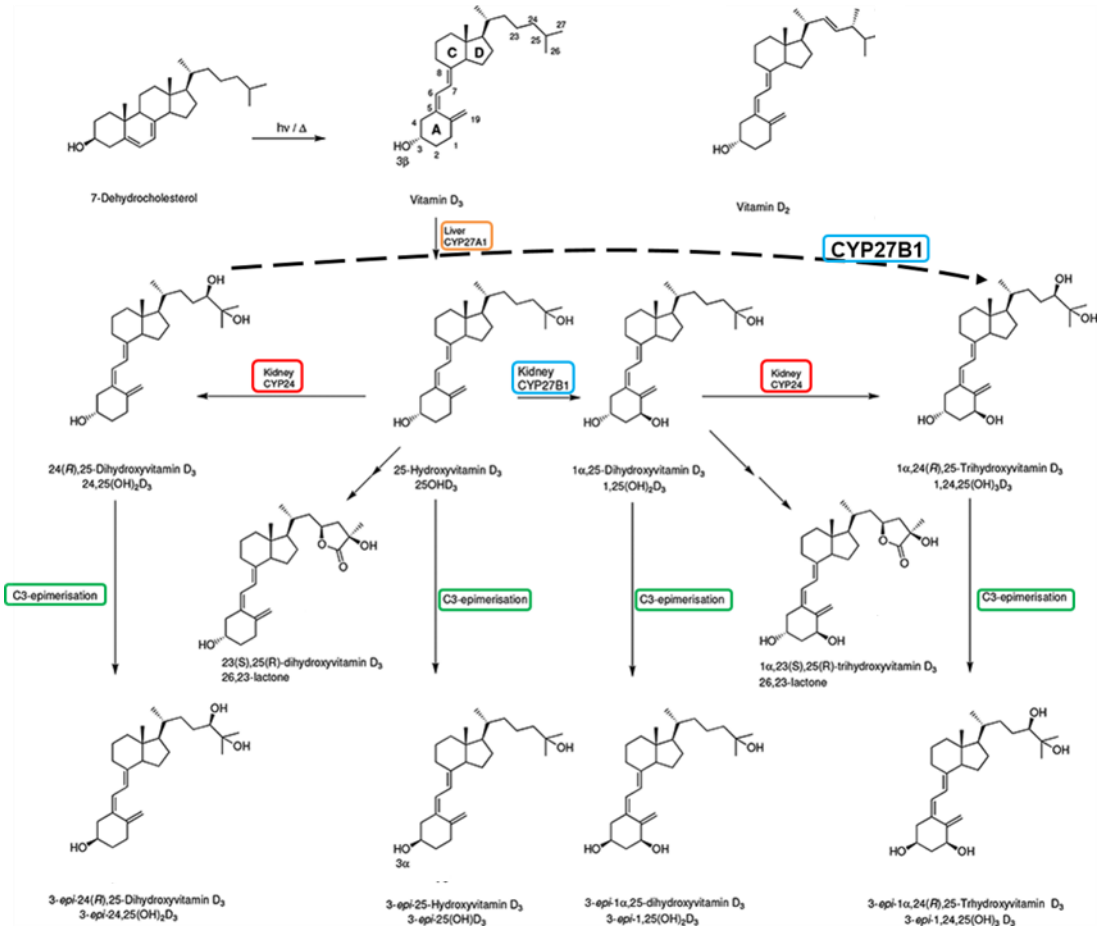

Figure 8.4 Overview of vitamin D metabolism
The vitamin D system is dynamic. Multiple hydroxylase enzymes act on multiple vitamin D substrates. 25-hydroxylase (CYP27A1) acts to metabolise vitamin D_2 and D_3 into 25(OH)D. 1α-hydroxylase (CYP27B1) acts to metabolise 25(OH)D into $1,25(OH)_2D$. 24-hydroxylase (CYP24A1) acts to catabolise 25(OH)D and $1,25(OH)_2D$. C3-epimerisation acts on multiple metabolites; if this enzyme increases, multiple metabolites are metabolised (Kattner & Volmer, 2017).

Specific vitamin D metabolite profiles have now been associated with certain diseases, suggesting that assessment of various vitamin D metabolites could aid in distinct diagnoses of vitamin D pathologies. In particular, the conditions associated with calcium/phosphate homeostasis may be associated with a different metabolite profile. Clinical research studies, and studies carried out in animals, have utilised the multiple vitamin D metabolite assay as a means of investigating diseases of calcium/phosphate homeostasis, for example idiopathic infantile hypercalcaemia (IIH) where there is impaired 24-hydroxylation of $1,25(OH)_2D$ (Molin *et al.*, 2015). This has led to an increased understanding of the roles of different vitamin D

metabolites, and with it, increased interest in the measurement of vitamin D metabolites.

A one-off measurement of a single vitamin D metabolite provides limited information as it may have been converted/catabolised to another metabolite. With increasing interest in vitamin D metabolites, the use of 25(OH)D, as a sole marker of vitamin D deficiency, has been questioned (Herrmann *et al.*, 2017). An absence of assay standardisation for most vitamin D metabolites currently affects interpretation; the same issue exists for the measurement of PTH, a marker routinely linked with vitamin D status (Sempos *et al.*, 2018).

8.2.2 What should we be testing to assess vitamin D status?

The 3-epimer of 25(OH)D and 1,25$(OH)_2$D

3-epi-25(OH)D was first discovered in children where concentrations were found to be high, when compared with those in adults (Singh *et al.*, 2006). Detectable 3-epi-25$(OH)D_3$ is thought to account for approximately 5% of 25(OH)D on average, but higher levels, accounting for as much as 50%, have been reported (Bailey *et al.*, 2013; Carter *et al.*, 2016; Cashman *et al.*, 2014; Chailurkit *et al.*, 2015; Jukic *et al.*, 2017; Lutsey *et al.*, 2015). Not all studies looking at 3-epi-25$(OH)D_3$ have found detectable levels in all individuals; it is not known if this relates to methodological shortcomings, or the vitamin D status of these populations (Bailey *et al.*, 2013; Lutsey *et al.*, 2015). 3-epi-25(OH)D, if included in 25(OH)D results, can lead to falsely high levels of 25(OH)D and therefore should be excluded when measuring total 25(OH)D (Shah *et al.*, 2018).

3-epi-25$(OH)D_3$ and 3-epi-1,25$(OH)_2D_3$ have been shown to bind to DBP at approximately 36-46% the affinity of 25(OH)D and 1,25$(OH)_2$D, and to VDR at approximately 2-3% the affinity of 25(OH)D and 1,25$(OH)_2$D (Kamao *et al.*, 2004). How 3-epi-25$(OH)D_3$ and 3-epi-1,25$(OH)_2D_3$ production is regulated and metabolised, and their overall importance in terms of vitamin D status, are as yet unknown (Al-Zohily *et al.*, 2020).

24,25$(OH)_2$D

The production of 24,25$(OH)_2$D and 1,24,25$(OH)_3$D is referred to as degradation or catabolism of vitamin D (Jones *et al.*, 2012). The enzyme, 24-hydroxylase

(CYP24A1), metabolises 25(OH)D to 24,25$(OH)_2$D, and 1,25$(OH)_2$D to 1,24,25$(OH)_3$D. The activity of 24-hydroxylase and 1α-hydroxylase (CYP27B1) should be reciprocal in order to control 1,25$(OH)_2$D, and its potent effects on calcium homeostasis. The loss of 24-hydroxylase activity (CYP24A1 function) results in increased levels of 1,25$(OH)_2$D and hypercalcaemia which in turn reduces PTH (Tebben, 2018). A negative feedback loop is provided by 1,25(OH)2D suppressing 1α-hydroxylase and inducing 24-hydroxylase activity (Prosser & Jones, 2004).

Serum 24,25$(OH)_2$D levels have been shown to be highly correlated with serum 25(OH)D, circulating at between 7 - 15% the amount of 25(OH)D (Wagner *et al.*, 2011; Wise *et al.*, 2017b). Measurement of 24,25$(OH)_2$D may offer further insight, and in turn, support clinical management of disorders relating to vitamin D deficiency, hypercalcaemia, and hypophosphataemia (Kaufmann *et al.*, 2014). The vitamin D metabolite ratio (VMR) of 25(OH)D to 24,25$(OH)_2$D is dramatically elevated in patients with CYP24A1 mutations (Tebben, 2018; Molin *et al.*, 2015; Kaufmann *et al.*, 2017) who have decreased catabolism of vitamin D. Figure 8.5 presents the biochemical effects as a result of loss-of-function of CYP24A1, along with the chemical structure of the vitamin D metabolites 25(OH)D, 1,25$(OH)_2$D and 24,25$(OH)_2$D. The 25(OH)D:24,25$(OH)_2$D VMR may offer benefits outside the patients with CYP24A1 mutations; 24-hydroxylase activity, and the 24,25$(OH)_2$D response to cholecalciferol supplementation, may vary between individuals; the clinical use of VMR is discussed further in sections 8.3 and 8.4.

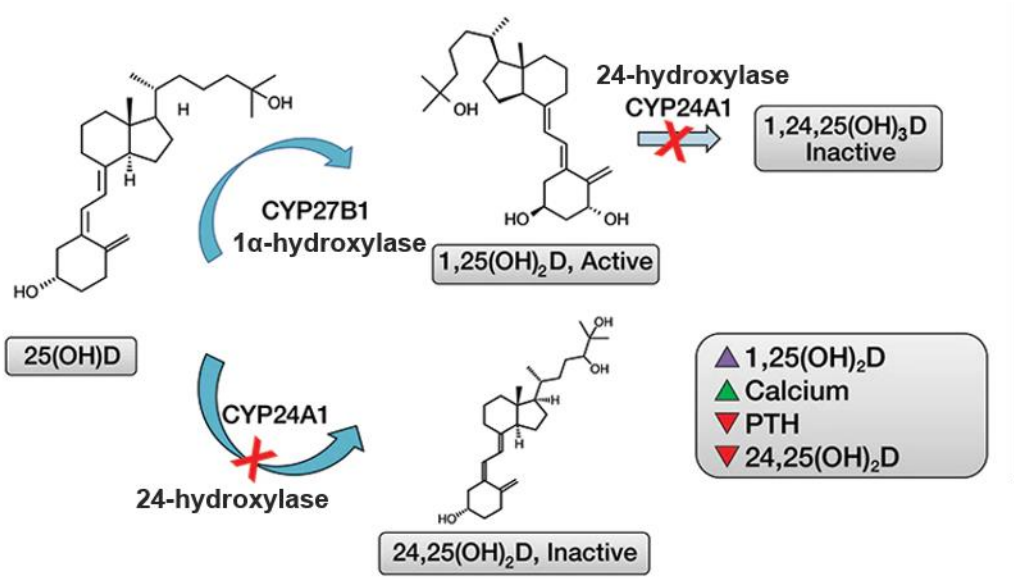

Figure 8.5 Loss of CYP24A1 enzyme function
25(OH)D is converted to $1,25(OH)_2D_3$ (CYP27B1) and $24,25(OH)_2D_3$ (CYP24A1). $1,25(OH)_2D$ is converted to $1,24,25(OH)_3D$ (CYP24A1). Calcium, phosphate, PTH, FGF23, and regulate the levels of $1,25(OH)_2D_3$ produced. The production of $24,25(OH)_2D_3$ and $1,24,25(OH)_3D$ is referred to as degradation; activity of CYP27B1 and CYP24A1 should reciprocal in order to control $1,25(OH)_2D_3$, and in turn serum calcium. The loss of CYP24A1 function results in increased $1,25(OH)_2D_3$ and hypercalcaemia which in turn reduces PTH synthesis and secretion (Tebben, 2018).

Vitamin D Binding Protein (DBP)

Vitamin D metabolites are lipid soluble compounds that circulate bound to transport proteins. A small proportion of 25(OH)D (<10%) can bind with low affinity to the abundant serum protein albumin. However, the majority of vitamin D metabolites circulate bound to a specific binding globulin, DBP (Bouillon & Pauwels, 2018; Chun, 2012). DBP binds vitamin D metabolites with greater affinity than albumin, and DBP has a higher affinity for 25(OH)D than catabolic metabolites such as $24,25(OH)_2D$, with $1,25(OH)_2D$ showing lower affinity, and an even lower affinity again for vitamin D itself; vitamin D_2 metabolites bind to DBP slightly less well than vitamin D_3 metabolites (Bouillon & Pauwels, 2018; Chun, 2012).

DBP can be measured using immunoassays and LC–MS/MS (Denburg *et al.*, 2016; Hoofnagle *et al.*, 2015). Due to the polymorphic nature of DBP, an immunoassay using polyclonal antibodies is preferred; immunoassays using monoclonal antibodies can result in falsely low DBP levels in individuals with certain isoforms

(for example the Gc1f isoform predominantly present in people of African ethnicity) (Nielson *et al.*, 2016b). Accurate measurement of DBP is important because it defines the proportion of vitamin D metabolites that are bound to DBP, and therefore the proportion of vitamin D metabolites, notably 25(OH)D, that are not bound to DBP – the level of 'free' 25(OH)D. Serum levels of DBP have attracted recent attention because of the 'free hormone hypothesis' which states that it is the free fraction of lipid soluble molecules such as vitamin D that access target cells by passive diffusion (Chun & Nielson, 2018). Several studies have suggested that the free serum form of 25(OH)D has greater biological relevance than total 25(OH)D (Bhan *et al.*, 2012; Chun *et al.*, 2019).

The free fraction of 25(OH)D

Is free serum 25(OH)D a better marker of vitamin D status than conventional total 25(OH)D? The hypothesis that only the unbound (free) fraction can enter many cells, and thus exert biological effects is attracting attention, and it is suggested that this form of vitamin D drives many of its nonclassical actions (Chun *et al.*, 2014; Mendel, 1992).

Approximately 85% of 25(OH)D is bound to DBP, and 15% to serum albumin; the free fraction accounts for <1% (Bhan *et al.*, 2012; Nielson *et al.*, 2016a; Nielson *et al.*, 2016b). Bioavailable 25(OH)D refers to the combined total of free, and albumin bound, 25(OH)D (Malmstroem *et al.*, 2017). Free 25(OH)D is usually estimated by using total serum 25(OH)D and DBP; the accuracy of this is unclear (Malmstroem *et al.*, 2017). Free 25(OH)D can also be measured directly using ELISA kits (Heureux *et al.*, 2017; Malmstroem *et al.*, 2017). The accuracy of this direct measurement is the subject of debate; there is currently no standardised assay for the measurement of DBP or free 25(OH)D (Chun & Nielson, 2018).

In healthy individuals', free vitamin D and 25(OH)D are shown to strongly correlate, implying total 25(OH)D is predictive of free 25(OH)D (Aloia *et al.*, 2015; Bikle *et al.*, 1986; Nielson *et al.*, 2016b). Whether conditions that impact on DBP affect this correlation, meaning free 25(OH)D is more clinically useful than total 25(OH)D for certain populations, have been considered (Chun & Nielson, 2018). Results from studies to date have been mixed; free 25(OH)D has been considered a superior marker to 25(OH)D by some (Shieh *et al.*, 2016; Shieh *et al.*, 2018), and others have demonstrated it does not correlate better with serum bone markers than total 25(OH)D (Bikle *et al.*, 2017a; Chun & Nielson, 2018; Jemielita *et al.*, 2016).

At present the utilisation of free 25(OH)D, as part of the assessment of vitamin D status, is unclear, and further research is needed (Bikle *et al.*, 2017b).

Summary

Limited studies exist looking at alternative markers of vitamin D status; evidence to date suggests free 25(OH)D is unlikely to offer additional information to conventional measurement of total 25(OH)D. 24,25$(OH)_2$D, and the ratio of this metabolite, to 25(OH)D, has been shown to be of value in certain clinical situations, in particular hypercalcaemia. This is explored further in the following section.

8.3 Vitamin D metabolite ratio

Individuals with hypercalcaemia of an unknown cause, and those with a family history of hypercalcaemia, may benefit from multiple vitamin D metabolite profiling and vitamin D metabolite ratio (VMR) assessment. VMR may offer further insight into vitamin D metabolism and its biological role within these conditions.

To date the most documented use, and a prime example, of 24,25$(OH)_2$D measurement and VMR profiling within disease; is in the diagnosis of idiopathic infantile hypercalcaemia (IIH). IHH covers at least 3 known conditions: **i)** Lightwood syndrome, caused by CYP24A1 loss-of-function mutations (Lightwood & Stapleton, 1953; Schlingmann *et al.*, 2011), **ii)** Williams-Beuren syndrome (WBS) caused by a deletion of genes on chromosome 7 which code for elastin (Twite *et al.*, 2019; Williams *et al.*, 1961), and **iii)** Loss-of-function mutations of the sodium-phosphate co-transporter, also referred to as SLC34A1 (Schlingmann *et al.*, 2016)

Loss-of-function mutations of CYP24A1 are one of the main genetic causes of hypercalcaemia (Schlingmann *et al.*, 2011). 24,25$(OH)_2$D is the main product of CYP24A1 found in the blood; it is a reliable measure of 24-hydroxylase activity *in vivo* and considered proportional to the level of 25(OH)D (Cashman *et al.*, 2015; Tang *et al.*, 2017). Serum 24,25$(OH)_2$D concentration is low in most IIH patients with CYP24A1 mutations, despite untreated IIH patients having elevated 25(OH)D levels (Molin *et al.*, 2015). A more reliable indicator of IIH due to CYP24A1 mutations is the ratio of metabolite to substrate, VMR, in this instance 25(OH)D:24,25$(OH)_2$D

VMR. Using the VMR improves interpretation of serum 24,25$(OH)_2$D, by ruling out a low 24,25$(OH)_2$D level secondary to lack of substrate (25(OH)D). In normal individuals with a serum 25(OH)D level between 50-200nmol/L, serum 24,25$(OH)_2$D levels are shown to be 5-20nmol/L with a 25(OH)D:24,25$(OH)_2$D VMR of 5-25 (Kaufmann *et al.*, 2014). A 25(OH)D:24,25$(OH)_2$D VMR >80 is associated with heterozygous or biallelic CYP24A1 mutations (two mutations of different points on the same allele, or mutations of both alleles from the one gene) (Kaufmann *et al.*, 2014). 25(OH)D:24,25$(OH)_2$D VMR is shown to be normal in the instance of WBS and sodium-phosphate co-transporter defects (SLC34A1), meaning CYP24A1 mutations can be ruled out by multi-metabolite analysis (Kaufmann *et al.*, 2017). The assessment of 25(OH)D:24,25$(OH)_2$D VMR clinically, may facilitate rapid diagnosis of CYP24A1 mutations, as well as ruling them out and aiding diagnosis and timely treatment of SLC34A1; which has been shown to respond quickly to phosphate supplementation (Schlingmann *et al.*, 2016).

A higher 1,25$(OH)_2$D:25(OH)D ratio was associated with reduced risk of aggressiveness of prostate cancer in African-American men (Ramakrishnan *et al.*, 2019). 1,25$(OH)_2$D:25(OH)D has also been explored as a diagnostic tool in cases of sarcoidosis. A study of 59 patients in France found a 1,25$(OH)_2$D:25(OH)D ratio >3.5 was predictive of sarcoidosis with a 68% sensitivity and a 78% specificity (Rohmer *et al.*, 2020). A recent cross sectional study of 464 patients in Qatar investigated VMR as a predictor of diabetes complications. A lower 1,25$(OH)_2$D:25(OH)D ratio was associated with hypertension ($P<0.001$), dyslipidaemia ($P<0.001$), diabetic retinopathy ($P<0.001$), diabetic neuropathy ($P<0.001$), coronary artery disease ($P=0.001$) and stroke ($P<0.05$) (Ahmed *et al.*, 2020).

It is thought that 24,25$(OH)_2$D is affected by seasonal changes in a similar way to 25(OH)D, with one study demonstrating a 2-fold increase in the peak of UK summer (Macdonald *et al.*, 2017). However the 25(OH)D:24,25$(OH)_2$D VMR is less prone to seasonal variation; thus, offering interpretation using fixed intervals regardless of time of year (Tang *et al.*, 2019). A moderate elevation of 25(OH)D:24,25$(OH)_2$D VMR due to reduced activity of 24-hydroxylase is seen in CKD and bone disorders (Bosworth *et al.*, 2012; Edouard *et al.*, 2012; Stubbs *et al.*, 2014). Evidence suggests there may also be value of using 25(OH)D:24,25$(OH)_2$D in assessment and management of fracture risk and CKD risk (Figueres *et al.*, 2015; Ginsberg *et al.*, 2018).

Tang and colleagues (2019) demonstrated that low 25(OH)D (<50nmol/L), normal $1,25(OH)_2D$ and high $1,25(OH)_2D$:$24,25(OH)_2D$ was associated with a statistically significantly higher serum PTH. Therefore, it is anticipated that treatment with active analogues, for suppression of PTH, would increase the $1,25(OH)_2D$:$24,25(OH)_2D$ VMR and therefore be counterproductive. Whereas treatment focusing on optimising 25(OH)D would induce 1α-hydroxylase and 24-hydroxylase, increasing $1,25(OH)_2D$ and $24,25(OH)_2D$ in a regulated fashion, in turn reducing the $1,25(OH)_2D$:$24,25(OH)_2D$ VMR. Measurement of vitamin D metabolites and calculation of the VMR could therefore offer insight into the prevention and management of SHPT in CKD.

8.4 Vitamin D metabolites and VMR - results from sub-study one

As discussed in Chapter 1 section 1.6.3, one of the pathophysiological consequences of CKD is the reduced renal capacity to synthesise $1,25(OH)_2D$ due to reduced renal 1α-hydroxylase activity. This is well acknowledged; to date routine treatment, particularly in ESRD, circumvents impaired 1α-hydroxylation by use of $1,25(OH)_2D$ analogues or 1α-hydroxylated forms of vitamin D for the management of calcium and PTH balance. As demonstrated by the results of the sub-study, people with ESRD receiving haemodialysis are still able to synthesise $1,25(OH)_2D$ when 25(OH)D availability is optimised (Figure 4.12, section 4.4).

The use and relevance of $1,25(OH)_2D$:25(OH)D VMR in CKD is yet to be established. $1,25(OH)_2D$:25(OH)D has shown to be lower in the CKD population (eGFR <60ml/min), and to respond less to vitamin D supplementation in comparison to a those with an eGFR ≥60mls/min/1.73m^2 (Batacchi *et al.*, 2017).

Compared with 1α-hydroxylase, relatively little attention has been given to 24-hydroxylase activity (vitamin D catabolism) in CKD. Expression of 24-hydroxylase and 1α-hydroxylase needs to be reciprocal in order to balance production and catabolism, and control $1,25(OH)_2D$ and calcium levels (Jones, 2011). Similar to CYP27B1, CYP24A1 is found in vitamin D responsive tissues in the body (St-Arnaud & Jones, 2018). It is induced by $1,25(OH)_2D$, FGF23, and suppressed by PTH (Kaufmann *et al.*, 2015; St-Arnaud & Jones, 2018). Given FGF23 and PTH are both increased in CKD, an effect on vitamin D catabolism is anticipated.

The most abundant product of vitamin D catabolism by CYP24A1 is $24,25(OH)_2D$; measurement of $24,25(OH)_2D_3$ therefore offers insight into CYP24A1 function. To investigate this, serum $24,25(OH)_2D_3$ was analysed in the sub-study patients at baseline and 12 months. Serum $24,25(OH)_2D_3$ results are presented along with $25(OH)D_3$, $1,25(OH)_2D_3$, and the metabolite ratios $1,25(OH)_2D_3$:$25(OH)D_3$, $25(OH)D_3$:$24,25(OH)_2D_3$, and $1,25(OH)_2D_3$:$24,25(OH)_2D_3$. Table 8.2 presents the results for all sub-study patients that had baseline and 12 months data available for all metabolites (n = 54). A statistically significant increase was seen from baseline to 12 months in all 3 metabolites: 25(OH)D, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$. A statistically significant reduction in $1,25(OH)_2D_3$:$25(OH)D_3$ VMR occurred, $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR reduced but the change did not reach significance. There was also no statistically significant change in $25(OH)D_3$:$24,25(OH)_2D_3$ VMR. Patients that were already 25(OH)D replete at baseline, and those that did not reach optimal serum 25(OH)D are included in the results presented in Table 8.2A. These patients were then excluded, and the same analysis repeated to see if effects were related to change in serum 25(OH)D levels and status, rather than cholecalciferol supplementation (regardless of effect), meaning any effects were less likely to have occurred due to chance, n = 47 (Table 8.2B). With the exception of $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR whereby the reduction seen now reached significance, the results remained reflective of the whole sub-study cohort. A statistically significant increase was seen from T0 to T12 in all 3 metabolites. A statistically significant reduction in $1,25(OH)_2D_3$:$25(OH)D_3$ VMR and $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR occurred following repletion, but no significant change was seen in 25(OH)D3:$24,25(OH)_2D_3$ (Table 8.2 - A and B).

(A)

Metabolite / VMR	Baseline (T0) (median, range, IQR)	12 months (T12) (median, range, IQR)	Wilcoxon signed rank test
$25(OH)D_3$ (nmol/L)	35.1 (9.2-144.8, 23.0-47.5)	119.9 (18.5-196.4, 99.5-143.3)	P <0.001
$1,25(OH)_2D_3$ (pmol/L)	48.3 (0.0-96.9, 35.9-57.9)	96.2 (37.1-275.4, 77.1-130.6)	P <0.001
$24,25(OH)_2D_3$ (nmol/L)	3.8 (0.0-17, 2.3-6.0)	12.3 (2.2-43.1, 9-16.4)	P <0.001
$1,25(OH)_2D_3$: $25(OH)D_3$	1.2 (0.0-5.0, 0.8-2.1)	0.9 (0.4-2.3, 0.6-1.1)	P = 0.010
25(OH)D3: $24,25(OH)_2D_3$	9.1 (1.1-24.0, 7.0-12.4)	10.3 (2.6-20.2,8-12.9)	P = 0.698
$1,25(OH)_2D_3$: $24,25(OH)_2D_3$	10.4 (0.0-67.8, 5.8-19.4)	7.9 (2.6-37.8, 5.6-11.9)	P = 0.053

(B)

Metabolite / VMR	Baseline (T0) (median, range, IQR)	12 months (T12) (median, range, IQR)	Wilcoxon signed rank test
$25(OH)D_3$ (nmol/L)	39.6 (11.9-72.6, 28.4-51.5)	130.0 (75.4-184.2, 107.9-149.3)	P <0.001
$1,25(OH)_2D_3$ (pmol/L)	48.3 (0.0-96.9, 36.3-57.8)	96.2 (55.0-275.4, 78.7-128.0)	P <0.001
$24,25(OH)_2D_3$ (nmol/L)	3.9 (1.1-17.0, 2.3-6.0)	12.5 (6.1-43.1, 10.1-16.6)	P <0.001
$1,25(OH)_2D_3$: $25(OH)D_3$	1.4 (0.0-5.0, 0.9-2.3)	0.8 (0.4-2.3, 0.6-1.0)	P = 0.001
25(OH)D3: $24,25(OH)_2D_3$	8.7 (1.1-24.0, 6.8-12.2)	9.9 (3.2-20.2, 7.6-12.5)	P = 0.664
$1,25(OH)_2D_3$: $24,25(OH)_2D_3$	11.5 (0.0-67.8, 5.9-20.6)	7.8 (2.6-25.0, 5.0-10.9)	P = 0.011

Table 8.2 Vitamin D metabolites and ratios at baseline and 12 months.
(A) Serum $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were measured using LC-MS/MS in the sub-study subjects at baseline, pre cholecalciferol supplementation (T0), and again at 12 months (T12), complete data was available for 54 subjects. A statistically significant increase was seen from T0 to T12 in all 3 metabolites. A statistically significant reduction in $1,25(OH)_2D_3$:$25(OH)D_3$ occurred but no significant change was seen from baseline to 12 months in $25(OH)D_3$:$24,25(OH)_2D_3$, or $1,25(OH)_2D_3$:$24,25(OH)_2D_3$. **(B)** Data was stratified to exclude patients already replete at baseline, and those that failed to adequately replete, n = 47. With the exception of $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ whereby the reduction seen now reached significance, results remained reflective of the whole sub-study cohort; a statistically significant increase was seen from T0 to T12 in all 3 metabolites.

Figure 8.6A presents the change in serum 25(OH)D from baseline to 12 months, the median level increased from 39.6nmol/L (range 11.9-72.6, IQR 28.4-51.5) to 130.0nmol/L (range 75.4-184.2, IQR 107.9-149.3), P <0.001. Median serum $24,25(OH)_2D_3$ increased from 3.9nmol/L (range 1.1-17.0, IQR 2.3-6.0) to 12.5nmol/L (range 6.1-43.1, IQR 10.1-16.6), P <0.001 (Figure 8.6B). The increase in $24,25(OH)_2D_3$ had no effect on the 25(OH)D:24(OH)2D3 VMR; this remained unchanged from baseline to 12 months: 8.7 (range 1.1-24.0, IQR 6.8-12.2) vs. 9.9 (range 3.2-20.2, IQR 7.6-12.5) respectively, P=.664 (Figure 8.6C). This suggests $24,25(OH)_2D_3$ increased proportionately to 25(OH)D. **Statistically** significant changes in the other VMR were seen. $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR reduced from T0 to T12: median 11.5 (range 0.0-67.8, IQR 5.9-20.6) vs. 7.8 (range 2.6-25.0, IQR 5.0-10.9) respectively, P = 0.011 (Figure 8.6E). A **statistically** significant reduction was also seen for $1,25(OH)_2D_3$:$25(OH)D_3$ VMR from baseline to 12 months: median 1.4 (range 0.0-5.0, IQR 0.9-2.3) vs. 0.8 (range 0.4-2.3, IQR 0.6-1.0), respectively, P = 0.001 (Figure 8.6F). Serum $1,25(OH)_2D_3$ significantly increased from 48.3pmol/L (range 0.0-96.9, IQR 36.3-57.8) to 96.2pmol/L (range 55.0-275.4, IQR 78.7-128.0), P <0.001 (Figure 8.6D). The results demonstrate changes in serum 25(OH)D and $24,25(OH)_2D_3$ are disproportionate to $1,25(OH)_2D_3$ increases, further demonstrating the tight regulation of $1,25(OH)_2D_3$ synthesis and secretion.

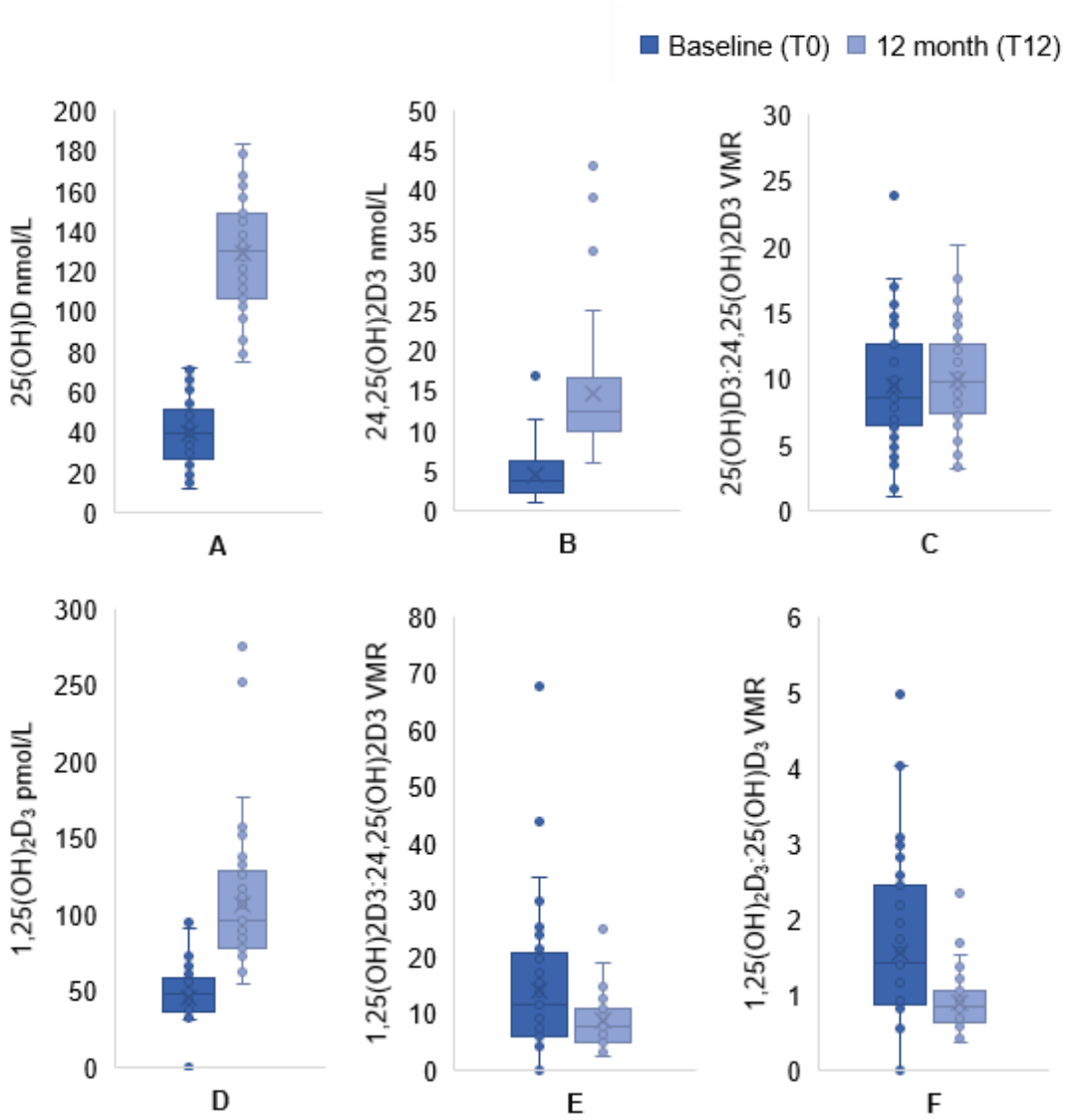

Figure 8.6 serum 25(OH)D, $24,25(OH)_2D_3$, 25(OH)D3:$24,25(OH)_2D_3$ VMR, $1,25(OH)_2D_3$, $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR, and $1,25(OH)_2D_3$:$25(OH)D_3$ VMR at baseline and 12 months.

Serum 25(OH)D, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were measured using LC-MS/MS and $25(OH)D_3$:$24,25(OH)_2D_3$, $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ and $1,25(OH)_2D_3$:$25(OH)D_3$ calculated in the sub-study subjects at baseline, pre cholecalciferol supplementation (T0), and again at 12 months (T12). Patients that were already vitamin D replete at baseline, and those that did not achieve serum 25(OH)D ≥75nmol/L at T12 were excluded. **(A)** Serum 25(OH)D increased: 40.1nmol/L (11.9-72.6, 28.4-51.5) vs. 129.2nmol/L (75.4-184.2, 107.9-149.3), P <0.001. **B)** Serum $24,25(OH)_2D_3$ increased: 3.9nmol/L (1.1-17.0, 2.3-6.0) vs. 12.5nmol (6.1-43.1, 10.1-16.6), P <0.001. **(C)** No significant change in $25(OH)D_3$:$24,25(OH)_2D_3$ VMR: 8.7 (1.1-24.0, 6.8-12.2) vs. 9.9 (3.2-20.2, 7.6-12.5) respectively, P = 0.664. **(D)** Serum $1,25(OH)_2D_3$ increased 48.3pmol/L (0.0-96.9, 36.3-57.8) vs. 96.2pmol/L (55.0-275.4, 78.7-128.0), <0.001. **(E)** $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR reduced : 11.5 (0.0-67.8, 5.9-20.6) vs. 7.8 (2.6-25.0, 5.0-10.9) P=0.011. **(F)** $1,25(OH)_2D_3$:$25(OH)D_3$ VMR also reduced: 1.4 (0.0-5.0, 0.9-2.3) vs. 0.8 (0.4-2.3, 0.6-1.0), P=0.001. Wilcoxon signed rank test. Data represent median (range and IQR), n = 47.

Relationships between the vitamin D metabolites and the VMRs were explored. A statistically significant correlation was seen between $25(OH)D_3$ and $24,25(OH)_2D_3$ at baseline and 12 months, ρ = 0.511, P = <0.001 (T0) and ρ = 0.294, P = 0.45 (T12) (Figure 8.7A and B). There was no correlation between $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ at either time point, ρ = 0.075, P = 0.616 (T0) and ρ = 0.005, P = 0.972 (T12) (Figure 8.8A and B).

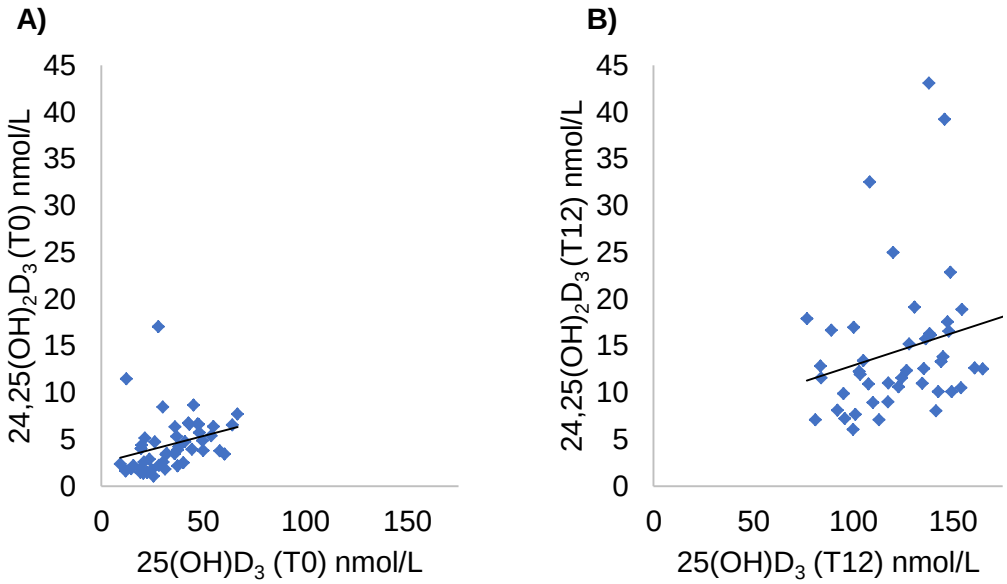

Figure 8.7 Correlation between mean $25(OH)D_3$ and $24,25(OH)_2D_3$ at A) baseline (T0), and B) 12 months (T12).
$25(OH)D_3$ and $24,25(OH)_2D_3$ were measured using LC-MS/MS in the sub-study subjects at baseline, pre cholecalciferol supplementation (T0), and again at 12 months (T12). Patients that were already vitamin D replete at baseline, and those that did not achieve serum 25(OH)D ≥75nmol/L were excluded. A statistically significant positive correlation was seen between serum $25(OH)D_3$ and $24,25(OH)_2D_3$ at baseline and this remained at 12 months, ρ = 0.511, P = <0.001 (T0) and ρ = 0.294, P = 0.45 (T12). Spearman's ρ (n=47).

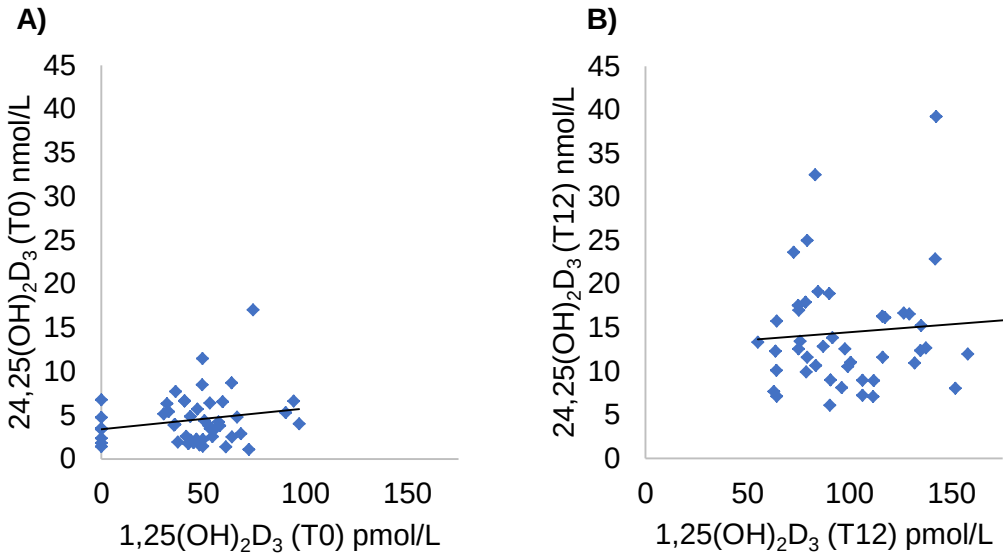

Figure 8.8 Correlation between mean $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ at baseline (A) and 12 months (B).
$24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were measured using LC-MS/MS in the sub-study subjects at baseline, pre cholecalciferol supplementation (T0), and again at 12 months (T12). Patients that were already vitamin D replete at baseline, and those that did not achieve serum 25(OH)D ≥75nmol/L were excluded. No correlation was seen between serum $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ at baseline or 12 months, ρ = 0.075, P = 0.616 (T0) and ρ = 0.005, P = 0.972 (T12). Spearman's ρ (n=47).

The relationship between $25(OH)D_3$ and $25(OH)D_3$:$24,25(OH)_2D_3$, VMR and $25(OH)D_3$ and $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR was explored and no significant correlation was seen at baseline or 12 months, ρ = 0.266, P = 0.071 (T0) and ρ = 0.187, P = 0.209 (T12), and ρ = -0.247, P = 0.095 (T0) and ρ = -0.174, P = 0.242 (T12) respectively (Figure 8.9 A,B,C and D).

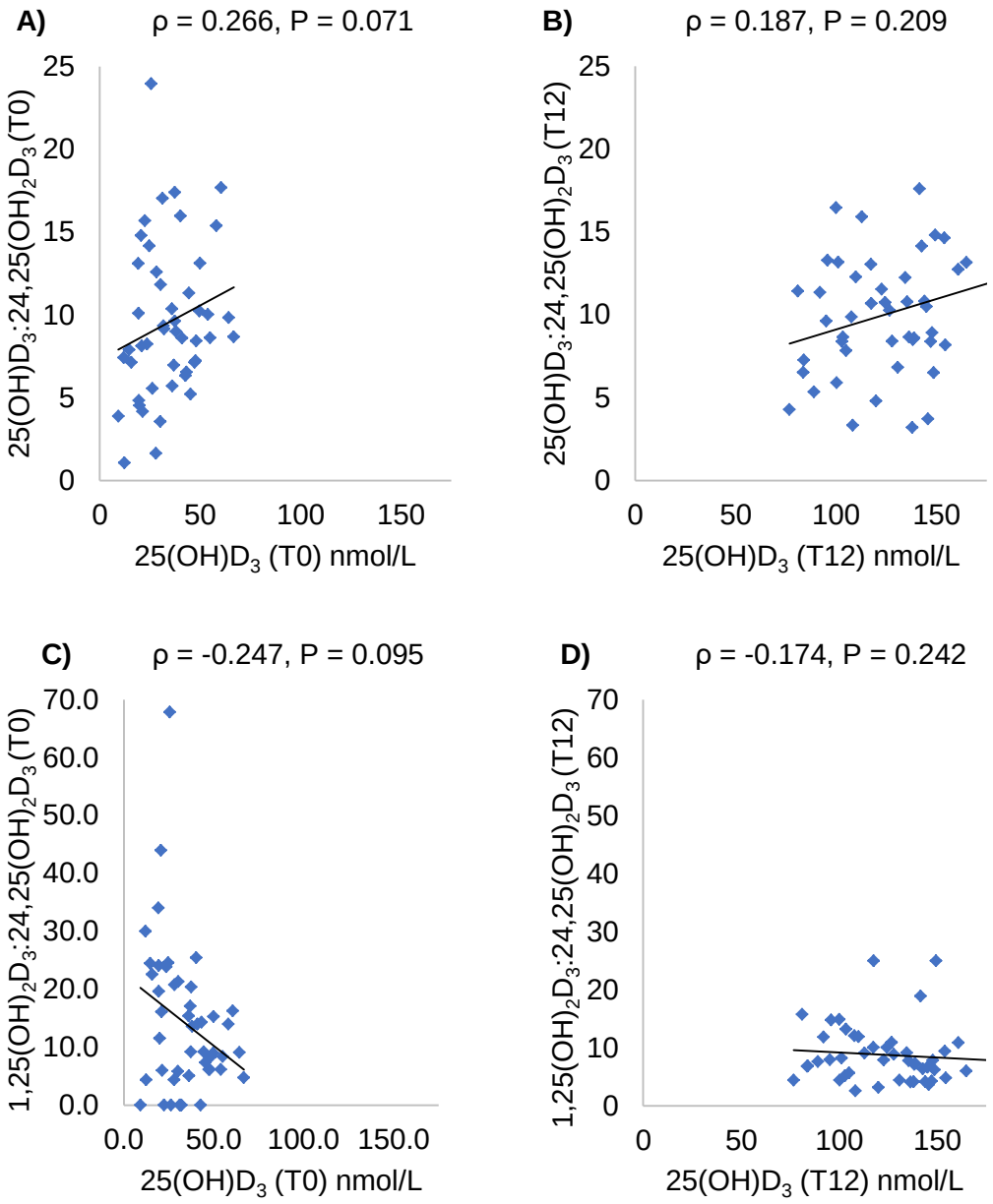

Figure 8.9 Correlation between mean $25(OH)D_3$:$24,25(OH)_2D_3$ and $25(OH)D_3$, and mean $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ and $25(OH)D_3$ at baseline and 12 months. $25(OH)_2D_3$, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$ were measured using LC-MS/MS in the sub-study subjects at baseline, pre cholecalciferol supplementation (T0), and again at 12 months (T12). $25(OH)D_3$:$24,25(OH)_2D_3$ and $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR were calculated and the relationship between $25(OH)D_3$ and VMRs explored; no significant correlations were seen. Patients that were already vitamin D replete at baseline, and those that did not achieve serum $25(OH)D_3$ ≥75nmol/L were excluded. Spearman's ρ (n=47).

8.4.3 PTH and VMR

Serum concentrations of 1,25$(OH)_2$D suppress PTH synthesis and secretion as part of the fundamental feedback regulation of calcium homeostasis (Haussler *et al.*, 2010). However, increased serum 25(OH)D is also associated with suppression of circulating PTH (Romagnoli *et al.*, 2008), suggesting that local synthesis of 1,25$(OH)_2$D via parathyroid expression of CYP27B1 may also play a role in regulating PTH synthesis (Kawahara *et al.*, 2008). Vitamin D deficiency is associated with an increase in the 1,25$(OH)_2$D:24,25$(OH)_2$D VMR demonstrating that as substrate (25(OH)D) availability diminishes, 1,25$(OH)_2$D synthesis is favoured over 24,25$(OH)_2$D production (Tang *et al.,* 2019). This may also further underline the importance of 25(OH)D vs. 1,25$(OH)_2$D as the principal inhibitor of PTH expression, with lower 25(OH)D resulting in elevated PTH and increased 1,25$(OH)_2$D. Young healthy individuals with serum 25(OH)D ≤50nmol/L, normal 1,25$(OH)_2$D, but high 1,25$(OH)_2$D:24,25$(OH)_2$D VMR have statistically significantly higher PTH levels than those with a 25(OH)D >50nmol/L and lower 1,25$(OH)_2$D:24,25$(OH)_2$D VMR (n = 940) (Tang *et al.,* 2019). These findings suggest 24,25$(OH)_2$D may have a role beyond catabolism. Increased 24,25$(OH)_2$D production may inhibit 25(OH)D and 1,25$(OH)_2$D activity, particularly extra-renal 1,25$(OH)_2$D synthesis, to down regulate PTH secretion whilst maintaining 1,25$(OH)_2$D at a concentration sufficient for calcium homeostasis (Tang *et al.,* 2019). Low 24,25$(OH)_2$D could stimulate PTH production enhancing the anabolic effects of vitamin D metabolism (Tang *et al.,* 2019). Alternatively, or additionally, increased 1,25$(OH)_2$D:24,25$(OH)_2$D VMR may indicate increased VDR sensitivity and thus more VDR induced expression of CYP24A1. Finally, it is important to acknowledge recent studies showing that 24,25$(OH)_2$D is able to signal independently of the VDR via the membrane receptor FAM57B2 (Martineau *et al.*, 2018). This mechanism has been shown to influence bone fracture repair but may also be associated with other physiological responses.

PTH results were explored in relation to 25$(OH)D_3$ and 1,25$(OH)_2D_3$:24,25$(OH)_2$D VMR in the sub-study one cohort to see if results are affected by the altered vitamin D metabolism, and SHPT present in ESRD (n = 47). Patients with low levels of 25$(OH)D_3$ (T0) and higher 1,25$(OH)_2D_3$:24,25$(OH)_2$D VMR had higher median PTH concentration (*ns*) (Table 8.3). In the presence of serum 25$(OH)D_3$ repletion (T12), there was a statistically significant difference in PTH concentration between 1,25$(OH)_2D_3$:24,25$(OH)_2$D VMR groups, P = 0.050 (Table 8.3). Overall higher PTH

exists with higher $1,25(OH)_2D_3$:$24,25(OH)_2D$ VMR supporting the findings of Tang and colleagues (2019).

PTH, pmol/L (median, range and IQR)		25(OH)D_3 (nmol/L) T0		25(OH)D_3 (nmol/L) T12
		<30	30-74	≥75nmol/L
$1,25(OH)_2D_3$: $24,25(OH)_2D$ VMR	>20	45 (5-91, 28-43)	9 (4-16, 7-13)	102 (41-162, 71-132)
	10-20	22 (18-41, 20-32)	26 (2-68, 8-46)	41 (9-66, 30-52)
	<10	17 (2-74,12-35)	26 (7-147, 20-32)	20 (3-78, 13-37)
Kruskal-Wallis		P = 0.338	P = 0.142	P = 0.050
25(OH)D_3: $24,25(OH)_2D$ VMR	>20	55 **	*	41 **
	10-20	27 (5-63, 15-38)	20 (4-147, 13-48)	34 (6-162, 16-48)
	<10	33 (2-91, 17-54)	24 (2-80,15-30)	17 (3-78, 12-40)
Kruskal-Wallis		P = 0.589	P = 0.711	P = 0.323

Table 8.3 PTH concentrations in categories of vitamin D status and increasing $1,25(OH)_2D_3$:$24,25(OH)_2D$ and 25(OH)D:$24,25(OH)_2D$ VMRs.
Cholecalciferol supplementation was initiated at baseline (T0). PTH was measured as part of standard care and serum 25(OH)D_3, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were measured by LC-MS/MS as T0 and 12 months (T12). PTH concentration decreased significantly from high to low $1,25(OH)_2D_3$:$24,25(OH)_2D_3$ VMR at 12 months in patients with serum 25(OH)D_3 ≥75nmol/L. There was no statistically significant difference in PTH concentration between $1,25(OH)_2D_3$:$24,25(OH)_2D$ and 25(OH)D_3:$24,25(OH)_2D$ VMR groups at baseline, or 25(OH)D_3:$24,25(OH)_2D$ VMR groups at 12 months. Median (range, IQR). n = 47. * = no PTH values fell within this group, **single value therefore no range reported.

To further investigate PTH in relation to 25(OH)D, results from the main study cohort (PTH and total 25(OH)D) were explored at baseline (T0) and 12 months (T12). 25(OH)D and 25(OH)D:PTH VMR results were compared according to PTH groupings. PTH groups were defined as follows: over-suppressed (<8pmol/L), on

target (8-38pmol/L), high (39-84pmol/L) and very high (≥85pmol/L). There was a statistically significant difference in 25(OH)D concentration between PTH groups at baseline in the presence of deficiency (Table 8.4A), n = 323, but not at 12 months when most patients were serum 25(OH)D replete (Table 8.4B), n =246. There was a statistically significant difference in the 25(OH)D:PTH VMR between PTH groups at both baseline and at 12 months (Table 8.4 – A and B).

(A)

T0 (n)	PTH <8 (23)	PTH 8-38 (193)	PTH 39-84 (67)	PTH ≥85 (40)	Kruskal Wallis
25(OH)D nmol/L	30 (0-135 21-52)	21 (0-140 12-34)	26 (0-93 12-38)	18 (0-87 12-38)	P = 0.042
25(OH)D:PTH (median, range and IQR)	13.00 (0.00-48.00 4.53-17.92)	1.00 (0.00-11.20 0.50-2.08)	0.46 (0.00-1.94 0.20-0.64)	0.14 (0-0.94 0.22-0.62)	P <0.001

(B)

T12 (n)	PTH <8 (25)	PTH 8-38 (134)	PTH 39-84 (62)	PTH ≥85 (25)	Kruskal Wallis
25(OH)D nmol/L	117 (63-159 98-138)	108 (14-175 79-134)	115 (17-168 96-137)	109 (49-172 78-133)	P =0.211
25(OH)D:PTH (median, range and IQR)	27.62 (9.14-53.00 19.43-37.00	6.20 (1.27-17.88 3.59-8.28	2.20 (0.42-3.82 1.70-2.88)	0.79 (0.48-1.31 0.79-0.93)	P <0.001

Table 8.4 Serum 25(OH)D and 25(OH)D:PTH ratio in categories of increasing PTH concentration.
Cholecalciferol supplementation was initiated at baseline and PTH and 25(OH)D were measured every 3 months as part of standard routine care. Baseline (T0) and 12 months (T12) 25(OH)D and 25(OH)D:PTH VMR results were compared according to PTH groupings. PTH groups were as follows: over-suppressed (<8pmol/L), on target (8-38pmol/L), high (39-84pmol/L) and very high (≥85pmol/L). There was a statistically significant difference between serum 25(OH)D concentration between PTH groups at baseline in the presence of deficiency (A), n = 323, but not at 12 months (B), n =246. There was a statistically significant difference in the 25(OH)D:PTH VMR between PTH groups at baseline (A) and at 12 months (B). Median (range, IQR).

These data indicate a sensitive relationship between serum 25(OH)D and PTH at baseline (T0), with suppression of PTH to <8pmol/L achieved with 25(OH)D of 30 nmol/L and 25(OH)D:PTH ratio of 13.00. By contrast, at T12 median serum 25(OH)D was elevated to >100 nmol/L but suppression of PTH to <8pmol/L was only achieved with a 25(OH)D:PTH ratio of 27.62. It therefore appears that although serum 25(OH)D is linked to PTH levels, there may be a threshold above which no

further benefit is achieved in terms of PTH suppression. Likewise, some patients appear to be resistant to the PTH effects of elevated 25(OH)D as 25 patients had PTH >85pmol/L despite repleted 25(OH)D (109 nmol/L) at T12. $1,25(OH)_2D$ data was not available for these patients to determine if this was due to lack of $1,25(OH)_2D$ synthesis and secretion. One contributory factor could be loss of VDR expression and activation due to parathyroid hyperplasia (McCann & Beto, 2010).

8.4.4 The 3-epimer of 25(OH)D – results from sub-study one

Levels 3-epi-25(OH)D_3 was measured in sub-study patients at baseline and 12 months (n=55). Detectable levels were found in 100% of subjects (55 of 55). Data was analysed for all patients that also had total 25(OH)D data (in addition to 3-epi-25(OH)D_3) available (n=53). At baseline mean 3-epi-25(OH)D_3 levels were 3.7±2.1nmol/L (median 3.2nmol/L, range 0.5-10.8nmol/L); this increased to 6.4±3.0nmol/L (median 6.1nmol/L, range 2.4-15.3nmol/L) at 12 months, P = <0.001. The percentage of 3-epi-25(OH)D_3 to total 25(OH)D significantly reduced following cholecalciferol supplementation; the concentration of epi-25(OH)D_3 was 9.7% of total 25(OH)D on average at baseline and 5.7% at 12 months, P = <0.001, range 1.3-28.9% and 1.7-37.8% respectively. Results are presented in Table 8.5.

	3-epi-25(OH)D_3 T0 (nmol/L)	3-epi-25(OH)D_3 T12 (nmol/L)	Wilcoxon signed rank	% of 3-epi-25(OH)D_3 to 25(OH)D T0	% of 3-epi-25(OH)D_3 to 25(OH)D T12	Wilcoxon signed rank
Mean ±SD	3.7±2.1	6.4±3.0	P = <0.001	9.7±6.4	5.7±5.0	P = <0.001
Median (range, IQR)	3.2 (0.5-10.8, 2.1-4.5)	6.2 (2.4-15.3, 4.3-7.8)		7.6 (1.3-28.9, 5.6-10.1)	4.6 (1.7-37.8, 3.6-6.8)	

Table 8.5 3-epi-25(OH)D_3 concentrations of the sub study patients
3-epi-25OHD_3 was measured using LC-MS/MS in the sub-study subjects at baseline, pre cholecalciferol supplementation (T0), and again 12 months after the introduction of routine cholecalciferol supplementation (T12). 3-epi-25(OH)D_3 concentration increased significantly from T0 to T12 (P = <0.001) but the percentage of 3-epi-25(OH)D_3 to total 25(OH)D significantly reduced (P = <0.001), n=53.

8.5 Discussion

It is likely that as LC-MS/MS technology advances and clinical research continues to shed light on interpretation and utilisation of vitamin D metabolites, the expansion of multi-metabolite assays into the clinical setting will be possible. At present measurement of a single metabolite, 25(OH)D, remains the marker of vitamin D status, and it is still unclear whether free 25(OH)D offers increased diagnostic value. The limitations of 25(OH)D in the clinical setting largely fall down to the assay. The results presented here, comparing the immunoassay against LC-MS/MS, demonstrate negative bias; the Elecsys immunoassay underestimated serum 25(OH)D.

The Vitamin D Standardisation Programme (VDSP) facilitates standardisation of clinical and research assays (NIH, 2017). A recent study run by the VDSP sought to assess whether 25(OH)D assay performance has improved, through comparison of 25(OH)D results, obtained from 15 different laboratories using 8 immunoassays and 8 LC-MS/MS assays on the same 50 serum samples; (Wise *et al.,* 2017a). Results were compared against reference measurement procedures and VDSP performance criteria (coefficient of variation (CV) ≤10% and bias ≤5%). All LC-MS/MS assays met the VDSP CV criteria (range 3.3 – 8.9%), and 75% met the bias criteria (range -6 – 7%), whereas only 50% of immunoassays met CV criteria (range 4.3 – 21.9%), and 38% bias criteria (range -20 – 23%). Study findings confirm standardisation of assays is warranted. The results presented in section 8.1 show the Elecsys immunoassay has a bias of -13.1 to -15.2, therefore VDSP bias criteria (≤5%) were not met.

Vitamin D treatment is predominantly led by vitamin D status categories rather than exact values therefore it is important that any assay variation does not cause misclassification of vitamin D status. Retrospective standardisation of data from the Irish National Adult and Nutrition Survey (NANS) using VDSP protocols resulted in prevalence of vitamin D deficiency (serum 25(OH)D <30nmol/L) increasing from 6.5% to 11.4% (n = 1118); a misdiagnosis in 55 people (Cashman *et al.,* 2013). In order to validate findings, the authors re-analysed a proportion of samples (n=99), using LC-MS/MS; this supported the findings, showing a 11.2% prevalence of deficiency (Cashman *et al.,* 2013).

Even in the presence of ESRD, where vitamin D metabolism is altered, it is evident that repletion of 25(OH)D offers repletion of other vitamin D metabolites; namely $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. The vitamin D metabolite, and VMR, response to cholecalciferol supplementation is summarised in Table 8.6.

Metabolite / VMR	$25(OH)D_3$ repletion response	Explanation of response
$25(OH)D_3$ (nmol/L)	Increase	↑ D3 = ↑ 25-hydroxylation = ↑ $25(OH)D_3$
$1,25(OH)_2D_3$ (pmol/L)	Increase	↑ $25(OH)D_3$ = ↑ 1α-hydroxylation = ↑ $1,25(OH)_2D_3$
$24,25(OH)_2D_3$ (nmol/L)	Increase	1. ↑ $25(OH)D_3$ = more substrate availability = ↑$24,25(OH)_2D_3$ 2. ↑ $25(OH)D_3$ = ↑ $1,25(OH)_2D_3$ = ↑ 24-hydroxylase activity = ↑$24,25(OH)_2D_3$
$25(OH)D_3$: $24,25(OH)_2D_3$	No change	$25(OH)D_3$ and $24,25(OH)_2D_3$ increase proportionately
$1,25(OH)_2D_3$: $25(OH)D_3$	Reduce	1. ↑ $25(OH)D_3$ 2. 1α-hydroxylation tightly regulated 3. $1,25(OH)_2D$ and $25(OH)D_3$ do not ↑ proportionately 4. ↓ ratio of $1,25(OH)_2D_3$:$25(OH)D_3$
$1,25(OH)_2D_3$: $24,25(OH)_2D_3$	Reduce	1. ↑ $25(OH)D_3$ 2. ↑ $24,25(OH)_2D_3$ 3. 1α-hydroxylation tightly regulated 4. $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ do not ↑ proportionately 5. ↓ ratio of $1,25(OH)_2D_3$:$24,25(OH)_2D_3$

Table 8.6 The vitamin D metabolite, and VMR, response to serum $25(OH)D_3$ repletion.

VMR is used to present the concentration of one metabolite in relation to another; changes in the VMR in response to serum changes in serum 25(OH)D and/or $1,25(OH)_2D_3$ give insight into vitamin D metabolism. $1,25(OH)_2D$, $24,25(OH)_2D_3$, $25(OH)D$:$24,25(OH)_2D_3$ VMR and $1,25(OH)_2D$:$24,25(OH)_2D_3$ VMR may be valuable diagnostic measures in patients presenting with unexplained hypercalcaemia. The use of VMR is shown to be useful in states of altered vitamin D metabolism, for example the $1,25(OH)_2D$:$25(OH)D$ VMR can help diagnose sarcoidosis and the $25(OH)D$:$24,25(OH)_2D_3$ VMR can be used to diagnose loss of CYP24A1 function (Dinour *et al.*, 2013; Hilderson *et al.*, 2014; Kowalska *et al.*, 2021; Rohmer *et al.*, 2020). The evaluation of $24,25(OH)_2D_3$ as a single metabolite does not confirm disturbed CYP24A1 function because the concentration depends on 25(OH)D availability. Low $24,25(OH)_2D_3$ could simply reflect low 25(OH)D. Whereas

25(OH)D: $24,25(OH)_2D_3$ VMR is a viable marker of altered 24-hydroxylase activity (Kowalska *et al.*, 2021).

The use of metabolite ratios to aid clinical diagnosis is not new. Urinary steroid metabolite profiles, for example urinary cortisol:cortisone ratio, have been used in the diagnosis of congenital steroid disorders (for example Cushing's syndrome) for decades (Antonelli *et al.*, 2014; Quinkler & Stewart, 2003; Shackleton, 1993; Soro *et al.*, 1995; Stewart *et al.*, 1988; Storbeck *et al.*, 2019). The use of steroid metabolomics, within routine diagnostic testing of disorders of adrenal steroid synthesis and metabolism, is being considered (Storbeck *et al.*, 2019). Whilst research is steering towards more comprehensive analysis of metabolites (Tuckey *et al.*, 2019), the wider usefulness of multiple vitamin D metabolites and application of their ratios clinically is yet to be determined. At present, the problem is knowing when to measure (what clinical situations the multiple vitamin D metabolite profile is useful for) and how to measure. Despite vitamin D multi-metabolite assay analysis not being performed in clinical laboratories, clinical samples can be tested for $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ in the UK. Professor. William Fraser at The University of East Anglia is director of the Supra-regional Assay Service for bone metabolism and calcium homeostasis and carries out multi-metabolite profiling to aid clinical diagnosis.

Chapter 9 Discussion and conclusion

The introduction to this thesis highlighted the high prevalence of vitamin D deficiency, and the absence of specific recommendations for vitamin D (ergocalciferol or cholecalciferol) supplementation, in ESRD.

This Ph.D study provides the largest study to date, investigating the effects of vitamin D supplementation in HD patients. A multicentre RCT is currently underway in the UK, investigating the effects of cholecalciferol on survival (Hiemstra, 2015); recruitment for this is still ongoing, and the study is expected to run until at least 2023. To my knowledge, no other UK based study, investigating cholecalciferol use in ESRD, has been carried out. The largest cohort study, published to date, took place in Portugal, and included 158 HD patients; the largest RCT was carried out in the US, using ergocalciferol rather than cholecalciferol, and included 105 HD patients (Matias *et al.,* 2010; Bhan *et al.,* 2015). The limited number of studies to date, and small study size, has meant an optimal 25(OH)D target in ESRD, and how to achieve it, has yet to be defined (Jean *et al.*, 2017).

9.1 Introduction of 25(OH)D screening and cholecalciferol supplementation

The Endocrine Society (2011), and K/DOQI (2003), serum 25(OH)D target of 75nmol/L was adopted for our HD population, and a systematic review was undertaken in order to establish what dose of cholecalciferol would be needed to achieve it.

Data revealed low serum 25(OH)D levels in 94% (309 of 328) of UHCW HD patients; a prevalence similar to reports by other studies (Saab *et al.*, 2007; Tokmak *et al.*, 2008; Wolf *et al.*, 2007). The initial supplementation protocol of 40,000IU weekly for 12 weeks, for repletion, followed by 20,000IU weekly, for maintenance, resulted in serum 25(OH)D levels increasing to >150nmol/L (the clinical guideline upper limit is 150nmol/L) in 12.3% of patients (35 of 285) at 3 months, and 24.6% at 6 months (65 of 264). In response, the clinical guideline was reviewed and the maintenance dose of cholecalciferol was reduced from 20,000 units per week to 20,000 units every other week. Due to the long half-life of cholecalciferol (~2

months), serum 25(OH)D levels are considered to peak at 14 days (Armas *et al.*, 2004; Mawer *et al.*, 1971), the impact of the initial repletion dose would continue beyond its withdrawal. A high dose loading phase followed by a lower, less frequent, maintenance dose has been associated with reduced efficacy, perhaps due to increased initial catabolism by 24-hydroxylase (CYP24A1), and risk of initial overdosing (Courbebaisse *et al.*, 2009; Tokmak *et al.*, 2008). However, adequate repletion is not always achieved without a loading phase (Tangpricha & Wasse, 2014). The UHCW guideline had a conservative upper limit of 150nmol/L, 250nmol/L is considered safe (Holick, 2007). Due to the limitations of the 25(OH)D assay used at UHCW, it is unable to detect levels above 175nmol/L, 175nmol/L is the highest concentration the guideline could adopt without risking not knowing an individuals' serum 25(OH)D concentration. If 200nmol/L had been a feasible upper target, the results may have been different, and the original version of the supplementation guideline, may well have remained.

9.2 Efficacy of supplementation

The supplementation regimen was effective at increasing and maintaining serum 25(OH)D ≥75nmol/L at a population level; between 83% (219 of 264) and 93% (105 of 113) of patients had serum 25(OH)D levels ≥75nmol/L from month 6 to 15. This level of repletion success was in keeping with the studies used to inform the guideline (Jean *et al.,* 2009; Daroux *et al.,* 2012; Ozkurt *et al.,* 2013; Bucharles *et al.,* 2012; Armas *et al.,* 2013). However, other studies have shown an inferior repletion success of 0-62% (Jakopin *et al.,* 2014; Armas *et al.,* 2012; Massart *et al.,* 2014; Tokmak *et al.,* 2008). Reasons for this are believed to relate to varying dose regimens, shorter intervention periods, and poor compliance (Jean *et al.,* 2017). However other influences on serum 25(OH)D response cannot be ruled out. Such factors include BMI, weight, body fat, waist circumference, ethnicity, and genetics (Aloia *et al.*, 2008; Barger-Lux *et al.*, 1998; Blum *et al.*, 2008; Gallagher *et al.*, 2013; Gallagher *et al.*, 2012; Giusti *et al.*, 2010; Manousaki *et al.*, 2020; Mazahery *et al.*, 2015).

25(OH)D has been shown to increase 20% less in people with BMI ≥30 kg/m² compared with BMI <25 kg/m² (Blum *et al.*, 2008). Aloia and colleagues (2008) reported African Americans require 50% higher doses than white Americans to achieve 25(OH)D concentrations ≥75 nmol/L by 18 weeks. In this study the African

Americans had lower baseline 25(OH)D levels, making it difficult to decipher the impact of ethnicity (Aloia *et al.*, 2008). Recently, the genome wide association study (GWAS) has demontrated that genetic background can influence 25(OH)D response by 5-7.5% (Manousaki *et al.*, 2020). This may go some way to explaining the variable individual response, to both vitamin D_2 and D_3, seen in a 12 month study of a predominantly Caucasian population (95%), aged >65 years, regardless of good compliance ≥91% (Binkley *et al.*, 2011). These studies further illustrate the complexity of vitamin D metabolism and question whether a standard cholecalciferol dose can be effective in everyone.

The aim of the study was to investigate if a one dose fits all supplementation strategy could be successfully applied in clinical care. Results demonstrate this approach is effective in the majority. More thorough investigation into the exact reasons why optimal levels were not achieved in 100% of patients was beyond the scope of this study. In this cohort of patients, it could be attributed to high BMI, older age, and ethnic background. Whether a guideline that incorporates more individualised dosing could improve this percentage is an interesting question, and one that is not clearly answered by existing literature (Mazahery & von Hurst, 2015).

9.3 Safety

Cholecalciferol supplementation was shown to be safe. Mean corrected calcium increased, but remained within the target range, and positively, the incidences of hypocalcaemia significantly reduced. These results differ from other studies which have shown cholecalciferol has no effect on serum corrected calcium (Mose *et al.,* 2014; Delanaye *et al.,* 2013; Seibert *et al.,* 2012; Marckmann *et al.,* 2012; Armas *et al.,* 2012; Wasse *et al.,* 2012; Obi *et al.* 2020). This could be due to the longer follow up period in this study, better compliance, lack of exclusion criteria, or the high usage of active vitamin D analogues. 71% (249 of 350) of UHCW HD patients were prescribed an active analogue at baseline whereas other studies have excluded patients that have had active analogues within 1-6 months of the study start (Massart *et al.*, 2014; Mieczkowski *et al.*, 2014). Assessing safety of cholecalciferol through analysis of urinary calcium, a more sensitive marker, has been carried out by others (Kimball *et al.*, 2011; Leaf *et al.*, 2012); however, in ESRD where many patients are anuric, this is not feasible.

PTH is the dominant regulator of calcium homeostasis, not 25(OH)D; therefore, as expected, there was no correlation between 25(OH)D and corrected calcium pre, or post, serum 25(OH)D repletion. Once 25(OH)D repletion was achieved, a statistically significant increase in $1,25(OH)_2D$ occurred, and a statistically significant correlation between corrected calcium and $1,25(OH)_2D$ was seen. This finding is interesting as it demonstrates that in the presence of ESRD, where 1α-hydroxylase is known to be reduced, 1α-hydroxylation is able to occur and facilitate calcium homeostasis. The results support existing literature, showing that calcium is not directly related to 25(OH)D, but rather indirectly through the actions of $1,25(OH)_2D$. Production of $1,25(OH)_2D$ is tightly regulated so optimising 25(OH)D through cholecalciferol supplementation will not result in unlimited $1,25(OH)_2D$ and severe hypercalcaemia.

Management of SHPT in ESRD has historically focused on active vitamin D analogues. Whilst the latest KDIGO recommendations suggest serum 25(OH)D may be checked and, if low, supplemented in CKD patients, including those on dialysis (KDIGO, 2017), clinicians remain sceptical about the safety of cholecalciferol in the presence of already prescribed active vitamin D analogues. Although studies have demonstrated that the use of concurrent therapies is safe (Mose *et al.,* 2014; Delanaye *et al.,* 2013; Seibert *et al.,* 2012) there remains an absence of large trials and clear guidance, to direct clinicians in knowing what dose of cholecalciferol should be used. This Ph.D study demonstrated that a repletion dose of 40,000IU weekly, and maintenance dose of 20,000IU fortnightly is both effective and safe. Unlike certain other studies, where active analogues have been discontinued prior to initiation of cholecalciferol (Massart *et al.*, 2014; Mieczkowski *et al.*, 2014, Jean *et al.,* 2009), the patients in this study remained on active analogue therapy, and this continued to be adjusted by the renal physicians according to corrected calcium and PTH levels. Concurrent cholecalciferol and active analogue treatment were shown to be safe; 25(OH)D should therefore be repleted in ESRD regardless of active analogue use.

9.4 Chronic kidney disease – mineral bone disorders

Results suggest that cholecalciferol may offer improved management of CKD-MBD markers, at least in those with the highest PTH and lowest corrected calcium levels pre supplementation. Results from existing studies of cholecalciferol

supplementation in HD patients have varied from demonstrating no effect on PTH, calcium and phosphate, to showing a beneficial effect on PTH, and unfavourable effect on phosphate (Marckmann *et al.,* 2012; Armas *et al.,* 2012; Wasse *et al.,* 2012; Obi *et al.,* 2020; Mose *et al.,* 2014; Tokmak *et al.,* 2008; Saab *et al.,* 2007; Matias *et al.,* 2010; Jean *et al.,* 2009; Delanaye *et al.,* 2013).

The most comparable study of cholecalciferol supplementation in HD patients to date (n = 107), in terms of dose (100,000IU per month), serum 25(OH)D ≥75nmol/L repletion success (91%) and follow up period (15 months) was an open labelled, single centre, intervention study, carried out by Jean and colleagues in France (2009). Increased serum 25(OH)D from 32±13 at baseline to 105.8±27nmol/L at 15 months ($P < 0.001$) resulted in a 35.6% reduction in PTH ($P < 0.05$). There was no change in phosphate or calcium. Patients receiving active analogues were excluded from this study.

Another open labelled, single centre, intervention study, carried out in 30 HD patients in the US using a weekly cholecalciferol dose of 20,000IU, for 3 months, saw a 91% (serum 25(OH)D ≥75nmol/L) repletion success yet no effect on calcium, PTH or phosphate (Armas *et al.,* 2013). Whereas a RCT of the same study size carried out in Belgium, using 25,000IU a fortnight, resulted in a repletion success of 75% at 12 months resulting in a 37% reduction in PTH ($P = 0.02$) but no change in calcium and phosphate (Delanaye *et al.,* 2013). Results suggest a longer follow up period is needed in order to see benefits of cholecalciferol supplementation on PTH levels in HD patients.

Baseline serum corrected calcium in the three studies discussed above was median 2.30nmol/L (IQR 2.15-2.45) (Armas *et al.,* 2013), mean 2.24±0.12nmol/L (Jean *et al.,* 2009) and mean 2.18±0.15nmol/L (Delanaye *et al.,* 2013). Two of the studies reported lower baseline serum calcium levels compared with the UHCW HD patient study cohort (mean 2.29±0.13). Despite this, there was no statistically significant change in serum corrected calcium following cholecalciferol supplementation reported (Armas *et al.,* 2013; Delanaye *et al.,* 2013). The lack of significance may relate to the small study size; both studies reported data on 30 participants.

To explore data from this PhD further, grouped analysis was carried out for each parameter. Interestingly, corrected calcium, phosphate and PTH significantly reduced in patients with the highest baseline levels of each parameter. Therefore,

although an increase in corrected calcium and phosphate occurred overall, if levels were already high, they did not increase further; instead, they reduced. This reinforces the safety of cholecalciferol supplementation, as well as suggesting a role in optimising CKD-MBD management. Further support for this role is the statistically significant increase in PTH seen in those with low PTH concentration at baseline; implying cholecalciferol does not cause the same risk of over suppression and adynamic bone disease, as active vitamin D analogue use. This has not been identified in previous studies.

9.5 Anaemia

The active form of vitamin D, $1,25(OH)_2D$, has been shown to have anti-inflammatory effects (Wu *et al.*, 2011). Therefore, it was anticipated that improving serum 25(OH)D, and concomitant conversion to $1,25(OH)_2D$, would reduce EPO requirements. This was demonstrated by a statistically significant, albeit small, reduction in monthy EPO usage following cholecalciferol supplementation. Given the complex nature of renal anaemia and the confounding variables, it is difficult to decipher the exact impact of improved serum 25(OH)D on EPO use. High doses of EPO, to target higher Hb concentrations, are associated with increased morbidity and mortality risk; clinicians may have reduced EPO, or opted against increasing EPO, for this reason, rather than a reduced requirement due to improved serum 25(OH)D (KDOQI, 2007; Phrommintikul *et al.*, 2007; Szczech *et al.*, 2008; Vaziri, 2008).

The lack of control arm prevents clear conclusions from being drawn. Two RCTs looking at the effects of vitamin D supplementation in HD patients found increased serum 25(OH)D had no effect on EPO use/resistance, ferritin or Hb (Bhan *et al.*, 2015; Obi *et al.*, 2020). An increase in total iron binding capacity was seen in one study, $P < 0.05$ (Bhan *et al.*, 2015). Existing data linking increased 25(OH)D with reduced ESA use, are either observational, or small, non-randomised, studies (Saab *et al.,* 2007; Lac *et al.,* 2010; Kiss *et al.,* 2011; Rianthavorn & Boonyapapong, 2013).

Results here demonstrate that 25(OH)D repletion positively impacts on ferritin, increasing concentrations in patients, apart from those, in whom, ferritin levels were already too high. A reduction in ferritin was seen in those with the highest baseline

(ferritin) levels. Similarly, whilst there was no change in hepcidin overall, levels were reduced in those with the highest hepcidin concentration at baseline. Given both hepcidin and ferritin are inflammatory markers (Andrews, 2004; Rambod *et al.*, 2008), these results may be due to the anti-inflammatory actions of increased serum 25(OH)D. However, $1,25(OH)_2D$ is a transcription suppressor of hepcidin gene expression, and consequently vitamin D may exert direct effects on hepcidin levels (Bacchetta *et al.*, 2012).

The only RCT investigating the effects of cholecalciferol supplementation in HD patients on anaemia and hepcidin as primary endpoints, demonstrated an increase in median hepcidin in the intervention arm, however the study size was small (<58 patients received cholecalciferol) and the follow up period was only 6 month (Obi *et al.*, 2020). A significant increase in serum hepcidin was reported between the cholecalciferol group and control group at day 3 ($P < 0.005$), and month 6 ($P < 0.05$). However, the distribution of data (lower IQR in the cholecalciferol arm) suggests hepcidin levels were lower at 6 months and hepcidin decreased in some patients (Figure 9.1 and Table 9.1) (Obi *et al.,* 2020). Serum 25(OH)D concentration increased in the cholecalciferol group from 24.7nmol/L (IQR 20.0-31.0) at baseline to 59.7 (IQR 48.7-70.6) and 57.9nmol/L (IQR 50.9-72.1) at month 3 and 6, respectively. Serum 25(OH)D concentration was statistically significantly higher in the intervention group than placebo group at month 3 and 6 ($P<0.001$) Table 9.1. The intervention group did not reach 25(OH)D repletion based on ES guidelines (Holick *et al.,* 2011).

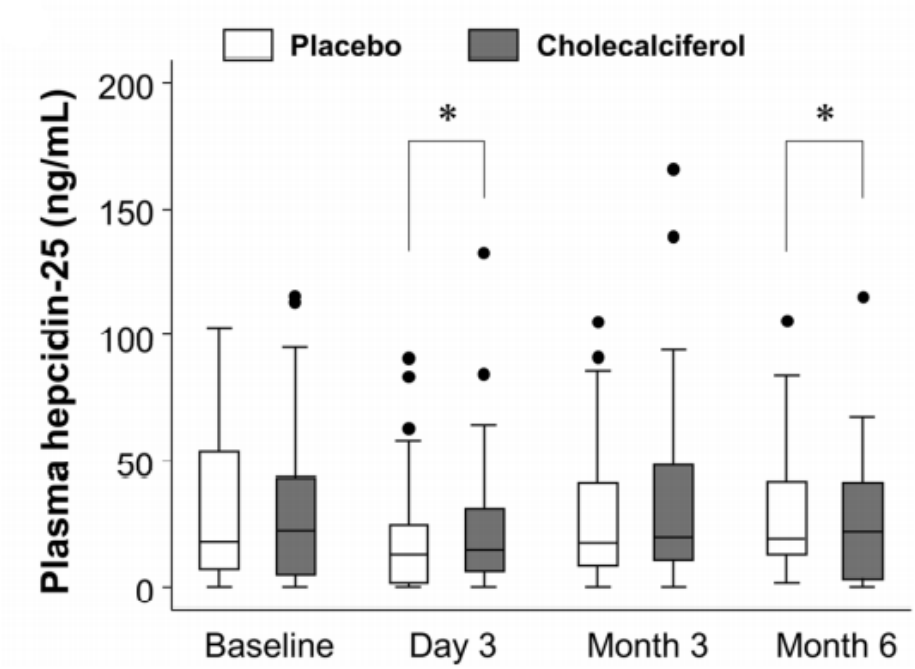

Figure 9.1 Changes in hepcidin concentration in placebo and cholecalciferol groups.
Median hepcidin was higher in the cholecalciferol arm vs. placebo at day 3 and month 6 with adjustment for baseline values *P <0.05 (Obi *et al.,* 2020).

	Group	Baseline	Day 3		Month 3		Month 6	
Hepcidin (ng/mL)	Placebo	18.0 (7.5-54.0)	13.4 (2.1-24.9)	P = 0.004	17.8 (9.1-41.6)	P = 0.5	19.7 (13.5-42.0)	P = 0.04
	D3	22.8 (5.2-43.1)	15.1 (6.4-31.2)		20.1 (11.1-48.1)		22.0 (3.9-41.4)	
Serum 25(OH)D nmol/L	Placebo	30.2 (24.0-37.7)	33.7 (22.5-38.4)	difference not reported	32.2 (26.5-41.4)	P <0.001	32.9 (30.5-39.9)	P < 0.001
	D3	24.7 (20.0-31.0)	34.1 (28.0-41.7)		59.7 (48.7-70.6)		57.9 (50.9-72.1)	

Table 9.1 Changes in serum 25(OH)D and hepcidin concentration in placebo and cholecalciferol groups.
Serum 25(OH)D concentration was **statistically** significantly higher in the cholecalciferol group (D3) than placebo group at month 3 and 6. Median hepcidin was higher in the D3 group vs. placebo at day 3 and month 6 with adjustment for baseline values. The IQR for hepcidin at month 6 was lower in the D3 arm compared with placebo, 3.9-41.4ng/mL vs. 13.5-42.0ng/mL (Obi *et al.,* 2020).

Like many anaemia markers, interpreting the hepcidin response (to increased 25(OH)D) is difficult due to confounding variables, including EPO use, which is considered to effect serum hepcidin concentration (Shoji *et al.*, 2013). As such, it is difficult to decipher the exact impact of improved serum 25(OH)D on anaemia management. More research, in the form of large RCTs, is warranted, in order to confirm the benefits seen in this study.

9.6 Health-related quality of life

Dialysis treatment success was traditionally represented by survival; yet the importance of HRQOL as an outcome measure for dialysis treatment, is increasingly being accepted (Aguiar *et al.*, 2019; Grincenkov *et al.*, 2013; Kušleikaitė *et al.*, 2010; Mapes, 2004). Multiple factors are considered to affect HRQOL in ESRD, including clinical manifestations of disease, treatment burden and side-effects, as well as social relationships (Valderrábano *et al.*, 2001).

This study did not show any improvement in HRQOL measures in response to cholecalciferol supplementation. Complete follow up data for all 4 time points was only available for 50 patients; study size is a limiting factor. HRQOL has been shown to deteriorate overtime in HD patients, as demonstrated by a 4 year study of 183 HD patients in Lithuania (Kušleikaitė *et al.*, 2010). The fact the HRQOL of the current study population did not worsen, may therefore be a positive outcome; vitamin D supplementation may mitigate HD patients' decline. The comparatively short follow up period, and absence of control arm, prevents this from being clearly determined.

As discussed in section 1.9 there is some evidence supporting a role for vitamin D supplementation in improving HRQOL. Evidence in CKD populations is lacking, yet numerous studies have looked at vitamin D and HRQOL in a range of populations (Hoffmann *et al.*, 2015). These studies have largely been observational, providing correlations between vitamin D and HRQOL; low serum 25(OH)D levels are linked with muscle atrophy, increased falls, musculoskeletal pain, and poorer physical function, all of which impact on HRQOL (Boudville *et al.*, 2010). In the absence of supplementation, vitamin D is largely provided in response to skin sunlight exposure; higher levels are expected in people that spend more time outdoors'. This limits the interpretation of observational data and asks the question - is better

HRQOL directly related to higher serum 25(OH)D or is it a coincidence, with better HRQOL scores representing individuals being healthier and able to go outside more?

Intervention studies using vitamin D supplementation for the purpose of improving HRQOL are lacking. HRQOL is often a secondary end-point and as such studies may not be adequately powered to provide meaningful results. Heterogenicity exists, within current research data, in terms of the study populations, as well as type, dose, and duration of vitamin D; and the HRQOL tools used (Hoffmann *et al.*, 2015). This, in addition to limitations of study design and lack of control for confounding factors has led to inconsistent findings (Hoffmann *et al.*, 2015).

Efforts to improve HRQOL are needed, not solely because HRQOL is a recognised outcome measure in ESRD, but moreover, poor HRQOL is associated with increased hospital admissions and mortality (Bossola *et al.*, 2017; Grincenkov *et al.*, 2013; Kušleikaitė *et al.*, 2010; López Revuelta *et al.*, 2004; Mapes, 2004; Thong *et al.*, 2008). Whether improved HRQOL can be achieved with improved serum 25(OH)D remains unknown. There is a large RCT currently underway in the UK, using cholecalciferol supplementation in ESRD, and investigating HRQOL as a secondary endpoint; this study aims to recruit 4200 participants and is the largest vitamin D supplementation study in ESRD to date (Hiemstra, 2015). The outcome of this study will hopefully provide greater insight, yet an adequately powered intervention study with HRQOL as the primary end point is warranted.

9.7 Vitamin D metabolites

Vitamin D metabolism is known to be altered in ESRD. Results from this study demonstrate that repletion of 25(OH)D offers repletion of other vitamin D metabolites; in particular $1,25(OH)_2D_3$, and also $24,25(OH)_2D_3$. At present, measurement of serum 25(OH)D concentration remains the sole marker of vitamin D status. Existing evidence has failed to demonstrate that other measures, such as free 25(OH)D (25(OH)D not bound to DBP or albumin), offer increased diagnostic value. The results presented here, offer new insight into vitamin D metabolism in ESRD, specifically demonstrating the need for 25(OH)D repletion in the treatment of $1,25(OH)_2D$ deficiency. $1,25(OH)_2D$, $24,25(OH)_2D_3$, and $25(OH)D_3$:$24,25(OH)_2D_3$ VMR appear to be valuable diagnostic measures in patients presenting with

hypercalcaemia, yet the clinical usefulness of the multi-metabolite assay, in ESRD, remains unknown. Furthermore, it is now clear that many other, previously unrecognised, vitamin D metabolites may contribute to the biological activity of vitamin D (Jenkinson, 2019).

The data presented demonstrate that the immunoassay results generated by a routine hospital analytical chemistry service were statistically different to data generated by LC-MS/MS analysis in a dedicated mass spectrometry research laboratory. Others have demonstrated the immunoassay gives a misdiagnosis of vitamin D deficiency in ~11% of subjects (Cashman *et al.,* 2013). Health professionals should bear this in mind when interpreting 25(OH)D results.

9.8 Conclusion

Patients with ESRD have historically received active vitamin D analogues alone because there has been an acceptance that (in the presence of renal insufficiency and elevated FGF23) there is a lack of 1α-hydroxylase enzyme activity for the conversion of 25(OH)D to $1,25(OH)_2D$. The focus has been on CKD-MBD management for which $1,25(OH)_2D$ was considered the important vitamin D form.

Increasingly evidence suggests vitamin D has many health benefits beyond the scope of bone mineral health, and these effects may be dependent on the repletion of 25(OH)D. The results presented here show that cholecalciferol can be used effectively to treat both 25(OH)D and $1,25(OH)_2D$ deficiency. This results in clinical benefits, without the same hypercalcaemic risks associated with active vitamin D analogues. Despite this knowledge, as yet benefits have not translated into national supplementation guidelines for the CKD population.

This study is limited by the absence of a control group; a randomised controlled trial is required to prove effects. Without a control arm, firm conclusions (on possible benefits) cannot be drawn, thus the clinical meaningfulness of outcomes is affected. However, results presented here are from the real-life setting. It is therefore anticipated that the UHCW cholecalciferol supplementation guideline could be adopted elsewhere without adaptation. Whilst further research is needed, similar improvements in vitamin D metabolite profile, in particular increases in both serum 25(OH)D and $1,25(OH)_2D$, and an improvement in SHPT and anaemia, would be anticipated if all patients with ESRD were 25(OH)D replete.

9.9 Questions for future research studies

New approaches to the assessment of vitamin D status bring with them further questions. It is important that research not only seeks to improve clinical outcomes, but also strives to improve science through investigation into the potential mechanisms involved.

The development of national or international clinical practice guidelines relies on a strong evidence base to inform recommendations. Although, where evidence is lacking, guidelines may make recommendations based on expert opinion, this should not be aspired to (Uhlig, 2011). Management of CKD-MBD (including management of vitamin D deficiency) has been an area of controversy in terms of guideline development due to lack of evidence and conflicting views of key opinion leaders (Levin & Wheeler, 2015; Uhlig, 2011). Recommendations for further research should be specific in order to help inform research strategies (Levin & Wheeler, 2015).

The use of placebo in vitamin D trials in becoming increasing difficult ethically due to growing recognition for potential benefits of vitamin D supplementation, along with increased awareness surrounding risks of deficiency. Given everyone in the UK is recommended to take 400IU of vitamin D daily, at least from October to March (SACN 2016), it is anticipated that any RCT would need to include this dose in the control arm. Therefore, more focus is needed to investigate clinical outcomes based on specific serum 25(OH)D concentrations/targets.

A RCT (SIMPLIFIED) is currently underway in the UK, giving cholecalciferol supplementation to HD and PD patients (Hiemstra, 2015). New vitamin D studies in ESRD are likely unfeasible until SIMPLIFIED has concluded. This is due to challenges relating to applying for funding until the current RCT outcomes are known, and also recruitment challenges due to many UK renal units already being involved in a cholecalciferol trial.

The results of the Ph.D study demonstrate that cholecalciferol supplementation given to HD patients as part of routine care, in a one rule fits all approach, effectively and safely, repletes serum 25(OH)D in ~90% of patients. Research

questions that could be answered from retrospective investigation of the current study cohort are:

1. Who are the patients that did not adequately replete?
    a. Did this group have specific characteristics (for example ethnicity or high BMI)
    b. Did these patients actually receive the cholecalciferol; were there prescribing oversights? or were these patients hypercalcaemic and cholecalciferol was intentionally not prescribed?

2. Did active vitamin D analogue use alter during the study follow up period; was there a reduction in use?

A question that requires further research, for which the study details are yet to be determined, is:

3. In ESRD, does the increase in serum 1,25$(OH)_2$D, following 25(OH)D repletion, occur as a result of renal synthesis of 1,25$(OH)_2$D; or is compensatory extra-renal synthesis?

Questions that require further research in the form of a large multicentre RCT include:

4. Does serum 25(OH)D repletion in ESRD reduce EPO requirements?
    a. Are iron markers improved?
    b. What is the mechanism responsible?

5. Can serum 25(OH)D repletion manage SHPT in ESRD without the need for active vitamin D analogues?
    c. Does this reduce risk of mortality?
    d. Does this reduce hyperphosphataemia and hypercalcaemia?
    e. Does this reduce calcification and cardiovascular disease risk?

6. Can serum 25(OH)D repletion, achieved and maintained following the initial CKD diagnosis, prevent or minimise severity of SHPT?
    a. Does this reduce onset and occurrence of hyperphosphataemia and hypocalcaemia/hypercalcaemia?

b. Does this prevent the need for active analogue therapy?
c. Does this reduce calcification and cardiovascular disease risk?
d. Does this reduce, or delay, the requirement for EPO?
e. Does this reduce mortality?

Whilst the SIMPLIFIED trial is not aiming to address any of these questions, it is capturing all study data using routine data sources (Bond *et al.*, 2015). Therefore question 4 could be answered by the SIMPLIFIED study cohort through utilisation of Renal Patient View and UK Renal Registry (UKRR) data. Questions 3, 5 and 6 necessitate new studies. The exact study needed to answer question 3 is unknown at present. Question 5 could possibly be answered through an extension to the SIMPLIFIED study, in which the active analogue use is removed from the intervention arm. Question 6 requires a CKD (not ESRD) cohort, and the study would be very involved in terms of set up, management and length of follow up. However, given optimisation of serum 25(OH)D prior to diagnosis of SHPT, may eliminate the need for calcimimetics or parathyroidectomy (Jean *et al.*, 2010), targeting earlier CKD stages may confer the most clinical benefit.

The Ph.D is clinically focussed, yet to give clinical guidance there is a requirement to understand more at the molecular level. More knowledge is needed about the vitamin D metabolites, their signalling pathways, and their interactions with FGF23 and PTH in the context of CKD.

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Appendices

Appendix A Coventry and Warwickshire vitamin D treatment guidelines, based on expert consensus 2013

Serum Vitamin D (25-OHD) level	Diagnosis and management strategy	Product to use	Treatment dose and frequency	Cost per 4 weeks of treatment
75nmol/L or more	**Optimal levels:** No action required	Lifestyle advice if appropriate		
50-74nmol/L	**Adequate:** Lifestyle advice	Lifestyle advice *And if required* - self treatment with purchased supplement, 400 - 800 units daily or maintenance treatment.		N/a
30-50nmol/L (with symptoms of bone disease)	**Insufficiency:** High dose treatment for **5 weeks** followed by long term maintenance.	**Bio-vitamin D3**	60,000 units weekly	**£3.18**
		HuxD3	60,000 units weekly	**£2.51**
Less than 30 nmol/L	**Deficiency:** High dose treatment for **5 weeks** followed by long term maintenance.	**ProD$_3$**	60,000 units weekly	**£9.60**
Maintenance therapy		**Fultium**	800-1600 units daily	**£3.36 - £6.72**
		Desunin	800-1600 units daily	**£3.36 - £6.72**
		Calceos* ***or suitable equivalent***	1 tablet bd	**£3.38**

Appendix B Cholecalciferol prescribing page within the dialysis prescription book

Vitamin D supplementation; Colecalciferol (Fultium D3)

Vitamin D supplementation guidelines for haemodialysis patients can be found on the e-library, if you have any queries please contact Sharon Huish (Dietitian) on extension 26151 or Dr Simon Fletcher (Consultant) on extension 28294. Vitamin D levels should be monitored according to guidance within the relevant clinical guideline. Unless there is a clinical indication or exceptional circumstance; blood vitamin D levels should not be repeated within a 3 month period.

Vitamin D level	Colecalciferol Dose
< 50nmol/L	40,000 units weekly
50 - 75nmol/L	20,000 units alternate weeks
76-150nmol/L	If not already taking colecalciferol, and colecalciferol was not previously prescribed and stopped - no indication to start. If taking colecalciferol already - maintain levels on maintenance dose of 20,000 units alternate weeks
> 150nmol/L	STOP colecalciferol, recheck vitamin D level in 3 months and if <150nmol/L restart 20,000 units alternate weeks

Drug: Colecalciferol	**Route: oral**	**Dose**
Frequency:	Date	Signature, Print Name and Bleep Number
Drug: Colecalciferol	**Route: oral**	**Dose**
Frequency:	Date	Signature, Print Name and Bleep Number
Drug: Colecalciferol	**Route: oral**	**Dose**
Frequency:	Date	Signature, Print Name, and Bleep Number

Administration

Date	**Time**	**Initial**	**Date**	**Time**	**Initial**	**Date**	**Time**	**Initial**

Appendix C UHCW Research, Development and Innovation study approval letter

Research, Development and Innovation Department
Director of R,D&I: Professor Chris Imray - Tel: 024 7696 5264
Head of R,D&I: Ceri Jones - Tel: 024 7696 6198
R,D&I Operations Manager: Tammy Holmes – Tel: 024 7696 6196
Research Portfolio Development Manager: Deborah Griggs - Tel: 024 7696 4958
Research Associate - Governance: Isabella Petrie - Tel: 024 7696 6069
R,D&I Administration Specialist: Hannah Williamson- Tel 024 7696 4995
R,D&I Administration Specialist: Kim Holmes – Tel 024 7696 6202
R,D&I Senior Administration Officer: Sonia Kandola – Tel 024 7696 6199

07 November 2014

Mrs Sharon Huish
Nutrition and Dietetics Department
2nd Floor Rotunda, University Hospital
Coventry
CV2 2DX

Dear Sharon

Study Title: Evaluation of the effectiveness of Cholecalciferol Supplementation in Haemodialysis Patients: The Impact on Erythropoietin (EPO) Requirements and Quality of Life

Thank you for submitting the above study for consideration by the Research Development and Innovation Office. I am pleased to inform you that your study has been approved.

To meet national recruitment targets, you need to ensure that you recruit the first patient into this study by* 06 December 2014 *Please contact the R,D&I team if you need support in order to achieve this target.

Approved Documents:
The documents approved for use in this study are:

Document	Version	Date
Protocol	1.1	04/11/2014
Participant information sheet (PIS)	-	27/06/2014
Participant consent form	-	30/06/2014
Validated Questionnaire – SF36	-	11/06/2014
Validated Questionnaire – EQ-5D-DL	-	11/06/2014

Conditions of Approval:

- Should you wish to make any changes to the documents listed above, you must obtain R,D&I approval prior to use.
- An Annual Progress Report (APR) should be submitted to the main research ethics committee (REC) once a year throughout the trial or on request by R,D&I. The first report is due on 07 November 2015. In addition, for CTIMP studies, a Development Safety Update Report (DSUR) should be submitted to the MHRA and the REC once a year. Guidance on the DSUR can be found in SOP 41 'Preparation and Submission of Annual Progress Reports and Development Safety Update Reports'.
- Notification of any serious breaches of GCP or the trial protocol must be reported to the RD&I Department and a DATIX Clinical Adverse Event form completed within 24 hours of any suspected breach being identified and confirmed.

Sponsorship & Indemnity:

Your research is covered by NHS indemnity as set out in HSG(96)48.

Your project may be subject to ad hoc audit by our department to ensure these standards are being met.

May I take this opportunity to remind you that, as a researcher, you must ensure that your research is conducted in a way that protects the dignity, rights, safety and well-being of participants. Trust RD&I Approval assumes that you have read and understand the Research Governance Framework and accept that your responsibilities as a researcher are to comply with it, the Data Protection and Health & Safety Acts.

The Trust wishes you every success with your project.

Yours sincerely

[redacted]

Ceri Jones
Head of Research, Development and Innovation

Gail Evans, Research Nurse, UHCW NHS Trust
Sue Hewins, Research Nurse, UHCW NHS Trust
Becky Chadwick, Sponsor Representative
Isabella Petrie, Sponsor Representative
Sonia Kandola, Sponsor Representative
Dr Simon Fletcher, Academic Supervisor
Dr Rosemary Bland, Academic Supervisor
Professor Janet Dunn, Academic Supervisor

Appendix D Information forms for sub-study (health-related quality of life questionnaires and non-routine bloods samples)

Study: Evaluation of Vitamin D Supplementation in Haemodialysis Patients

Lead Researcher: Mrs Sharon Huish (Specialist Renal Dietitian)
Study Consultant: Dr Simon Fletcher (Consultant Nephrologist)

Patient Information Sheet

Nearly all dialysis patients in the UK have low vitamin D blood levels.

In view of many health benefits that have been linked with vitamin D, UHCW plan to introduce supplementation as part of the routine care for all patients having haemodialysis. If your blood results show that you require vitamin D then supplements will be given to you during your dialysis sessions. This will not affect your diet or lifestyle, any of your medications, or any of your other vitamins. Your renal consultant and healthcare team will monitor your blood vitamin D levels and requirement for supplements on an ongoing basis.

We would like to monitor the effectiveness of the change in your routine vitamins. To do this we will be looking at your renal blood results and your blood pressure readings. We also want to gather information about how you feel and will therefore be inviting you to complete some questionnaires.

Before you decide whether you are happy to be involved it is important for you to understand more about it. Please take time to read the following information carefully and ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this research study?

EPO (Erythropoietin) is the injection that nearly all dialysis patients have for anaemia. Reduced EPO medication dose and improved quality of life have been linked with better health outcomes.

There is some evidence to suggest that nutritional vitamin D (the type of vitamin D made by your skin when it is exposed to the sun) may help to reduce the amount of EPO your body needs and may also help to improve your quality of life and how you feel. This research study will help confirm this.

What if I am not having EPO injections?

If you do not require EPO injections it is because your kidneys are still producing enough EPO. Vitamin D may still help your body use your own EPO better and therefore we would still like you to be involved in this study.

What is involved in taking part?
You will be asked to complete 2 short questionnaires just before you start taking vitamin D supplements and then again once every 4-5 months over the following 12-18 months. These questionnaires will be given to you when you attend your dialysis sessions and will take approximately 10 minutes to complete.

The information from these questionnaires will be collected and linked with your blood pressure readings and your renal blood results. You will not be required to have any additional blood tests or blood pressure monitoring if you take part in this study.

Is this study confidential?
All information collected in this study will be kept confidential at all times. The collected information will only be used for the purpose of this study. You will not be individually identifiable from the records we keep. Your name will not be disclosed outside of the hospital.

Information will be stored on a secure database within the hospital and Warwick University. Only the research team will have access to this.

What will happen to the results from this study?
The results from this study will be shared with the patients via the renal patient forum and kidney courier and also published in a medical journal. You will not be identified in any publication or report.

Do I have to take part?
Completing the questionnaires will be entirely voluntary. We will describe the study and go through this information sheet, which we will then give you to keep. If you agree to take part then you will be asked to sign a consent form. You are free to decline to complete the questionnaires at anytime. A decision not to take part at any time will not affect the standard of care you receive.

The actual vitamin D supplements are not part of our study; if you are shown to require these vitamins based on your blood results then you will receive them regardless of whether or not you take part.

Who has reviewed this study protocol (the plan for this study)?
This study has been reviewed by, and had approval from, the British Renal Society who are helping to fund the study and The Research Ethics Committee (REC). Research studies can only be conducted if approval has been obtained from the REC.

Contact for further information:

Lead Researcher: Sharon Huish
Nutrition and Dietetics Department
2nd Floor Rotunda
University Hospital
Coventry
CV2 2DX
Telephone 02476 966 151

Thank you for taking the time to read this information sheet. Please ask any questions if you need to.

Study: **Effect of Vitamin D Supplementation on Markers of Iron Availability and Inflammation in Haemodialysis Patients**

Lead Researcher: Mrs Sharon Huish (Specialist Renal Dietitian)

Study Consultant: Dr Simon Fletcher (Consultant Nephrologist)

Patient Information Sheet

Nearly all dialysis patients in the UK have low vitamin D blood levels.

In view of many health benefits that have been linked with vitamin D, UHCW plan to introduce supplementation as part of the routine care for all patients having haemodialysis. If your blood results show that you require vitamin D then supplements will be given to you during your dialysis sessions. This will not affect your diet or lifestyle, any of your medications, or any of your other vitamins. Your renal consultant and healthcare team will monitor your blood vitamin D levels and requirement for supplements on an ongoing basis.

Why have I been asked to be take part?

We are asking haemodialysis patients at UHCW to take part. We would like to study the effects of this change in your routine vitamins on blood markers of inflammation and iron.

Before you decide whether you are happy to be involved it is important for you to understand more about it. Please take time to read the following information carefully and ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this research study?

EPO (Erythropoietin) is the injection that nearly all dialysis patients have for anaemia. Reduced EPO medication dose and improved quality of life have been linked with better health outcomes.

There is some evidence to suggest that nutritional vitamin D (the type of vitamin D made by your skin when it is exposed to the sun) may help to reduce the amount of EPO your body needs through improved iron transport and also reduced levels of inflammation. This research study will help confirm this potential mechanism.

What is involved in taking part?

This study will take place over a year. You would be seen by a research nurse at the start of the study and again 12 months later. You would be seen when you attend for dialysis and you will not be required to make any additional visits. At each research nurse visit two blood samples will be taken. These will both be frozen for analysis of markers of iron transport and inflammation at the end of the study.

The results from these blood samples will be linked with your routine renal blood results.

Appendix E Consent forms for sub-study (health-related quality of life questionnaires and non-routine bloods samples)

University Hospitals Coventry and Warwickshire NHS Trust

CONSENT FORM

Participant Identification Number for this trial:

Title of Project**: Evaluation of Vitamin D Supplementation in Haemodialysis Patients**

Name of Researcher: Mrs Sharon Huish (Specialist Renal Dietitian)

Study Consultant: Dr Simon Fletcher (Consultant Nephrologist

Please initial box

1 I confirm that I have read the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2 I understand that my participation is voluntary and that I am free to decline to complete the questionnaires on any occasion without giving any reason, without my medical care or legal rights being affected. ☐

3 I understand that information from the questionnaire will be linked to my routine renal blood results and blood pressure readings. ☐

4 I agree to take part in the above study. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

University Hospitals Coventry and Warwickshire NHS Trust

CONSENT FORM

Participant Identification Number for this trial:

Title of Project**: Effect of Vitamin D Supplementation on Markers of Iron Availability and Inflammation in Haemodialysis Patients**

Name of Researcher: Mrs Sharon Huish (Specialist Renal Dietitian)

Study Consultant: Dr Simon Fletcher (Consultant Nephrologist

Please initial box

1 I confirm that I have read the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2 I understand that my participation is voluntary and that I am free to decline to give blood samples on any occasion, without giving any reason, without my medical care or legal rights being affected. ☐

3 I understand that some blood will be stored for this study and may be used for future research. ☐

4. I understand that study results will be linked to my routine renal blood results. ☐

5. I agree to take part in the above study. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix F SF-36 and EQ-5D Questionnaire

Your Health and Well-Being

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.
Thank you for completing this survey!

For each of the following questions, please tick the one box that best describes your answer.

1. In general, would you say your health is:

Excellent	Very good	Good	Fair	Poor
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

2. <u>Compared to one year ago</u>, how would you rate your health in general <u>now</u>?

Much better now than one year ago	Somewhat better now than one year ago	About the same as one year ago	Somewhat worse now than one year ago	Much worse now than one year ago
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	☐ 1	☐ 2	☐ 3
b Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	☐ 1	☐ 2	☐ 3
c Lifting or carrying groceries	☐ 1	☐ 2	☐ 3
d Climbing several flights of stairs	☐ 1	☐ 2	☐ 3
e Climbing one flight of stairs	☐ 1	☐ 2	☐ 3
f Bending, kneeling, or stooping	☐ 1	☐ 2	☐ 3
g Walking more than a mile	☐ 1	☐ 2	☐ 3
h Walking several hundred yards	☐ 1	☐ 2	☐ 3
i Walking one hundred yards	☐ 1	☐ 2	☐ 3
j Bathing or dressing yourself	☐ 1	☐ 2	☐ 3

4. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Cut down on the amount of time you spent on work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b Accomplished less than you would like	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c Were limited in the kind of work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d Had difficulty performing the work or other activities (for example, it took extra effort)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

5. **During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?**

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Cut down on the amount of time you spent on work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b. Accomplished less than you would like	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c. Did work or other activities less carefully than usual	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

6. **During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?**

Not at all	Slightly	Moderately	Quite a bit	Extremely
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

7. **How much bodily pain have you had during the past 4 weeks?**

None	Very mild	Mild	Moderate	Severe	Very severe
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	☐ 6

8. **During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?**

Not at all	A little bit	Moderately	Quite a bit	Extremely
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks…

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Did you feel full of life?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b. Have you been very nervous?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c. Have you felt so down in the dumps that nothing could cheer you up?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d. Have you felt calm and peaceful?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
e. Did you have a lot of energy?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
f. Have you felt downhearted and low?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
g. Did you feel worn out?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
h. Have you been happy?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
i. Did you feel tired?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

All of the time	Most of the time	Some of the time	A little of the time	None of the time
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

11. How TRUE or FALSE is <u>each</u> of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
a. I seem to get ill more easily than other people	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b. I am as healthy as anybody I know	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c. I expect my health to get worse	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d. My health is excellent	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

Thank you for completing these questions!

Health Questionnaire

English version for the UK

Under each heading, please tick the ONE box that best describes your health TODAY

MOBILITY

I have no problems in walking about ❑
I have slight problems in walking about ❑
I have moderate problems in walking about ❑
I have severe problems in walking about ❑
I am unable to walk about ❑

SELF-CARE

I have no problems washing or dressing myself ❑
I have slight problems washing or dressing myself ❑
I have moderate problems washing or dressing myself ❑
I have severe problems washing or dressing myself ❑
I am unable to wash or dress myself ❑

USUAL ACTIVITIES *(e.g. work, study, housework, family or leisure activities)*

I have no problems doing my usual activities ❑
I have slight problems doing my usual activities ❑
I have moderate problems doing my usual activities ❑
I have severe problems doing my usual activities ❑
I am unable to do my usual activities ❑

PAIN / DISCOMFORT

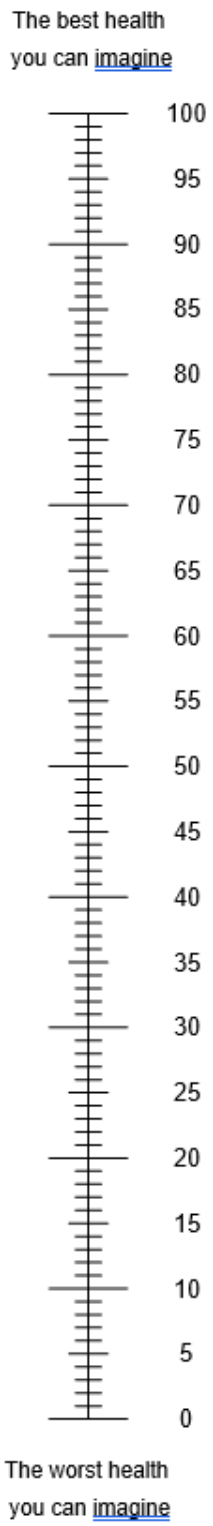
I have no pain or discomfort ❑
I have slight pain or discomfort ❑
I have moderate pain or discomfort ❑
I have severe pain or discomfort ❑
I have extreme pain or discomfort ❑

ANXIETY / DEPRESSION

I am not anxious or depressed ❑
I am slightly anxious or depressed ❑
I am moderately anxious or depressed ❑
I am severely anxious or depressed ❑
I am extremely anxious or depressed ❑

- We would like to know how good or bad your health is TODAY.
- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine.
  0 means the worst health you can imagine.
- Mark an X on the scale to indicate how your health is TODAY.
- Now, please write the number you marked on the scale in the box below.

YOUR HEALTH TODAY = ☐



Appendix G Journal publications

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Prevalence and treatment of hypovitaminosis D in the haemodialysis population of Coventry

Sharon A. Huish[a,d,*], Simon Fletcher[b], Janet A. Dunn[d], Martin Hewison[c], Rosemary Bland[b,d]

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ABSTRACT

Low serum 25(OH)D and associated bone and non-bone related problems are not well appreciated in end stage renal disease (ESRD). Vitamin D treatment strategies in the UK currently focus almost exclusively on calcitriol [1,25$(OH)_2$D], alfacalcidol or paricalcitol. In ESRD hypovitaminosis D is associated with bone loss, muscle weakness, falls, fractures and increased inflammation. National guidelines changed in 2014 and now recommend the diagnosis and treatment of low serum 25(OH)D in all patients with glomerular filtration rate (GFR) less than 30 ml/min/1.73m^2. However as yet there are no standardized guidelines for dosage, frequency and monitoring in ESRD patients. Following a systematic review of the literature we developed a clinical guideline for cholecalciferol supplementation at University Hospitals of Coventry and Warwickshire, UK. The guideline recommends 40,000IU cholecalciferol weekly for patients with 25(OH)D <50 nmol/L and 20,000IU weekly for patients with 25(OH)D 50–75nmol/L; to be continued long term unless levels increase to ≥150 nmol/L. To date we have measured 25(OH)D levels in 385 in-center haemodialysis patients. Virtually all patients (95%) had serum 25(OH)D levels <75 nmol/L (65% deficient, <30 nmol/L; 30% insufficient, 30–74 nmol/L). Only 5% of patients had optimal levels (≥75 nmol/L). Our data indicates that hypovitaminosis D is prevalent in the haemodialysis population in Coventry and Warwickshire and this is likely to reflect UK haemodialysis patients, highlighting the need for a national supplementation guideline.

Contents

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E-mail address: [redacted] (S.A. Huish).

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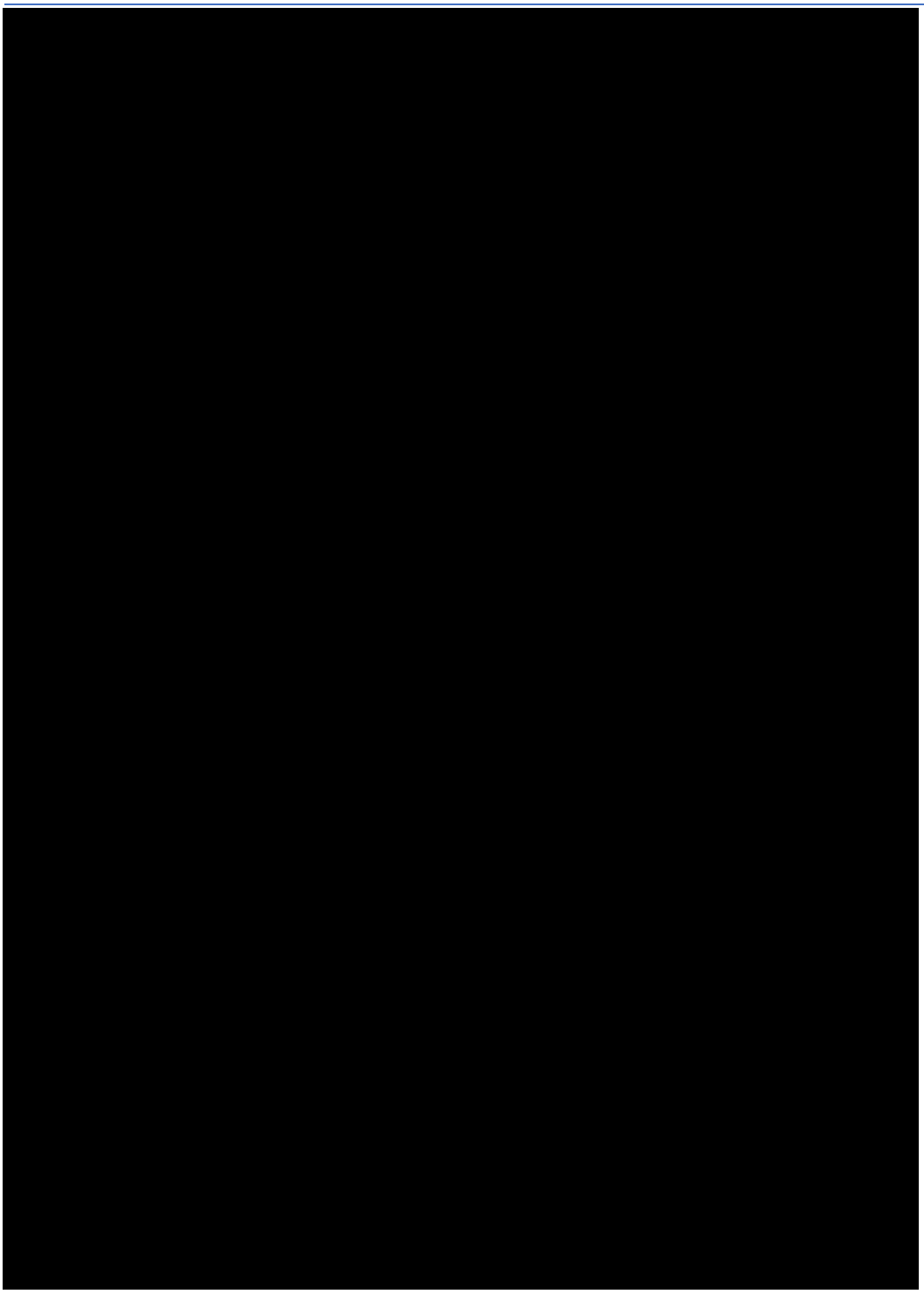
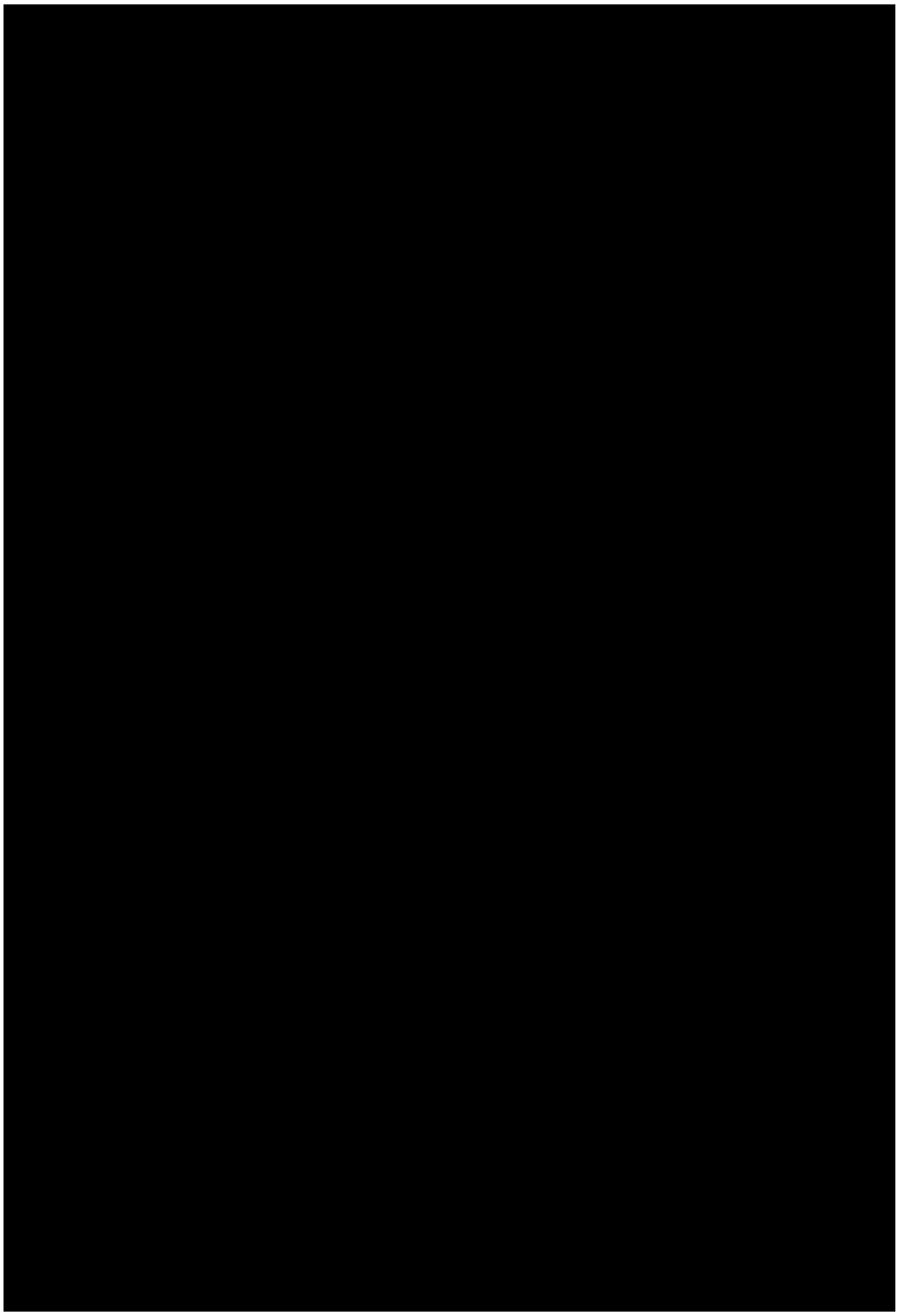
0960-0760/





2.139. The Addition of Cholecalciferol in the Management of Secondary Hyperparathyroidism; A Haemodialysis Case Report

Parker, S.A.; Ting, S.M.; Fletcher, S.; Zehnder, D.; Bland, R.

Background: Secondary hyperparathyroidism (SHPT), a complication of Chronic Kidney Disease (CKD) is associated with increased fracture and cardiovascular risk. Routine treatment includes phosphate restriction ± binding agents and vitamin D receptor activators (VDRa) such as calcitriol [1,25(OH)D], alfacalcidol or paricalcitol. Those refractory to standard treatment are treated with calcimimetics (allosteric activators of the calcium sensing receptor) or if appropriate parathyroidectomy. Management with VDRa and calcimimetics is often complicated by hyperphosphataemia and hypercalcaemia. We report a case of a 43 year female of African-Caribbean origin with severe SHPT in the presence of hungry bone syndrome due to chronically depleted bone mineral stores. Having end stage renal disease (ESRD) since 2002 she was initially managed on peritoneal dialysis and transferred to haemodialysis in 2004. The patient refused parathyroidectomy. Calcimimetic treatment was titrated up to maximum dose and used alongside high dose VDRa, high calcium dialysate and high dose phosphate

supplementation. This resulted in some improvement in serum iPTH (intact PTH) but not to within, or near, target (8–38 pmol/L). At this point the patient's vitamin D status was assessed as insufficient and vitamin D_3 supplementation commenced.

Methods: In April 2011 the patient's serum 25(OH)D was measured as 65 nmol/L (optimal is ≥75 nmol/L). At this time the patient had a serum iPTH of 222 pmol/L, alkaline phosphatase (ALP) 4075 U/L (target 35–105 U/L), adjusted calcium of 2.28 nmol/L (target 2.10–2.58 nmol/L) and phosphate of 0.65 nmol/L (target 1.1–1.7 nmol/L). The patient was being managed with; calcitriol 6mcg/day, phosphate sandoz 2 tablets thrice daily, calcium sandoz 2 tablets thrice daily, and the calcimimetic cinacalcet 180 mg/day. Vitamin D_3 20,000 IU/week was added and bone and iPTH levels were monitored monthly. We report 3 time points; time zero (T0), 6 months (T6) and 15 months (T15). ALP and iPTH were measured using Roche modular assays.

Results: At T0 despite being on a maximal dose of cinacalcet and a high VDRa dose the patient's iPTH remained high (222 pmol/L). However, addition of vitamin D_3 resulted in a reduction of both iPTH and ALP levels. After 6 months (T6) of vitamin D_3 supplementation serum iPTH had reduced by 29% to 158 pmol/L and ALP by 26% to 3008 IU/L. Adjusted calcium and phosphate levels remained stable at 2.35 mmol/L and 0.65 mmol/L respectively. At T15 serum 25(OH)D was 103 nmol/L (37% increase from T0). There had been a continued decrease in iPTH and ALP levels. Serum iPTH had reduced further to 63 pmol/L (72% reduction from T0) and ALP had decreased to 1955 IU/L (52% reduction from T0). Adjusted calcium had risen slightly (2.55 mmol/L; 8.5% increase from T0), and phosphate was reduced (0.26 mmol/L; 60% reduction from T0). The decrease in serum phosphate may be attributed to documented concordance problems with phosphate supplementation rather than a consequence of improved serum 25(OH)D.

Conclusions: Management of renal bone disease in ESRD has traditionally focused on treatment with VDRa. Improving serum 25(OH)D levels to >75 nmol/L may offer further benefit through promoting synthesis of local 1,25(OH)D in the parathyroid tissues, particularly in the presence of cinacalcet resistance. Assessment and correction of vitamin D status should be considered in addition to the current routine treatment of secondary hyperparathyroidism.

2.140. Vitamin D Deficiency in Advanced Chronic Kidney Disease Is not Corrected after Kidney Transplantation

Parker, S.; Ting, S.M.; Petchey, M.; Higgins, R.; Fletcher, S.; Zehnder, D.; Bland, R.

Background: Supplementation with calcitriol (1,25-dihydroxy vitamin D; or analogue) is used routinely in patients with chronic kidney disease (CKD). However this form of supplementation overlooks 25-hydroxy vitamin D [25(OH)D] deficiency, which is rarely screened for or treated. Vitamin D deficiency correlates with falling estimated glomerular filtration rate (eGFR) and is seen in up to 95% of people with end stage renal disease. This may be due to; reduced sunlight exposure, uraemia effecting liver hydroxylation, poor diet or higher activity of 24-hydroxylase. Successful kidney transplantation may therefore improve circulating 25(OH)D levels.

Methods: We assessed serum 25(OH)D in renal transplant patients within 4 weeks prior to kidney transplantation in parallel to CKD patients (CKD5) and a group of healthy medication-controlled hypertensive subjects at time zero (T0) and 1 year later (T1). 25(OH)D was measured using the Elecsys Vitamin D Total Assay (Roche). Statistical analysis was performed using Wilcoxon matched pairs, Kruskal-Wallis and Spearman correlation (SPSS). Data represent mean ± SEM.

Results: In total 99 patients were assessed. 32 who received a kidney transplant (mean age 45.2 ± 2.59 years; male 59%; BMI 24.7 ± 0.62 kg/m^2), 33 patients with CKD (mean age 47.6 ± 2.34 years; male 73%; BMI 27.4 ± 1.00 kg/m^2) and 34 in the hypertensive group (mean age 55.2 ± 1.24 years; male 44%; BMI 28.0 ± 0.58 kg/m^2). At T0 a significant number of patients in each group had low serum 25(OH)D levels. As expected there was no significant difference in the levels between the CKD group and the patients just prior to transplant (34.6 ± 2.7 nmol/L *vs.* 35.6 ± 3.1 nmol/L respectively). 100% of patients had levels <75 nmol/L and 38.5% of patients were deficient (<30 nmol/L). In contrast the basal 25(OH)D level was significantly greater in the hypertensive group (62.4 ± 5.2 nmol/L; $p < 0.001$) with 32.4% having optimal levels (≥75 nmol/L) and only 17.6% were deficient. Levels were measured again one year later (T1). In the hypertensive patients serum 25(OH)D had not altered significantly (T1, 66.0 ± 5.0 nmol/L *vs.* T0, 62.4 ± 5.2 nmol/L). However, it was apparent that there were now differences between the CKD and transplanted patients. 25(OH)D levels in the CKD group had decreased still further (27.3 ± 3.2 nmol/L; $p < 0.05$) and were now significantly lower than the transplant patients ($p < 0.05$) and significantly more were deficient (66.7%; $p < 0.05$). In contrast, 25(OH)D levels in the patients who had received a transplant showed a small non-significant increase to 45.5 ± 4.6 nmol/L. However, this was still significantly lower than the hypertensive group ($p = 0.004$). Although levels increased in 47% of patients, only 15.6% achieved levels ≥75 nmol/L and 28.1% remained deficient. Post-transplant the 25(OH)D levels correlated with PTH ($p < 0.05$), but not eGFR. At T0, 56% of this group of patients were receiving alphacalcidol/calcitriol, and this reduced to 15.6% post-transplant. Although not positively correlated, it was interesting to note that of the 15 patients whose 25(OH)D levels increased only two remained on alphacalcidol/calcitriol.

Conclusions: All patients with CKD had low levels of serum 25(OH)D. In 47% of patients levels increased post-transplantation, but did not achieve those seen in the hypertensive group. Therefore, although kidney transplant may improve 25(OH)D levels in some patients they still remain insufficient and supplementation should be considered.

Appendix H Abstracts from oral presentations

The International Vitamin D Workshop (Barcelona, Spain 2018)

VITAMIN D SUPPLEMENTATION CORRECTS 1,25-DIHYDROXYVITAMIN D DEFICIENCY AND REDUCES ERYTHROPOIETIN REQUIREMENT IN HAEMODIALYSIS PATIENTS.

S Huish[1,2], C Jenkinson[3], P Mistry[2], S Fletcher[1], J Dunn[2], M Hewison[3] and R Bland[1,2]

[1]University Hospitals of Coventry and Warwickshire (UHCW) NHS Trust, Coventry, UK, [2]The University of Warwick, Coventry, UK, [3]The University of Birmingham, Birmingham, UK

Reduced renal 25 hydroxyvitamin D-1α hydroxylase in end stage renal disease (ESRD) results in low serum 1,25$(OH)_2$D. Thought to be due to reduced renal cell function together with elevated serum fibroblast growth factor 23 (FGF23); routine treatment includes calcitriol, alfacalcidol or paricalcitol. However, this overlooks 25(OH)D deficiency which is common in ESRD and associated with increased erythropoietin (EPO) requirement. UHCW NHS Trust introduced routine cholecalciferol supplementation for all in-centre haemodialysis patients (n=350) in 2014 (cholecalciferol supplementation: serum 25(OH)D <20ng/L 40,000IU for 3 months, ≥20ng/L 20,000IU fortnightly, >60ng/L stop and recheck in 3 months). Data was collected for 12 months prior to (T-12-T-1) and 15 months post (T0-T15) introduction of cholecalciferol. Serum 25(OH)D was measured 3 monthly (T0 -T15). This study reports the effectiveness of cholecalciferol supplementation and the impact on EPO usage. Multiple serum vitamin D metabolites were measured using liquid chromatography-tandem mass spectrometry at T0 and T12 in a subset of patients (n=33). Data are mean (±SD), or median (range). 64% of patients were vitamin D deficient and 94% insufficient (25(OH)D<12ng/L and <30ng/L) at T0. Vitamin D repletion (25(OH)D ≥30ng/L) was achieved in 76.5% of patients by T3. Serum 25(OH)D increased following supplementation (T0 *vs.* T15; 8.8 (0-56) to 50ng/L (22-70) *(*p<0.001*).* Average serum calcium increased following supplementation (T-12-T-1 *versus* T4-T15; 2.29±0.14 to 2.34±0.14mmol/L, p<0.001*).* Monthly EPO usage decreased from 141.30±127.16μg (T-12-T-1) to 139.34±139.58μg (T4-T15) (p<0.05; n=264). Subset analysis revealed serum 1,25$(OH)_2D_3$ increased in 94% of patients from 20.6pmol/L (6-39) at T0 to 34.7pmol/L (15.5-73.9) at T12 (p<0.001). At T0 serum 25$(OH)D_3$ correlated with 24,25$(OH)_2D_3$, but in the presence of adequate 25$(OH)D_3$ (T12) 25$(OH)D_3$ correlated with 3-epi-25$(OH)D_3$ (p<0.05). 1,25$(OH)_2D_3$ correlated with calcium and 24,25$(OH)_2D_3$ at T0 and T12 (p<0.05). 25$(OH)D_3$ was not correlated with calcium at either time point. This study indicates that in the presence of adequate substrate the ability to synthesise 1,25$(OH)_2D_3$ is maintained in ESRD. Cholecalciferol significantly increased serum 25(OH)D and 1,25$(OH)_2D_3$ and was associated with a reduction in EPO requirements. Cholecalciferol may prove effective in aiding both anaemia, and bone and mineral disorder management in renal disease.

UK Kidney Week (Harrogate, 2018

Evaluating the impact of routine colecalciferol on erythropoietin (EPO) requirements

Sharon Huish[1,2], Pankaj Mistry[2], Simon Fletcher[1], Janet Dunn[2], and Rosemary Bland[1,2]

[1]University Hospitals of Coventry and Warwickshire NHS Trust, Coventry, UK, [2]The University of Warwick, Coventry, UK.

Background
Vitamin D deficiency and insufficiency (serum 25(OH)D <30nmol/L and <75nmol/L respectively) is prevalent in haemodialysis patients, and is associated with increased erythropoietin (EPO) requirements. Lack of renal 25-hydroxyvitamin D-1α hydroxylase (CYP27B1) for the conversion of 25(OH)D to active 1,25(OH)D is well acknowledged in end stage renal disease (ESRD) with the routine use of active vitamin D analogues. However, this overlooks 25(OH)D deficiency. Following the development of a new local clinical guideline, UHCW NHS Trust introduced colecalciferol supplementation to all in-centre haemodialysis patients as part of standard routine care (colecalciferol supplementation: serum 25OHD <50nmol/L repletion dose of 40,000IU for 3 months, ≥50nmol/L maintenance dose of 20,000IU fortnightly, >150nmol/L stop and recheck in 3 months). This provided a unique opportunity to investigate potential benefits of adequate serum 25(OH)D.
Study Aim
To assess whether increased serum 25(OH)D levels result in an improved response to EPO, measured by a reduction in mean EPO usage.
Method
Data from all 350 patients receiving in-centre haemodialysis across Coventry and Warwickshire was included in this study. Retrospective data looking at total monthly EPO dose received and serum haemoglobin was collected for 12 months prior to the introduction of colecalciferol (T-12 to T-1). The same data was collected prospectively for 15 months post introduction of colecalciferol (T0-T15). The 15 month prospective observational period was chosen to allow collection of 12 months of data after vitamin D repletion had been achieved (3 months to achieve repletion, followed by 12 months post repletion). Monthly serum calcium (corrected for albumin) and 3 monthly serum 25(OH)D was measured throughout the 15 month prospective follow up period. All aspects of the study received NHS ethical approval (reference numbers 14/NS/1012 and 14/EE/10). The primary outcome measure was the reduction of mean total monthly EPO dose needed to maintain haemoglobin within the target range of 100-120g/L according to UHCW renal anaemia guidelines. Wilcoxon signed rank test was used to test the null hypothesis that there is no difference in EPO dose after vitamin D supplementation is introduced.
Results
EPO dosage data was analysed for all patients at all monthly timepoints where their serum haemoglobin levels fell between 100-120g/L (n=264). Data showed that the total EPO use fell following vitamin D supplementation. Total monthly EPO (mcg) received during the 12 months prior to the introduction of vitamin D supplementation (T-12 to T-1) was compared to total monthly EPO (mcg) received during the 12 months post vitamin D repletion (T4 to T15). In months T-12 to T-1 mean EPO use was 141.30mcg (median 112.1mcg) which was reduced to 139.34 mcg (median 95.0mcg) post vitamin D repletion *($p = 0.0258$).* Mean serum 25(OH)D increased from 27.41±25.28nmol/L at T0 to 120±27.09nmol/L at T15 *($P < 0.0001$).* Vitamin D

repletion (serum 25(OH)D ≥75nmol/L) was achieved in 76.5% of patients by T3. Mean serum 25(OH)D continued to increase from T3 to T6 and was adequately maintained throughout the duration of the study.

Conclusion

Hypovitaminosis D is prevalent in the haemodialysis population in Coventry and this is likely to reflect UK haemodialysis patients. The vitamin D supplementation guideline developed at UHCW for all haemodialysis patients, is both safe and effective. Complementing 1,25(OH)D analogue treatment with colecalciferol may have a role in improving EPO response and thus in the management of renal anaemia.

This work was supported by the British Renal Society in collaboration with the British Kidney Patient Association

Appendix I Posters

Poster presented at UK Kidney Week 2020

Evaluating the impact of routine colecalciferol on secondary hyperparathyroidism: are renal guidelines missing something?

Sharon Huish[1,2,3], Simon Fletcher[1], Janet Dunn[3], and Rosemary Bland[3]

[1]University Hospitals of Coventry and Warwickshire NHS Trust, Coventry [2]Department of Nutrition and Dietetics, Royal Devon and Exeter NHS Foundation Trust, Exeter
[3]Warwick Medical School, The University of Warwick, Coventry.

1. Reduced vitamin D synthesis in haemodialysis patients

Vitamin D deficiency and insufficiency (serum 25(OH)D <30nmol/L and <75nmol/L respectively) is prevalent in haemodialysis (HD) patients and associated with secondary hyperparathyroidism (SHPT). Reduced renal synthesis of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) from 25-hydroxyvitamin D [25(OH)D] in end stage renal disease (ESRD) results in low serum $1,25(OH)_2D$ (figure 1). Treatment strategies have therefore focussed on $1,25(OH)_2D$ or its synthetic analogues, alfacalcidol or paricalcitol (VDR activators; VDRa). However this overlooks 25(OH)D deficiency, which is common in ESRD, and may impact on the management of SHPT.

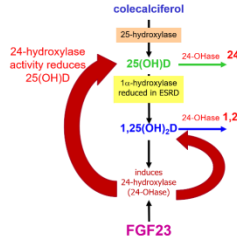


Figure 1. Why might vitamin D metabolites be reduced in ESRD? Patients with ESRD have low 25(OH)D levels and reduced renal 1α-hydroxylase activity reduces $1,25(OH)_2D$. Induction of 24-hydroxylase (24-Ohase) activity by VDRa and/or fibroblast growth factor 23 (FGF23), which is raised in ESRD, results in metabolism (and so reduces levels) of both $1,25(OH)_2D$ and 25(OH)D.

Serum 25(OH)D (nmol/L)	Vitamin D status	Basal vitamin D levels (% of patients)
≥ 75	Optimal (replete)	6 (n=19; median 97nmol/L)
30-74	Insufficient	13 (n=100; median 39nmol/L)
< 30	Deficient	65 (n=209, median 15nmol/L)

Table 1. Vitamin D deficiency in HD patients is common. Vitamin D thresholds and % of patients in each category (n=328). 94% of patients had serum 25(OH)D levels <75nmol/L. Only 19 patients (6%) had optimal levels (≥75nmol/L).

2. Study design

Since early 2015, all patients having in-centre HD at University Hospitals Coventry and Warwickshire (UHCW) NHS Trust are routinely screened, and if indicated, treated for vitamin D deficiency (table 2). In the study reported here retrospective data looking at PTH levels was collected for 12 months prior to the introduction of colecalciferol (T-12 to T-1) and prospectively for 15 months post introduction of colecalciferol (T0 to T15). This allowed 3 months to achieve serum 25(OH)D repletion, followed by 12 months post repletion (T4 to T15). Patients with insufficient data, and those that had a parathyroidectomy prior to, or during the study, were excluded. The number included in the final analysis was 280. Serum calcium and 25(OH)D data was also collected. NHS ethical approval was received.

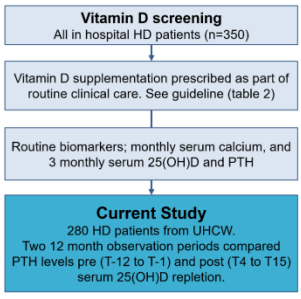


Figure 2 (left). Overview of study design.

Table 2 (below). Clinical guideline for colecalciferol supplementation in haemodialysis patients. (Fultium-D3 20,000IU capsules). Colecalciferol is prescribed by the patient's renal consultant within their dialysis prescription book and administration is overseen by nursing staff.

Serum 25(OH)D	Colecalciferol Dose
<50nmol/L	40,000 units weekly
50 - 74nmol/L	20,000 units alternate weeks
75 - 150nmol/L	If not already taking colecalciferol, no indication to start. If taking colecalciferol already - maintain levels on maintenance dose of 20,000 units alternate weeks
> 150nmol/L	STOP colecalciferol, recheck vitamin D level in 3 months and if <150nmol/L restart 20,000 units alternate weeks

3. Results

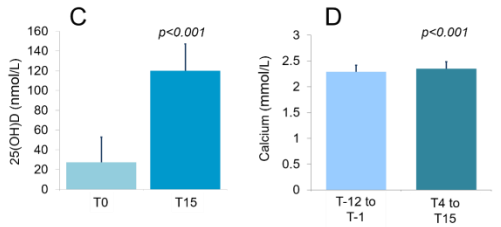
Whole cohort, and grouped analysis was carried out using a related samples Wilcoxon signed rank test to compare mean PTH pre (T-12 to T-1) against mean PTH post (T4 to T15) serum 25(OH)D repletion. Data were grouped, according to mean PTH levels pre vitamin D supplementation (T-12 to T-1) as follows: over-suppressed (<8.4pmol/L), on target (8.4-37.8pmol/L), high (37.9-85pmol/L) and very high (>85pmol/L). The PTH cut offs for the groups were decided based on the UK Renal Association targets for HD patients at the time the data was collected (2016), which was 2-9 times the upper-normal limit of the local laboratory reference range.

A

Gender / ethnicity	Numbers % (n)	Age (years) median (range)	HD vintage (years) median (range)
Female	39 (138)	69 (24-95)	2.4 (0-17.5)
Male	61 (212)		
Caucasian	74 (260)		

B

PTH Grouping (pmol/L)	No. of results	PTH T -12 to T-1 (mean ± SD)	PTH T4 – T15 (mean ± SD)	Change	Wilcoxon signed rank test
All patients	280	41.2 ± 38.7	37.2 ± 35.3	Decrease	P = .123
Over-suppressed <8.4	28	5.8 ± 1.8	14.5 ± 8.5	Increase	P <0.001
8.4-37.8 On target	147	22.0 ± 8.5	24.1 ± 15.5	Increase	P = .513
37.8-84.9 High	71	52.2 ± 13.5	46.5 ± 24.4	Decrease	P = .020
≥85.0 Very high	34	130.7 ± 26.7	92.9 ± 59.8	Decrease	P = .001



(A) Baseline characteristics of the study population (n=350). **(B)** Serum PTH levels pre (T-12 to T-1) and post (T4 to T15) serum 25(OH)D repletion. The grouped analysis revealed no difference in PTH for the patients that already had a mean PTH within target range at baseline, but a significant difference was seen in the over-suppressed, high and very high PTH groups. Data indicate that those with the highest serum PTH pre colecalciferol supplementation are likely to have the most significant PTH reduction. **(C)** Graphical illustration of the change in serum 25(OH)D. Basal 25(OH)D levels were low in 94% of patients. Colecalciferol supplementation effectively increased serum 25(OH)D from 27.4±25.3nmol/L at T0 to 120.0±27.1nmol/L at T15 *(p<0.001)*. **(D)** Serum corrected calcium levels. Mean serum calcium increased from 2.29±0.13mmol/L (T-12 to T-1) to 2.35±0.13mmol/L (T4 to T15) but remained well within target range *(p<0.001)*. No hypercalcaemia was directly associated with colecalciferol supplementation. B, C and D data represents mean±SD.

4. Discussion points

- Low serum 25(OH)D levels are prevalent in HD patients.
- The colecalciferol supplementation guideline developed for haemodialysis patients at UHCW is both effective and safe.
- This study indicates that patients with the highest serum PTH levels are likely to have the most significant PTH reduction following normalisation of serum 25(OH)D.
- Complementing $1,25(OH)_2D$ analogue treatment with colecalciferol may prove effective in aiding the management of SHPT.

University Hospitals Coventry and Warwickshire NHS Trust · BRS British Renal Society · Royal Devon and Exeter NHS Foundation Trust · WARWICK

International Vitamin D Conference (Delft, Netherlands 2015)

Prevalence and treatment of hypovitaminosis D in the haemodialysis population of Coventry.

Sharon Huish[1,4], Simon Fletcher[2], Janet Dunn[4], Martin Hewsion[3] and Rosemary Bland[4].

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1. Introduction

Vitamin D [25(OH)D] deficiency is not well understood in end stage renal disease (ESRD). UK treatment strategies for vitamin D deficiency in this population have historically focused on calcium and hyperparathyroidism management with the sole use of vitamin D receptor activators (VDRa); 1,25-dihydroxyvitamin D (calcitriol, [1,25(OH)$_2$D]), alfacalcidol or paricalcitol overlooking the requirement for adequate 25(OH)D. In ESRD low serum 25(OH)D levels are associated with bone loss, muscle weakness, falls, fractures and increased inflammation. In response to increasing evidence the national CKD (chronic kidney disease) guidelines changed in 2014 and now recommend the diagnosis and treatment of 25(OH)D deficiency and insufficiency in those with a glomerular filtration rate (GFR) <30mls/min, and where required concurrent vitamin D (colecalciferol or ergocalciferol) and VDRa treatment. However as yet there are no standardised guidelines for dosage, frequency and monitoring in ESRD. Consequently response to new guidance has been varied and slow.

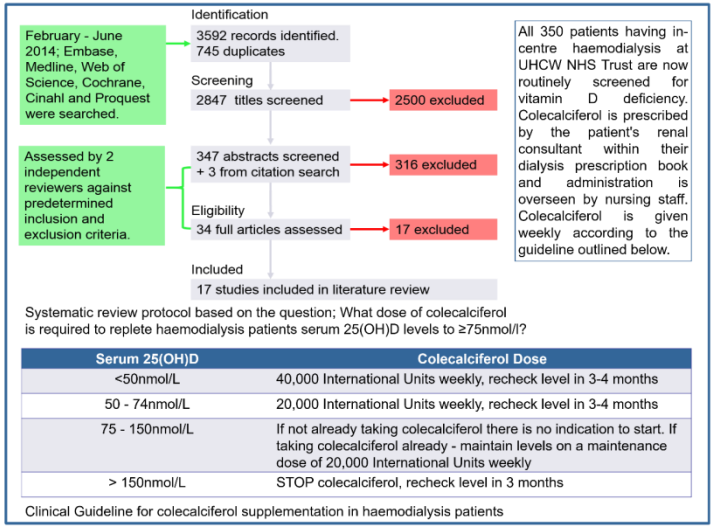
2. Study aims

- To develop a clinical guideline for colecalciferol supplementation in ESRD at the University Hospitals of Coventry and Warwickshire (UHCW) in order to replete serum 25(OH)D levels to ≥75nmol/l;
- To assess the extent of vitamin D insufficiency/deficiency in the haemodialysis population of Coventry.

3. Study design

- A systematic review was undertaken to determine the colecalciferol dose required to safely and effectively replete serum 25(OH)D to ≥75nmol/l in haemodialysis patients. This was used to develop a clinical guideline.
- 25(OH)D levels were measured using Elecsys Vitamin D Total Assay (Roche). Supplementation was commenced following the new guideline (see section 4)

4. Development of Clinical Guideline



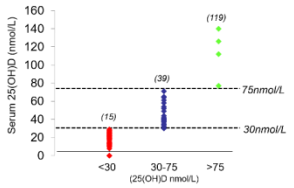
All 350 patients having in-centre haemodialysis at UHCW NHS Trust are now routinely screened for vitamin D deficiency. Colecalciferol is prescribed by the patient's renal consultant within their dialysis prescription book and administration is overseen by nursing staff. Colecalciferol is given weekly according to the guideline outlined below.

Systematic review protocol based on the question; What dose of colecalciferol is required to replete haemodialysis patients serum 25(OH)D levels to ≥75nmol/l?

Serum 25(OH)D	Colecalciferol Dose
<50nmol/L	40,000 International Units weekly, recheck level in 3-4 months
50 - 74nmol/L	20,000 International Units weekly, recheck level in 3-4 months
75 - 150nmol/L	If not already taking colecalciferol there is no indication to start. If taking colecalciferol already - maintain levels on a maintenance dose of 20,000 International Units weekly
> 150nmol/L	STOP colecalciferol, recheck level in 3 months

Clinical Guideline for colecalciferol supplementation in haemodialysis patients

5. Prevalence of hypovitaminosis D



Graph showing serum 25(OH)D levels of the first 100 of our 350 haemodialysis patients screened (…male, mean aged….). Virtually all patients (96%) had serum 25(OH)D levels <75nmol/L (63% deficient, <30nmol/L; 33% insufficient, 30-74nmol/L). Only 4 patients had optimal levels (≥75nmol/L).

Data represents individual patient values grouped into deficient (<30nmol/L), insufficient (30-74nmol/L) and optimal (≥ 75nmol/L) *(median)*.

6. Discussion points

1. Hypovitaminosis D is prevalent in the haemodialysis population of Coventry, this is likely to reflect the UK.
2. This may be due to; reduced sunlight exposure, uraemia effecting skin synthesis and liver hydroxylation, and poor diet. Increased 24-hydroxylase activity induced by VDRa use and increased FGF23 could also be key (figure 1).
3. Our data highlight the need for clear national supplementation guidelines.
4. We have produced a local guideline to ensure deficiency is identified and appropriately treated in our patients.

FUTURE WORK: Serum 25(OH)D levels are being measured every 4 months initially but this will be reviewed in the longer term once the efficacy and maintenance data over an 18month prospective period has been appraised. Biochemical and qualitative data is being collected for future analysis.

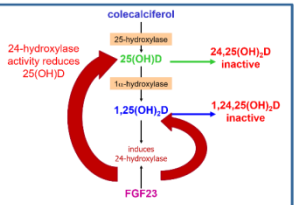


Figure 1.
Induction of 24-hydroxylase activity by VDRa and/or FGF23 (raised in ESRD) results in metabolism of both 1,25(OH)$_2$D and 25(OH)D.

Vitamin D deficiency in CKD patients is not corrected by transplantation; should routine supplementation be considered at all stages of CKD?

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[1]Dept. of Nutrition & Dietetics, [2]Dept. of Nephrology, UHCW, [3]Biochemistry Department [4]Warwick Medical School, Coventry, UK

1. INTRODUCTION

Production of 1,25-dihydroxyvitamin D (calcitriol; 1,25D) from 25-hydroxvitamin D (calcidiol; 25D) is catalysed by 25D-1α-hydroxylase. Supplementation with 1,25D or an analogue is routine in CKD. However this overlooks 25D deficiency, which is seen in up to 95% of people with end stage renal disease and correlates with falling eGFR.

Serum 25D (nmol/L)	Vitamin D status
≥ 75	Optimal (replete)
30-74	Insufficient
< 30	Deficient

Table 1. Vitamin D thresholds

AIM: To determine the vitamin D status of patients with CKD stage 5 pre- and post-transplantation and to assess whether successful kidney transplantation improves circulating 25D levels.

2. Study design

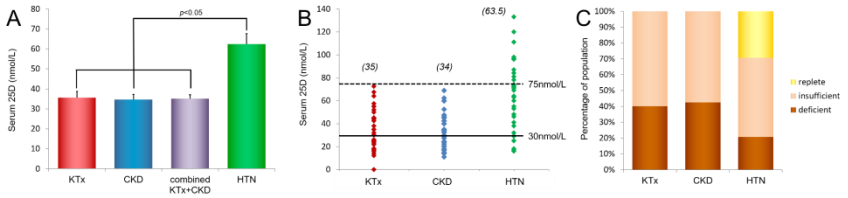
Serum 25D was assessed in renal transplant patients within 4 weeks prior to kidney transplantation (KTx) in parallel to CKD5 patients (CKD; GFR ≤15) and healthy medication-controlled hypertensive subjects (HTN; GFR >60) at time zero (T0) and 1 year later (T1). 25D was measured using the Elecsys Vitamin D Total Assay (Roche). Vitamin D thresholds are outlined in table 1. Statistical analysis was performed using Wilcoxon matched pairs, Kruskal-Wallis and Spearman correlation (SPSS). Data represent mean ± SEM or median.

Table 2. Baseline characteristics of the study population. Data represents mean ±SEM

	KTx *(n=32)*	CKD *(n=33)*	HTN *(n=34)*
Age	45.2 ± 2.59	47.6 ± 2.34	55.2 ± 1.24
Male (%)	59	73	44
BMI (kg/m²)	24.7 ± 0.62	27.4 ± 1.00	28.0 ± 0.58
Caucasian (%)	97	67	82

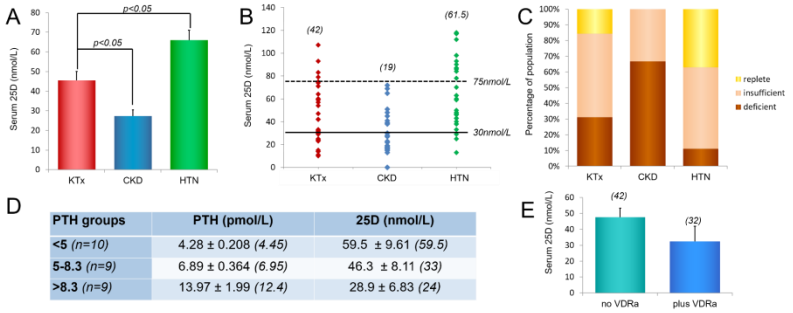
3. Advanced chronic kidney disease is associated with low serum 25D

(A) Basal serum 25D at T0 prior to kidney transplant (KTx) and in CKD stage 5 (CKD) was significantly lower than that in a healthy hypertensive population (HTN).
(B and C) 100% of the KTx and CKD patients had levels <75nmol/L and 43% were deficient (<30nmol/L). In contrast in the HTN group 29.4% were replete and only 20.6% were deficient.
Data represents the mean ± SEM *(median)*.



4. Kidney transplantation does not correct 25D insufficiency

(A) Serum 25D at 1 year (T1) post-kidney transplant (KTx) and in CKD stage 5 (CKD) remained significantly lower than that in a healthy hypertensive population (HTN) and levels in the CKD group had decreased still further.
(B and C) Although levels increased in 47% of KTx patients and the mean levels showed a small non-significant increase to 45.5 ± 4.6nmol/L, only 15.6% (5 pts) achieved levels ≥75nmol/L and 31.2% remained deficient.
(D) Post-transplantation PTH was correlated with serum 25D but not eGFR. Spearman correlation $p<0.01$
(E) At T0, 56% of KTx were receiving alphacalcidol/calcitriol (VDRa). This reduced to 15.6% post-transplant. Of the 15 patients whose 25D levels increased only two remained on alphacalcidol/calcitriol.
Data represents the mean ± SEM *(median)*



D

PTH groups	PTH (pmol/L)	25D (nmol/L)
<5 *(n=10)*	4.28 ± 0.208 *(4.45)*	59.5 ± 9.61 *(59.5)*
5-8.3 *(n=9)*	6.89 ± 0.364 *(6.95)*	46.3 ± 8.11 *(33)*
>8.3 *(n=9)*	13.97 ± 1.99 *(12.4)*	28.9 ± 6.83 *(24)*

5. Discussion and conclusion

All patients with CKD had low levels of serum 25D. This may be due to; reduced sunlight exposure, uraemia effecting liver hydroxylation or poor diet. We would also suggest that increased activity of 24-hydroxylase, possibly as a consequence of supplementation with a VDR activator (VDRa) and removal of 25D (Figure 1), could be key. Although kidney transplant improved 25D levels in some patients they still remain insufficient. This, along with the advice for these patients to avoid skin sun exposure due to immunosuppressive therapy means routine long term supplementation should be considered.

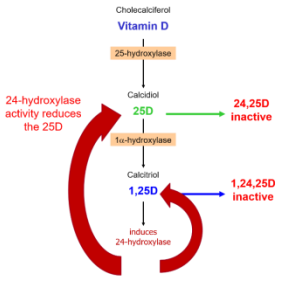
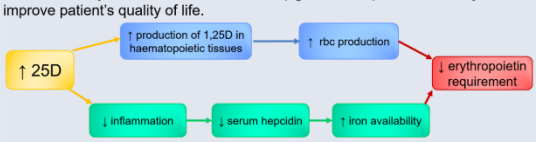


Figure 1.
Induction of 24-hydroxylase activity by VDRa results in metabolism of both 1,25D and 25D.

6. Possible clinical implications of low serum 25D

Low serum 25D will impact on extra-renal synthesis of 1,25D which is necessary for regulation of the immune system and inflammation. Increased inflammation is common in CKD and is associated with resistance to erythropoietin (EPO) and increased hepcidin; a key regulator of iron homeostasis. We suggest that vitamin D repletion will reduce inflammation and so reduce hepcidin which will improve iron availability and EPO effectiveness (figure below) and this may in turn improve patient's quality of life.



KDIGO guidelines suggest vitamin D supplementation should be considered in all stages of CKD including stage 5D. Given the potential clinical benefits we will be implementing routine screening and vitamin D supplementation of all haemodialysis and transplant patients at UHCW, Coventry. We plan to develop robust supplementation guidelines and evaluate the effectiveness through studying the impact of vitamin D repletion on EPO use and quality of life.

Warwick Medical School
Medical and Life Sciences Research Fund
The University of Warwick
British Heart Foundation
University Hospitals Coventry and Warwickshire NHS Trust

British Endocrine Society (Edinburgh, UK 2017)

1,25-dihydroxyvitamin D deficiency in haemodialysis patients is corrected by vitamin D supplementation.

Sharon Huish[1,2], Carl Jenkinson[3], Simon Fletcher[1], Janet Dunn[2], Martin Hewison[3] and Rosemary Bland[1,2]
[1]University Hospitals of Coventry and Warwickshire NHS Trust, Coventry, [2]The University of Warwick, Coventry, [3]The University of Birmingham, Birmingham

1. Reduced vitamin D synthesis in haemodialysis patients

Reduced renal synthesis of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) from 25-hydroxyvitamin D [25(OH)D] in end stage renal disease (ESRD) results in low serum $1,25(OH)_2D$. This appears to be due to reduced renal cell function and elevated serum fibroblast growth factor 23 (FGF23) (figure 1). Treatment strategies have therefore focussed on $1,25(OH)_2D$ or its synthetic analogues, alfacalcidol or paricalcitol (VDR activators; VDRa). However this overlooks 25(OH)D deficiency, which is common in ESRD.

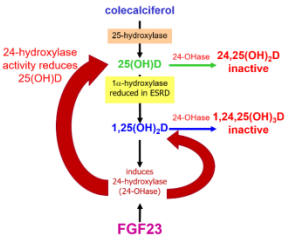


Figure 1. Why might vitamin D metabolites be reduced in ESRD? Patients with ESRD have low 25(OH)D levels and reduced renal 1α-hydroxylase activity reduces $1,25(OH)_2D$. Induction of 24-hydroxylase (24-Ohase*)* activity by VDRa and/or FGF23 (raised in ESRD) results in metabolism (and so reduces levels) of both $1,25(OH)_2D$ and 25(OH)D.

Production of $24,25(OH)_2D$ is considered an indicator of 24-OHase activity.

Serum 25(OH)D (nmol/L)	Vitamin D status	Basal vitamin D levels (% of patients)
≥ 75	Optimal (replete)	5 (n=21; median 102nmol/L)
30-74	Insufficient	13 (n=115; median 39nmol/L)
< 30	Deficient	65 (n=249, median 16nmol/L)

Table 1. Vitamin D deficiency in HD patients is common. Vitamin D thresholds and % of patients in each category (n=385). 95% of patients had serum 25(OH)D levels <75nmol/L. Only 21 patients (5%) had optimal levels (≥75nmol/L.

2. Study design

We have shown previously that vitamin D deficiency is prevalent in ESRD (table 1). Consequently all patients having in-centre haemodialysis (HD) at University Hospitals Coventry and Warwickshire (UHCW) NHS Trust are routinely screened for vitamin D deficiency. Patients are monitored and data collected routinely as outlined in figure 2. In the study reported here multiple vitamin D metabolites were assessed in a subset of 33 HD patients at baseline and 1 year after the introduction of vitamin D supplementation into standard routine care.

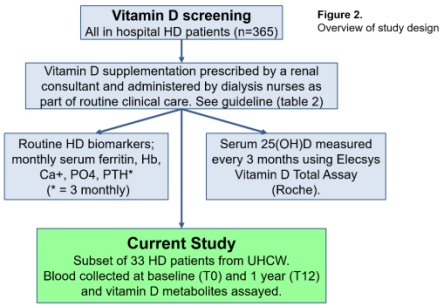


Figure 2. Overview of study design

Serum 25(OH)D	Colecalciferol Dose
<50nmol/L	40,000 units weekly
50 - 74nmol/L	20,000 units alternate weeks
75 - 150nmol/L	If not already taking colecalciferol, no indication to start. If taking colecalciferol already - maintain levels on maintenance dose of 20,000 units alternate weeks
> 150nmol/L	STOP colecalciferol, recheck vitamin D level in 3 months and if <150nmol/L restart 20,000 units alternate weeks

Table 2. Clinical guideline for colecalciferol supplementation in haemodialysis patients. (Fultium-D3 20,000IU capsules). Colecalciferol is prescribed by the patient's renal consultant within their dialysis prescription book and administration is overseen by nursing staff

3. $1,25(OH)_2D$ is corrected by colecalciferol

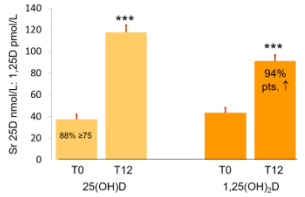
A

Gender	numbers % (n)	Age (years)		Baseline weight (kg)		HD vintage (years)	
		Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)
Female	48 (16)						
Male	52 (17)	63.3±14.96	97.5 (27-85)	86±24.9	79.7 (45.4-144.4)	4.40±3.30	4.37 (0.24-11.6)
Caucasian	88 (29)						

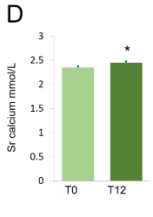
B

	Basal values (T0)		12 months (T12)		p
	Mean ± SEM	Median (range)	Mean ± SEM	Median (range)	
25(OH)D (nmol/L)	37.4 ± 4.38	31 (9.2-145.0)	117.7 ± 6.61	123 (18.5-196.7)	<0.001
$1,25(OH)_2D$ (pmol/L)	43.4 ± 4.75	52.8 (30.7-94.3)	91.2 ± 5.3	84 (37.1-177.3)	<0.001

C



D



A. Baseline characteristics of the study population. **B.** Serum 25(OH)D and $1,25(OH)_2D$ levels of the patient population at T0 and T12. Basal $1,25(OH)_2D$ levels were low in all patients (ideal reference range 55-139pmol/L) **C.** Graphical illustration of the change in serum 25(OH)D and $1,25(OH)_2D$ levels from basal levels (T0) to replete (T12) **D.** Serum corrected calcium levels. No hypercalcaemia was associated with colecalciferol supplementation. C and D data represents mean±SEM; * p<0.05, *** p<0.001 T0 *vs.* T12.

METHODS

- Multiple serum vitamin D metabolites were measured using liquid chromatography-tandem mass spectrometry Samples were extracted by SLE extraction and a Waters ACQUITY UPLC – Xevo TQ-S mass spectrometer was used for analysis.
- Statistical analysis was performed using paired t-tests and Pearson correlations (SPSS).

4. Low 25(OH)D limits synthesis of $1,25(OH)_2D$ in ESRD

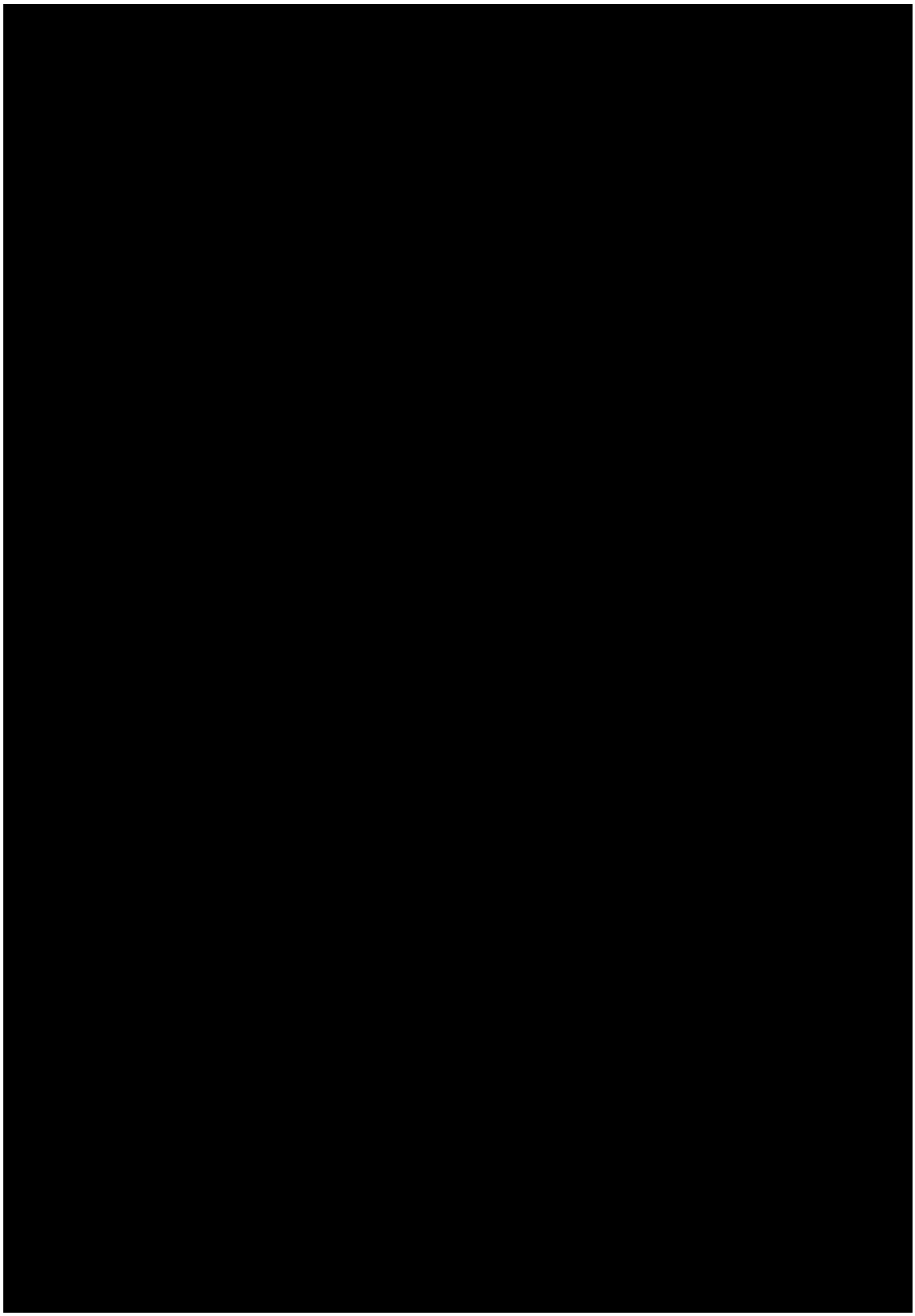
Vitamin D status	Correlations		Significance	? Normal vitamin D metabolism
	Vitamin D metabolite	? correlated with these metabolites/Ca^{2+}		
25(OH)D deficient (T0)	25(OH)D	$1,25(OH)_2D$	<0.05	No
		$24,25(OH)_2D$	<0.01	No
		calcium	ns	Yes
	$1,25(OH)_2D$	$24,25(OH)_2D$	<0.01	Yes
		calcium	ns	No
25(OH)D replete (T12)	25(OH)D	$1,25(OH)_2D$	ns	Yes
		$24,25(OH)_2D$	ns	Yes
		calcium	ns	Yes
	$1,25(OH)_2D$	$24,25(OH)_2D$	<0.01	Yes
		calcium	<0.01	Yes

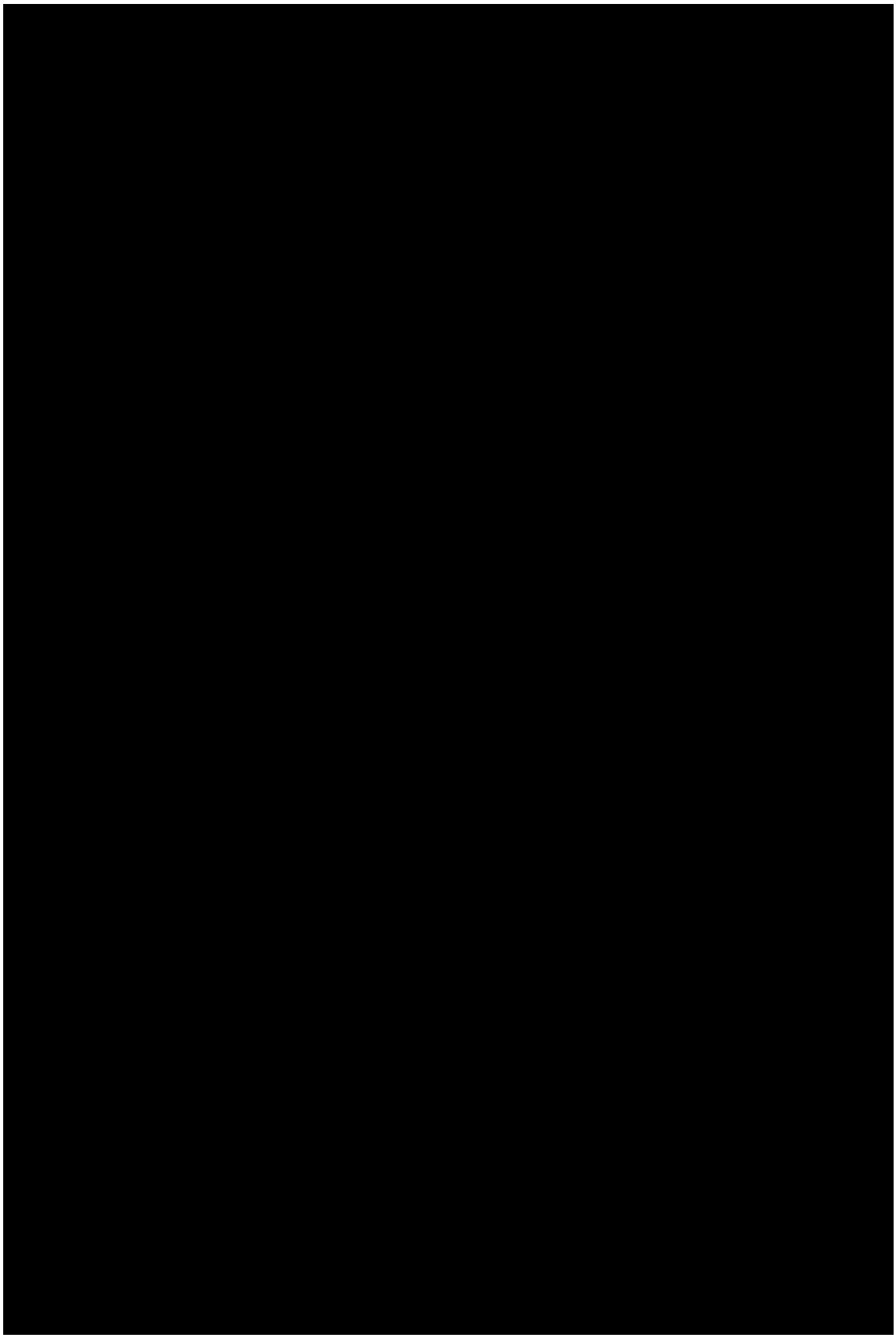
Correlation between vitamin D metabolites. Once 25(OH)D was replete (T12) although correlation between $1,25(OH)_2D$ and $24,25(OH)_2D$ was maintained (p<0.001), neither correlated with serum 25(OH)D, suggesting serum 25(OH)D was limiting $1,25(OH)_2D$ synthesis at T0 (25(OH)D is normally in excess and so non-limiting). Vitamin D metabolism appeared to be normalised post-repletion.

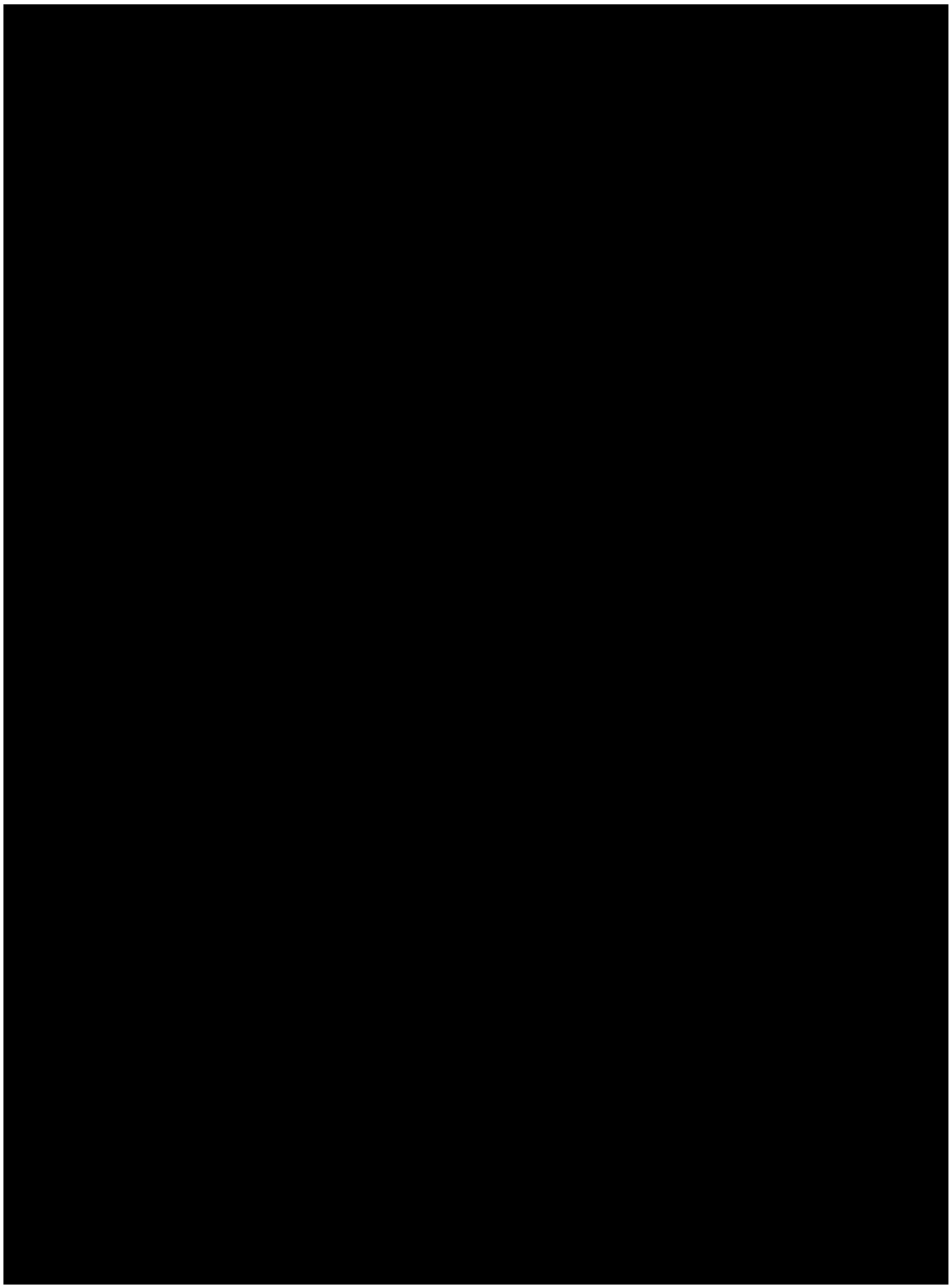
5. Conclusions and discussion points

- These data suggest that low serum 25(OH)D limited $1,25(OH)_2D$ synthesis at T0.
- Vitamin D repletion (colecalciferol supplementation) in haemodialysis patients was safe and significantly increased serum $1,25(OH)_2D$. Demonstrating that kidneys with a low GFR and elevated FGF23 retain the ability to synthesise $1,25(OH)_2D$ in a substrate-dependent fashion.
- Complementing $1,25(OH)_2D$ analogue treatment with colecalciferol may prove effective in managing bone and mineral disorders associated with renal disease.

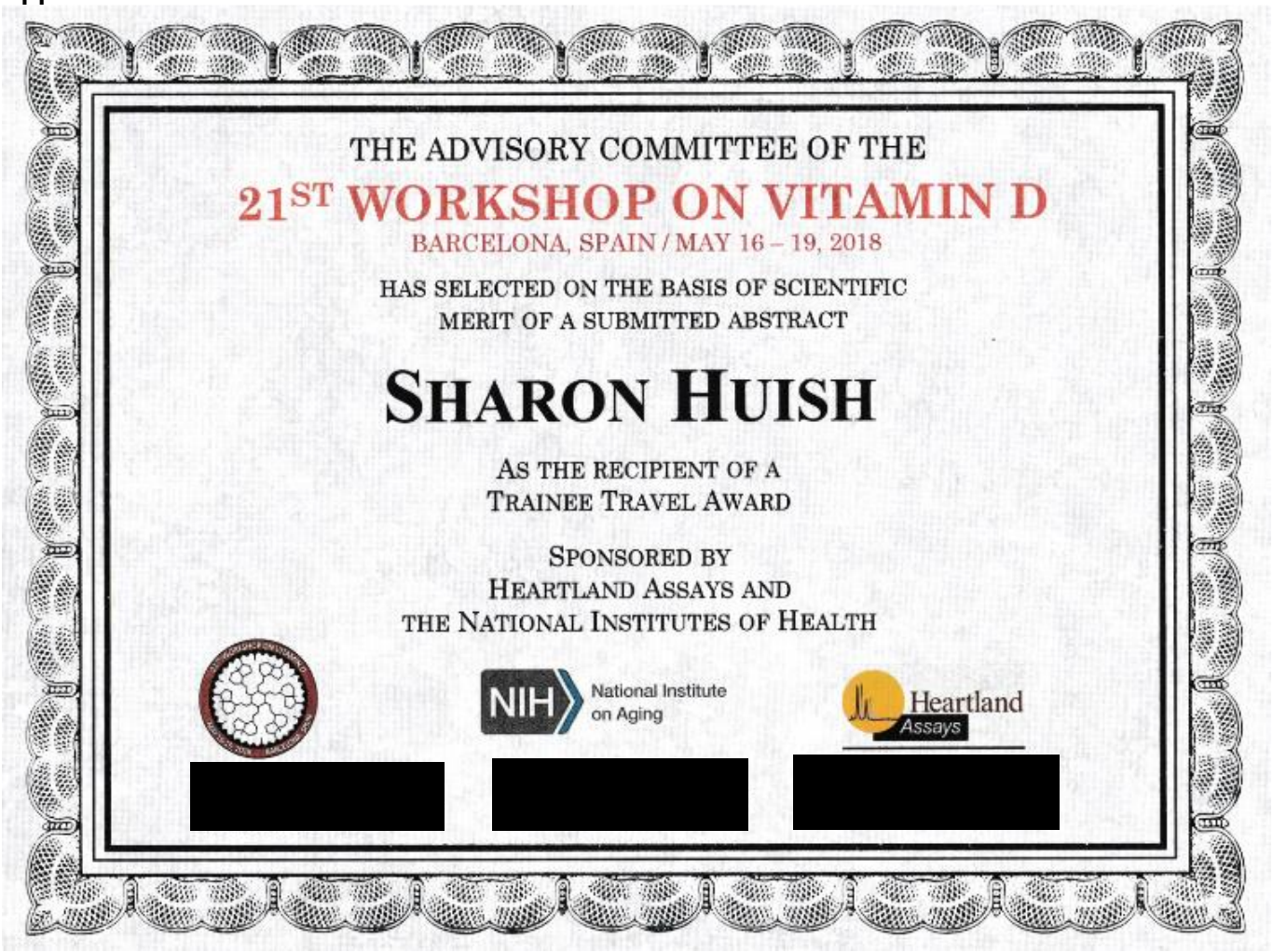
Appendix J Complete Nutrition magazine article







Appendix K Trainee travel award – certificate of scientific merit



THE ADVISORY COMMITTEE OF THE

21ST WORKSHOP ON VITAMIN D

BARCELONA, SPAIN / MAY 16 – 19, 2018

HAS SELECTED ON THE BASIS OF SCIENTIFIC MERIT OF A SUBMITTED ABSTRACT

SHARON HUISH

AS THE RECIPIENT OF A TRAINEE TRAVEL AWARD

SPONSORED BY HEARTLAND ASSAYS AND THE NATIONAL INSTITUTES OF HEALTH

NIH National Institute on Aging

Heartland Assays

[redacted] [redacted] [redacted]

Appendix L British Renal Society British Kidney Patient Association grant application outcome letter

BRITISH KIDNEY Patient ASSOCIATION
improving life for kidney patients

BRITISH RENAL SOCIETY MULTI-PROFESSIONAL TEAM

Sharon Parker
University Hospital Coventry & Warwickshire NHS Trust
Nutrition & Dietetics Department
2nd Floor, Rotunda
Clifford Brige Road
Walsgrave, Coventry, CV2 2DX

7th January 2014

Dear Miss Parker,

Ref: Evaluating the impact of routine cholecalciferol on Erythropoietin (EPO) Requirements

Thank you for submitting a proposal to the BRS Research Committee. Each application was judged by the BRS Research Committee according to the criteria listed in the "information for applicants" and with the benefit of reports from independent referees.

The Research committee reviewed your grant application and gave it a favourable opinion. Indeed your resilience to amend, take advice and revise your previous submission to a higher quality was commended by the committee members. However the maximum research award allocated for your study was **£17,500.** The committee felt unable to fund the Hepciden assays (£6,500). Please can you confirm you are happy to receive this lower award? If the award is to be accepted we require you to revise your study costs and submit an amended budget identifying clearly what has been altered or changed within the research plan and costs to accommodate this allocated funding

Please send us a copy of ethical approval when available, if you have not already done so. We are unable to pay out the award until we are in receipt of this**. You must take up the Grant Award within 12 months of the date of this offer.**

Grant recipients will be expected to complete a progress report at six monthly intervals and a final progress report will be required at the end of the project. These should include mention of all abstracts and publications arising from the funded project. We do not consider the research to be complete until it has been published.
Please acknowledge that this work was funded by BRS/BKPA in any presentations or publications arising from this award.
Recipients will be expected to present their work at a BRS Conference. If you are attending the UK Kidney Week 2014 Conference in Glasgow, you should plan to go to the Research Forum session on Thursday 1st May at 16.00 where previous grant recipients will be presenting their work.

Two years after the completion of your project we would ask you to send us more information about your research to demonstrate the impact it has had in changing patterns of care locally and nationally, and what benefit it has been to patients. This is an important component of the research committee's feedback and also helps in our aim to attract funding into multi-professional patient centred research.

In future years, we may approach you to be a member of an advisory panel to help applicants submit a successful grant application. This is with a view to attracting a wide range of individuals and professions into the research arena and we hope you will agree to assist the BRS in this way.

The amount of your grant will be paid upon receipt of an invoice to the British Renal Society for the full amount and after our receipt of your ethical approval letter.

Finally, we enclose comments received from the lead reviewer and extracts of the reviews that were presented to the committee for your information.
We hope you find them helpful.

Yours Sincerely,

[redacted]

Simon Ball
BRS President

[redacted]

Paula Ormandy
Chair, BRS Research for Renal Committee

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