

Vitamin D and endothelial function in chronic kidney disease

Submitted in partial fulfilment of the requirements for

the degree of

Doctor of Medicine (Research)

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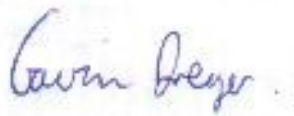
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Details of collaborations and acknowledgements

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ABSTRACT

Background

Vitamin D deficiency in patients with chronic kidney disease, measured by reduced serum concentrations of 25 hydroxy vitamin D, is highly prevalent and associated with both endothelial dysfunction and an increased risk of cardiovascular disease. Observational studies in chronic kidney disease have demonstrated that vitamin D therapy reduces the risk of cardiovascular disease. In patients with chronic kidney disease and concomitant vitamin D deficiency, the effect of vitamin D therapy on endothelial function, which is associated with cardiovascular disease, is poorly understood. The mechanism by which vitamin D affects endothelial function is unclear.

Methods

Presented in this thesis, two studies have addressed these issues:

1. A double blind, randomized controlled trial evaluating the effect of ergocalciferol compared to placebo on microcirculatory endothelial function in patients with non-dialysis chronic kidney disease and concomitant vitamin D deficiency
2. *In vitro* and *in vivo* experiments to determine the mechanistic effect of ergocalciferol on endothelial function in an experimental model of uraemia.

Results

In the clinical study, ergocalciferol increased vitamin D serum concentrations and improved microcirculatory endothelial function measured by laser Doppler flowmetry after iontophoresis of acetylcholine. Oxidative stress measured by skin

autofluorescence for advanced glycation end products did not change in the ergocalciferol group but increased significantly in the placebo group.

Ergocalciferol increased endothelial nitric oxide synthase expression and activity in cultured human endothelial cells and improved endothelial function in an *in vivo* model of mild uraemia.

The findings from the *in vivo* and clinical studies occurred independently of changes in blood pressure, conduit artery function, serum calcium, phosphate and parathyroid hormone supporting *in vitro* findings that ergocalciferol acts directly on the endothelium.

Conclusion

Ergocalciferol improved endothelial function in both rodent and human subjects with chronic kidney disease. Experimental evidence suggests this effect occurs through an endothelium dependent mechanism involving changes in the upregulation and function of endothelial nitric oxide synthase.

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ABBREVIATIONS

25 (OH) D	25 hydroxy vitamin D
ACE-I	Angiotensin converting enzyme inhibitor
ACh	Acetylcholine
ADMA	Asymmetrical dimethyl arginine
aPWV	Aortic pulse wave velocity
CAD	Coronary artery disease
CI	Confidence interval
CKD	Chronic kidney disease
cMRI	Cardiac magnetic resonance imaging
CV	Cardiovascular
CVD	Cardiovascular Disease
CRP	C-reactive protein
DM	Diabetes mellitus
EDHF	Endothelium-derived hyperpolarizing factor
EM	Ethnic minority
eNOS	Endothelial nitric oxide synthase
ESKD	End stage kidney disease
FCD	Functional capillary density
FMD	Flow mediated vasodilatation
FGF-23	Fibroblast growth factor 23
GFR	The glomerular filtration rate
HR	Hazard ratio
hs CRP	Highly sensitive C-reactive protein
IL	Interleukin
IMT	Intima-medial thickness
IU	International units
KDIGO	Kidney Disease Improving Global Outcomes
LDF	Laser Doppler flowmetry
LDI	Laser Doppler imaging

L-NAME	N-omega-nitro-L-arginine methyl ester
LVM	Left ventricular mass
LVMi	Left ventricular mass index
MAP	Mean arterial pressure
MFI	Microvascular flow index
NO	Nitric oxide
NOS	Nitric oxide synthase
P:CR	Protein creatinine ratio
PE	Phenylephrine
PORH	Post-occlusive reactive hyperaemia
PTH	Parathyroid hormone
PWV	Pulse wave velocity
RAAS	Renin-angiotensin-aldosterone system
RRT	Renal replacement therapy
SDF	Side stream dark field imaging
SHR	Spontaneously hypertensive rats
SNP	Sodium Nitroprusside
SNx	Sub-total nephrectomy
SpNO	Spermine NONOate
VDBP	Vitamin D binding protein
VDD	Vitamin D deficiency
VDI	Vitamin D insufficiency
VDR	Vitamin D receptor
VDRA	Vitamin D receptor activator
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
UVB	Ultra Violet B

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Vitamin D

1.1.1 Historical aspects of vitamin D

The history of the use of vitamin D in medical practice extends back as far as the early 19th century when sun exposure was recognised as an important preventative and potentially curative treatment for rickets¹. Almost 100 years later, the use of radiation from a mercury arc lamp for one hour three times a week was shown to increase long bone mineralisation in children with rickets based on radiographic evidence². These findings could not be replicated in children not exposed to mercury arc lamp radiation, leading to the conclusion that ultraviolet (UV) radiation was essential for the treatment of rickets. In 1918, rickets in puppies was treated with the use of cod liver oil and in 1922, the potential factor leading to the amelioration of rickets was termed vitamin D³⁻⁴. This prompted the exposure of certain food stuffs, notably yeast, to UV light which was subsequently demonstrated to have anti-rachitic properties.

When the structure of vitamin D was identified and its synthesis from yeast achieved, it was eventually added to milk at a standard dose of 400 international units (IU) per quarter gallon. This represents the first known fortification of food with vitamin D¹. However, differences in the vitamin D extracted from yeast and that contained in cod liver oil became apparent when chickens with rickets were treated with both vitamin D obtained from yeast and cod liver oil. Yeast extracted vitamin D exerted no anti-rachitic properties whereas cod liver oil was associated with an improvement in rickets. Given these two types of vitamin D were synthesized differently and had potentially differing biological effects, they were named vitamin D₂ (synthesized artificially from yeast) and vitamin D₃ (synthesized intrinsically from skin). Since this original

delineation of vitamin D compounds, the understanding of the synthesis, actions and pharmaceutical compounds of vitamin D has expanded significantly.

1.1.2 Vitamin D compounds and metabolism

Vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol) are referred to as nutritional forms of vitamin D. Originally, ergocalciferol and cholecalciferol were considered biologically equivalent, however there is now evidence that cholecalciferol is more potent than ergocalciferol⁵. Both compounds are equally efficaciously absorbed and equally converted to 25 hydroxy vitamin D (25 (OH) D) in the liver. However, ergocalciferol upregulates a range of enzymes which causes the degradation of both ingested ergocalciferol and intrinsic cholecalciferol⁶. Despite this, ergocalciferol can be safely used to replete circulating stores of vitamin D⁷.

Vitamin D is generated in humans by the conversion of 7-dehydrocholesterol to previtamin D₃ in the epidermis which is then rapidly metabolised to vitamin D₃. This conversion occurs after exposure of the skin to ultra-violet B (UVB) light between a wavelength of 290-315 nm. To generate a sufficient store of vitamin D such that it can usefully released during winter months when sunlight exposure is ineffective at converting 7-dehydrocholesterol to previtamin D₃, exposure of bare arms and legs to the correct incident angle of sunlight between the hours of 10am and 3pm twice a week is sufficient depending on season, skin tone and latitude¹.

Between the months of October to April, the incident angle of sunlight on the skin is inadequate to generate the formation of previtamin D₃ in 90% of the UK, most of Western Europe and 50% of North America⁸. UVB from sunlight also results in the degradation of any excessive cholecalciferol, thus preventing vitamin D toxicity⁹.

Approximately 50% of vitamin D production is due to conversion of the vitamin D₃ precursor to cholecalciferol with the additional component coming from dietary sources including oily fish and mushrooms¹⁰.

Vitamin D is transported through the circulation to the liver in chylomicrons where it undergoes 25 hydroxylation in the liver to 25 (OH) D, a process primarily mediated by the mitochondrial P450 enzyme in hepatic tissue (CYP27A1) but which can also be mediated through other enzymatic pathways including the microsomal cytochrome P450 enzymes CYP2R1, CYP3A4 and CYP2J3¹¹. In the systemic circulation, approximately 85-90% of 25 (OH) D is bound to vitamin D binding protein (VDBP), with 10-15% bound to albumin and <1% available in the free form¹²⁻¹³. The 25 (OH) D and VDBP complex is filtered at the glomerulus and reabsorbed by the proximal tubule, a process which is mediated by the endocytic receptors megalin and cubulin¹⁴. 25 (OH) D is released into the mitochondria after degradation by proximal tubular lysosomes where it undergoes the final hydroxylation step, mediated by kidney 1-OHase activity (CYP27B1), to 1,25 (OH)₂ D₃ before returning to the systemic circulation¹⁵.

The conversion of 25 (OH) D to 1,25 (OH)₂ D₃ is increased in the presence of hypocalcaemia, hypophosphataemia and elevated serum concentrations of parathyroid hormone (PTH) and decreased by elevated serum concentrations of fibroblast growth factor 23 (FGF-23), 1,25 (OH)₂ D₃ and serum phosphate¹⁶. 1,25 (OH)₂ D₃ reduces the activity of CYP27B1¹⁰ and also induces the expression of 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) which metabolises both 25 (OH) D and 1,25 (OH)₂ D₃ to the inactive calcitriolic acid¹⁷. Thus, 1,25 (OH)₂ D₃ acts to prevent significant vitamin D toxicity through a negative feedback mechanism. Table 1 describes the major vitamin D compounds, analogues and their respective function and clinical utility.

Table 1 Summary of the most common vitamin D compounds, nomenclature, chemistry and clinical applications.

Generic name	Chemical name	Molecular formula	Type of vitamin D compound	Clinical use
Previtamin D	7-Dehydro cholesterol	$C_{27}H_{44}O$	Vitamin D ₃ precursor	Vitamin D ₃ precursor
Vitamin D ₂	Ergocalciferol	$C_{28}H_{44}O_2$	Nutritional vitamin D	Vitamin D supplement
Vitamin D ₃	Cholecalciferol	$C_{27}H_{44}O$	Nutritional vitamin D	Vitamin D supplement
25-hydroxy vitamin D (25 (OH) D)	Calcidiol	$C_{27}H_{44}O_2$	25 hydroxylated vitamin D	Major metabolite of vitamin D
1,25 hydroxy vitamin D (1,25 (OH) ₂ D ₃)	Calcitriol	$C_{27}H_{44}O_3$	Active vitamin D metabolite	Major active vitamin D metabolite
24,25 hydroxy vitamin D	Calcitroic acid	$C_{23}H_{34}O_4$	Inactivated vitamin D metabolite	Inactivated vitamin D metabolite
1-alpha calcidol	1-hydroxy cholecalciferol	$C_{27}H_{44}O_2$	Synthetic vitamin D ₃ analogue	PTH suppression, increase calcium in CKD
Paricalcitol	19-nor-1,25-(OH) ₂ -vitamin D ₂	$C_{27}H_{44}O_3$	Synthetic 1,25-dihydroxy ergocalciferol analogue	PTH suppression, increase calcium in CKD
Doxercalciferol	1-Hydroxy vitamin D ₂	$C_{28}H_{44}O_2$	Synthetic vitamin D ₂ analogue	PTH suppression, increase calcium in CKD

1,25 (OH)₂ D₃ has a range of biological effects which are predominantly involved with the regulation of calcium and phosphate (Figure 1). Specifically, 1,25 (OH)₂ D₃ increases the absorption of calcium and phosphate from the intestine, a process which is mediated through the interaction of 1,25 (OH)₂ D₃ and the vitamin D receptor (VDR) in the intestinal lumen which increases the expression of the intestinal epithelial calcium channel transient receptor potential vanilloid type 6 (TRPV6)^{9 18}. 1,25 (OH)₂ D₃ acts on renal tubules to promote the reabsorption of calcium and phosphate as well as mobilising calcium stores from bone through the stimulation of pre-osteoclasts to differentiate into mature osteoclasts. This process is mediated through the binding of 1,25 (OH)₂ D₃ to its receptor on osteoblasts causing an increase in the expression of the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)⁹. Preosteoclasts which express receptor activator of NF-κB (RANK) bind to RANKL and mature into differentiated osteoclasts which causes calcium and phosphate release from bones into the systemic circulation⁹. Parathyroid hormone (PTH) stimulates the kidneys to reabsorb calcium and increases renal production of 1,25 (OH)₂ D₃ as well as stimulating calcium reabsorption from bone through the RANK/RANKL pathway. These mechanisms are the primary regulators of serum calcium and phosphate. Significant abnormalities of vitamin D synthesis, actions and regulatory pathways occur in CKD and are discussed in section 1.2.4.

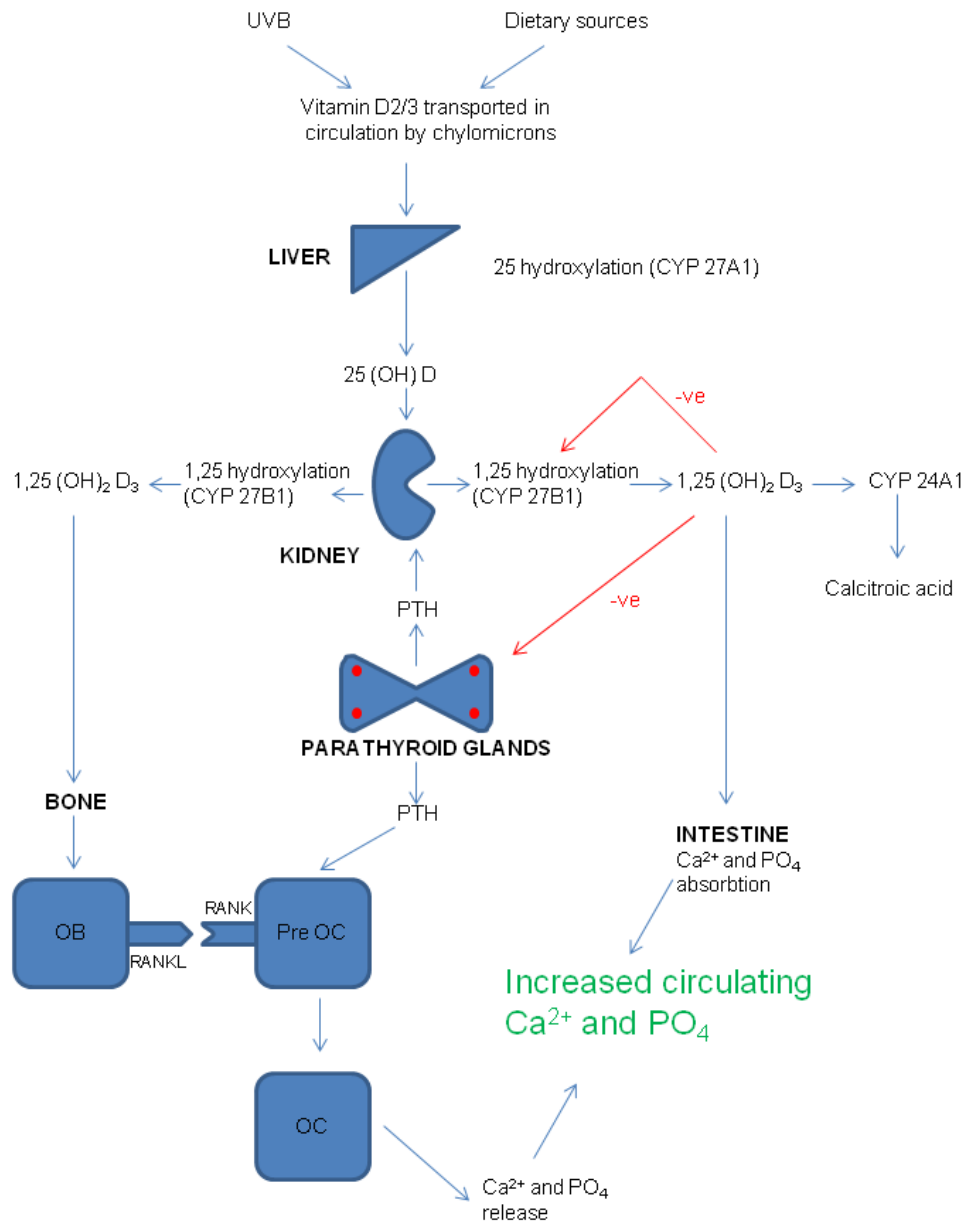


Figure 1 Metabolism and calcaemic effects of vitamin D. Vitamin D is generated by UVB exposure or through dietary intake. 25-hydroxylation of circulating vitamin D occurs in the liver before conversion to 1,25(OH)₂D₃ occurs in the kidney or peripheral tissues. Circulating concentrations of calcium and phosphate are increased through reabsorption from bone and intestine. 1,25(OH)₂D₃ inhibits the effect of CYP27B1 and PTH (red arrows). Adapted from Holick 2006¹

UVB – ultraviolet B radiation, PTH – parathyroid hormone, OB – osteoblast, OC – osteoclasts – RANK - receptor activator of NF-κB, RANKL - receptor activator of nuclear factor-κB (NF-κB) ligand

25 (OH) D is the most abundant form of vitamin D in the body (1000x greater concentration than 1,25 (OH)₂ D₃)¹¹ and is used as the routine biochemical assay target for vitamin D status in clinical practice. This is due to its long half-life (2-3 weeks compared to 3-4 hours for 1,25 (OH)₂ D₃), its more reproducible assay^{8 10 19}, better correlation with markers of bone metabolism than serum 1,25 (OH)₂ D₃ concentrations¹¹ and since serum 25 (OH) D concentrations reflect vitamin D intake from both food and sunlight¹⁰. Given that 25 (OH) D is the substrate for extra renal CYP27B1¹¹, this adds strength to the argument that serum 25 (OH) D concentrations should be carefully monitored and maintained.

1.1.3 Pleiotropic actions of vitamin D

The effect of 1,25 (OH)₂ D₃ is exerted through the activation of the vitamin D receptor (VDR) which is universally expressed in almost all tissues in humans²⁰⁻²¹ and is reported to control the activation of approximately 200 genes¹⁴. In humans, the VDR is coded for on chromosome 12 and is formed from 427 amino acids²². 1,25 (OH)₂ D₃ is transported to the target cell bound to VDBP, crosses the cell membrane and binds to the VDR inside the nucleus¹¹. Target cells expressing extra renal CYP27B1 can uptake VDBP bound 25 (OH) D and internally convert it to 1,25 (OH)₂ D₃ via intracellular CYP27B1. The VDR binds 1,25 (OH)₂ D₃ with 100 times more affinity than 25 (OH) D which indicates the importance of local intracellular 1,25 (OH)₂ D₃ in the process of gene transcription²³.

The final step in VDR activation involves the binding of ligand activated VDR to the retinoic acid X receptor (RXR)^{9 22}. The ligand bound and activated VDR only binds to nuclear elements of vitamin D responsive genes via a specific vitamin D response element (VDRE) which is located upstream of vitamin D response genes. The

activated VDR is then involved with the recruitment of positive or negative transcription factors which activate or inactivate gene transcription^{11 22}.

Through these mechanisms and due to the presence of extra renal CYP27B1, vitamin D has a number of pleotropic effects including activity in macrophage cells, pancreatic, breast, and prostatic tissue^{9 24} (Figure 2). Macrophages, when stimulated by *Mycobacterium tuberculosis* (MTB), upregulate the VDR and CYP27B1. If there is sufficient substrate (25 (OH) D) for extra renal CYP27B1 available, intracellular 1,25 (OH)₂ D₃ upregulates production of cathelicidin, a peptide that can destroy MTB. The overall effect of 1,25 (OH)₂ D₃ on the immune system involves increased microbicidal capacity as well as reducing the risk of auto-immune diseases¹⁸. Evidence for this latter phenomenon is provided by epidemiological studies that link auto-immune conditions such as multiple sclerosis²⁵⁻²⁶ and type 1 diabetes mellitus (DM)²⁷ to lower concentrations of vitamin D.

There is increasing epidemiological evidence that the incidence of some malignancies, including breast, colon and prostate, is reduced when exposure to sunlight is increased^{18 23}. 1,25 (OH)₂ D₃ is thought to mediate anti-cancer effects through the regulation of multiple genes which control cell proliferation (p21 and p27) and the inhibition of genes that increase angiogenesis and promote apoptosis^{9 18}. However, there remains uncertainty about the effect of vitamin D on the reduction of cancer incidence which requires further investigation¹⁸.

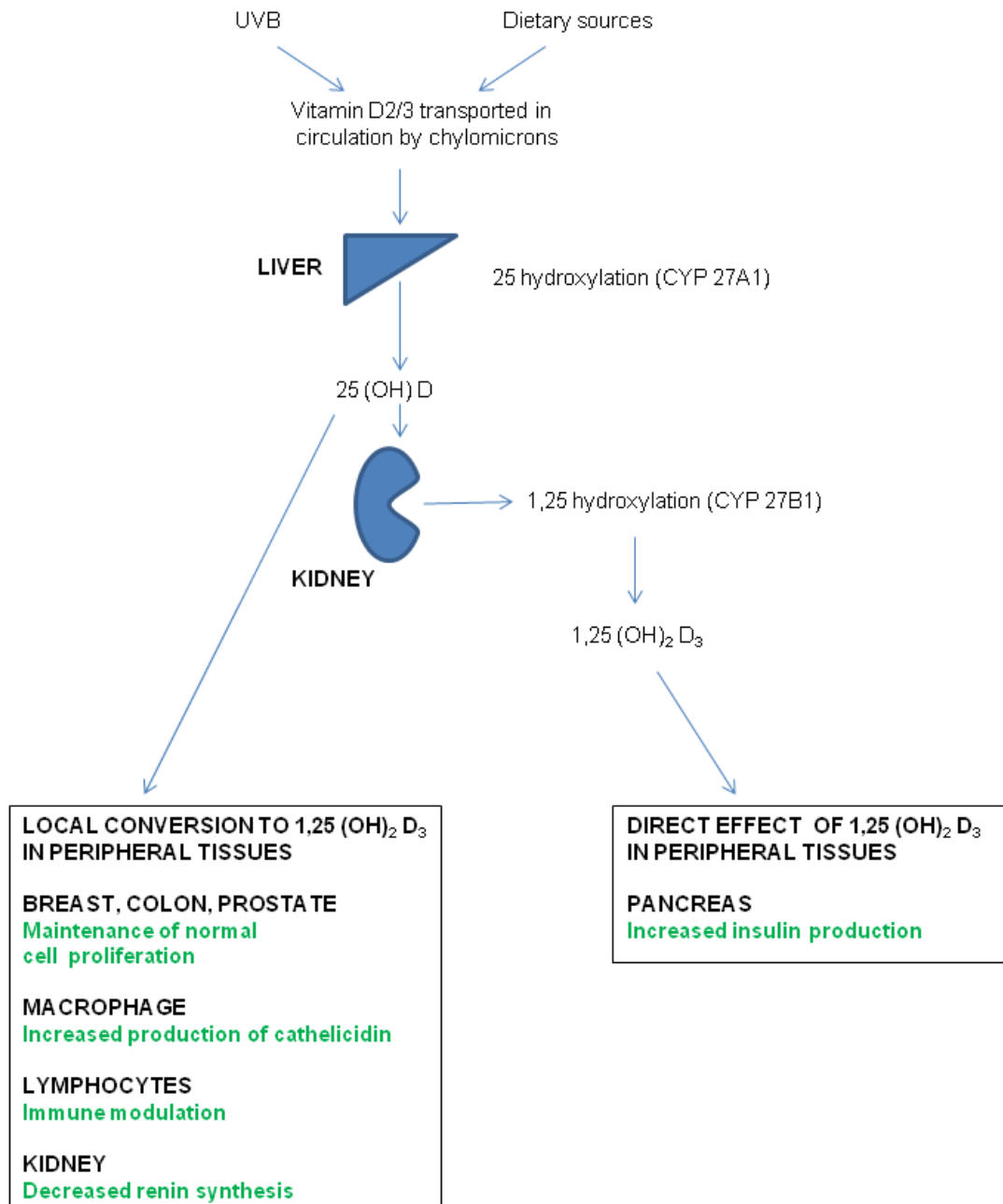


Figure 2 Pleiotropic effects of vitamin D. 25(OH)D is converted to 1,25(OH)₂D₃ in pancreatic, endothelial, kidney and B and T lymphocytic cells. 1,25(OH)₂D₃ has a direct effect on breast, colon, prostate and macrophage cells. Adapted from Holick 2006¹

Vitamin D mediates at least part of its effects on the renin-angiotensin-aldosterone system (RAAS) with circulating serum concentrations of both 25 (OH) D and 1,25 (OH)₂ D₃ having been shown to negatively correlate with serum concentrations of renin²⁸. In a rat model of experimental uraemia induced by 5/6th nephrectomy in which animals were treated with paricalcitol or control, there was a reduction in renin and vascular endothelial growth factor (VEGF) mRNA, as well as reduced serum concentrations of renin and angiotensin in the remaining renal mass²⁹. These findings occurred independently of changes in calcium and phosphate and were additionally associated with reductions in proteinuria, hypertension and progression of renal disease. Therefore, VDD is associated with abnormalities in the RAAS system which, in rat models of experimental uraemia, can be partly overcome by therapeutic intervention with activated vitamin D compounds²⁹.

The findings from pre-clinical studies that vitamin D can reduce proteinuria and modulate the RAAS system has been evaluated in clinical studies. Agarwal *et al.*³⁰ examined data from 220 patients with CKD stage 3-4 and secondary hyperparathyroidism enrolled into 3 clinical trials across 46 sites in the USA and Poland. Patients were randomized to either paricalcitol (mean dose 9.5 µg per week) or placebo. Proteinuria was reduced in the active treatment group compared to placebo (p=0.04) independently of DM, pharmacological suppression of the RAAS system, age, sex, ethnicity or degree of hypertension. However, this study used a semi quantitative measure of proteinuria in the form of a urine dipstick rather than a more precise laboratory measure of proteinuria as well as including patients enrolled into 3 separate clinical trials and so the results must be interpreted with caution.

Paricalcitol has been studied as an intervention for left ventricular hypertrophy (LVH) in patients with CKD. In two randomized controlled trials by Thadani *et al.*³¹ and Wang *et al.*³² comparing paricalcitol to placebo in patients with stage 3-5 CKD, LVH was assessed by cardiac magnetic resonance imaging (cMRI) after 48-52 weeks of therapy. Both studies were relatively small (n=227 in the Thadani *et al.*³¹ study, n=60 in the Wang *et al.*³² study) but after nearly 1 year of follow up in each study, neither study demonstrated a significant difference in left ventricular mass (LVM) or size but in both studies, PTH was reduced in the paricalcitol group. However, in the study by Thadani *et al.*³¹, left atrial size, which is associated with improved long term survival in patients treated with haemodialysis³³, reduced in the paricalcitol treated group and therefore longer term follow up in this study may have demonstrated a reduction in mortality.

1.1.4 Vitamin D deficiency and the effect of vitamin D supplementation on all cause survival

Vitamin D deficiency may occur as a result of inadequate nutrition, intestinal malabsorption, or lack of exposure to sunlight. The highest risk groups for VDD include ethnic minority groups with darker coloration of the skin, the elderly, especially those in institutions including long term care facilities, individuals with limited sunlight exposure, obese individuals and individuals taking drugs which adversely affect the metabolism of vitamin D^{8 34}.

Worldwide, VDD is now considered a new medical syndrome. However, there is no consensus opinion on the optimum serum 25 (OH) D concentration⁹ making an understanding of the epidemiology of VDD challenging. Several authors have proposed that VDD exists when serum 25 (OH) D concentrations are < 50 nmol/L and

recommended targets for serum concentration of 25 (OH) D vary between 50-75 nmol/L³⁵⁻³⁸. These conclusions are largely drawn from optimisation of calcium transport and suppression of PTH, data which cannot be readily applied to reduction in cancer risk or cardiovascular (CV) mortality in kidney disease.

A recent Cochrane review of 50 trials including 94,148 participants³⁴ examining the effect of vitamin D therapy for prevention of mortality in adults identified that only cholecalciferol reduced all-cause mortality (relative risk (RR) 0.97, 95% CI 0.94 - 1.00) while ergocalciferol, alfacalcidol and calcitriol had no mortality benefit and that these compounds increased the risk of hypercalcaemia. The finding that cholecalciferol but not ergocalciferol reduces all cause mortality has also been reported by Chowdhury *et al.*³⁹ in a large meta-analysis of the effect of vitamin D on all-cause mortality in 880,128 adults (RR cholecalciferol 0.89, 95% CI 0.80 to 0.99, RR ergocalciferol 1.04, 95% CI 0.97 to 1.11). In a meta-analysis by Autier *et al.*⁴⁰, the effect of vitamin D supplementation on all-cause mortality in 18 studies including 57,311 patients either supplemented or not with a heterogeneity of vitamin D compounds demonstrated a slight reduction in all-cause mortality in patients supplemented with vitamin D (RR for death in supplemented patients 0.93, 95% CI 0.87 - 0.99). In contrast to this, an umbrella review of meta-analyses, systematic reviews and randomized controlled trials of serum concentrations of 25 (OH) D and any outcome concluded that there is little convincing evidence for the overall beneficial effect of vitamin D therapy⁴¹. The effect of VDD on morbidity and mortality in patients with CKD is specifically discussed in section 1.2.6.

The existing evidence for the association between serum concentrations of 25 (OH) D may need to be reconsidered in light of a new paradigm for the measurement of serum

vitamin D concentrations suggested by Powe *et al.*¹³. This study demonstrated that in Black compared to White community dwelling adults in the USA, two common single nucleotide polymorphisms of the VDBP involving the rs7041 and rs4588 polymorphisms may explain lower serum concentrations of total 25 (OH) D in Black compared to White patients. This study demonstrated lower levels of VDBP and lower serum 25 (OH) D concentrations in Blacks but that there were equivalent serum concentrations of bioavailable 25 (OH) D in both Blacks and Whites while Black patients tended to have higher bone mineral density and a lower risk of fractures. Thus, standard measurement of serum 25 (OH) D concentrations may not accurately represent vitamin D status and may lead to the over classification of VDD in Black populations.

1.1.5 The effect of ethnicity on vitamin D metabolism and replacement

The effect of ethnicity on serum 25 (OH) D concentrations and therefore the differential survival of ethnic minority groups is particularly important since ethnic minority groups are likely to be at a higher risk of VDD due to darker skin tone, a diet which may predispose to VDD, biological and sociological aspects of vitamin D metabolism^{9 42-43}. Ethnic variations in the structure of the VDR have been described⁴⁴, however, the functional consequences of VDR mutations in ethnic groups and specifically how environmental factors interact with VDR polymorphisms has yet to be fully ascertained⁴⁵.

Tareen *et al.*⁴⁶ compared the prevalence of VDD in CKD and non-CKD patients comparing ethnicity in both groups. This study used observational data from the National Health and Nutrition Examination Survey (NHANES) study⁴⁷ and excluded

patients taking high dose ergocalciferol but included those taking “routine” vitamin D supplements containing lower doses of ergocalciferol (although the details of these supplements are not specified). In both CKD and non-CKD patients not taking vitamin D supplements, Black and Hispanic ethnicities were associated with a lower mean serum 25 (OH) D concentration (White non-CKD 78.8 nmol/L, White CKD 80.8 nmol/L, Black non-CKD 48.0 nmol/L, Black CKD 57.6 nmol/L, Hispanic non-CKD 62.7 nmol/L, Hispanic CKD 59.0 nmol/L) and a higher prevalence of VDD (White non-CKD 48.3% White CKD 54.6%, Black non-CKD 86.4%, Black CKD 85.1%, Hispanic non-CKD 65.9%, Hispanic CKD 73.5%).

Sanchez *et al.*⁴⁸, in a prospective study of 184 patients (mean age 67.2 years, 48.9 % female, mean eGFR 36.0 mL/min) treated with ergocalciferol for VDD in CKD according to Kidney Disease Outcomes and Quality Initiative (K/DOQI) guidelines⁴⁹, demonstrated that Hispanic compared to Caucasian ethnicity was independently associated with failure to achieve recommended serum 25 (OH) D concentrations of > 75 nmol/L (adjusted odds ratio (OR) for achieving vitamin D repletion in Hispanics 0.25, 95% CI 0.10 – 0.62, p=0.0032).

In addition to risk factors associated with low serum concentrations of 25 (OH) D, Awumey *et al.*⁴² demonstrated increased activity of CYP24A1 in skin fibroblasts of Asian Indian immigrants to the USA compared to a control Caucasian population which they postulated may explain the lower serum concentrations of 25 (OH) D in South Asian patients in their study. Their analysis was conducted in patients without significant kidney disease but support the hypothesis that genetic variants in ethnic minority patients may play a significant role in determining vitamin D status and the likely response to therapy.

1.1.6 Summary

The understanding of vitamin D biology and its effect as a hormone involved in the regulation of calcium homeostasis has evolved to include an understanding of the pleiotropic effects of vitamin D. Epidemiological and scientific evidence have provided insights into the extra renal synthesis and metabolism of vitamin D which includes evidence suggesting that vitamin D replacement may reduce all-cause mortality. The effect of ethnicity on vitamin D status has emerged as an important facet of vitamin D biology and recent evidence has indicated that standard serum measures of vitamin D may not fully describe vitamin D status in ethnic minority groups rendering these groups vulnerable to misdiagnosis and inadequate treatment for VDD.

1.2 Chronic kidney disease

1.2.1 Background

CKD has previously been defined as a progressive, irreversible loss of kidney function. More recently, the definition of CKD has been revised and the classification is now based on the estimated glomerular filtration rate (eGFR)⁴⁹⁻⁵⁰ (Table 2). The glomerular filtration rate (GFR) can be measured by investigations such as inulin clearance, creatinine clearance and radio-isotopic techniques. However, these investigations are expensive and invasive. There are several estimating equations for the GFR in routine use, the most commonly used being the 4 variable Modification of Diet in Renal Disease (MDRD) equation which requires a measure of the patient's serum creatinine, age, gender and ethnicity⁵¹ from which the GFR is estimated. This equation was used at the time of designing the experiments for this thesis. However, new estimating have been introduced and include the CKD EPI equation and variants of this equation using serum creatinine alone or serum creatinine combined with serum cystatin C⁵².

Table 2 Current classification of CKD. Reproduced from Kidney Disease Improving Global Outcomes (KDIGO) 2012 guidelines⁵³ Kidney damage is defined as the presence of albuminuria (urine albumin to creatinine ratio > 30, urine sediment abnormalities, structural renal abnormalities on imaging or a history of kidney transplantation). Abnormalities must be present at least twice over a period of greater than 3 months for the diagnosis of CKD to be confirmed

Degree of kidney damage	Equivalent stage of CKD	Abnormal parameters
Evidence of kidney damage (one or more) and GFR \geq 60 mL/min/1.73m ²	Stage 1 CKD – GFR \geq 90 mL/min/1.73m ² Stage 2 CKD – GFR 60-90 mL/min/1.73m ²	<ul style="list-style-type: none"> • Albumin excretion ratio \geq 30mg/24 h <ul style="list-style-type: none"> • Urine albumin to creatinine ratio \geq30mg/g • Abnormal urine sediment • Tubular disorders causing electrolyte abnormalities • Structural abnormalities detected by kidney biopsy or imaging of the kidney • Previous renal transplant
Reduced glomerular filtration rate	Stage 3a CKD – GFR 45-60 mL/min/1.73m ² Stage 3b CKD – GFR 30- 45 mL/min/1.73m ² Stage 4 CKD – GFR 15-30 mL/min/1.73m ² Stage 5 CKD – GFR \leq 15 mL/min/1.73m ²	GFR < 60 mL/min/1.73m ²

1.2.2 The epidemiology of chronic kidney disease

The prevalence of CKD varies globally and even within geographical regions. Two studies from the USA estimate the prevalence of CKD stage 3-5 from between 4.7% and as high as 19.1% with differing ethnic profiles of the populations accounting for the wide discrepancy⁵⁴⁻⁵⁵. Globally, the prevalence of CKD is as high as 12.4% in Kinshasa (Democratic Republic of Congo)⁵⁶ and 19.4% in Northern India⁵⁷. Many of the prevalence studies for CKD have only used one measure of serum creatinine and urine dipstick rendering their prevalence estimates less accurate than might have been obtained if two measures of urine and creatinine had been obtained.

In the United Kingdom (UK) the population prevalence of CKD has been estimated as 8.5%, however only 0.63% of subjects reported their ethnicity in this study and thus the ethnic specific prevalence of CKD in the UK remains unclear⁵⁸. The prevalence of CKD in the UK is as high as 18-22% in populations with DM and hypertension respectively⁵⁸⁻⁶⁰. Ethnic differences in the prevalence of CKD in the UK in high risk populations have been described. In a study from east London, an area of high multi-ethnicity and deprivation, among diabetic patients with CKD⁵⁹, South Asian and Black ethnicities were associated with an increased OR for stage 4-5 CKD compared to White ethnicity (South Asian OR 1.54 (95% CI 1.26 – 1.88), Black OR 1.39 (95% CI 1.06 – 1.81)). A study set in the same area that evaluated the prevalence of CKD in patients with hypertension across different ethnicity groups⁶⁰ demonstrated that South Asian (OR 1.53, 95% CI 1.21 - 1.89) but not Black ethnicity (OR 1.02, 95% CI 0.78 - 1.27) was associated with more severe CKD (stage 4-5). Both these studies were cross sectional in nature and are prone to the problems associated with this study design including missing eGFR data and details of the severity of DM and hypertension. However,

these studies do highlight the important effect of non-White ethnicity on the prevalence of CKD in high risk populations in multi-ethnic areas of the UK.

1.2.3 The pathology and epidemiology of cardiovascular disease in chronic kidney disease

The anatomical pattern of CVD in CKD differs to that conferred by traditional risk factors seen in the general population. Arterial stiffness⁷⁵, vascular calcification⁷⁶ and left ventricular hypertrophy⁷⁷ dominate in CKD. Left ventricular hypertrophy is a strong predictor of mortality in patients treated with dialysis^{69 78} and a prevalent co-morbidity in patients with non-dialysis requiring CKD with over 50% of patients developing LVH when the eGFR is < 30 mL/min⁷⁷. These anatomical differences result in different patterns of cause of death from CVD in the general and CKD population, with cardiac failure, dysrhythmia and sudden cardiac arrest more prevalent in patients with CKD⁷⁹.

Progression to ESKD in patients with CKD is far less common than death from CVD, the risk of which is increased even in mild CKD⁶¹⁻⁶² and accounts for approximately 40% of all deaths in patients with ESKD⁵⁷⁻⁵⁸. Furthermore, as CKD progresses, the coexistence of traditional CV risk factors increases⁶³. CKD, even in its earlier stages, is now recognized to be as strong a CV risk factor as DM and hypertension⁶⁴.

In a study from the USA, the hazard ratio (HR) of death from any cause in patients with an eGFR <15 mL/min compared to individuals with an eGFR of ≥60 mL/min was 5.0 (95% CI 5.4 - 6.5) and the HR for CV events was 3.4 (95% CI 3.1 - 3.8)^{2 65}. In a post-hoc analysis of the Hypertension Optimal Treatment trial, the adjusted risk ratios for a major CV event (CV death or all-cause mortality) were all significantly increased ($p < 0.001$) in patients with an eGFR <60 mL/min⁶⁶.

Culleton *et al.*⁶⁷ examined 15 year follow up data from the Framingham heart study during which, in 6,233 study participants, there were 1,000 CV events and 1,406 deaths. In women, CKD (defined as a serum creatinine between 136 and 265 $\mu\text{mol/L}$) was not associated with either an increased risk for CVD (HR 1.04, 95% CI 0.79 - 1.37) or all-cause mortality (HR 1.08, 95% CI 0.87 - 1.34). In men, CKD was not associated with CVD (HR 1.17, 95% CI 0.88 - 1.57) but was associated with increased all-cause mortality (HR 1.31, 95% CI 1.02 - 1.67). This study has the significant advantage of long term follow-up, robust definitions of CVD and due to the nature of the cohort, limited missing data ensuring that key covariates were included in adjusted models for outcomes. However, the study was conducted before the current classification of CKD based on the eGFR and it is thus limited by the relatively crude definition of CKD based primarily on a range of serum creatinine rather than the precise stage of CKD.

Data from the Second National Health and Nutrition Examination Mortality Survey⁶⁸, which followed 6,354 individuals over 16 years, demonstrated that in subjects with a low eGFR (< 70 mL/min) compared to a normal eGFR (> 90 mL/min), there was an increased HR for death from CVD (HR 1.68, 95% CI 1.33 - 2.13) and all-cause mortality (HR 1.51, 95% CI 1.19 - 1.91). However, the study did not include an assessment of non-fatal CV events including cardiac dysrhythmia, a major cause of death in CKD which has been included in other studies of CVD in CKD⁶⁹.

The increased risk of CVD in CKD has been established in different ethnic groups, age groups and geographical areas⁷⁰. Studies from Canada⁷¹ and Taiwan⁷² identified a reduction in life expectancy from CVD in patients aged > 30 years as the stage of CKD worsened. In the Canadian study⁷⁰, for stage 3a, 3b, 4 and 5 CKD, life expectancy was

reduced respectively by 1.3, 7, 12.5 and 16.7 years. In Taiwan⁷², the reduction in life expectancy in the same stages of CKD was 2.1, 8.8, 17.8 and 21.3 years.

End stage kidney disease treated with dialysis confers a significantly higher risk of death than in age matched members of the general population^{69 73}. In France, the standardised mortality ratio of dialysis patients aged 18-44 compared to the general population in the first year of initiating dialysis was 26.7⁷³. The high CV mortality in ESKD is independent of age^{65 69} with young dialysis patients having a near 100-fold increase in risk of CV death than an age and sex matched individual from the general population⁶⁹ (Figure 3). Data from the Renal Registry UK report from 2012⁷⁴ have demonstrated that in patients aged 30-34 years receiving dialysis for ESKD, the mortality rate compared to the general population was 18-fold higher with CVD accounting for 22% of all deaths in patients receiving dialysis.

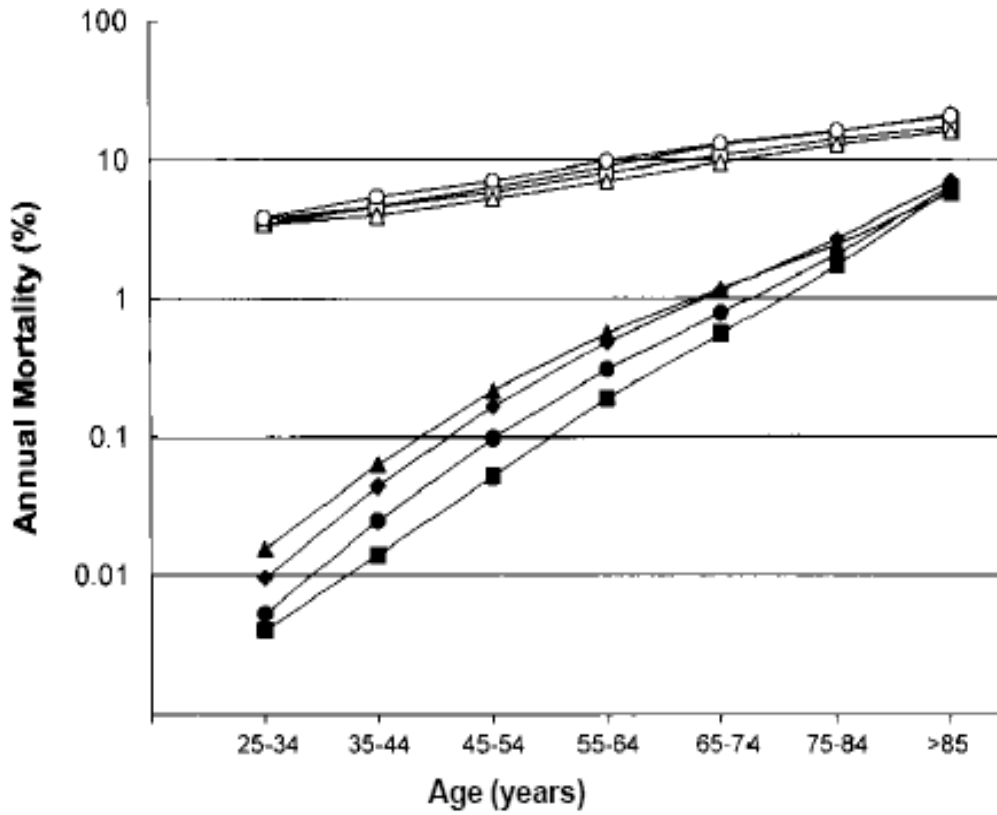
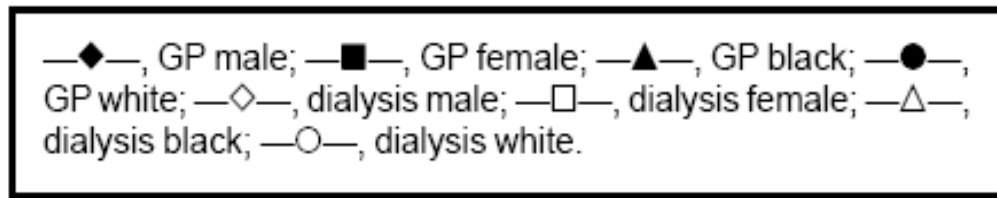


Figure 3 Cardiovascular disease in end stage kidney disease – from Foley *et al.*⁶⁹
 The observed effects on mortality of gender, ethnicity and age in the general population are no longer apparent when compared to patients receiving dialysis therapy indicating the significant effect of dialysis requiring ESKD on increased mortality compared to the general population.

1.2.4 Vitamin D metabolism and vitamin D receptor activation in chronic kidney disease

CKD affects vitamin D synthesis, metabolism and activity through reduced serum concentrations of 25 (OH) D, reduced production of 1,25 (OH)₂ D₃ and resistance to circulating vitamin D¹⁴. The combination of these mechanisms results in abnormalities of bone mineral metabolism and affects the pleotropic actions of vitamin D.

Patients with all stages of CKD including ESKD are vulnerable to VDD for multiple reasons. Skin synthesis of vitamin D is reduced due to lower exposure to sunlight and hyper pigmentation of the skin in patients with CKD⁷⁶⁻⁷⁷. Uraemia interferes with the normal skin synthesis of vitamin D from exposure to UV sunlight⁷⁸ and, through its associated effect on appetite, reduces the intake of foods rich in vitamin D and the absorption of vitamin D from the intestinal tract⁷⁹. Uraemia has also been associated with reduced hepatic metabolism of 25 (OH) D which has been shown to be mediated through the effect of PTH in downregulating the activity of liver CYP450 isoforms including CYP27A1⁸⁰.

In CKD, 1,25 (OH)₂ D₃ metabolism is affected by reduced availability of its substrate (25 (OH) D) for conversion and reduction in renal mass, a consequence of progressive kidney disease, which also leads to reduced availability and activity of the converting enzyme CYP27B1. However, the function of extra-renal CYP27B1 may be regulated differently to the renal version of the enzyme. This hypothesis is supported by a study by Jean *et al.*⁸¹ who treated 43 ESKD patients treated with haemodialysis with VDD (serum 25 (OH) D concentration < 75 nmol/L, mean age 72.6 years, mean duration on dialysis 71 months) with 400-1200 IU/day of cholecalciferol for 6 months. At the end of

the study period, serum concentrations of both 25 (OH) D and 1,25, (OH)₂ D₃ increased significantly (25 (OH) D - 27.8 to 118 nmol/L (p < 0.001), 1,25 (OH)₂D₃ 7.7 to 30.5 pmol/L (p < 0.001)) and a positive correlation between 25 (OH) D and 1,25 (OH)₂D₃ was observed (p=0.02). These findings indicate that even in patients with an extended period of time on dialysis, extra renal CYP27B1 activity is sufficient to increase serum concentrations of 1,25 (OH)₂ D₃.

In addition to reduced availability of CYP27B1, systemic acidosis, the uraemic milieu and elevated serum concentrations of phosphate and FGF-23 reduce the activity of CYP27B1⁸²⁻⁸⁴. Inactivation of 1,25 (OH)₂ D₃ is increased by the upregulation of CYP24A1 which in CKD is a consequence of elevated serum concentrations of PTH and FGF-23⁸². Low serum concentrations of 1,25 (OH)₂ D₃, reduced binding of 1,25 (OH)₂ D₃ to the VDR and reduced binding of this whole complex to the nuclear vitamin D response element result in resistance to vitamin D⁸⁵⁻⁸⁶. In addition, the overall expression of the VDR is reduced in both uraemia and in uraemic induced hyperphosphataemia, the latter of which also modulates VDR mediated gene transcription⁸⁷. In advanced CKD, there is decreased expression of the VDR in parathyroid tissue resulting in a reduced response to vitamin D therapy⁸⁸.

1.2.5 The epidemiology of vitamin D deficiency in chronic kidney disease

The majority of the epidemiological data on VDD in CKD is described in patients receiving dialysis, with fewer studies examining the prevalence of VDD in earlier stages of CKD. A cross sectional study of 201 subjects by LaClair *et al.*⁸⁹ examined serum 25 (OH) D concentrations in patients with CKD stage 3-4 in 12 geographically distinct regions of the USA. 71% of patients with stage 3 and 83% of patients with stage 4 CKD had insufficient serum 25 (OH) D concentrations (<75 nmol/L). This study is in

line with the findings that low serum concentrations of 25 (OH) D are present in patients even with mild abnormalities before abnormal serum concentrations of bone markers such as calcium, PTH and phosphate are evident⁹⁰⁻⁹¹.

Even in geographical locations where exposure to sunlight is high, VDD in CKD is common. Cuppari *et al.*⁹² studied 144 patients with stage 2-5 CKD living in a solar rich environment in Brazil. Severe VDD was rare (only found in 1 patient) but vitamin D insufficiency (VDI) defined as a serum 25 (OH) D concentration of 40-75 nmol/L was present in 39.6% of patients and serum 25 (OH) D concentrations exhibited a downward linear trend as CKD advanced. This study highlights the importance of ambient sunlight as source of vitamin D. In addition to increased ambient sunlight, the younger age, relative preservation of renal function, exclusion of diabetic patients and low numbers of patients with significant proteinuria in this study may explain higher serum concentrations of 25 (OH) D compared to other studies.

Zehnder *et al.*⁹³ have demonstrated that the earlier stages of CKD are associated with reduced serum concentrations of both 25 (OH) D and 1,25 (OH)₂ D₃ as well as a reduction in the ratio between 1,25 (OH)₂ D₃ and 25 (OH) D. In this study, 249 patients with CKD (defined as serum creatinine > 130 µmol/L) were compared to 79 age and sex matched controls. Serum concentrations of 25 (OH) D and 1,25 (OH)₂ D₃ were lower in patients with CKD compared to healthy controls (25 (OH) D 42.1 vs. 60.4 nmol/L p<0.0001, 1,25 (OH)₂ D₃ 58.2 vs. 119.5 pmol/L, p<0.0001). In addition, serum concentrations of both 25 (OH) D (p<0.01) and 1,25 (OH)₂ D₃ (p<0.001) were negatively correlated with kidney function measured by serum cystatin C. The ratio of 1,25 (OH)₂ D₃ to 25 (OH) D was higher in healthy controls than patients with CKD and this ratio was negatively correlated with kidney function (p<0.001). This study did not

record the exact nature of vitamin D supplementation in patients with CKD or healthy controls, however, the findings indicate that even in the early stages of CKD, there are significant abnormalities of vitamin D metabolism.

VDD in patients receiving dialysis is a very common clinical entity. In a study of 825 incident haemodialysis patients from over 500 dialysis centres in the USA, Wolf *et al.*⁹⁴ established that 78% of patients had a serum 25 (OH) D concentration < 75 nmol/L and 18% had a serum 25 (OH) D concentration < 25 nmol/L. Gonzalez *et al.*⁹⁵ examined serum 25 (OH) D concentrations in a cohort of dialysis and non-dialysed CKD patients. The prevalence of VDD was high in both groups but significantly higher and almost universally present in patients treated with dialysis in whom the prevalence of VDD was 97% compared to 86% in non-dialysed CKD patients. Saab *et al.*⁹⁶ in a study of 131 haemodialysis patients in the USA, have reported the prevalence of VDD as 92% and Taskapan *et al.*⁹⁷, in 273 peritoneal dialysis patients in Greece and Turkey, have reported the prevalence of VDD as 96%.

1.2.6 The association between vitamin D status and morbidity and mortality in chronic kidney disease

Observational studies have provided evidence that VDD is associated with CV morbidity and mortality in both dialysis and non-dialysis requiring CKD. Wolf *et al.*⁹⁴ evaluated 825 incident haemodialysis patients from the USA to ascertain the effect of vitamin D status on CV and all-cause mortality. Patients who died within 90 days of starting haemodialysis (n=175) were compared to patients who survived beyond 90 days (n=725). The lowest tertile of 25 (OH) D serum concentration (<25 nmol/L) but not 1,25 (OH)₂ D₃ (<6 pg/mL) was strongly associated with all cause and CV mortality within 90 days of starting dialysis (lowest tertile of 25 (OH) D all-cause mortality HR 1.9,

95% CI 1.3 – 2.9, CV mortality HR 1.9, 95% CI 1.0 – 3.4). These findings were independent of residual renal function, nutritional status and markers of bone mineral metabolism.

Drechsler *et al.*⁹⁸ described the effect of VDD on CV mortality (defined as a composite of CV events and death from an unknown cause) in 762 incident dialysis patients (both haemodialysis and peritoneal dialysis) from the Netherlands (61% male, mean age 59 years). An adjusted Cox regression analysis comparing patients with a serum 25 (OH) D concentration of less than or greater than 25 nmol/L demonstrated increased CV mortality in the low vitamin D group after 6 months (HR 2.7, 95% CI 1.1 - 6.5) and at 3 years (HR 1.7, 95% CI 1.1 - 2.7) with no effect of vitamin D status seen on non-CV mortality. The effect of VDD was modified by PTH with low serum 25 (OH) D concentrations only affecting CV mortality in patients with a PTH > 123 pmol/L (HR compared to patients with a PTH < 123 pmol/L 3.37, 95% CI 1.64 – 6.91). The same authors report the risk of sudden cardiac death in haemodialysis patients in a post-hoc analysis of the 4D study which examined the effect of atorvastatin compared to placebo in dialysis patients in Germany⁹⁹. Their analysis demonstrated that the risk of sudden cardiac death is increased in patients with serum 25 (OH) D concentrations < 25 nmol/L compared to serum concentrations > 75 nmol/L (HR 2.99, 95% CI 1.39 – 6.40) even after adjusting for age, sex, atorvastatin use, bone mineral parameters, blood pressure, smoking and dialysis vintage. Similar findings have been described by Anand *et al.*¹⁰⁰ who demonstrated that in 256 incident haemodialysis patients followed up for a mean of 3.8 years, patients with a serum 25 (OH) D concentration <27 nmol/L (the lowest tertile of vitamin D status in the study) had a higher incidence of all-cause mortality (HR 1.75, 95% CI 1.03 - 2.97).

In a meta-analysis of 10 studies which included dialysis (7 studies, n=3,441) and non-dialysis requiring CKD patients (3 studies, n=3,412), Pilz *et al.*¹⁰¹ demonstrated that increasing serum concentrations of 25 (OH) D were associated with a reduction in CV mortality. An increase in serum concentrations of 25 (OH) D by 25 nmol/L was associated with an overall reduction in the relative risk of mortality of 0.86 (95% CI 0.82 - 0.91). In the three studies that only evaluated non-dialysis requiring CKD¹⁰²⁻¹⁰⁴, the highest risk for all-cause mortality was consistently demonstrated in the lowest category of VDD although these studies differed in size and populations studied.

Mehrotra *et al.*¹⁰³ have evaluated a large cohort of non-dialysis requiring CKD patients (n=3,011, mean age 54 years, stage 1-4 CKD). Compared to their reference group of a serum 25 (OH) D concentration > 75 nmol/L, the lowest tertile of 25 (OH) D status (< 37.5nmol/L) had an increase in the relative risk of all-cause mortality of 1.56 (95% CI 1.12 - 2.18). The middle tertile (37.5-75 nmol/L) had an increased risk but this failed to attain statistical significance (RR 1.17, 95% CI 0.99 - 1.38).

The benefit of higher serum 25 (OH) D concentrations on co-morbidity endpoints in CKD has also been demonstrated. In 2 separate observational studies of patients with non-dialysis requiring CKD, for every 25 nmol/L reduction in serum 25 (OH) D concentration, there was a 23% increase in the risk of developing coronary artery calcification¹⁰⁵ and a 25% increased risk for more rapid progression of CKD¹⁰⁶.

1.2.7 Active vitamin D compounds and morbidity and mortality in chronic kidney disease

A number of early trials comparing nutritional vitamin D replacement to calcitriol therapy in patients with CKD demonstrated that calcitriol was more effective at lowering PTH at

the cost of a higher incidence of hypercalcaemia and, in separate studies, that nutritional vitamin D replacement therapy did not normalize bone histology after 18 months of treatment¹⁰⁷⁻¹⁰⁸. These small studies led to a preference for the use of calcitriol over nutritional vitamin D in patients with CKD and ESKD treated with dialysis. Therapy with active vitamin D has subsequently been associated with a reduction in all-cause mortality in patients receiving haemodialysis treatment¹⁰⁹⁻¹¹².

Two of the largest studies evaluating the effect of active vitamin D therapy on all-cause mortality in dialysis patients have demonstrated that there are differential effects on mortality when comparing treatment with injectable vitamin D compared to no treatment and that paricalcitol compared to calcitriol therapy differentially reduces all-cause mortality. In the first study¹⁰⁹, 37,173 incident ESKD patients treated with haemodialysis who received no injectable active vitamin D compound compared to any injectable active vitamin D compound were followed up for 2 years. The two groups differed at baseline with the no treatment group having a higher PTH serum concentration compared to the treatment group (354 compared to 179 pg/mL, $p < 0.01$). Using a Cox regression model, the risk of all-cause mortality was reduced in the group receiving any injectable vitamin D compound (HR 0.80, 95% CI 0.76 – 0.83).

In the second observational study by Teng *et al.*¹¹³, the authors evaluated the differential effect of paricalcitol compared to calcitriol in 67,399 prevalent ESKD patients treated with haemodialysis over 3 years. Using a Cox regression model, there was a 16% (95% CI 10 - 21%, $p < 0.001$) greater reduction in mortality with paricalcitol compared to calcitriol after 36 months follow up. When patients were switched from calcitriol to paricalcitol and vice-versa, after 2 years of follow up, the patients who switched to paricalcitol had a significant 2 year survival advantage (73% compared to

64%, $p=0.04$). Both of these studies were not randomized but they provided early evidence for the potential benefit of activated vitamin D therapy in patients treated with haemodialysis and that there may be differential effects on mortality reduction based on the choice of injectable agent.

In a retrospective cohort analysis of 58,058 prevalent ESKD patients treated with haemodialysis followed-up for 2 years, Kalantar-Zadeh *et al.*¹¹⁴ demonstrated a U shaped association with all-cause mortality and paricalcitol use. This phenomenon may be due to the higher PTH seen at baseline in the high dose paricalcitol group which may have been an independent risk factor for mortality rather than therapy with high dose paricalcitol.

Similar findings were obtained by Shoji *et al.*¹¹¹ and Melamed *et al.*¹¹⁵ in smaller, prospective studies of activated vitamin D compounds and survival. Shoji *et al.*¹¹¹ evaluated oral alfacalcidol compared to no therapy in 242 haemodialysis patients and found that over 5 years, therapy with alfacalcidol was associated with a reduction in CV mortality HR 0.38 (95% CI 0.25 – 0.58). Melamed *et al.*¹¹⁵ evaluated 1,007 incident haemo and peritoneal dialysis patients over 2.5 years and compared any use of calcitriol to no use. Patients treated with calcitriol had a reduction in all-cause mortality but the upper confidence interval was equal to 1 (HR 0.74, 95% CI 0.56 – 1.00).

Tentori *et al.*¹¹⁰ evaluated the differential effects of three vitamin D analogues (paricalcitol, doxercalciferol and calcitriol) compared to no vitamin D therapy. In their study of 7,731 incident haemodialysis patients with a median of 37 weeks follow-up, they found no significant difference in all-cause mortality between the different analogues but they identified that in patients not receiving any vitamin D analogue

compared to any analogue, the risk of all-cause mortality was higher HR 1.20 (95% CI 1.10 – 1.32). There was no difference observed between calcitriol and paricalcitol in contrast to the study by Teng *et al.*¹¹³ and this could be explained by the shorter follow and lower number of outcome events in the Tentori study¹¹⁰.

These studies and others are the subject of a meta-analysis by Duranton *et al.*¹¹⁶ which evaluated 10 studies, comparing the effect of activated vitamin D compounds or no vitamin D treatment with survival in haemodialysis patients. The overall effect of vitamin D on all-cause mortality was a risk reduction of 0.73 (95% CI 0.64 - 0.83). This meta-analysis also included 3 studies that have evaluated the effect of active vitamin D compounds on survival in non-dialysis requiring CKD. Shoben *et al.*¹¹⁷ in an observational study of 1,418 patients with CKD 3-4 followed up for a mean 1.9 years demonstrated that calcitriol use was associated with a 26% lower risk for death (95% CI 5 - 42% p=0.016) but that there was an increased risk of hypercalcaemia after treatment with calcitriol. Kovesdy *et al.*¹¹⁸ studied 520 male US veterans with stage 3-5 CKD treated or untreated with calcitriol over a median follow up of 2.1 years. Patients treated with calcitriol had a lower incidence of death or dialysis initiation compared to untreated patients (RR 0.69, 95% CI 0.55 - 0.86, p=0.001). Sugiura *et al.*¹¹⁹, in a retrospective cohort study, evaluated 665 patients with non-dialysis requiring CKD, 107 treated with 0.25 – 0.5 µg per day of alfacalcidol and 558 who received no vitamin D compound. During the follow up period of nearly 6 years in the alfacalcidol group compared to 3.5 years in the non-treatment group, in a Cox regression model, CV events (HR 0.52, 95% CI 0.30 – 0.89, p=0.017) but not death from CVD (HR 0.80 0.44 – 1.46 p=0.46) were significantly lower in the alfacalcidol group. The meta analysis¹¹⁶ of these 3 studies for the effect of active vitamin D on all-cause mortality in non-dialysis

requiring CKD showed an overall beneficial effect of alfacalcidol (HR 0.73, 95% CI 0.55 - 0.98, p=0.04)¹¹⁶.

1.2.8 Nutritional vitamin D compounds and morbidity and mortality in chronic kidney disease

The K/DOQI guidelines⁴⁹ have advocated the use of high dose ergocalciferol therapy for patients with CKD stage 3-4 and VDD with the specific dose regimen based on serum concentrations of 25 (OH) D (Table 3). There have been a number of studies examining the effect of nutritional vitamin D in CKD and ESKD patients but the majority of these studies have primarily focused on changes in biochemical parameters and have not addressed CV events or mortality endpoints¹²⁰⁻¹²⁹. The endpoints of these studies varied as much as their designs (randomized trials, observational cohorts, retrospective analyses) and included the effect of nutritional vitamin D therapy on serum concentrations of PTH^{124 128 130-131}, 1,25 (OH)₂ D₃^{81 132}, serum calcium¹²² and HbA1c¹²². These studies were generally small, and did not include mechanistic analyses of the effect of nutritional vitamin D or have long enough follow-up periods to detect clinically relevant CV events or their surrogates.

A systematic review¹³³ of studies of nutritional vitamin D compounds in patients with CKD and ESKD identified an overall beneficial effect of therapy with vitamin D resulting in a reduction in PTH (mean difference 41.7 pg/mL (95% CI 55.8 - 72.7) and increase in serum 25 (OH) D concentrations (mean difference 60.3 nmol/L (95% CI 49 – 71.5 nmol/L). None of the 22 studies included an assessment of the effect of therapy on CV endpoints or mortality.

Table 3 The treatment of vitamin D deficiency in chronic kidney disease. Reproduced from the Kidney Disease Outcomes and Quality Initiative (K/DOQI) guidelines⁴⁹ for the use of high dose ergocalciferol therapy for patients with CKD and vitamin D deficiency. The specific dose regimen is based on serum concentrations of 25 (OH) D

Serum concentration of 25 (OH) D ng/mL (nmol/L)	Category of vitamin D status	Recommended dose of oral ergocalciferol	Recommended duration of therapy with ergocalciferol	Interval for measurement of serum concentrations of 25 (OH) D
<5 (<12)	Severe VDD	50,000 IU/week for 12 weeks, then monthly or 500,000 IU as a single dose IM	6 months	Confirm adherence. Measure serum concentrations of 25 (OH) D after 6 months
5-15 (12-37)	Mild VDD	50,000 IU/week for 4 weeks then 50,000 IU/month	6 months	Measure serum concentrations of 25 (OH) D after 6 months
16-30 (40-75)	VDI	50,000 IU/month	6 months	No specific recommendations

VDD – vitamin D deficiency, VDI – vitamin D insufficiency, IU –international units, IM – intra-muscular

Two studies have evaluated the effect of ergocalciferol on mortality in patients with CKD. Sprague *et al.*¹³⁴ conducted an observational study of 204 patients with CKD (mean eGFR 31.4 mL/min, mean age 73 years) of whom 160 received 50,000 IU of ergocalciferol weekly for 12 weeks and then monthly compared to no intervention. Patients were followed up for an average of 27 months. The likelihood for the combination of all-cause mortality and dialysis initiation was significantly lower in treated compared to untreated patients (OR 0.11, 95% CI 0.11 - 0.74, p=0.024).

Lishmanov *et al.*¹³⁵, in a retrospective cohort study, evaluated the effect of ergocalciferol therapy on 126 men with a mean age of 70 years with stage 3-4 CKD from a US Veterans cohort. Ninety patients received ergocalciferol based on their initial serum concentration of 25 (OH) D in a dose sufficient to increase serum 25 (OH) D concentrations by greater than or equal to 25% after 6 months of therapy with the remaining patients (n=36) acting as controls. The primary outcome was a composite of CV events and death from CVD. Serum 25 (OH) D concentrations were higher in the ergocalciferol group (85.6 nmol/L vs 40.5 nmol/L, p<0.001). In an adjusted logistic regression analysis, treatment with ergocalciferol predicted a lower OR for the composite endpoint (OR 0.37, 95% CI 0.14–1.00, p=0.05). Despite the small size and retrospective nature of this cohort and borderline statistical significance, this study demonstrates the potential reduction in CV events that can be achieved by supplementing patients with CKD and VDD with ergocalciferol. Notably, the benefit of ergocalciferol was independent of changes in serum PTH suggesting mechanisms other than the amelioration of secondary hyperparathyroidism were responsible for their observations. The nature of the population (elderly American veterans) means these results are difficult to generalise to other populations with CKD.

1.2.9 Summary

CKD is a global health problem resulting in an increase risk of CV events. The metabolism, action and absolute serum concentrations of vitamin D are significantly altered in CKD which exposes patients with all stages of CKD to a high burden of CVD. Observational studies have provided evidence for the beneficial effect of vitamin D in reducing CV morbidity and mortality in CKD. The heterogeneous nature of existing studies in terms of design (observational or interventional) populations (dialysis compared to non-dialysis requiring CKD) and intervention (nutritional compared to active vitamin D) means that the optimum treatment strategy for VDD in CKD remains poorly defined. Specifically, the mechanistic pathway by which vitamin D is exerting a beneficial effect remains unclear.

1.3 Endothelial function in health and in chronic kidney disease

1.3.1 Background

The endothelium is located between the muscular wall of all blood vessels and the blood stream. It responds to stimuli including pressure, shear stress, and hormonal agents which mediate relaxation and contraction of the underlying vascular muscular wall. These stimuli prompt endothelial cells to release agents that regulate vasomotor function, trigger local inflammatory reactions and affect vascular homeostasis¹³⁶.

Endothelial dysfunction, a consequence of the imbalance between relaxing and contractile factors, results in reduced vasodilatation, a pro-inflammatory state, leucocyte adherence and pro-thrombotic consequences that predispose to atherosclerotic plaque formation¹³⁷. These processes are in turn associated with hypertension¹³⁸, coronary artery disease¹³⁹, chronic heart failure¹⁴⁰, and peripheral

artery disease¹⁴¹. Risk factors for endothelial dysfunction include traditional (immobility, smoking, DM, hypertension, dyslipidaemia) and non-traditional parameters (CKD, vasculitis, depression, hyperhomocysteinaemia and vitamin D deficiency)¹³⁷.

Discovered over 30 years ago, the principle mediator of endothelial vasodilatation is nitric oxide (NO)¹³⁶. NO is synthesized from L-arginine by nitric oxide synthase (NOS), using oxygen and nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates¹⁴². NO maintains both the vascular smooth muscle tone and its non-proliferative state as well as reducing platelet and leucocyte adhesion. These effects are modulated by the variable effect of NO on cellular DNA synthesis to either increase or decrease the expression of proteins which modulate vascular smooth muscle cell (VSMC) proliferation¹⁴³ and leucocyte adhesion¹⁴⁴. The vasodilating effect of NO is mediated by increasing cyclic guanosine monophosphate (cGMP) in adjacent VSMC which results in a reduction in basal vascular tone¹⁴². Figure 4 demonstrates pathways of VSMC relaxation.

NOS exists in three forms – inducible NOS (iNOS) which is upregulated in response to pathological stimuli including bacterial infection, neuronal NOS (nNOS) present in neurons and endothelial NOS (eNOS). The latter two forms are present under normal physiological conditions whereas iNOS is upregulated in response to various stimuli including acute infection. NOS requires a critical co-factor, tetrahydrobiopterin, to effectively synthesize NO and deficiency of this co-factor leads to dysregulation and uncoupling of NOS function resulting in the production of oxidant factors including hydrogen peroxide and superoxide¹³⁷.

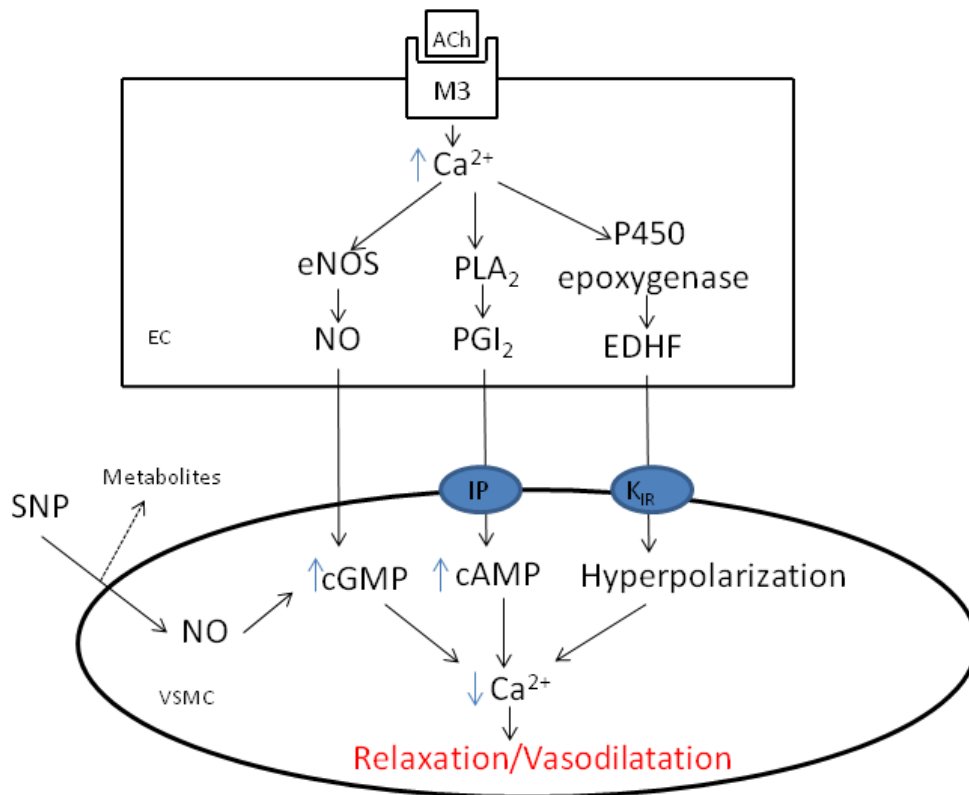


Figure 4 Mechanisms of vascular smooth muscle cell vasodilatation. ACh binds to the muscarinic type 3 receptor (M3) causing increased intracellular calcium in the EC. This in turn stimulates eNOS, phospholipase A2 and P450 epoxygenase. The overall consequence of this is VSMC relaxation and endothelial vasodilatation mediated by reduced Ca^{2+} in VSMC. This occurs through the direct stimulation of cGMP by NO which can additionally enter VSMC directly via the NO donor SNP. Additional VSMC relaxation occurs as a result of reduced intracellular VSMC Ca^{2+} mediated via the stimulation of the VSMC IP receptor by PGI_2 leading to an increase in VSMC cAMP as well as VSMC hyperpolarization mediated by the effect of EDHF on the VSMC K_{IR} .

EC – endothelial cell, VSMC – vascular smooth muscle cell, eNOS endothelial nitric oxide synthase, PLA_2 phospholipase A₂, PGI_2 – prostaglandin I₂, EDHF – endothelium derived hyperpolarizing factor, SNP – sodium nitroprusside, ACh – acetylcholine, NO – nitric oxide, IP – prostanoid receptor, K_{IR} inwardly rectifying potassium channel, cGMP – cyclic guanosine monophosphate, cAMP – cyclic adenosine monophosphate.

eNOS is expressed primarily on endothelial cells but has been identified in other tissues including cardiac myocytes, placenta and platelets¹⁴⁵. The function of eNOS is regulated by various molecules including intracellular calcium. Vascular wall shear stress is a key activator of eNOS, mediated through phosphorylation, a process which can additionally be induced by exposure of the endothelium to insulin, oestrogen and VEGF¹⁴². Phosphorylation of eNOS increases the sensitivity of eNOS to calcium¹⁴⁶. When the calcium-calmodulin complex binds to eNOS, the flow of electrons from NADPH in the reductase domain to the haem moiety in the oxygenase domain of eNOS occurs. This process facilitates the generation of NO from L-arginine¹⁴².

Endothelial contractile responses are mediated by endothelin-1 and angiotensin-II which induce leucocyte and platelet activation, promote vasoconstriction and a pro-thrombotic milieu¹³⁷. One of the critical roles of NO is to oppose the contractile forces of endothelin-1 and angiotensin-II¹³⁷. Reduced endothelial vasodilatory responses are mainly caused by reduced NO generation due to down-regulation of eNOS. This process has been shown to be multi-factorial and includes genetic factors, abnormalities of tetrahydropterin biology and the influence of inflammatory markers including C reactive protein (CRP)¹⁴⁷⁻¹⁴⁹.

The understanding of endothelial biology has advanced recently to include the role of nitrite (NO_2^-) which was initially considered a biologically inert by-product of the processes which generate NO. Nitrite is now considered both a store of NO and as a physiologically active molecule capable of generating endothelial vasodilatation¹⁵⁰⁻¹⁵¹. Nitrite has two major sources, generated both as a consequence of the conversion of L-arginine to NO and through dietary intake which includes ingestion of both nitrite and nitrate¹⁵². Dietary nitrate is converted to nitrite under the influence of anaerobic

bacteria located on the dorsal surface of the tongue and this nitrite is then either converted to NO in the acidic conditions of the stomach or enters the systemic circulation¹⁵³. NO generation from nitrite is mediated by eNOS and is more likely under the influence of specific physiological conditions including hypoxia¹⁵⁴. Recently, the role of xanthine oxidoreductase as the converter of nitrite to NO in acidotic conditions has become evident¹⁵⁰.

Pre-clinical and clinical evidence for the beneficial effect of nitrite and the role of the endothelium in its synthesis is emerging. Using an isolated rat Langendorff heart preparation, Webb *et al.*¹⁵³ demonstrated that nitrite to NO conversion was effectively abolished when the endothelium in Langendorff hearts was removed. The same group have demonstrated that dietary nitrate in the form of beetroot juice lowers blood pressure, inhibits platelet aggregation and improves endothelial function¹⁵³. In this open label study which used a cross over design, 30 healthy volunteers received either 500 mL of beetroot juice or a water control. The peak reduction in systolic BP (10.4 ± 3.0 mmHg) occurred 2.5 h after ingestion of beetroot juice and occurred synchronously with the peak nitrite concentrations. In the same study, nitrate ingestion improved the response to post ischaemic endothelial function of the forearm assessed by brachial artery flow mediated vasodilatation (FMD) compared to controls ($p < 0.05$).

1.3.2 Techniques to evaluate endothelial function

Endothelial function and the endothelial response to various stimuli can be measured in large conduit arteries and peripheral microcirculatory beds. Structural and functional assessments of large conduit arteries (brachial, carotid and femoral) can be obtained by using ultrasound imaging to evaluate atherosclerotic occlusive lesions as well as arterial intima medial thickness (IMT).

1.3.2.1 Techniques to measure conduit artery endothelial function

Pulse wave velocity (PWV) and FMD are the most common techniques used to measure conduit artery endothelial function. Brachial artery FMD is a technique which measures the vasodilatory response of the brachial artery (percentage increase in the diameter of the brachial artery after exposure to stimulus assessed by ultrasound) to a range of stimuli, including induction of ischaemia and subsequent reperfusion by occlusion of the brachial artery, local heating of the skin and sublingual nitroglycerin administration.

PWV measures the transit time in m/s of one cardiac pulse wave cycle between two fixed points in the arterial tree. PWV is measured by the use of two recording probes sited over conduit arteries (often carotid, brachial or femoral) which measure speed of transit of pulse waves. The distance between the two sites is measured and PWV can be calculated from these values. Pathological conditions including atherosclerosis lead to stiffening of large conduit arteries which results in an increase in pulse wave transit time which is reflected as an increase in measured PWV¹⁵⁵. Increasing PWV has been associated with a higher risk of CV events in hypertensive patients and an increase in mortality in patients with stages 2-4 CKD¹⁵⁶⁻¹⁵⁷.

1.3.2.2 Techniques to measure microcirculatory endothelial function

Endothelial function in the microcirculation is an area of increasing interest. The microcirculation is defined as any blood vessel < 150µM in diameter located in tissue parenchyma¹⁵⁸. Maintaining blood flow to the critical central microcirculatory organ beds, the main site of oxygen and nutrient exchange between blood and tissues, is an essential part of the maintenance of normal physiology. Disruption to this process, as a

consequence of microcirculatory endothelial dysfunction, has the potential to cause significant disruption to normal homeostasis.

While more critical central microcirculatory beds, for example cardiac and renal, are less anatomically accessible, the response of the dermal microcirculation can be ascertained using bedside techniques. Dermal microvessels have been shown to reflect microcirculatory endothelial function in renal, retinal and cardiac microcirculatory beds¹⁵⁹⁻¹⁶¹. Consequently through relatively simple and non-invasive techniques, the skin microcirculation can provide a representative peripheral window onto the more functionally relevant central microcirculation¹⁶²⁻¹⁶³.

The response of dermal microcirculatory endothelial function can be quantitatively assessed through techniques including Laser Doppler Imaging (LDI) and Laser Doppler Flowmetry (LDF)¹⁶². LDF measures erythrocyte flux in dermal microvessels generated by direct contact of laser probes with the skin surface with the output measured as perfusion in arbitrary units. LDI is a non-contact technique which produces a visual, differential colour speckle output of skin perfusion over a much larger area (up to 50x50cm) than LDF.

Dermal microcirculatory endothelial function can be assessed by the response of microcirculatory vessels to physical stimuli such as reactive hyperaemia, local cooling or heating of the skin and pharmacological therapy, most commonly through the delivery of acetylcholine (ACh) and sodium nitroprusside (SNP). The assessment of the microcirculatory endothelial response to ACh and SNP involves the local delivery of very small doses of these compounds into the dermal microcirculation using an iontophoresis apparatus. Laser Doppler probes measure the change in the flux of

erythrocytes in a fixed volume of dermal tissue in response to these drugs. This technique has the significant advantage of not requiring the delivery of vasoactive compounds to the systemic circulation.

1.3.2.3 The relationship of skin microcirculatory function to central microcirculatory function

Coulon *et al.*¹⁶⁴ evaluated the correlation between renal perfusion, measured by the renal resistive index (RI) and both post-occlusive and post heating reactive hyperaemia of the forearm skin. They assessed 22 patients with systemic hypertension and 11 healthy controls. Post occlusive reactive hyperaemia was achieved by inflation of a blood pressure cuff to 200 mmHg for 3 min and local heating was achieved increasing the surface temperature of the skin to 44 °C. The authors also evaluated the correlation between the renal RI and the QKD interval. This is a measure of the onset of the QRS complex and the last sound detected in diastole using a microphone positioned over the brachial artery. This was achieved using a 24 h automated blood measure monitor linked to a 3 lead ECG. The QKD score is corrected for a standard systolic blood pressure of 100 mmHg and a pulse of 60 beats per minute and is reported as the QKD₁₀₀₋₆₀ with lower scores reflecting increased arterial stiffness.

The authors established that while basal dermal perfusion did not differ between the groups, the response of the dermal microcirculation after occlusion of the brachial artery and local skin heating (measured as % variation of LDF between post occlusive to peak perfusion after release of the blood pressure cuff) was impaired in hypertensive patients compared to controls ($p < 0.05$). In addition they demonstrated a significant negative correlation between renal RI and skin microcirculatory endothelial function in a univariate analysis. This finding was present when the renal RI was correlated to both

the percentage variation in resting to peak perfusion after release of the BP cuff ($r=-0.44$, $p=0.01$) and the percentage variation in perfusion immediately after occlusion of the brachial artery to peak perfusion after release of the cuff ($r=-0.42$, $p=0.02$). The QKD_{100-60} correlated negatively with the renal RI ($r=-0.44$, $p=0.01$).

This study indicates an important association between skin and renal perfusion indices but is limited by the small numbers and by the fact that 91% of hypertensive patients were taking anti-hypertensive drugs which the authors indicate may have affected both the dermal response to stimulus and the renal RI. Additionally, the mean systolic blood pressure of the hypertensive group was 133.8 mmHg which while significantly higher than controls (111.0 mmHg $p<0.0001$), would not be considered clinically significant hypertension.

Agarwal *et al.*¹⁶⁵ assessed the function of skin microcirculation and the presence or absence of coronary artery disease (CAD). Forty eight patients with angiographically proven CAD were compared to 25 age and sex matched controls without evidence of CAD. Skin microcirculatory function was assessed by iontophoresis of ACh, local heating to 41 °C and occlusion of the brachial artery. The difference between baseline and maximum perfusion was measured in arbitrary perfusion units by LDF and adjusted for BMI, presence of DM, smoking status, waist circumference and systolic blood pressure. Change in perfusion was higher in controls compared to patients with CAD when assessed by iontophoresis of ACh ($p=0.023$) and local skin heating ($p=0.032$) but not post occlusive reactive hyperaemia (PORH, $p=0.21$). However the time to peak perfusion was higher in patients with CAD compared to healthy controls after PORH ($p=0.01$) but not iontophoresis of ACH or local skin heating.

Shamin-Uzzaman *et al.*¹⁶⁰ evaluated 24 patients with established CAD and 24 healthy controls using brachial artery FMD and LDI in combination with PORH. Brachial artery FMD was attenuated in patients with CAD compared to controls ($1.85 \pm 4.29\%$ in CAD compared to $4.30 \pm 4.00\%$ in controls, $p=0.05$). Both change in peak perfusion from baseline (CAD $294 \pm 290\%$; controls $501 \pm 344\%$, $p=0.04$) and time to maximum response (CAD 16.84 ± 9.61 s; controls 9.13 ± 4.43 s, $p=0.001$) were improved in controls compared to patients with CAD. Using a receiver operator curve analysis, the LDI time to peak response was a better predictor of CAD than brachial artery FMD (FMD sensitivity 71.4%, specificity 73.9%, LDI sensitivity 73.7%, specificity 91.3%).

The relationship between skin and retinal microcirculation has been assessed by Tur *et al.*¹⁶¹ in 25 patients with type 2 DM and 25 non-diabetic controls. Using skin perfusion measured by LDF after PORH, they established that peak blood flow but not time to peak blood flow was higher in controls compared to patients with DM. In a subgroup analysis of patients with DM comparing those with proliferative or no retinopathy, the ratio of peak to time to peak perfusion (with lower values reflecting impaired microcirculatory function) was significantly lower in the retinopathy group (31.4 ± 3.9) compared to the non-retinopathy group (50.9 ± 10.9 , $p<0.05$).

While these studies indicate the utility of skin microcirculatory function as a means to assess microcirculatory function in less accessible central beds, none of these studies reported the degree of renal dysfunction or proteinuria making it difficult to generalise the results to patients with kidney disease. In addition, these studies demonstrate that when comparing control to disease groups, resting perfusion (albeit measured with a variety of methods) is similar and differences in microcirculatory function only become apparent when the endothelium is stimulated by either delivery of ACH, PORH or local

heating. The variety of techniques used to measure peripheral microcirculatory function and the lack of consensus about the optimal technique has so far prevented the development of a unified diagnostic approach for the assessment of skin microcirculatory function.

A newer technique, side stream dark field imaging of the microcirculation, involves real time video capture of capillary blood flow using a small camera which directly images a capillary bed, usually the sublingual microcirculation¹⁶⁶ (see section 2.2.14.1 for technique details). This technique provides a semi quantitative measure of capillary blood flow and capillary density and thus provides both a structural and functional analysis of the microcirculation¹⁶⁶. While predominantly used in studies of sepsis¹⁶⁷⁻¹⁶⁸ and peri-operative medicine¹⁶⁹⁻¹⁷⁰, Reynolds *et al.*¹⁷¹ have recently evaluated the sublingual microcirculatory parameters in healthy volunteers and those with liver cirrhosis, DM and stage 5 CKD. Comparing 20 patients per group (n=18 in the cirrhosis group), there were no significant differences in the primary outcome measure of microvascular flow index (MFI) between the comparison groups (p=0.14) nor were there any difference in perfused vessel density (p=0.08) or proportion of perfused vessels (p=0.46). The mean MFI was higher in patients with CKD stage 5 compared to healthy controls under 25 years of age (MFI CKD – 3.0, MFI controls 2.85). While this difference was not statistically significant, the authors postulate that a larger study may have shown a significant difference. SDF imaging has the significant advantage of providing different anatomical and physiological microcirculatory parameters compared to LDF or LDI and therefore complements an assessment of the microcirculation by other techniques. However, to date, no studies have evaluated the correlation between SDF imaging and either skin microcirculatory parameters or the function of central microcirculatory beds.

1.3.3 Endothelial dysfunction and cardiovascular events

Endothelial dysfunction has been established as a pathogenic factor in the full spectrum of CVD and is a predictor of future CV morbidity and mortality¹⁷². In a study of 308 patients with and without angiographically demonstrated CAD, pharmacological stimulation of the coronary circulation with ACh and SNP demonstrated that impaired relaxation of the coronary microcirculation was associated with an increased incidence of acute ischaemic coronary events¹⁷³. Failure of relaxation of the coronary microcirculation was an independent risk factor for ischaemia after multivariable analysis including adjustment for the presence of baseline CAD.

Tatematsu *et al.*⁷⁵ have identified specific endothelial abnormalities in the coronary arteries in an *in vivo* model of CKD. In this study, dogs undergoing 5/6th nephrectomy had a blunted coronary artery vasodilatory response to stimulation with ACh. Endothelial nitric oxide synthase (eNOS) and dimethyl arginine dimethylaminohydrolase-II (DDAH-II), a degrading enzyme for asymmetrical dimethyl arginine (ADMA), were downregulated in coronary artery cells from 5/6th nephrectomised animals and were postulated as mediators of the observed endothelial abnormalities.

Evaluating the peripheral circulation can provide prognostic information on future CV events. In 73 patients with angina, Neunteufl *et al.*¹⁷⁴ demonstrated a brachial artery FMD of <10% was associated with an increased need for coronary revascularization over a 5 year follow up period after multivariable analysis¹⁷⁴. Gocke *et al.*¹⁷⁵, in a study of 199 patients with peripheral vascular disease, found that there was an increased OR of 9.5 (95% CI 2.3 - 40) for major CV events (death, stroke or myocardial infarction) in

patients with an FMD of <8.1%. In 152 patients with coronary artery disease¹⁷⁶ followed up for 34 months, Chan *et al.*¹⁷⁶ identified that increased carotid artery plaque area and reduced FMD of the brachial artery after administration of sublingual nitroglycerin were associated with an increased risk of CV events.

Endothelial function measured by the digital response to reactive hyperaemia using the Endo-PAT 2000 device has been shown to predict CV events. In a study by Matsuzawa *et al.*¹⁷⁷, 105 CV events over a total of 1,468 person years of follow up were observed in 528 stable patients classified as high risk for future CV events. In a Cox regression analysis, digital arterial function was associated with a reduction in risk for CV events with a 0.1 increase in the reactive hyperaemia index resulting in a reduction in the HR for CV events of 0.76 (95% CI 0.67 – 0.85). When this information was added to more traditional risk models including the Framingham Heart Study risk prediction for CV events, the accuracy of CV event prediction improved by 27.5%. This finding indicates the importance of endothelial dysfunction in improving the prediction of CV events.

1.3.4 Endothelial function and vitamin D

The role of vitamin D in the prevention of an atherosclerotic phenotype in the endothelium is primarily mediated through the conversion of 25 (OH) D to 1,25 (OH)₂ D₃ in endothelial cells which then acts to reduce endothelial adhesion molecule expression and cytokine induced endothelial cell activation⁴⁴. Endothelial cells have been shown to express CYP27B1 which is involved in the proliferation of endothelial cells, peripheral conversion of 25 (OH) D to 1,25 (OH)₂ D₃ and may regulate the adhesion of monocytes to endothelial cells, a process which is in turn modulated by inflammatory cytokines²⁴. Rahman *et al.*¹⁷⁸, using wild type and VDR knock-out mice have shown

that reduced VDR stimulation increases metalomatrix proteinases (MMP) 2 and 9 and reduces tissue inhibitors of MMP, the combined effect of which results in vascular wall remodelling, a pro thrombotic environment and an increase in cellular hypertrophy in cardiac myocytes. Thus, vitamin D at the level of the endothelium plays a key role in preventing endothelial dysfunction and progressive vascular disease. In addition to these mechanisms, Wu-Wong *et al.*¹⁷⁹ have identified that gene transcription (assessed by real time polymerase chain reaction (RT-PCR) and microarray analysis) was modulated in human coronary VSMC after 30 h of incubation with the active vitamin D compounds paricalcitol and calcitriol. Both drugs inhibited cell proliferation and paricalcitol was found to regulate genes involved in VSMC cell proliferation (IGF1, WT1 and TGF- β 3). Calcitriol and paricalcitol were found to upregulate the type-B endothelin receptor which increases endothelial NO and thus vessel relaxation.

The coexistence of the VDR and extra renal CYP27B1 in endothelial tissues²⁴ has prompted the use of vitamin D as a potential therapeutic agent in endothelial dysfunction in pre-clinical and clinical studies. Borges *et al.*¹⁸⁰ have evaluated the effect of cholecalciferol in the spontaneously hypertensive rat (SHR). After treatment with 0.125 μ g/kg of cholecalciferol for 6 weeks, blood pressure and endothelial relaxant responses to ACh were normalized in cholecalciferol treated rats and this response was due to the effect of endothelium-derived hyperpolarizing factor (EDHF) whereas in non-cholecalciferol treated SHR, the relaxant effect of ACh was mediated by NO. In a separate study by the same authors¹⁸¹, the mechanism of this effect was demonstrated to be the restoration of function of calcium dependent K⁺ channels in VSMC. In this study, SHR, normotensive Wistar rats (NWR) and normotensive Wistar-Kyoto (WKY) animals were treated with cholecalciferol for 6 weeks via oral gavage at a dose of 0.125 μ g/kg. Systolic blood pressure, endothelium contractile response and resting

membrane potential were measured at 6 weeks. Cholecalciferol treated SHR had a lower blood pressure and resting perfusion pressure of the mesenteric arterial bed than non-treated SHR and either treated or untreated WKY ($p < 0.05$). In SHR treated with cholecalciferol, there was a significant reduction in the contractile response to adrenaline. However, this effect was abolished after the addition of 100 nmol/L of apamin, an inhibitor of calcium dependent K^+ channels. This indicates that the blood pressure lowering effect of cholecalciferol in SHR is due to recovery of calcium-dependent K^+ channels which have previously been shown to be abnormal in SHR¹⁸². The resting membrane potential (RMP) of the mesenteric bed of SHR was higher than both NWR and WKY both with and without intact endothelium and this was normalised in SHR treated with cholecalciferol. In addition, in mesenteric arterial rings where the endothelium was removed, the magnitude of endothelial hyperpolarization induced by adrenaline and the α_2 -adrenergic agonists UK 14,304 was higher in cholecalciferol SHR than in non-treated SHR.

Kalliovalkama *et al.*¹⁸³ and Jolma *et al.*¹⁸⁴ have made similar observations about the importance of calcium-dependent K^+ channels in uraemia. Using WKY rats exposed to 5/6th nephrectomy, but not treated with any form of vitamin D, Kalliovalkama *et al.*¹⁸³ established that NO mediated vasodilatation in uraemic compared to control rats was impaired in mesenteric arteries. The addition of apamin and charybdotoxin, (both inhibitors of calcium-dependent K^+ channels) to control and uraemic animals in which NO mediated vasodilatation was inhibited by L-NAME, had significantly different results. In the uraemic group, the addition of apamin and charybdotoxin had no effect on vasodilatation whereas in the control group, these compounds significantly inhibited the vasodilatory response of mesenteric rings. This indicates that decreased endothelium dependent vasodilatation in the 5/6th nephrectomy group was potentially due to

the impaired function of calcium-dependent K^+ channels. Blood pressure in the control and uraemic animals was not significantly different and the authors suggest that uraemia itself may be responsible for the differences they observed. Jolma *et al.*¹⁸⁴ used a very similar experimental design but in their experiments, 4 weeks after 5/6th nephrectomy, some animals were fed a high calcium diet (3% compared to 0.3% calcium content). The addition of extra calcium to the diet decreased serum concentrations of PTH and phosphate and increased ionised calcium but did not affect blood pressure. This was associated with enhanced vasodilatory function in response to ACh and improvement in calcium-dependent K^+ channel mediated vasodilatation.

Borges *et al.*¹⁸¹ have elucidated the receptor through which cholecalciferol may be exerting its beneficial effects on endothelial function in SHR and in the studies of uraemia by Kalliovalkama *et al.*¹⁸³ and Jolma *et al.*¹⁸⁴, this receptor also appears to be the calcium-dependent K^+ channel. The exact mechanism of the recovery of calcium-dependent K^+ channels in SHR after exposure to cholecalciferol, which could be a transcriptional or post translational modification of the channel, has not been elucidated, nor has the effect of cholecalciferol or other vitamin D compounds on calcium-dependent K^+ channel in uraemia. The finding by Jolma *et al.*¹⁸⁴ that increasing dietary calcium improves endothelial vasodilatory response has a number of potential explanations including the suppression of PTH and phosphate, rather than a direct effect of calcium itself on vascular tone. However, these studies^{181 183} highlight the multi-faceted endothelial vasodilatory mechanisms and the potential for therapeutic intervention with vitamin D compounds.

The effect of vitamin D on endothelial vasodilatory response has been assessed by Wong *et al.*¹⁸⁵ who exposed aortic rings from SHR to 1,25 (OH)₂ D₃ in organ baths

before manipulating vascular tone. The addition of 1,25 (OH)₂ D₃ improved endothelial vasodilatory function compared to the absence of the drug when aortic rings were stimulated by ACh. The authors identified that 1,25 (OH)₂ D₃ reduces endothelial dysfunction by reducing the cytosolic-free calcium concentration in endothelial cells but does not affect VSMC. Thus, there is evidence to suggest that different vitamin D compounds may exert a beneficial effect on endothelial function through different cellular pathways.

Andrukhova *et al.*¹⁸⁶ have evaluated the effect of calcitriol on eNOS synthesis and activity in VDR knock-out mice. VDR knock-out mice demonstrated lower concentrations of NO as a consequence of reduced expression of eNOS which resulted in endothelial dysfunction measured by increased aortic stiffness, remodelling of the aorta and systolic and diastolic cardiac dysfunction. In the same study, aortic tissue from wild type mice was treated with 10⁻⁷ M 1,25 (OH)₂ D₃ which resulted in a four-fold increase in eNOS mRNA. 25 (OH) D at a concentration of 10⁻⁷ M was also associated with an increase in aortic eNOS mRNA although the increase was lower (2.5-fold) than that achieved with 1,25 (OH)₂ D₃. Both of these findings were absent in VDR knock-out mice indicating the key role of the VDR in the regulation of NOS synthesis.

Experiments by Molinari *et al.*¹⁸⁷ supplement these findings. In cultured HUVEC cells, 1,25 (OH)₂ D₃ was shown in a dose dependent manner to increase NO production a process which was mediated by the phosphorylation of intracellular enzymes including eNOS, p38, AKT and ERK all of which are recognised to be part of intracellular pathways that lead to the synthesis of NO. The peak in NO production occurred after 1 minute of incubation with 1,25 (OH)₂ D₃. The addition of L-NAME and the VDR antagonist ZK159222 abrogated the production of NO indicating key roles for eNOS

and the signalling induced by the VDR in the production of NO at the cellular level. The same authors have demonstrated that in porcine endothelial cells, 1,25 (OH)₂ D₃ increases endothelial cell proliferation and that this process is mediated by NO, as evidenced by the significant reduction of endothelial cell proliferation in the presence of L-NAME¹⁸⁸.

1.3.4.1 Clinical studies of the effect of vitamin D on endothelial function

In clinical studies of both healthy subjects and patients with disease entities (other than CKD) with concomitant vitamin D deficiency, vitamin D therapy has produced conflicting results on endothelial function. Tarcin *et al.*¹⁸⁹ studied twenty three healthy, subjects with serum 25 (OH) D concentrations < 25 nmol/L who were compared to a control group with serum 25 (OH) D concentrations > 75 nmol/L. Brachial artery FMD was impaired at baseline in vitamin D deficient patients (7% vs 11.2% p=0.001) who were then supplemented with 300,000 IU of cholecalciferol intra-muscularly for 3 months. Serum 25 (OH) D concentrations increased from 20.4 nmol/L to 116.9 nmol/L after treatment with cholecalciferol and were unchanged in the control group. The repeat measures of FMD improved to 10.4% with no change in controls (p=0.02)¹⁸⁹.

Shab-Bidar *et al.*¹⁹⁰, conducted a randomized controlled trial of 100 patients with type 2 DM and normal kidney function allocated to daily cholecalciferol fortified yogurt (1000 IU/day, n=50) compared to unfortified yogurt (n=50) over 12 weeks. In the fortified group, there was a reduction in circulating markers of endothelial dysfunction (endothelin-1, E Selectin and MMP-9, p<0.05 for all). Sugden *et al.*¹⁹¹, in a randomized controlled trial of type 2 diabetic patients with preserved kidney function (creatinine clearance > 80 mL/min in both groups) and VDD (serum 25 (OH) D concentrations < 50 nmol/L), demonstrated that 8 weeks after a single dose of 100,000 IU of ergocalciferol,

brachial artery FMD improved by 2.3% with a relatively modest increase in serum 25 (OH) D concentrations of 15.3 nmol/L in the treatment compared to placebo group ($p=0.02$). This effect occurred without concomitant changes in PTH and insulin sensitivity suggesting that ergocalciferol had a direct effect at the level of the endothelium.

In a similar study by Yiu *et al.*¹⁹², 100 patients with type 2 DM were randomized to 5,000 IU of cholecalciferol ($n=50$) or placebo ($n=50$) daily for 12 weeks. Despite a rise in serum 25 (OH) D concentrations after 12 weeks by 87 nmol/L in the treatment group, FMD, highly sensitive C reactive protein (hs CRP), circulating endothelial progenitor cells and measures of oxidative stress did not differ between the groups. Witham *et al.*¹⁹³ randomized patients with type 2 DM and serum 25 (OH) D concentrations < 100 nmol/L to either placebo ($n=22$), a single dose of 100,000 IU cholecalciferol ($n=19$) or 200,000 IU of cholecalciferol ($n=20$). All patients at enrolment had a serum 25 (OH) D concentration between 40 and 50 nmol/L. The maximum serum 25 (OH) D concentration was achieved in the 200,000 IU dose group (79 nmol/L vs 63 nmol/L in the 100,000 IU group and 54 nmol/L in the placebo group, $p<0.001$). Brachial artery FMD, HbA1c and insulin resistance were assessed at 8 weeks and did not differ between treatment groups although blood pressure and B type natriuretic peptide were lower in both treatment groups.

Witham *et al.*¹⁹⁴ treated south Asian women in the UK with low serum 25 (OH) D concentrations with cholecalciferol and found no effect of cholecalciferol on microcirculatory function assessed by iontophoresis of ACh and SNP combined with LDF after 8 weeks. Subjects in this trial, which excluded patients with an eGFR < 40 mL/min, received one dose of 100,000 IU of cholecalciferol only. The rise in serum 25

(OH) D concentrations was small (16 nmol/L 95% CI 11 - 21 nmol/L) and this, combined with a single high dose compared to sustained therapy with vitamin D, may explain the lack of change in endothelial function.

Vitamin D supplementation has been evaluated as a modifier of endothelial function in conditions including human immunodeficiency virus (HIV) infection¹⁹⁵, peripheral vascular disease¹⁹⁶, coronary artery disease¹⁹⁷ and in patients who have had a myocardial infarction¹⁹⁸. FMD was the primary measure of endothelial function in these studies but other surrogate markers including the reactive hyperaemia index using fingertip plethysmography, hs CRP, von Willebrand factor and E selectin were evaluated differentially across these studies. All of these studies failed to find a significant beneficial effect of vitamin D on endothelial function and other surrogate marker of CV health. The heterogeneity of patient populations studied, choice of clinical end points and vitamin D replacement strategies combined with the small size of these studies makes interpreting these results difficult.

1.3.5 Endothelial dysfunction in chronic kidney disease

Uraemia affects endothelial function through a number of mechanisms. L-arginine, the substrate for NO synthesis by eNOS, is reduced in CKD due to impaired conversion of citrulline to arginine in the proximal renal tubule, an effect mediated by the reduced renal mass in CKD¹⁹⁹. The expression of all renal isoforms of NOS is decreased in CKD²⁰⁰. The mechanism for this includes increased intracellular calcium content as a consequence of secondary hyperparathyroidism. This hypothesis was supported by Vaziri *et al.* who demonstrated that, in a 5/6th nephrectomy model of uraemia in rats, parathyroidectomy normalized the expression of eNOS in aortic and remnant renal tissue²⁰⁰. In a model of renal mass reduction by 5/6th nephrectomy, Aiello *et al.*²⁰¹

demonstrated that NO synthesis in the kidney was reduced as a consequence primarily of the downregulation of the iNOS isoform and this is likely to have contributed to the progressive decline in renal function²⁰¹. Findings from a study by Nakayama *et al.*²⁰² of eNOS knock-out mice compared to control animals, both exposed to renal mass reduction by a 5/6th nephrectomy, have demonstrated that lack of eNOS is associated with progressive kidney disease and that renal mass reduction is associated with a reduction in the number of endothelial cells in remnant renal tissue.

In contrast to the reduction of NO in renal tissue in CKD, NO availability in the systemic circulation has been shown to increase in CKD in *in vitro*²⁰³⁻²⁰⁴, *in vivo*²⁰¹ and human studies²⁰¹. It is postulated that the increased systemic NO is a key mediator of excessive bleeding in CKD mediated by NO induced platelet dysfunction²⁰⁵. Aiello *et al.*²⁰¹ have demonstrated that in rats subjected to 5/6th nephrectomy, eNOS expression in the thoracic aorta was increased and that this might contribute to the increase in systemic NO availability which is postulated to provide some protection against the hypertension induced by renal mass reduction. However, Vaziri *et al.*²⁰⁰ and Kim *et al.*²⁰⁶ did not replicate the finding of increased eNOS in large conduit vessels of rats after sub-total nephrectomy (SNx). This may in part be due to the differential effects of models of renal failure in rats on systemic hypertension. eNOS expression may have been upregulated by Aiello *et al.*²⁰¹ since their model of 5/6th nephrectomy induced hypertension compared to the model of Vaziri *et al.*²⁰⁰ and Kim *et al.*²⁰⁶ which used renal artery ligation rather than subtotal nephrectomy which is less likely to induce hypertension.

In addition to the downregulation of eNOS and reduction in concentrations of its substrate L-arginine, the inhibition of eNOS by ADMA is exacerbated in CKD since the

kidneys normally clear ADMA from the circulation. Accordingly, ADMA has been identified as an independent predictor of CVD and endothelial dysfunction in CKD²⁰⁷ and haemodialysis patients²⁰⁸⁻²⁰⁹.

Endothelial repair, mediated by bone marrow released endothelial progenitor cells (EPC), is impaired in CKD which is associated with lower concentrations of circulating EPC which have impaired functional ability²¹⁰⁻²¹². P-cresyl, a uraemic toxin, has been implicated in the pathogenesis of endothelial dysfunction in CKD. In two studies by Dou *et al.*, elevated concentrations of p-cresyl, predicted endothelial dysfunction and mortality in haemodialysis patients²¹³⁻²¹⁴. Meijers *et al.*²¹⁵ studied 100 prevalent haemodialysis patients and identified a positive correlation between serum concentrations of p-cresyl and endothelial micro particles, reflecting endothelial damage. These findings were supported by *in vitro* studies of HUVEC cells in which p-cresyl in a dose dependent manner increased the presence of endothelial microparticles, which are released as a consequence of endothelial damage²¹⁵.

Endothelial function has been shown to predict the severity of renal damage in a rat model of 5/6th nephrectomy²¹⁶. Endothelial function assessed by vasodilatation of the nephrectomised renal arteries to ACh removed at the time of 5/6th nephrectomy correlated with subsequent renal damage after renal mass reduction (proteinuria and eGFR). Renal damage did not correlate with contraction to phenylephrine or stimulation with SNP suggesting that endothelial vasodilatory function rather than changes in smooth muscle structure and function were more predictive of future renal damage after 5/6th nephrectomy.

CKD is associated with a generalised inflammatory response that has been implicated in the pathogenesis of endothelial dysfunction. The role of CRP in endothelial dysfunction was assessed by Yilmaz *et al.*²¹⁷ who evaluated a cohort of 304 patients with stage 1-5 CKD. In this study, CRP increased in a linear fashion as stage of CKD worsened. After a median follow up of 40 months, there were 89 fatal and non-fatal CV events. C-reactive protein together with eGFR and PTH were associated with carotid IMT and brachial artery FMD. In a multivariable analysis, the HR for a CV event increased by 1.07 per mg/L increase in CRP (95% CI 1.04 – 1.10, p<0.001). This study excluded patients who were taking drugs that have been shown to modify endothelial function in CKD including ACE-I, ARB, statins and erythropoietin and so was not representative of the general CKD population. The cross sectional nature of the design means that it is difficult to interpret if a reduction in eGFR causes increased concentrations of CRP or vice-versa.

In addition to elevated concentrations of CRP, vascular adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E and P-selectin), have been shown to be increased in CKD²¹⁸⁻²¹⁹ and were associated carotid IMT and mortality²²⁰. These molecules regulate the initial attachment of circulating monocytes to the endothelium and are involved in their tethering and transmigration across the endothelium where they become actively involved in the atherogenic processes that eventually lead to vascular dysfunction

1.3.5.1 Clinical studies evaluating endothelial function in chronic kidney disease and end stage kidney disease

Clinical assessments of endothelial dysfunction in CKD, evaluating large conduit artery and microcirculatory endothelial function, support the pre-clinical findings of endothelial

dysfunction in CKD. Kawamoto *et al.*²²¹ examined the relationship between kidney function measured by the eGFR and PWV in 310 patients screened during a yearly health check. There was significant linear, inverse relationship between eGFR and PWV ($r=-0.317$, $p<0.001$). The effect of eGFR on PWV remained significant in a linear regression model after adjusting for traditional risk factors including age, sex, BMI, blood pressure and medication use for hypertension (β regression coefficient $=-0.171$, $p<0.001$).

Recio-Mayoral *et al.*²²² studied endothelial function and markers of inflammation in 154 patients with different stages of CKD (pre-dialysis, haemodialysis and post-transplant) and compared them to 65 age and sex matched controls with normal kidney function. FMD was reduced in haemodialysis patients compared to controls, pre-dialysis and post-transplant patients. In pre-dialysis patients, but not haemodialysis patients, eGFR correlated with FMD ($r=0.36$, $p=0.001$). In haemodialysis patients, C-reactive protein was found to correlate negatively with FMD ($r=-0.51$, $p<0.001$) and positively with carotid IMT ($r=0.50$, $p<0.001$). The same pattern of correlation was observed when patients with DM were excluded from the analysis. This study supports the hypothesis that CKD is associated with both endothelial dysfunction and inflammation. However, the study was cross sectional in nature, without long term follow up and can only support an association rather than determining the mechanistic pathway of endothelial dysfunction in CKD.

Landray *et al.*²²³ have described the association between CKD and circulating markers of endothelial dysfunction in a cross sectional study of 334 patients with CKD (creatinine $> 130 \mu\text{mol/L}$) compared to age and sex matched controls both with and without CAD. CRP, fibrinogen, von Willebrand factor and P-selectin were significantly

higher in patients with CKD compared to controls while albumin was significantly lower. In an adjusted linear regression analysis, there was a significant association between fibrinogen, von Willebrand factor, albumin, P-selectin but not CRP and worsening kidney function (measured by serum cystatin C concentration). These circulating markers of inflammation are associated with abnormalities of endothelial function but in this study, there was no functional assessment of the endothelium and the clinical significance of these factors is difficult to ascertain due to the cross sectional nature of the study. The authors postulate that even if these factors are causally associated with CVD, their effect is likely to be modest.

Endothelial dysfunction in CKD has been evaluated using techniques that examine peripheral microcirculatory beds. Cupisti *et al.* have investigated the dermal response to the iontophoretic delivery of ACh in 20 patients with CKD²²⁴. Uraemia itself (mean serum creatinine in the CKD group 600 µmol/L) in the absence of hypertension was not associated with abnormalities in LDF assessment of dermal microvessels. Since ACh has been shown to mediate endothelial derived NO production and that this process is blunted in CKD due to dysregulation of eNOS, this surprising finding may be due to the preserved prostanoid mediated vasodilatation in the peripheral circulation of CKD²²⁵⁻²²⁶. This study did not measure and therefore could not evaluate the effect of potential confounding variables in the CKD group including CRP and PTH.

The importance of PTH on endothelial function in patients with CKD has been demonstrated in a separate study by Rossi *et al.*²²⁷ who evaluated microcirculatory endothelial function in 32 non-dialysis requiring patients with stage 3-5 CKD and 32 age and sex matched controls using LDF after brachial artery occlusion to generate reactive hyperaemia. Control patients exhibited a significant increase in perfusion measured by

forearm dermal LDF compared to patients with CKD. The systolic blood pressure ($r=-0.45$, $p< 0.01$) and PTH ($r=-0.38$, $p=0.05$) correlated with impaired endothelial function in patients with CKD²²⁷.

Dermal microcirculatory endothelial function assessed by LDF has been shown to be abnormal in patients with ESKD in the absence of DM or established CVD. Stewart *et al.*²²⁸ studied 63 patients with ESKD and compared abnormalities of microcirculatory function by LDF and LDI after PORH in ESKD patients with DM and established CVD ($n=30$) compared to ESKD without evidence of DM or CVD ($n=33$). They demonstrated that patients with ESKD and the combination of DM and CVD had a distinct low frequency, oscillatory pattern in LDF waveforms, known to be consistent with endothelial cell dysfunction²²⁹, that was present in 50% of patients with ESKD but without DM or CVD. While this study was cross sectional in nature, the authors postulate that the presence of these specific waveforms could predict the development of DM or CVD in patients with ESKD and therefore may represent an opportunity for therapeutic interventions that reduce the risk or progression of DM or CVD.

Pannier *et al.*²³⁰ have demonstrated that endothelial dysfunction correlates with LVM, an important predictor of CV events in patients with ESKD²³¹. In this study of 60 stable patients treated with haemodialysis (mean age 54 ± 14 years, male/female ratio 1.3, mean dialysis vintage 103 ± 187 months), microcirculatory function was assessed by flow debt repayment (FDR) using venous plethysmography. This was calculated as the ratio between the excess hyperaemic flow (the area under the curve between the release of an occlusive BP cuff and duration of hyperaemia) and the flow debt (the area under the curve between the start and end of ischemia). LVM, measured by echocardiography, correlated positively with time to FDR ($r=0.35$, $p<0.01$). The findings

of Pannier *et al.*²³⁰ are in line with those of Poulidakos *et al.*²³² who studied 30 patients with CKD (17 pre-dialysis, 13 post-transplant) and 29 age and sex matched controls. FMD was significantly lower (3.2 vs. 6.1 % $p < 0.001$) while CRP (3.9 vs. 1.0 mg/L, $p < 0.001$) and left ventricular mass index (LVMI) quantified by cardiac were significantly higher in the CKD group ultrasound (146.1 vs. 105.3 g/m, $p < 0.001$). After adjusting for age, DM and smoking, FMD was negatively correlated with LVMI (β regression coefficient = -0.39, $p = 0.004$). Both these studies suggest that endothelial function is associated with LVH, although the cross sectional nature of the studies can only suggest but not confirm a causal association.

1.3.6 Summary

Endothelial function and dysfunction is a complex interaction of genetic, hormonal and biochemical factors many of which are disturbed in the presence of kidney disease. Vitamin D and the VDR modulate endothelial function and there is increasing evidence for the efficacy of vitamin D therapy, both nutritional and activated, in ameliorating endothelial dysfunction. There is significant evidence for endothelial dysfunction in patients with both CKD and ESKD however, existing studies have used a heterogeneous approach to quantifying endothelial dysfunction, either using non-specific serum markers or functional studies of the endothelium, making direct comparison between studies and different patient groups difficult. Currently, most clinical evidence involving an assessment of endothelial function is cross sectional without sufficient follow-up periods that allow for a robust evaluation of the true association of markers of endothelial dysfunction and the development of CV events in patients with CKD and ESKD.

1.4 The association between chronic kidney disease, vitamin D deficiency and endothelial dysfunction – epidemiology and therapeutic intervention with vitamin D

As discussed in section 1.2.5, vitamin D deficiency in CKD is highly prevalent and is related to CV morbidity and mortality in patients with both dialysis and non-dialysis requiring CKD. While the excess of CVD in CKD is multi-factorial, an understating of the relationship between VDD and endothelial dysfunction in CKD is an important epidemiological and therapeutic avenue to pursue as part of the broader effort to reduce CV morbidity and mortality in CKD. This approach is supported by the presence of the VDR on VSMC²³³ and endothelial cells²³⁴ which suggests that modification of endothelial function by vitamin D is a plausible biological mechanism.

The association between concomitant CKD and VDD on endothelial dysfunction has been investigated in both dialysis and non-dialysis requiring CKD. Chitalia *et al.*²³⁵ studied the relationship between serum 25 (OH) D concentrations and endothelial function in 50 patients with non-dialysis requiring CKD who were not taking vitamin D supplements. The mean age of the patients was 56 years with an equal distribution of males and females. The mean serum 25 (OH) D concentration was 53 ± 33 nmol/L and the mean eGFR was 38 ± 15 mL/min. There was a linear relationship between serum 25 (OH) D concentrations and FMD ($r=0.44$, $p=0.001$) which was preserved after adjusting for traditional CV risk factor including presence of hypertension, sex, age, BMI, smoking and hyperlipidaemia (adjusted regression coefficient $\beta = 0.452$, $p<0.002$). Patients were further analysed in two groups with either a serum 25 (OH) D concentration of greater or less than 37.5 nmol/L. The two groups had similar baseline

characteristics but FMD was lower in the low vitamin D compared to high vitamin D group ($4.4 \pm 2.5\%$ vs. $2.5 \pm 1.6\%$; $p = 0.007$).

Despite the exclusion of patients taking vitamin D supplements, the study involved patients with a wide range of serum 25 (OH) D concentrations (<10 nmol/L to > 125 nmol/L) and therefore examined endothelial function across a wide range of serum 25 (OH) D concentrations. Patients were excluded if they were receiving any form of renal replacement therapy (RRT), had DM, heart failure, active inflammation, a recent CV event, cancer or autoimmune diseases and while this means the results of the study cannot be readily applied across the entire spectrum of CKD patients, it has provided an evaluation of the effect of serum 25 (OH) D concentrations in the absence of likely confounding variables. The cross sectional nature of the design can only imply there is an association between serum 25 (OH) D concentrations and endothelial function in CKD rather than establishing that VDD directly causes endothelial dysfunction or the mechanism by which this may be occurring.

The association between serum 25 (OH) D concentrations and endothelial function in patients receiving haemodialysis has been evaluated by London *et al.*²³⁶ who studied 52 prevalent haemodialysis patients (mean age 58 ± 1.9 years) with a median dialysis vintage of 46 months and a minimum time on dialysis of 3 months. The patients in this study had not received vitamin D therapy and had all anti-hypertensive agents stopped 10 days before endothelium assessment were made. Endothelial function was measured by aortic PWV (n=52), brachial artery distensibility (BAD, a measure of arterial wall displacement through the cardiac cycle) (n=42) and brachial artery FMD (n=37) after hand warming of the non-fistula arm. Vitamin D deficiency (serum 25 (OH) D concentration < 81 nmol/L) was present in 90% of patients. Serum 25 (OH) D and

1,25 (OH)₂ D₃ concentrations correlated significantly and positively with FMD and BAD and negatively with aortic PWV in both uni and multivariate adjusted analyses (the latter adjusted for age and systolic BP). There were no significant associations between Ca²⁺, PO₄ or PTH and either aortic PWV, FMD or BAD.

1.4.1 The effect of vitamin D therapy on endothelial function in chronic kidney disease and coexisting vitamin D deficiency

1.4.1.1 Pre-clinical evidence

The beneficial effect of vitamin D therapy on endothelial biology and function has been evaluated in both *in vitro* and *in vivo* studies. Talmor *et al.* in two studies²³⁷⁻²³⁸ have evaluated the effect of calcitriol on the expression of eNOS and inflammatory markers in human umbilical vein endothelial cells (HUVEC) cultured with advanced glycation end products (AGE) (elevated in both uraemia and DM) and in an environment more similar to CKD in which cultures were incubated in a low calcium, high PTH and AGE rich environment. In both sets of experiments, there was a decrease in eNOS expression and function as well as an increase in pro inflammatory IL-6 and expression of the receptor of AGE (RAGE). The addition of calcitriol to culture media in both studies reversed these findings, improving eNOS expression and function and reducing IL-6 mRNA expression. The beneficial effect of calcitriol was mediated by blunting of the stimulatory effect of NF-κB.

Studies of the effect of vitamin D conducted in *in vivo* models have principally evaluated the effect of activated vitamin D compounds. Wu-Wong *et al.*²³⁹ evaluated the effect of paricalcitol and the calcimimetic drug cinacalcet on endothelial function in 5/6th nephrectomised rats. They report a dose dependent increase in thoracic aortic ring

relaxation after stimulation of aortic rings with ACh following treatment with increasing doses of paricalcitol. Near normalization of the ACh mediated endothelial relaxation occurred at a dose of 0.083 µg/kg of paricalcitol. This effect was independent of the suppression of PTH since cinacalcet suppressed PTH concentrations but did not affect arterial vasodilatory responses.

The effect of vitamin D in the form of calcitriol on the cardiac remodelling process was assessed by Koleganova *et al.*²⁴⁰. In this study, 5/6th nephrectomised rats were treated with calcitriol (6 ng/kg) or vehicle for 12 weeks immediately after 5/6th nephrectomy. Cardiac remodelling assessed by myocardial capillary deficit and interstitial fibrosis was ameliorated in calcitriol treated rats. This occurred in the absence of changes in blood pressure, activation of the RAAS axis and cardiac mass, through pathways involving reduced expression of pro-fibrotic mediators (TGF-β), reduced deposition of collagen I and III as well as upregulation of VEGF receptor 2.

Macrovascular function assessed by PWV and aortic calcification in uraemic rats has been shown to be differentially affected by Vitamin D receptor activators (VDRA), independently of Ca²⁺, PO₄ and the calcium phosphate product. In a study by Noonan *et al.*²⁴¹, 5/6th nephrectomised rats were randomized to either doxercalciferol or paricalcitol given intra-peritoneally 3 times per week for 6 weeks after 5/6th nephrectomy. Doxercalciferol increased PWV and aortic calcification whereas paricalcitol had no effect on PWV or aortic calcification. This study did not include an assessment of functional responses to endothelial vasodilating drugs such as ACh but does allude to the different mechanistic properties of VDRA compounds in a model of uraemia.

In experimental uraemia achieved by 5/6th nephrectomy, a novel VDRA, VS-105, has been shown by Wu-Wong *et al.*²⁴² to effectively suppress PTH without significant changes in Ca²⁺ or PO₄ and to improve endothelial vasodilatory capacity in response to ACh. Male, Sprague-Dawley rats were exposed to 5/6th nephrectomy and after 6 weeks were treated for 2 weeks with a thrice weekly oral or intra peritoneal dose of either vehicle or VS-105 (dose range 0.004–0.64 mg/kg). Endothelial function improved in a dose dependent manner after treatment with VS-105 with near complete normalization of the endothelial vasodilatory response to ACh occurring at a dose of 0.16 µg/kg. Treatment with 0.64 µg/kg of VS-105 was associated with a reduction in LVH to levels approaching the sham operated control animals.

1.4.1.2 Clinical evidence

The hypothesis that vitamin D can improve endothelial function in patients with both haemodialysis and non-dialysis requiring CKD was tested in a study by Marckmann *et al.*¹²⁰ which compared the effect of 8 weeks of 40,000 IU of weekly cholecalciferol to a placebo in patients with both non-dialysis requiring CKD (n=25) and ESKD treated with haemodialysis (n=27). Patients in the control and intervention group were similar at baseline including dialysis status, serum 25 (OH) D, 1,25 (OH)₂ D₃ and PTH, Ca²⁺ and PO₄ concentrations. There was a significant increase in serum 25 (OH) D concentrations in the treatment compared to control group (154.7 nmol/L vs 23.5 nmol/L, p<0.001) and a significant increase in 1,25 (OH)₂ D₃ concentrations (p<0.01) and reduction in PTH (p<0.001) although these changes were only observed in the non-dialysis CKD group. Despite the significant changes in serum vitamin D concentrations, there was no reduction in markers of endothelial dysfunction including D-dimer, von Willebrand factor, fibrinogen, IL 8 and CRP. Additionally, blood pressure, aortic pulse wave velocity (aPWV) and aortic augmentation index did not change

between the two groups. Specifically, there were no significant differential effects on these parameters when comparing treated and untreated patients in the dialysis and non-dialysis groups.

While this study evaluated biomarkers of endothelial function and conduit vessel parameters, it did not include an assessment of microcirculatory endothelial function. In addition the follow-up time was limited to 8 weeks which may have been too short to detect meaningful changes in endothelial function and was too short to assess the effect of vitamin D on CV outcomes. The study was powered to detect a 50% difference in serum PTH concentration after treatment but was not powered to evaluate other parameters. In addition, the authors report relatively low numbers of subjects who underwent assessment of some of the endothelial parameters with fewer than half of the patients in both the placebo and treatment groups evaluated for macrocirculatory parameters.

The direct effect of ergocalciferol on biomarkers of endothelial function in patients receiving chronic haemodialysis has been assessed in a case control study of 40 patients (mean age 64.4 years, 21 (52.5%) male) by Assimon *et al.*²¹⁹. Twenty haemodialysis patients receiving ergocalciferol (average dose 33,125 IU per month) were matched on age and ethnicity to 20 patients not receiving ergocalciferol. There were no significant differences in baseline parameters including dialysis vintage and both groups were receiving an equivalent dose of doxercalciferol (5 µg/week in the non-ergocalciferol group, 6 µg/week in the ergocalciferol group, $p=0.76$) Serum 25 (OH) D concentrations were higher in the ergocalciferol group (90.8 nmol/L compared to 60.2 nmol/L, $p=0.03$) but PTH was not significantly different between groups ($p=0.84$). In patients treated with ergocalciferol, biomarkers of endothelial function including

sVCAM-1 ($p=0.03$), sICAM-1 ($p=0.005$) and P-selectin ($p=0.02$) were lower than in patients not receiving ergocalciferol. There was no difference in inflammatory biomarkers including IL-6 and TNF- α , however serum concentrations of CRP were not measured. Despite the fact that this study has relatively low numbers in each comparison group, it lends support to the hypothesis that ergocalciferol *per se* has a specific and beneficial effect on biomarkers endothelial function. However, in keeping with other literature in this area, the functional response of the endothelium was not evaluated and therefore the reduction in serum concentrations of endothelial biomarkers in this study cannot be shown to correlate with the functional status of the endothelium. The case control design cannot determine a causal relationship between ergocalciferol and endothelial biomarkers and consequently it is difficult to draw definitive conclusions from this study about the effect of ergocalciferol in endothelial function.

Stubbs *et al.*²⁴³ evaluated the effect of cholecalciferol therapy in 7 prevalent haemodialysis patients who, after a 4 week washout from other VDRA compounds, received cholecalciferol 50,000 IU twice per week over 8 weeks, with a dose adjustment based on serum 25 (OH) D concentrations at 3 and 6 weeks. Serum 25 (OH) D concentrations increased after treatment with cholecalciferol from 35 nmol/L to 137 nmol/L ($p<0.0001$) and serum 1,25 (OH)₂ D₃ concentrations increased from 9.4 to 32.3 pg/mL ($p<0.0001$) but there was no significant change in serum PTH concentrations. Correspondingly, concentrations of IL-6, IL-8 and TNF- α fell although only the reduction in TNF- α attained statistical significance. CD16+ monocytes, implicated in the pathogenesis of CVD in ESKD, were found to express high levels of the VDR although the downstream consequences of this were not evaluated. Despite the small size of this study and lack of a comparison arm, an important finding was that

inflammatory biomarkers did change after a relatively short duration of cholecalciferol therapy and that the achieved serum 25 (OH) D concentration was higher than in previous studies. This suggests that the maximum beneficial response of vitamin D therapy on the endothelium may require significantly higher serum concentrations of 25 (OH) D than have previously been achieved in other studies.

Chitalia *et al.*²⁴⁴ have assessed the effect of high dose cholecalciferol on endothelial function in stage 3-4 CKD and VDD (defined as a serum 25 (OH) D concentration of < 75 nmol/L). In this non-randomized, open label study which excluded patients with DM, 26 patients (mean age 50 years, mean eGFR 41 ml/min, 73 % males) received 2 doses of 300,000 IU oral cholecalciferol at 8 week intervals. Endothelial function was assessed by FMD and measures of serum biomarkers associated with endothelial function. After 16 weeks of follow-up, serum concentrations of 25 (OH) D increased from 43 ± 16 nmol/L to 84 ± 29 nmol/L ($p < 0.001$) and PTH fell from 10.8 ± 8.6 to 7.4 ± 4.4 pmol/L, $p = 0.001$). Brachial artery FMD improved from $3.1 \pm 3.3\%$ to $6.1 \pm 3.7\%$ ($p = 0.001$) and there were significant reductions in serum concentrations of E-selectin, VCAM-1 and ICAM-1 but there no changes in blood pressure, eGFR, PWV or augmentation index. While this study was non-randomized and thus prone to bias, it demonstrates the efficacy of cholecalciferol in improving endothelial function in CKD in the absence of changes to macrocirculatory parameters. The specific mechanism of action of cholecalciferol on endothelial function was not elucidated and the study duration was too short to evaluate the effect of cholecalciferol on CV endpoints.

1.4.2 Summary

CKD and concomitant VDD are associated with endothelial dysfunction. Pre-clinical studies have demonstrated that vitamin D, in various formulations, can ameliorate

endothelial function. Clinical data so far has failed to demonstrate a clear benefit of vitamin D therapy on endothelial function in CKD but the studies have had short follow-up periods and did not specifically evaluate the functional response of the microcirculation to vitamin D, which may better reflect endothelial function in cardiac and renal microcirculatory beds. The studies by Assimon *et al.*²¹⁹, Stubbs *et al.*²⁴³ and Chitalia *et al.*²⁴⁴ do however point to a specific effect of ergocalciferol and cholecalciferol on biomarkers of endothelial function and this therapeutic avenue requires additional study to clarify the potential role of nutritional vitamin D on endothelial function in kidney disease.

1.5 Rationale for experimental procedures

The available scientific literature has identified that CKD and concomitant VDD is a common clinical entity that results in an increased risk of CV morbidity and mortality⁹⁴
101 109-110 112-113 245. Endothelial dysfunction is a complex interplay of traditional and non-traditional risk factors and there is evidence that endothelial dysfunction is impaired in VDD, CKD and when both CKD and VDD coexist^{222 230 235-236}. Therapeutic intervention with vitamin D has the potential to ameliorate endothelial dysfunction in non-CKD and CKD populations. However, there are significant gaps in the current understanding of the optimum approach to address endothelial dysfunction when CKD and VDD co-exist. This is critically important given the high prevalence of VDD in CKD which is strongly associated with CVD.

Peripheral microcirculatory endothelial function has been established as a window into the more critical central microcirculatory beds including in the kidneys and myocardium and has been identified as a predictor of CV events in CKD^{159-161 246}. Therefore, a key aspect of endothelial function in CKD and concomitant VDD is the assessment of and

therapeutic interventions to improve microcirculatory endothelial function. This approach has the potential to reduce CV morbidity and mortality in CKD and concomitant VDD.

Existing evidence has provided an indication that nutritional vitamin D compounds may have a beneficial effect on endothelial function^{219 243-244} but further studies are required to confirm the effect of nutritional vitamin D on microcirculatory endothelial function in CKD and the mechanistic pathway through which this may occur. A recent review¹⁶² has highlighted the need for further assessments of microcirculatory endothelial dysfunction in patients with CKD as a method for predicting adverse CV outcomes. To date, current clinical research regarding vitamin D and CKD is mainly observational and primarily focuses on the suppression of PTH rather than the pleotropic effects of vitamin D therapy in CKD. A prospective, randomized controlled trial of vitamin D therapy in CKD patients, with a particular focus on microcirculatory endothelial dysfunction, has yet to be conducted.

Investigating endothelial dysfunction in the earlier stages of CKD is logical since the function of the vascular endothelium is already abnormal and is likely to be more responsive to therapeutic intervention compared to ESKD. Therapeutic intervention with nutritional vitamin D compounds compared to active vitamin D compounds in early CKD and concomitant VDD is appealing for several reasons. At the time of experimental design, ergocalciferol was recommended for the treatment of VDD in CKD⁴⁹ and has subsequently been recommended by Nigwekar *et al.*¹⁴ and in the KDIGO guidelines for CKD bone mineral disorders²⁴⁷. Ergocalciferol is cheap (compared to the expense of newer active vitamin D receptor activators), safe, and well tolerated and can be effectively used to increase serum 25 (OH) D concentrations in

CKD^{7 128}. However, double blind, randomized controlled trials of the effect of ergocalciferol in early CKD on endothelial function, an important surrogate for CV events, are conspicuously absent from the scientific literature. Equally, the mechanistic pathway through which nutritional vitamin D compounds affect endothelial function in non-dialysis requiring CKD has not been adequately described.

1.6 Hypothesis

The experimental work conducted and presented in this thesis has been designed to further the understanding of the effect of vitamin D in the form of ergocalciferol on microcirculatory endothelial function in patients with CKD and concomitant VDD as well as the potential mechanistic pathways through which ergocalciferol may be acting. Accordingly, two experimental chapters address these issues.

1. The effect on the microcirculation of ergocalciferol versus placebo in chronic kidney disease stage 3-4 and vitamin D deficiency: a pilot, double blind, randomized controlled trial

This study is designed to evaluate the effect of oral ergocalciferol compared to a placebo over 6 months on microcirculatory endothelial function in patients with CKD and concomitant VDD. The primary outcome is change in microcirculatory endothelial function measured by LDF after the iontophoresis of ACh. Secondary endpoints include measures of oxidative stress assessed by skin autofluorescence for AGE products, capillary density and flow in the sublingual microcirculation, bone mineral parameters, macrovascular parameters and left ventricular hypertrophy.

2. The effect of ergocalciferol on endothelial function in an *in vitro* and *in vivo* experimental model

These experiments describe the effect of ergocalciferol in cultured human aortic endothelial cells, specifically evaluating the effect of ergocalciferol on eNOS expression and function. An evaluation of the effect of ergocalciferol on endothelial function and associated mechanistic pathways in an *in vivo* model of experimental mild uraemia is described.

CHAPTER 2

THE EFFECT ON THE MICROCIRCULATION OF ERGOCALCIFEROL VERSUS PLACEBO IN CHRONIC KIDNEY DISEASE STAGE 3-4 AND VITAMIN D DEFICIENCY: A PILOT, DOUBLE BLIND, RANDOMIZED CONTROLLED TRIAL

2 Introduction

Many more patients with CKD die from CVD than progress to ESKD⁶¹. CV events are the major cause of morbidity and mortality in patients with ESKD receiving RRT⁶⁹. Traditional risk factors including age, hypertension, smoking and DM cannot completely explain the excess of CVD in patients with CKD. Vitamin D deficiency is a non-traditional CVD risk factor in patients with all stages of kidney disease²⁴⁸⁻²⁵⁰, is highly prevalent in patients with both CKD and ESKD^{89 122} and is associated with elevated CV morbidity and mortality in these patient groups^{101 105-106}.

Observational studies^{101 105-106 109-110 112 117 251-252} have determined that VDD is an important risk factor for CVD in CKD and have provided support for the protective role of VDRA in reducing the risk of CVD in patients with CKD and ESKD. However, these studies were heterogeneous in design, therapeutic intervention and patient populations (CKD and ESKD) and have not elucidated the mechanism by which vitamin D reduces CV risk in this patient group. Furthermore, these studies have principally focused on ESKD rather than CKD as well as the effect of synthetic analogues of vitamin D rather than ergocalciferol which, at the time of designing this study, was the recommended therapeutic approach for the treatment of VDD in CKD⁴⁹.

Clinical studies in patients without significant kidney disease, have demonstrated that endothelial function can be improved after treatment with nutritional forms of vitamin D¹⁸⁹⁻¹⁹¹. In pre-clinical⁷⁵ and clinical studies²³⁰, endothelial dysfunction has been identified as a non-traditional risk factor for CVD in CKD with improvements in endothelial function reflecting improved global vascular health and a reduced risk of CVD¹⁵⁵. The association between VDD and endothelial dysfunction in patients with

CKD and ESKD has been indicated in studies demonstrating a correlation between reducing concentrations of serum 25 (OH) D and a reduction in endothelial dysfunction (see section 1.4).

Interventions that target endothelial function in early CKD compared to more advanced or ESKD have the potential to manipulate and improve endothelial function at a stage of disease when the vasculature is more likely to be responsive to these therapies. As discussed in section 1.3.2.3, peripheral microcirculatory endothelial function predicts the function of the microcirculatory beds in renal and cardiac tissue and can be evaluated using non-invasive, bed side techniques that assess dermal capillary beds¹⁵⁹⁻

161 246

At the time this study was designed, there were no prospective, randomized controlled studies investigating the effect of vitamin D on microcirculatory endothelial in patients with CKD²⁵³. Assimon *et al.*²¹⁹, Marckmann *et al.*¹²⁰ and Chitalia *et al.*²⁴⁴ have evaluated the effect of nutritional vitamin D compounds on biomarkers and function of the endothelium in CKD and dialysis requiring ESKD but these studies have produced conflicting results. Thus, there is still a gap in the evidence for the efficacy of nutritional vitamin D compounds outside their influence on traditional markers of bone mineral metabolism and specifically the effect of these compounds on microcirculatory endothelial function.

In line with the unanswered questions in this area, a review of CV assessment in patients with CKD¹⁶² has highlighted the need for further assessments of microcirculatory endothelial dysfunction in patients with CKD as a method for predicting adverse CV outcomes. Given that the mechanism of the beneficial effect of vitamin D

on the reduction on CV morbidity and mortality in CKD remains unclear, there is a need to conduct randomized controlled trials which both conform to guidelines on replacement therapy for VDD in CKD and evaluate the effect of nutritional vitamin D compounds on microcirculatory endothelial function. These studies have the potential to confirm that microcirculatory endothelial function can improve after vitamin D therapy. Furthermore, studies of this kind could also elucidate clinical markers of endothelial dysfunction which could predict future CV events and can be conducted in a routine clinical environment.

We therefore conducted an exploratory, double blind, randomized, controlled trial to determine if therapy with ergocalciferol compared to placebo improves microcirculatory endothelial function in patients with CKD and concomitant vitamin D deficiency.

2.1 Hypothesis

The hypothesis of this study was that therapy with ergocalciferol compared to treatment with placebo in patients with non-diabetic CKD stage 3-4 and concomitant VDD improves microcirculatory endothelial function.

2.2 Methods

2.2.1 Study type and setting

The study design was a single centre, double blind, exploratory randomized controlled trial comparing oral ergocalciferol to placebo in patients with CKD stage 3-4 (estimated glomerular filtration rate (eGFR) 60-15 mL/min) and concomitant VDD (defined as a serum 25 (OH) D concentration of < 40 nmol/L). The study was conducted at the Royal London Hospital Kidney Unit, UK, between 1/5/2009 and 1/9/2010.

2.2.2 Trial registration and ethical approval

All patients and healthy volunteers provided written informed consent on enrolment to the study. The study was approved by the Medicines and Healthcare Regulatory Authority UK (MHRA reference - 14620/0021/001-0001, EUDRACT number 2008-008745-38) and was sponsored by Barts and the London NHS Trust. Ethical approval was obtained from the East London Research Ethics Committee (Reference 09/H0703/9). The trial was registered at clinicaltrials.gov (Clinical trials number - NCT00882401) and conducted in accordance with the Declaration of Helsinki (see appendices for full details of approval and registration documents).

2.2.3 Study design

A summary of the interventional study design is shown in Figure 5. After enrolment, subjects with CKD underwent a baseline clinical and laboratory assessment prior to commencement of the study drug (ergocalciferol or placebo). The total treatment duration for each subject was 6 months. Subjects were reviewed in a specialist study clinic which was run by the principle investigator (Dr Dreyer). Medical management of study patients was supervised by Professor M.M. Yaqoob (Chief investigator). Subjects were seen monthly for 6 months before being discharged back to their routine kidney disease clinic at which point the nephrologist normally in charge of their care took over all clinical decision making. Subjects underwent a routine clinical review during all study visits. All patients received dietary advice appropriate to their stage of CKD from specialist renal dietitians which included advice on dietary intake of calcium, phosphate, sodium, potassium and protein.

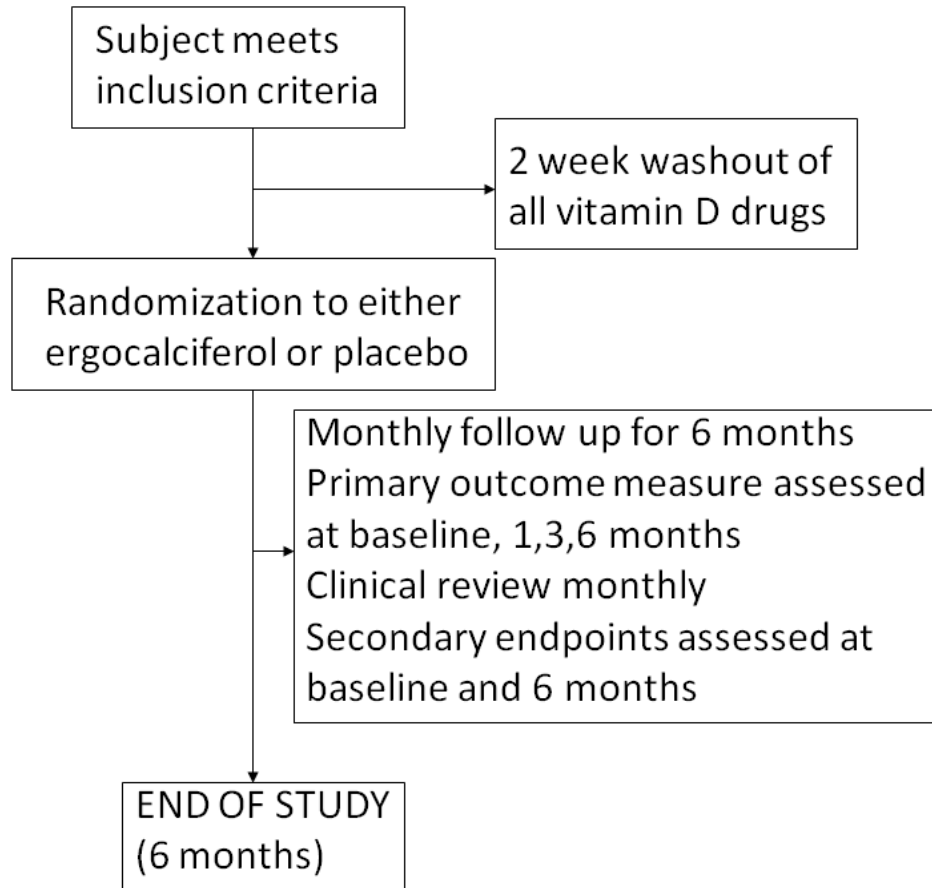


Figure 5 Diagrammatic representation of study procedures for patients with CKD stage 3-4 and concomitant vitamin D deficiency enrolled into the randomized controlled trial (see section 2.2.6 for details of intervention)

In addition, 15 healthy volunteers were recruited to compare baseline endothelial function between patients with and without CKD and to refine and optimize the diagnostic techniques used to assess the micro and macrocirculation (large conduit arteries) in the study. Healthy volunteers underwent iontophoresis of ACh and SNP combined with LDF, skin autofluorescence for AGE products, measures of aPWV, blood pressure and basic laboratory tests including serum 25 (OH) D and serum creatinine. Side stream dark field imaging of the sublingual microcirculation was not performed since the equipment was not available during the study period for healthy volunteers. Healthy volunteers received no therapeutic intervention and were evaluated before the randomized trial commenced.

2.2.4 Study endpoints

2.2.4.1 Primary outcome measure

The primary outcome measure was microcirculatory endothelial function assessed by LDF over forearm skin after iontophoresis of ACh and SNP.

2.2.4.2 Secondary outcome measure

Secondary outcome measures included skin AF for AGE products, side stream dark field imaging of the sublingual microcirculation as well as macrocirculatory parameters including blood pressure, pulse pressure, aPWV, LVMI and bone mineral parameters (calcium, phosphate and PTH)

2.2.5 Recruitment of study subjects

Patients with CKD stage 3-4 with a concomitant serum 25 (OH) D concentration of < 40 nmol/L were recruited from general outpatient kidney disease clinics at the Royal

London Hospital. Healthy volunteers were recruited by word of mouth and advertisements within the Royal London Hospital.

For patients with CKD the inclusion criteria were:

1. eGFR between 15 and 60 mL/min for at least 3 months prior to enrolment
2. Serum 25 (OH) D concentrations <40 nmol/L within 2 months of enrolment
3. No evidence of DM (fasting blood sugar <7.1, not taking any diabetic medication)
4. Not receiving any form of either haemodialysis or peritoneal dialysis
5. No dialysis therapy within the last 3 months
6. Age > 18 years and < 80 years
7. Patient agrees not use any medications (prescribed or over-the-counter including herbal remedies) judged to be clinically significant by the Principal Investigator during the course of the study.
8. Able to understand and sign the written Informed Consent form.
9. Able and willing to follow the protocol requirements.

The exclusion criteria were:

1. Currently receiving oral ergocalciferol at any dose
2. Received IM ergocalciferol therapy within last 3 months
3. Pacemaker or any other implanted cardiac device
4. Serum calcium above 2.6 mmol/L (the upper limit of the Royal London Hospital biochemistry laboratory⁴⁹)
5. Pregnant or lactating
6. Known hypersensitivity to ergocalciferol

7. Patient known to have a condition which predisposes to hypercalcaemia (multiple myeloma, sarcoidosis, other granulomatous disease)
8. Initial blood pressure of >160/100 mmHg
9. History of significant liver disease or cirrhosis
10. Anticipated requirement for dialysis in 6 months
11. Malabsorption, severe chronic diarrhoea, or ileostomy
12. Known diagnosis of hypervitaminosis D
13. Known to have renal calculi
14. Known to have systemic sclerosis, Raynaud's phenomenon or other disease associated with known microcirculatory dysfunction
15. Concurrent participation in any other research study

Women of childbearing potential were required to have a negative pregnancy test within 7 days before starting the study drug and, if the pregnancy test was negative, to use an effective method of contraception from the time of enrolment until 6 weeks after treatment discontinuation.

Healthy volunteers were included if they were between 18-70 years of age, had no acute or chronic medical conditions, were non-smokers and were not taking any medications. There was no restriction on serum 25 (OH) D concentrations for the recruitment of healthy volunteers.

2.2.6 Intervention

The dose of ergocalciferol delivered to the treatment arm was 50,000 IU weekly for one month starting on the day of randomisation followed by 50,000 IU monthly for 5 months resulting in a total dose of 450,000 IU over 6 months. The intervention was based on

guidelines from K/DOQI for the treatment of VDD in CKD⁴⁹. Ergocalciferol was also favoured for use in this study since it is plant derived and thus more appropriate for a multi-ethnic population with dietary restrictions that are served by the Royal London Hospital. The dose of ergocalciferol was standardized for all patients to ensure equal dosing of ergocalciferol over the duration of the study and to avoid the potential confounding effect of varying doses of ergocalciferol based on initial serum concentrations of 25 (OH) D. The control group intervention was a matching placebo given at the same dose schedule as ergocalciferol. To ensure blinding was achieved, ergocalciferol tablets (Sanofi Aventis, USA) were over-encapsulated into a red capsule and a matching placebo capsule containing lactose was manufactured by the pharmacy manufacturing department at Ipswich Hospitals NHS Trust. Compliance was assessed by a manual pill count at each study visit. A planned 2 week washout period for any vitamin D containing drugs or over the counter supplements that were likely to include vitamin D compounds e.g. multivitamins was included before randomisation, however, no subjects required vitamin D washout.

2.2.7 Randomisation procedures

The randomisation schedule was developed by an independent accountant. Sequentially numbered, sealed envelopes were used to achieve allocation concealment. Ergocalciferol or placebo were stored and dispensed to study patients in line with the randomization schedule by the Royal London Hospital clinical trial pharmacy team who were blinded to the intervention and allocation as were the remainder of the study team.

2.2.8 Criteria for withdrawal of subject

Withdrawal of study subjects and recruitment of new subjects to replace them was permitted if any of the following occurred:

1. Pregnancy occurring during the trial
2. Episode of symptomatic hypercalcaemia
3. Adverse reaction to any of the study drugs or procedures as judged by the principal investigator.
4. Patient request to withdraw for any reason
5. Permanent pace maker or other implantable cardiac device *in situ* or fitted during trial
6. Patients received dialysis therapy during trial duration
7. Patients underwent kidney transplantation during trial duration
8. Patients developed DM during trial duration
9. Failure to comply with study protocol, as judged by the principal investigator

2.2.9 Data collection

Data for the primary outcome measure and for skin AF was collected at baseline, 1, 3 and 6 months. Secondary endpoint measures were compared between baseline and 6 month follow up. Healthy volunteers underwent all study procedures except SDF imaging and cMRI. All study procedures except cMRI (performed at the London Chest Hospital) were performed at the Royal London Hospital Kidney Unit.

Study data were recorded and archived on a pre-designed case record form. Data was manually transferred to a master study data collection tool using Microsoft Excel and

was double entered to reduce transcribing errors. All data was held securely on password protected computers and was only accessible to the study team.

2.2.10 Medical care of study patients

All medical care, trial procedures and interventions were provided in the study clinic. Changes to non-trial medications were made based on clinical assessment of the Principal and Chief investigators. For example, blood pressure medications were adjusted if necessary to ensure patients were treated in line with existing CKD guidelines⁵⁰. The original treating nephrologist and primary care physician were notified in writing that a patient normally under their care had been enrolled into the study, were instructed not to prescribe any vitamin D containing medications for the duration of the study and to contact the study team to ensure any new prescription of medication or inter-current illness was notified.

2.2.11 Study procedures for the assessment of primary and secondary endpoints

Patients with CKD and healthy volunteers were instructed to wear loose clothing and avoid caffeine and nicotine for 12 h prior to assessment. Subjects were rested for 15 min in a temperature controlled room (20-21 °C) before study procedures were performed. Clinical assessments were conducted in the following order to avoid any systemic effect of drugs delivered during iontophoresis: SDF imaging of the sublingual microcirculation, skin AF, aPWV and iontophoresis.

2.2.12 Assessment of the primary outcome measure

Iontophoresis involves the delivery of charged particles to the local microcirculation, through the skin, using electrically repulsive forces. The most commonly used drugs to

generate a response from the endothelium are ACh and SNP. Sodium nitroprusside acts as an endothelium independent NO donor facilitating smooth muscle relaxation and thus vasodilatation whereas ACh provokes endothelium dependent vasodilatation (see section 1.3.1 and Figure 4). ACh response is therefore considered to reflect endothelial function whereas SNP is used as an assessment of maximal vasodilator response regardless of endothelial health.

LDF is a non-invasive technique which uses the Doppler principle to measure flux of erythrocytes in sub dermal capillaries. Laser Doppler probes continuously measure a defined volume of tissue (approximately 1mm³) and record their output as erythrocyte flux in this volume of tissue measured in arbitrary units. Increased erythrocyte flux after iontophoresis recorded by the laser Doppler probes reflects microcirculatory vasodilatation. The combination of these techniques is a validated method for studying endothelial function in the microcirculation in various pathologies including CKD^{224 254-255} and has been used to assess the effect of therapeutic interventions (including vitamin D) in randomized controlled trials^{194 256-258}.

2.2.13 Practical achievement of iontophoresis and recording of change in flux from baseline using laser Doppler flowmetry

The skin of the volar aspect of the non-dominant forearm was prepared by gentle wiping with an alcohol steret to remove a thin layer of dead skin which could theoretically interfere with laser Doppler assessment. The skin was left exposed to room air to dry for 60 s after wiping to avoid any vasoactive effects as a result of the cooling effect of the alcohol rub.

A diagrammatic representation of the iontophoretic equipment is shown in Figure 6. Two iontophoresis chambers (Moor Instruments, Axminster, Devon, UK) were adhered to the skin of the non-dominant forearm. The proximal chamber was located approximately 3 cm from the lower elbow crease and the chambers were separated by at least 5 cm to avoid the vasoactive response at one chamber being detected at the other chamber site.

Both chambers were sited to avoid hair, scars, freckles and visible or palpable blood vessels in order to maximize the chance of the laser probes obtaining a true recording from dermal microvessels. The Doppler probes (Moor Instruments, UK) were inserted into the centre of the iontophoresis chamber and approximately 100 μ l per chamber of a 1% solution of ACh (Novartis, Frimley, UK) or SNP (Mawdsley Brooks, Doncaster, UK) were introduced to the centre of the chamber via 2 holes in its upper surface. ACh was pre-diluted with water for injections and was inserted at the anode. SNP was pre-diluted with 5% dextrose and inserted at the cathode.

The laser probes were connected to a laser Doppler monitor (DRT4 – Moor instruments) which is in turn connected to a current delivery device (Moor Iontophoresis Controller (MIC) 1 – Moor instruments). The hardware is supported and controlled by integrated software (laser Doppler perfusion monitor v1.2, Moor Instruments) which allows for simultaneous laser Doppler recording and delivery of the iontophoretic protocol via the MIC 1 device. Doppler probes were calibrated weekly according to the manufacturer's instructions.

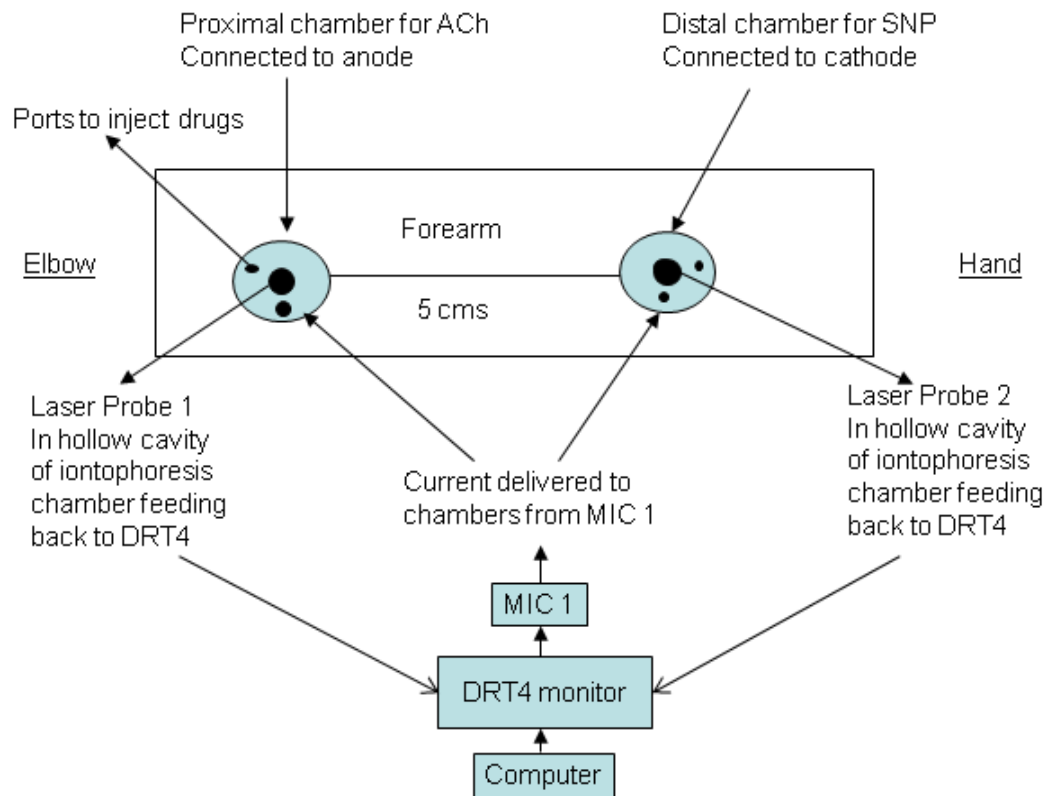


Figure 6 Diagrammatic representation of experimental iontophoretic equipment and connections. ACh – acetylcholine, SNP – sodium nitroprusside, MIC – Moor iontophoresis controller.

The iontophoretic protocol used a low current, sequential dose increases of ACh and SNP (Table 4) in order to avoid the galvanic effect of drug delivery itself on vasoactive changes in the microcirculation²⁵⁹. Baseline flux of the clinical trial subjects assessed by LDF, prior to the iontophoresis of either ACh or SNP, was measured as the mean of the flux recorded over the first epoch of 60 s after subjects had been acclimatised as described in section 2.2.11. Following the recording of baseline flux, a 1% solution of both ACh and SNP were iontophored on the volar aspect of the non-dominant forearm with a maximum achieved dose of 75 μ A after 7.5 min.

To eliminate baseline variability, relative percentage change from baseline flux to maximum flux after the maximum iontophoretic dose was the primary outcome measure^{194 254}. To assess that there were no significant changes in the level of baseline flux induced by either ergocalciferol or placebo (which may have had an effect on percentage change from baseline flux to maximum flux after the maximum iontophoretic dose), baseline flux was compared at randomisation and after 6 months of therapy in both groups (section 2.2.15.2). An example of the iontophoretic output is shown in Figure 7.

2.2.14 Secondary outcomes measures

2.2.14.1 Side stream dark field imaging of the sublingual microcirculation.

This is a non-invasive, real time, imaging tool to assess intra-vital capillary blood flow. This technique utilised the Microscan probe (Microscan Medical, Amsterdam, the Netherlands). Side stream dark field imaging is based on reflectance avoidance in which the illuminated light and reflected light travel via independent pathways. A small camera probe (0.7cm lens tip) with a replaceable sterile cap was placed under the tongue. The probe is circumferentially surrounded by green (530 nm) light emitting

diodes (LEDs). The light from the LEDs is absorbed and reflected by the haemoglobin of erythrocytes and results in the ability to observe the flowing cells in individual capillary vessels in the sublingual microcirculation (Figure 8 panel A). Three separate video images of 60 s duration were collected. Each image is taken from a separate area of the sublingual capillary bed by gentle repositioning of the camera tip (Figure 8 panel B).

Table 4 Iontophoretic protocol for patients with CKD and healthy volunteers.

Epoch	Current (μA)	ACh	SNP
1	0 (baseline)	60 s	60 s
2	10	30 s	30 s
3	0	60 s	60 s
4	20	30 s	30 s
5	0	60 s	60 s
6	35	30 s	30 s
7	0	60 s	60 s
8	50	30 s	30 s
9	0	60 s	60 s
10	75	30 s	30 s
11	0	60 s washout	60 s washout

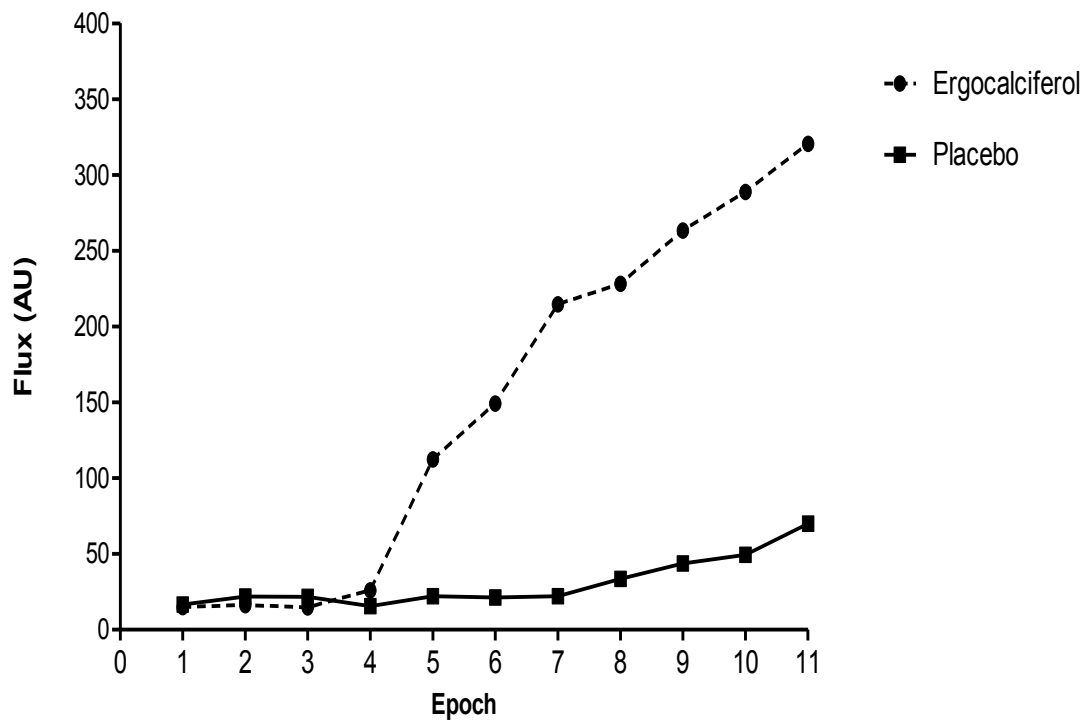
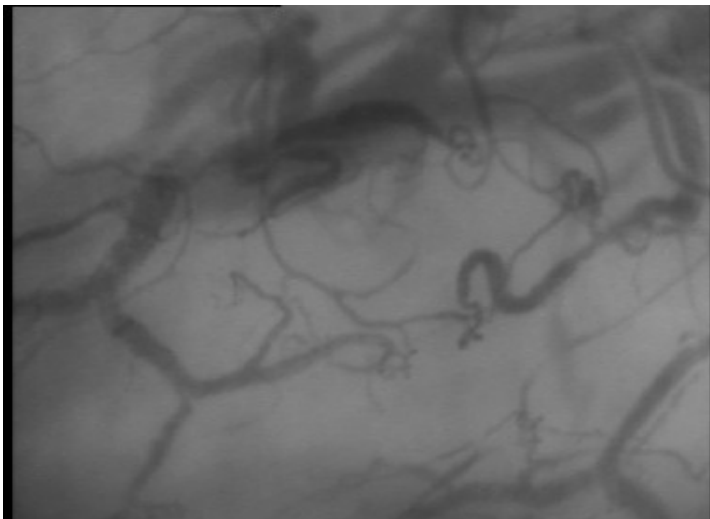
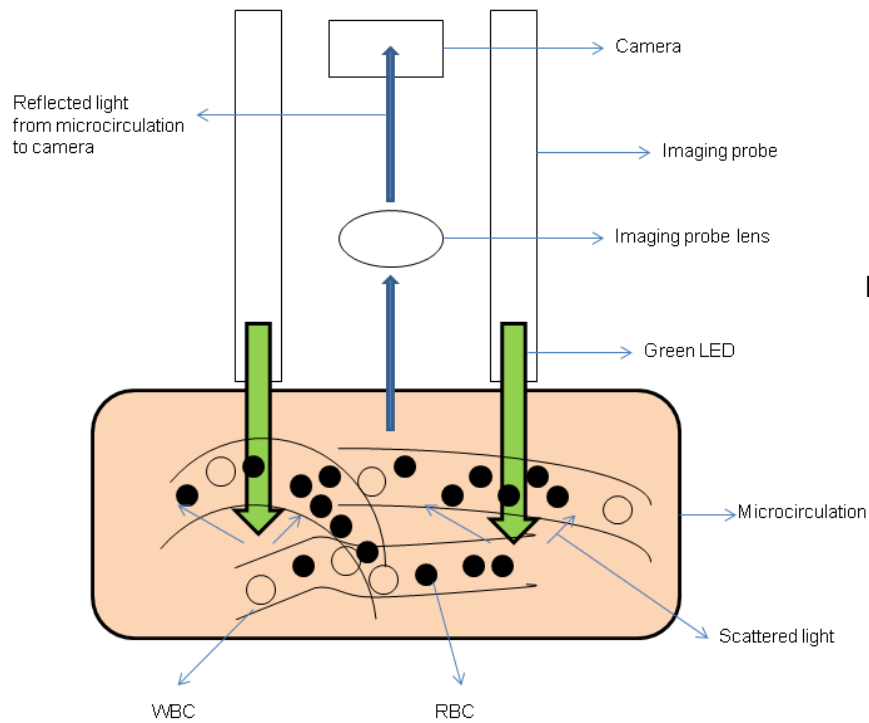


Figure 7 Examples of iontophoresis dose response curves. 6 months of treatment for 2 patients after delivery of ACh demonstrating greater relative increase in flux in the ergocalciferol compared to placebo treated patient. Flux measured in arbitrary units (AU)



Scale: — = 25 μ m

Figure 8 Reflectance avoidance in SDF imaging.

Panel A - Illuminating light (green arrow) and reflected light (blue arrow) travel by individual pathways.

Panel B - **Still image from SDF imaging video clips of the sublingual microcirculation.** Individual capillaries and flow of circulating red blood cells can be visualised in real time. LED – light emitting diode, RBC – red blood cell, WBC – white blood cell.

An analysis of the moving cells in the images permits the semi-quantitative measurement of red blood cell flow in the capillaries termed the MFI. Additionally, morphological characteristics of the microcirculation, such as the functional capillary density (FCD) and microcirculatory vessel morphology, can be measured using reflectance avoidance imaging. SDF images were scored and interpreted according to standard consensus guidelines¹⁶⁶.

2.2.14.2 Skin autofluorescence for tissue advanced glycation end products

This technique utilised the Diagnostics AGE reader (Diagnostics, Groningen, the Netherlands) which provides a non-invasive measure of AGE products in the skin. Advanced glycation end products correlate with measures of skin AF provided by the output from the AGE reader device. These levels are an independent risk predictor of microcirculatory complications and are predictors of CVD in kidney disease²⁶⁰⁻²⁶¹.

The principal of this technique is the differing reflectance and fluorescent properties of AGE moieties. The AGE reader consists of a small box, containing an excitation light source which emits light with wavelengths of 300–420 nm (peak intensity approximately 370 nm). To correct for differences in light absorption, skin AF is calculated by dividing the amount of emitted light intensity between 420 and 600 nm by the amount of excitation light intensity between 300 and 420 nm, expressed as arbitrary units²⁶¹.

Patients placed their forearm over the light emitting box. An area of the volar aspect of the forearm free from scars or tattoos was selected for measurement. The skin was gently cleaned with an alcohol swab. The mean of three separate readings of skin reflectance is converted to a measure of skin AF.

2.2.14.3 Aortic pulse wave velocity measurement

Patients were prepared as described in section 2.2.11 and in addition were reclined to approximately 15 degrees from horizontal. This technique utilised the Vicorder device (Skidmore Medical, Bristol, UK). For the measurement of the aPWV, a cuff was placed around the neck over the carotid artery as close to the sternal notch as was practical and a second cuff was placed around the upper thigh. These cuffs were connected to the control box which was in turn connected to a computer running the Vicorder software. With the patient recumbent at 15 degrees from the horizontal plane, the distance between the lower border of the neck cuff and the upper border of the thigh cuff is recorded, reflecting the approximate distance of the arterial segment being measured.

Both cuffs were inflated simultaneously to approximately 70 mmHg. The cuffs remained inflated for between 3 and 5 pulse transmission cycles. Pulse wave speed (transit time), the time taken in s for the pulse wave to travel between the 2 sensor cuffs was recorded and the aPWV was generated by dividing the distance between the supra sternal notch and the thigh cuff in cm by the transit time in s. Readings were taken from the left and right thigh and the mean value calculated as aPWV.

2.2.14.4 Cardiac magnetic resonance imaging

Studies were performed at the London Chest Hospital, supervised by Dr Mark Westwood using a Philips Achieva CV 1.5T. LVM was measured directly from the steady state free precession contiguous short axis cine stack (8mm slice thickness and 2mm inter slice gap, with whole LV coverage) using Philips MR WorkSpace software.

LVM is measured directly by this technique and was normalized to body surface area (calculated by the Dubois and Dubois formula²⁶²) to produce the LVMI.

2.2.14.5 Clinical measurements

Weight was measured on calibrated scales (Seca, London UK). Height was measured using a fixed, wall mounted device. Body mass index (BMI) was calculated as weight in kg divided by height in m². Blood pressure was measured using a standard mercury sphygmomanometer. The mean of the blood pressure on the left and right arm, each taken 5 min apart, with the patient seated and the cuff at heart level, was calculated. All equipment was checked and calibrated regularly in line with hospital guidelines. The principal investigator recorded and documented all clinical measures.

2.2.14.6 Biochemical measures

All biochemical tests were performed in the Royal London Hospital laboratory by a Roche modular unit analyser (F. Hoffmann-La Roche Ltd, London, UK). Serum 25 (OH) D concentrations were assessed by a quantitative ultra-performance liquid chromatography tandem mass spectrometry assay (UPLC-MS). Serum parathyroid hormone (PTH) concentrations were assessed by the Roche E170 intact PTH assay (Roche Diagnostics, West Sussex, UK). Serum creatinine was converted to the eGFR using the 4 variable Modification of Diet in Renal Disease (MDRD) equation which includes a correction factor for black ethnicity⁵¹.

2.2.15 Statistical analysis

2.2.15.1 Comparison of healthy volunteers and patients with chronic kidney disease

Baseline parameters for healthy volunteers and patients with CKD were tabulated and assessed for normality using tests of skewness and kurtosis. A comparison between the 2 groups was made using the Student's t test, Mann Whitney U test or Chi² test depending on the distribution or nature (continuous or categorical) of the data. A 2-tailed p value of < 0.05 was considered statistically significant. Analysis was conducted on Stata version 10 (www.stata.com) and GraphPad Prism software (version 5).

2.2.15.2 Analysis of clinical trial data

At the time of designing the study, there was insufficient available evidence of the effect of ergocalciferol therapy on microcirculatory parameters to undertake a standard power calculation. The hypothesis was that ergocalciferol would significantly improve the function of the endothelium in patients with CKD and concomitant VDD. Given the profoundly low concentrations of serum 25 (OH) D in this patient group, the expected rise in serum 25 (OH) D concentrations with ergocalciferol and predicted lack of change of serum 25 (OH) D concentrations in the placebo group, we estimated that 30% of patients in the placebo group and 80% of the patients in the ergocalciferol group would have an improvement in peripheral LDF after iontophoresis of ACh measured by relative change from baseline flux after 6 months of therapy. At 80% power and with a significance level of 0.05, this required 19 patients per study arm. An intention-to-treat analysis was performed.

The ergocalciferol and placebo groups were compared for similarity at baseline and after 6 months of therapy using the Student's t test for normally distributed variables, Mann Whitney tests for non-parametric data and Chi² tests or Fisher's exact test for proportions. The absolute difference in baseline flux, prior to the iontophoresis of ACh or SNP, was compared at randomisation and after 6 months of therapy in both groups by a paired t test. Differences in serum 25 (OH) D concentrations and change from baseline flux measured by LDF after iontophoresis were analysed using a two way repeated measures ANOVA test followed by Bonferroni post tests for comparisons at pre determined time points (1, 3 and 6 months). Forearm LDF is expressed as the percentage increase in flux from baseline after iontophoresis of ACh. The findings from the 2 way ANOVA with repeated analysis for the primary outcome measure were confirmed using a mixed effects model.

Four patients in the ergocalciferol group and 1 in the placebo group completed an initial 3 months on therapy before the end of the predetermined study period and this data was included in the analysis. The analysis of data at 6 months includes data from all remaining patients who completed the full follow up period. SDF imaging of the sublingual microcirculation was expressed as FCD and MFI as described previously¹⁶⁶. Differences between LVMI, SDF imaging, skin AF and bone mineral parameters were analysed using t tests and Mann Whitney tests based on the distribution of the data. A p value of <0.05 was considered statistically significant. Analysis was conducted on Stata version 10 (www.stata.com) and GraphPad Prism software version 5.

2.3 Results

2.3.1 Healthy volunteers

Table 5 summarises the differences between healthy volunteers and patients with CKD at baseline before treatment with ergocalciferol. Age, blood pressure and BMI were significantly lower in healthy controls compared to patients with CKD while serum 25 (OH) D concentrations were significantly higher in healthy volunteers. aPWV and skin AF were significantly higher in patients with CKD compared to healthy controls. There was no significant difference in percentage rise from baseline flux after either iontophoresis with either ACh or SNP between the two groups although the absolute value of percentage change in flux for both ACh and SNP was higher in healthy volunteers compared to the CKD group

2.3.2 Randomized trial data

Patient screening, enrolment and randomization are shown in Figure 9. Two patients were lost to follow up in the ergocalciferol arm and 1 in the placebo arm. One patient in the placebo arm developed DM during the trial and was withdrawn from the study according to pre specified withdrawal criteria. At baseline, the treatment and placebo groups were similar with respect to demographic, clinical (Table 6 and Table 7) and laboratory parameters (Table 8). Systolic blood pressure and mean pack years of smoking were higher in the placebo group but this did not reach statistical significance. All patients self-reported complete compliance and this was confirmed by manual inspection of study medication bottles at each visit.

Table 5 Comparison of baseline demographic, clinical and laboratory data between healthy volunteers and patients with CKD. Figures are mean (SD) or Number (%).

	Healthy volunteers (n=15)	CKD patients (n=38)	p value
Age (years)	32.0 (7.9)	47.0 (1.7)	<0.0001
Sex (% male)	9 (60%)	14 (73.7%)	0.15
Body mass index	25.2 (3.0)	29.9 (1.0)	0.004
Creatinine (µmol/L)	79 (14.0)	188 (11.0)	<0.0001
25 (OH) D (nmol/L)	60.7 (7.5)	30.3 (5.4)	<0.0001
eGFR (mL/min)	87.5 (6.3)	35.8 (2.4)	<0.0001
Systolic BP (mmHg)	106 (11)	117 (2)	<0.0001
Diastolic BP (mmHg)	65 (6)	70 (1)	0.002
MAP (mmHg)	77 (5)	86 (2)	0.003
Pulse pressure (mmHg)	41 (6)	46 (5)	0.016
% rise from baseline flux after IOP to ACh	1030 (178.9)	786.6 (107.7)	0.22
% rise from baseline flux after IOP to SNP	797.6 (153.1)	538.8 (90.6)	0.13
aPWV m/s	7.2 (0.9)	8.5 (0.2)	<0.0001
Skin AF (AU)	2.02 (0.48)	2.93 (0.15)	0.002

MAP = mean arterial pressure, IOP = iontophoresis, ACh = acetylcholine, SNP = sodium nitroprusside, aPWV = aortic pulse wave velocity, AF = autofluorescence, AU = arbitrary units, eGFR = estimated glomerular filtration rate.

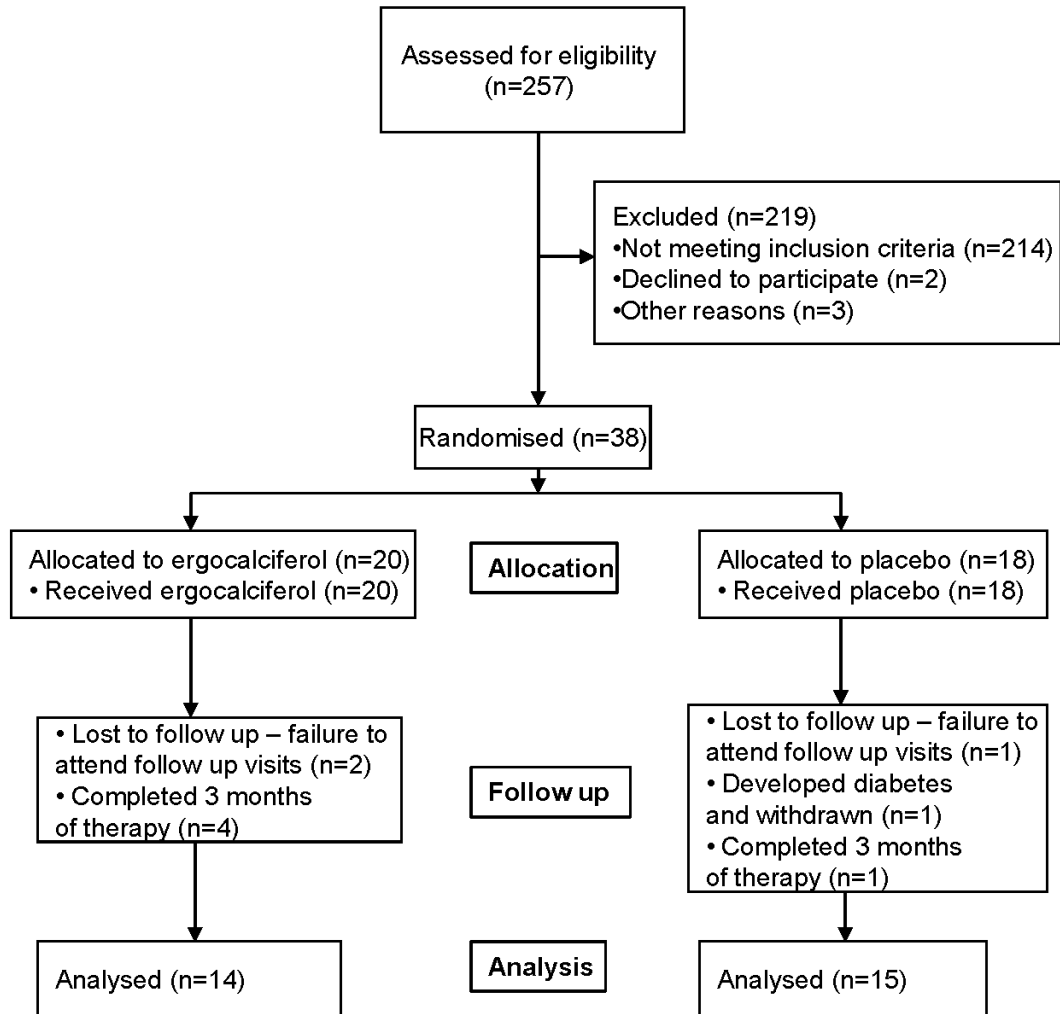


Figure 9 Recruitment and randomisation for subjects entering the clinical trial.

Table 6 Baseline demographic and clinical data for patients with CKD. Figures in brackets are standard deviation of the mean or % of total in treatment group.

	Ergocalciferol (n=20)	Placebo (n=18)	p value
Age years	45.8 (10.0)	48.8 (12.2)	0.39
Sex (% male)	14 (60.9%)	14 (73.7%)	0.22
Body mass index	30.4 (7.1)	29.2 (3.4)	0.51
Ethnicity			0.57
Caucasian	5 (21.8%)	6 (31.6%)	
Non-Caucasian	15 (78.2%)	12 (68.4%)	
Cause of CKD			0.74
Hypertension	5 (25%)	7 (38.9%)	
Glomerulonephritis	8 (40%)	5 (27.8%)	
ADPKD	2 (10%)	1 (5.6%)	
Other[~]	5 (25%)	5 (27.7%)	
Smoking status			0.59
Current smoker	1 (5%)	2 (11.1%)	
Never/Ex-smoker	19 (95%)	16 (88.9%)	
Presence of endovascular stent devices	0 (0%)	1 (5.6%)	0.47
ACE-I/ARB	16 (80%)	12 (66.7%)	0.33
β Blocker	7 (35%)	6 (33.3%)	0.57
Statin use	9 (45%)	7 (38.9%)	0.84
Anti platelet therapy	2 (10%)	3 (16.7%)	0.48
Folic acid	1 (5.0%)	1 (5.6%)	1.00
Nitrate containing medications[#]	0 (0%)	0 (0%)	1.00
Medications containing Vitamin D	0 (0%)	0 (0%)	1.00
Hypertension in past history	15 (65.2%)	11 (57.9%)	0.63

ADPKD – autosomal dominant polycystic kidney disease, ACE-I angiotensin converting enzyme inhibitor, ARB – angiotensin receptor blocker, MAP = mean arterial pressure

- any form of glyceryl trinitrate, isosorbide mononitrate, isosorbide dinitrate or other esters of nitric acid

~ - Additional causes of CKD in ergocalciferol group: tubulo-interstitial nephritis (n=1), reflux nephropathy (n=2), unknown (n=2). Placebo group: reflux nephropathy (n=2), ischaemic nephropathy presumed due to reno-vascular disease (n=1), unknown (n=2)

Table 7 Comparison of micro and macro circulatory parameters in the ergocalciferol and placebo groups at baseline. Figures in brackets are standard deviation of the mean.

	Ergocalciferol (n=20)	Placebo (n=18)	P value
Systolic BP (mmHg)	114 (10)	119 (10)	0.11
Diastolic BP (mmHg)	70 (8)	71 (7)	0.57
MAP (mmHg)	84 (8)	87 (8)	0.29
Pulse pressure (mmHg)	45 (7)	48 (6)	0.08
% rise from baseline flux after IOP to ACh	964.8 (170.0)	785.9 (121.3)	0.85
% rise from baseline flux after IOP to SNP	455.8 (117.7)	675.1 (152.2)	0.26
Skin AF (AU)	2.8 (0.9)	3.1 (0.9)	0.26
aPWV m/s	8.5 (1.1)	8.5 (1.5)	0.66
LVMI (g/m²)[#]	96.1 (36.3)	87.5 (174)	0.55

BP = blood pressure, MAP = mean arterial pressure, IOP = iontophoresis, ACh = acetylcholine, SNP = sodium nitroprusside, aPWV = aortic pulse wave velocity, AF = autofluorescence, AU = arbitrary units. LVMI – left ventricular mass index

n=13 scanned in ergocalciferol group, n=11 scanned in placebo group

Table 8 Baseline laboratory data for CKD patients randomized to either intervention or placebo. Figures in brackets are standard deviation of the mean.

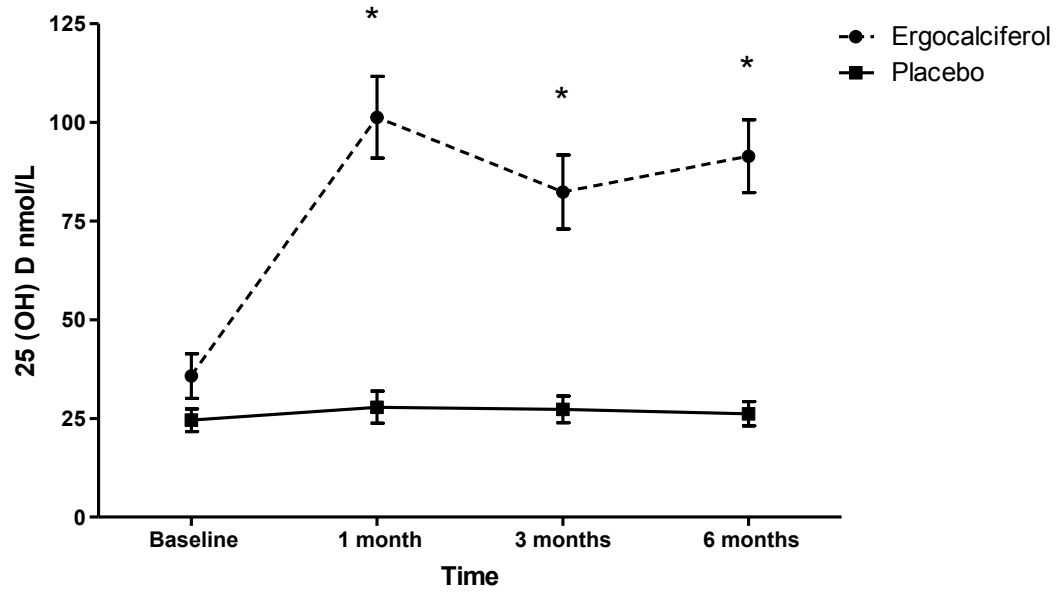
	Ergocalciferol (n=20)	Placebo (n=18)	P value
Creatinine (µmol/L)	203 (74)	176 (92.7)	0.60
eGFR (mL/min)	33.0 (13.5)	38.7 (15)	0.39
25 (OH) D (nmol/L)	35.7 (5.6)	24.5(2.8)	0.09
Hb (g/dL)	12.8 (1.8)	12.6 (1.4)	0.63
Calcium (mmol/L)	2.24 (0.1)	2.23 (0.2)	0.74
Phosphate (mmol/L)	1.2 (0.3)	1.1 (0.2)	0.16
PTH (pmol/L)	10.9 (8.7)	12.6 (11.0)	0.60
CRP (mg/L)	7.6 (17.2)	5.9 (9.8)	0.71
Urine P:CR	190.8 (276.4)	102.7 (147.0)	0.32
Total cholesterol (mmol/L)	5.0 (1.5)	4.8 (0.9)	0.36
LDL cholesterol (mmol/L)	3.2 (1.5)	2.7 (0.9)	0.20
HDL cholesterol (mmol/L)	1.6 (0.9)	1.3 (0.5)	0.17

eGFR = estimated glomerular filtration rate, Hb = haemoglobin, PTH = parathyroid hormone, CRP = C reactive protein, P:CR = protein: creatinine ratio, LDL – low density lipoprotein, HDL – high density lipoprotein

If patients had not taken a dose of the study medication before a specific clinical visit, they were asked to take the medication at the time of the study visit. This occurred once for each of 6 patients in the ergocalciferol group and 5 patients in the placebo group in the monthly dosing phase of the study. In the intensive weekly dosing phase, all patients had taken the correct number of tablets in the first month of the study although 4 patients in the ergocalciferol arm and 5 in the placebo had missed a scheduled dose by 1-2 days either side of the planned dose date. These minor differences were not considered to significantly affect the total dose of ergocalciferol received over the study period. No patients were taking nitrate containing medications that may have acted as vasodilators.

Serum 25 (OH) D concentrations were significantly higher in the ergocalciferol group compared to placebo group after 1 month of treatment and this difference was maintained over the study duration (repeated measures 2 way ANOVA $p < 0.0001$) (Figure 10). At 6 months, PTH concentration decreased in the ergocalciferol group but this did not attain statistical significance. There were no other statistically significant changes to key biochemical parameters during the study (Table 9)

Three patients in the ergocalciferol group and 1 in the placebo group required intensification of medication for blood pressure control which was achieved with increasing doses of medications that the patients were already taking.



Number at risk

	Baseline	1 month	3 months	6 months
Ergocalciferol	20	19	18	14
Placebo	18	18	16	15

Figure 10 Serum concentrations of 25 (OH) D in patients treated with ergocalciferol and placebo. Absolute values 25 (OH) D nmol/L baseline - ergocalciferol 35.7 placebo 24.5 ($p > 0.05$). 1 month - ergocalciferol 98.9, placebo 27.8 ($p < 0.0001$). 3 months - ergocalciferol 82.4, placebo 27.3 ($p < 0.0001$). 6 months - ergocalciferol 91.4, placebo 26.2 ($p < 0.0001$). p values are Bonferroni post-test following two way repeated measures ANOVA. (* = statistically significant)

Table 9 Laboratory results in both groups at baseline and after 6 months of therapy. Figures in brackets are standard deviation of the mean.

	Baseline			6 months		
	Ergocalciferol (n=20)	Placebo (n=18)	P value	Ergocalciferol (n=14)	Placebo (n=15)	P value
Creatinine (µmol/L)	203 (74)	176 (92.7)	0.60	212 (80)	203.2 (97.2)	0.80
eGFR (mL/min)	33.0 (13.5)	38.7 (15)	0.39	31.4 (10.6)	35.0 (14.5)	0.44
25 (OH) D nmol/L	35.7 (5.6)	24.5(2.8)	0.09	91.4 (6.4)	26.2 (3.2)	<0.0001
Hb (g/dL)	12.8 (1.8)	12.6 (1.4)	0.63	12.6 (2.1)	12.4 (1.3)	0.73
Calcium (mmol/L)	2.24 (0.1)	2.23 (0.2)	0.74	2.31 (0.2)	2.26 (0.2)	0.43
Phosphate (mmol/L)	1.2 (0.3)	1.1 (0.2)	0.16	1.2 (0.2)	1.1 (0.1)	0.98
PTH (pmol/L)	10.9 (8.7)	12.6 (11.0)	0.60	10.3 (7.9)	14.4 (10.2)	0.26
CRP (mg/L)	7.6 (17.2)	5.9 (9.8)	0.71	7.5 (15.0)	9.7 (19.8)	0.76
Urine P:CR	190.8 (276.4)	102.7 (147.0)	0.32	154.0 (210.3)	117.5 (126.3)	0.62
Total cholesterol (mmol/L)	5.0 (1.5)	4.8 (0.9)	0.36	5.0 (1.0)	4.5 (0.9)	0.21
LDL cholesterol (mmol/L)	3.2 (1.5)	2.7 (0.9)	0.20	2.6 (0.7)	2.3 (0.7)	0.24
HDL cholesterol (mmol/L)	1.6 (0.9)	1.3 (0.5)	0.17	1.4 (0.7)	1.3 (0.4)	0.67

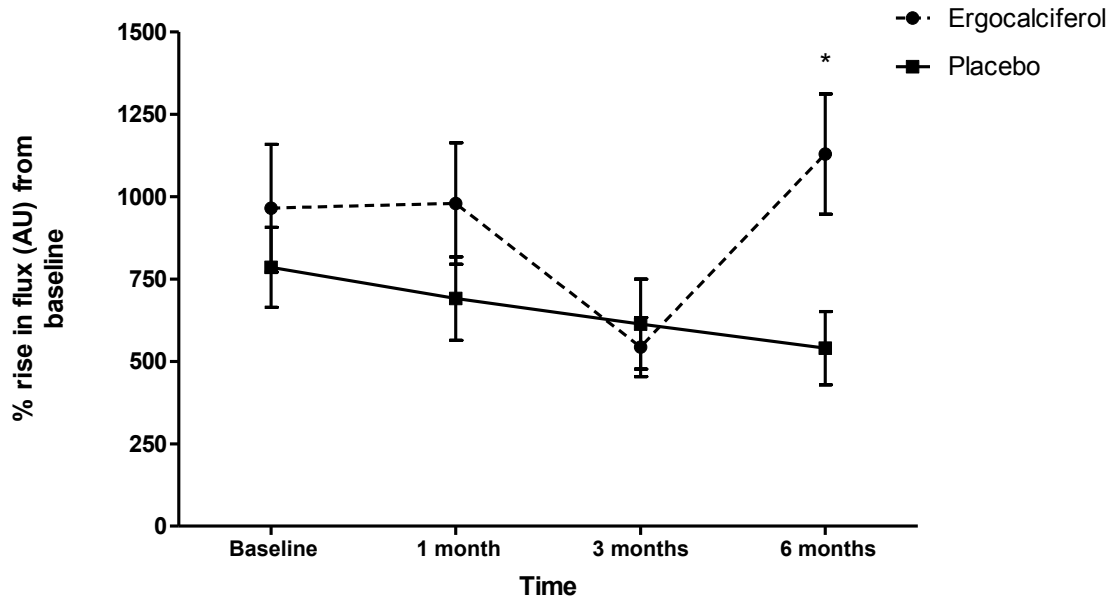
eGFR = estimated glomerular filtration rate, Hb = haemoglobin, PTH = parathyroid hormone, CRP = C reactive protein, P:CR = protein: creatinine ratio, LDL – low density lipoprotein, HDL – high density lipoprotein.

2.3.3 Primary outcome measure

There was no significant difference in the baseline value of flux, prior to the iontophoresis of ACh or SNP, between randomisation and 6 months follow up in either the placebo group (mean difference in flux -6.8 AU, 95% CI -20.8 – 7.2, $p=0.31$) or the ergocalciferol group (mean difference in flux -3.8 AU, 95% CI -13.5 – 5.9, $p=0.40$). Treatment with ergocalciferol compared to placebo was associated with a significant increase in change from baseline flux measured by LDF after iontophoresis of ACh (repeated measures 2 way ANOVA $p=0.03$) with a significant difference between treatment groups observed at 6 months (Bonferroni post-test $p=0.012$) (Figure 11). There were no significant differences in change from baseline flux after the iontophoresis of SNP between treatment groups at 6 months (repeated measures 2 way ANOVA, $p=0.18$) (Figure 12). The use of a mixed effects model did not change the significance of these findings (change from baseline after iontophoresis at 6 months follow up: ACh $p=0.03$, SNP $p=0.36$).

2.3.4 Secondary outcome measure

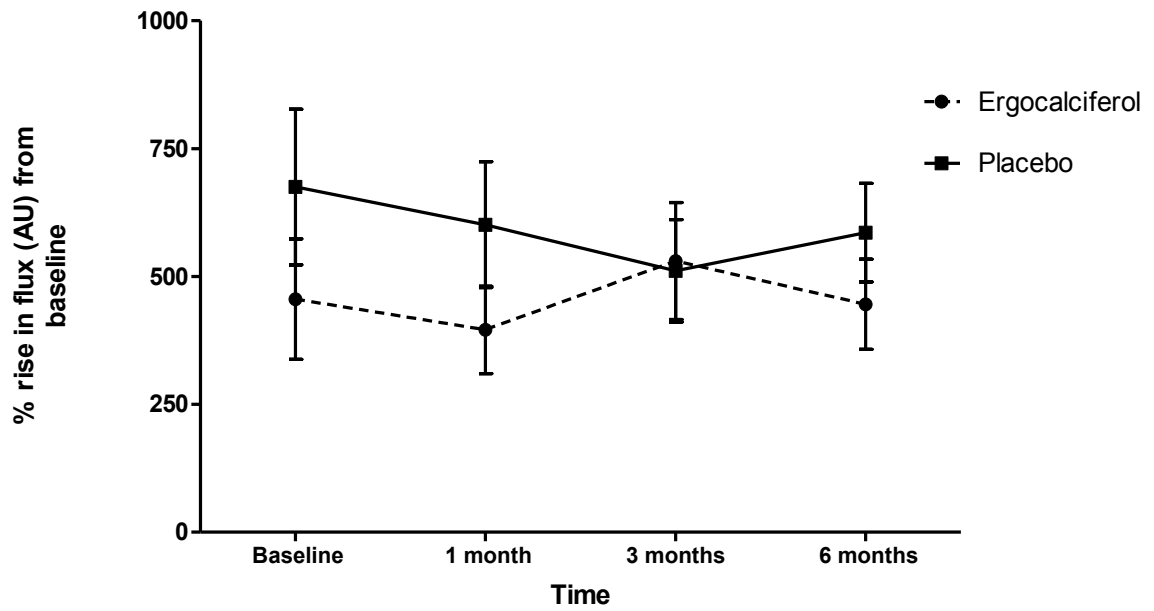
There was an overall increase in skin AF in the placebo group (repeated measures 2 way ANOVA $p=0.03$) with a significant difference observed between treatment groups at 6 months (Bonferroni post-test $p=0.02$, Figure 13). Skin AF did not change between baseline and 6 month follow up in the ergocalciferol group. There were no differences in the SDF imaging parameters of FCD or MFI at the end of the study period (Figure 14). Pulse pressure was significantly lower in patients treated with ergocalciferol after 6 months ($p=0.01$) but systolic, diastolic and MAP were similar between treatment groups. There were no differences in aPWV or LVMI after 6 months of therapy with either ergocalciferol or placebo (Table 10).



Number at risk

	Baseline	1 month	3 months	6 months
Ergocalciferol	20	19	18	14
Placebo	18	18	16	15

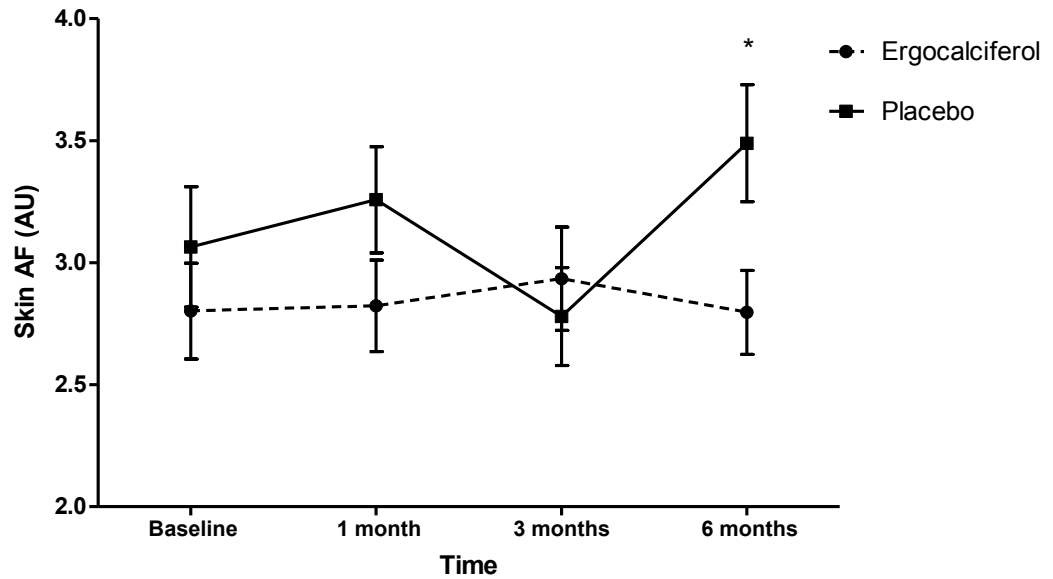
Figure 11 Percentage rise from baseline flux in arbitrary units (AU) after iontophoresis of ACh. Absolute values of percentage change in flux (AU): baseline - ergocalciferol 964.8, placebo 785.9 ($p>0.05$). 1 month - ergocalciferol 979.5, placebo 690.9 ($p>0.05$). 3 months – ergocalciferol 543.7, placebo 613.5 ($p>0.05$). 6 months – ergocalciferol 1130.0, placebo 540.6 ($p=0.012$). p values are Bonferroni post-test following two way repeated measures ANOVA. (* = statistically significant)



Number at risk

	Baseline	1 month	3 months	6 months
Ergocalciferol	20	19	18	14
Placebo	18	18	16	15

Figure 12 Percentage rise from baseline flux in arbitrary units (AU) after iontophoresis of SNP. Absolute values of percentage change in flux (AU): baseline - ergocalciferol 455.8, placebo 670.1 ($p > 0.05$). 1 month - ergocalciferol 395.5, placebo 601.3 ($p > 0.05$). 3 months – ergocalciferol 530.2, placebo 511.2 ($p > 0.05$). 6 months – ergocalciferol 445.7, placebo 585.9 ($p > 0.05$). p values are Bonferroni post-test following two way repeated measures ANOVA.



Number at risk

	Baseline	1 month	3 months	6 months
Ergocalciferol	20	19	18	14
Placebo	18	18	16	15

Figure 13 Change in skin AF between treatment groups over total study duration. Absolute values of percentage change in flux (AU): baseline - ergocalciferol 2.8, placebo 3.1 ($p>0.05$). 1 month - ergocalciferol 2.8, placebo 3.3 ($p>0.05$). 3 months – ergocalciferol 2.9, placebo 2.8 ($p>0.05$). 6 months – ergocalciferol 2.8, placebo 3.4 ($p=0.02$). p values are Bonferroni post-test following two way repeated measures ANOVA. (* = statistically significant)

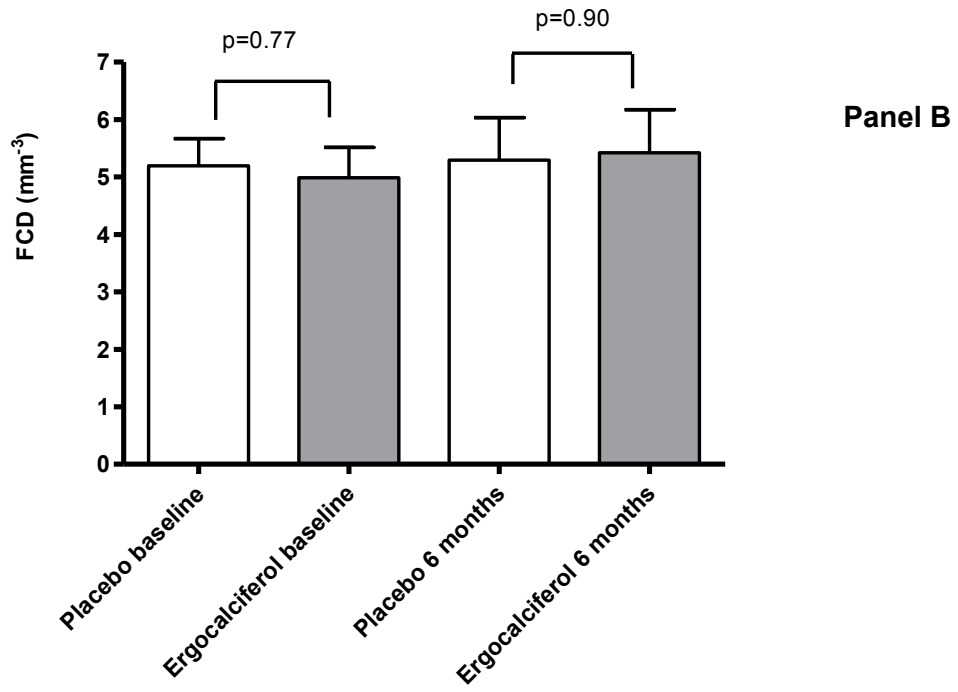
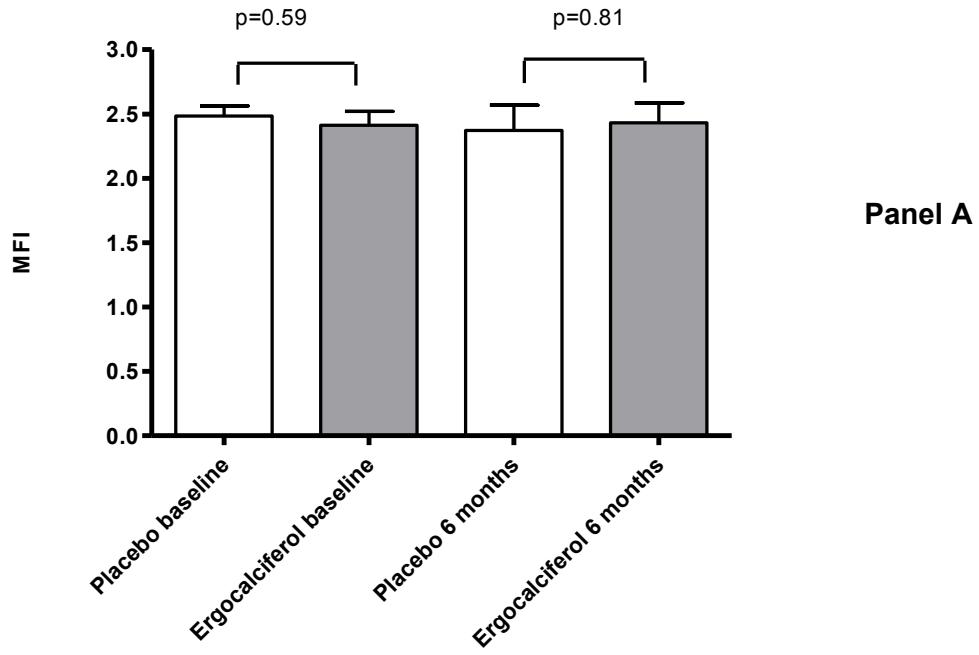


Figure 14 Sublingual microcirculatory parameters at baseline and after 6 months.

Panel A - Microvascular flow index (MFI)

Panel B - functional capillary density (FCD)

Table 10 Measures of macrovascular parameters in both groups at baseline and after 6 months of therapy. Figures in brackets are standard deviation of the mean.

	Baseline			6 months		
	Ergocalciferol (n=20)	Placebo (n=18)	P value	Ergocalciferol (n=14)	Placebo (n=15)	P value
Systolic BP (mmHg)	114 (10)	119 (10)	0.11	118 (10)	123 (15)	0.26
Diastolic BP (mmHg)	70 (8)	71 (7)	0.57	74 (6)	70 (9)	0.15
MAP (mmHg)	84 (8)	87 (8)	0.29	89 (7)	88 (10)	0.77
Pulse pressure (mmHg)	45 (7)	48 (6)	0.08	44 (8)	53 (12)	0.01
% rise from baseline flux after IOP to ACh	964.8 (170.0)	785.9 (121.3)	0.85	1130.0 (182.3)	540.6 (189.9)	0.012
% rise from baseline flux after IOP to SNP	455.8 (117.7)	670.1 (152.2)	0.26	445.7 (88.3)	585.9 (94.7)	0.28
Skin AF (AU)	2.8 (0.9)	3.1 (0.9)	0.26	2.8 (0.6)	3.4 (0.9)	0.02
aPWV (m/s)	8.5 (1.1)	8.5 (1.5)	0.66	8.4 (1.3)	8.5 (1.2)	0.78
LVMI (g/m²)	96.1 (36.3)	87.5 (17.4)	0.55	94.7 (28.4)	110 (54.3)	0.44

BP = blood pressure, MAP = mean arterial pressure, IOP – iontophoresis, ACh, acetylcholine, SNP, Sodium nitroprusside, AF – autofluorescence, aPWV = aortic pulse wave velocity, LVMI – left ventricular mass index,

2.3.5 Safety data

Three patients in each group experienced episodes of gout that resolved with paracetamol alone. The highest serum 25 (OH) D concentration recorded was 174 nmol/L which itself was not associated with concomitant hypercalcaemia. There were no recorded episodes of hypercalcaemia during the study (defined as a serum calcium > 2.6 mmol/L). The study drugs (ergocalciferol and placebo) were well tolerated and had no reported side effects.

2.4 Discussion

This study has demonstrated that endothelial function is impaired in patients with stage 3-4 CKD and concomitant VDD compared to healthy volunteers. Skin AF, aPWV and mean blood pressure were all higher in patients with CKD compared to healthy controls. Relative change from baseline flux measured by LDF after iontophoresis with ACh was numerically but not significantly higher in the healthy controls compared to patients with CKD. This finding has been previously reported by Cupisti *et al.*²²⁴ who demonstrated that maximal LDF response after iontophoresis with ACh and SNP was similar between healthy volunteers and patients with advanced CKD (median creatinine clearance 12 mL/min) but lower in patients in with essential hypertension who did not have CKD. This suggests that arterial hypertension rather than CKD may be a stronger risk for endothelial dysfunction.

The principal findings from the randomized controlled trial are that ergocalciferol therapy over 6 months in patients with CKD and concomitant VDD was associated with improved endothelium dependent microcirculatory function and that measures of tissue oxidative stress increased in the placebo group but did not change from baseline in the ergocalciferol group after 6 months of therapy. The increase in relative change of flux

from baseline after iontophoresis with ACh but not SNP in subjects treated with ergocalciferol indicates that improved microcirculatory function occurred through an endothelium dependent mechanism. The enhanced microcirculatory vasodilatation may explain the reduction in pulse pressure, a predictor of future CV events²⁶³, as a result of lower total systemic vascular resistance in peripheral microcirculatory beds. aPWV did not decrease in line with the reduction in pulse pressure and this finding may reflect the short duration of the study. Studies with a longer follow up duration are more likely to demonstrate a fall in PWV which may occur after a reduction in pulse pressure.

Skin AF, which is associated with reduced oxidative stress, was increased at 6 months in the placebo group but unchanged in the ergocalciferol group. Lower levels of oxidative stress lead to improved endothelial function and a reduced risk of future CV events²⁶¹. This is in line with previous experimental work that demonstrated the protective effect of calcitriol in human endothelial cells cultured in the presence of AGE products²³⁷⁻²³⁸.

Bone mineral parameters, kidney function, CRP, blood pressure and aPWV were similar at 6 month follow up suggesting that functional changes to the microcirculation occurred independently of these parameters and specifically, occurred independently of changes in large conduit arteries. After 6 months of therapy with ergocalciferol, there was no increase in the FCD or MFI within the sublingual microcirculation in either group. This implies that the observed improvements in endothelium dependent microcirculatory function in the ergocalciferol group did not involve the recruitment of extra functionally relevant capillaries or changes in blood flow but rather that the endothelium dependent function of the existing microcirculatory network was improved

by the direct effect of ergocalciferol. These findings suggest that ergocalciferol may have a specific mechanism of action within the microcirculation.

The present study is the first of its kind to explore the effect of vitamin D on microcirculatory function in patients with CKD and concomitant VDD. The exclusion of patients with DM allowed us to evaluate the effect of ergocalciferol on the microcirculation in CKD without the potentially confounding effect of DM on endothelial function. Given that the CKD patients in the present study were normotensive at baseline and that this level of blood pressure control was maintained throughout the study (as evidenced by similar measure of blood pressure at enrolment and at the end of study, combined with minimal changes in hypertensive medications), we have been able to assess the effect of ergocalciferol itself on endothelial function in CKD without the potential confounding effect of hypertension.

Prospective studies of the effect of nutritional vitamin D in patients with CKD have so far failed to show a beneficial effect of vitamin D on endpoints including LVMI, aPWV, blood pressure and inflammatory markers^{31 264}. Despite the prompt and sustained rise in serum 25 (OH) D concentrations, significant differences in key microcirculatory endpoints were only observed after 6 months of therapy with ergocalciferol. Previous clinical studies using high dose ergocalciferol or cholecalciferol in healthy and diabetic patients without significant kidney disease demonstrated improved microcirculatory function between 8-12 weeks¹⁸⁹⁻¹⁹¹. The delay in attainment of significantly improved endothelial function in the current study and in the study by Marckmann *et al.*¹²⁰, both of which achieved significant and rapid elevations in serum 25 (OH) D concentrations, may be a direct consequence of the uraemic milieu. Experimental cellular models of kidney disease demonstrated reduced expression of eNOS^{75 265 266} and increased

consumption of NO²⁰³. Therefore, the beneficial biological effects of vitamin D on the endothelium may require more time to overcome the endothelial dysfunction induced by uraemia.

In contrast to other studies^{120 133 267-268}, proteinuria and PTH in this study did not significantly reduce in the ergocalciferol group although there was a trend to an increase in PTH in the placebo group. The differences in vitamin D compounds, dose schedule, study duration and populations between those studies and ours may explain this variance. Similar to the studies by Thadani *et al.*³¹ and Wang *et al.*³² which evaluated the effect of paricalcitol on LVM in CKD, LVMI did not change between treatment groups in the study in this thesis. Of note, LVMI remained stable in the CKD group but increased in the placebo group but neither of these differences reached statistical significance which may reflect the small numbers of subjects undergoing cMRI. The enhanced peripheral microcirculatory vasodilatation and concomitant reduction in pulse pressure may explain these findings since the LV will theoretically work against a reduced after load if the peripheral vascular resistance is reduced.

2.4.1 Summary and conclusions

In patients with CKD stage 3-4 and concomitant vitamin D deficiency, high dose ergocalciferol therapy over 6 months improved microcirculatory endothelial function and maintained tissue oxidative stress at baseline levels compared to a significant increase in tissue oxidative stress levels observed in the placebo group. Ergocalciferol was well tolerated and resulted in no significant adverse side-effects. The primary endpoint of the study reflects global vascular health¹⁵⁵ and it is therefore logical to consider that the observed improvements in microcirculatory endothelial function will translate into improved clinical outcomes including reduction in CV events. To test this hypothesis,

studies in patients with CKD and concomitant VDD with longer follow up and adequately powered to detect CV end points are now required.

CHAPTER 3

**THE EFFECT OF ERGOCALCIFEROL ON ENDOTHELIAL
FUNCTION IN AN *IN VITRO* AND *IN VIVO* EXPERIMENTAL
MODEL**

3 Introduction

Vitamin D compounds, both nutritional and activated have been shown to improve endothelial function in a range of *in vitro*, *in vivo* and clinical experiments. The mechanism of action of vitamin D on endothelial cells is mediated by both genomic and non-genomic pathways. In cellular experiments by Talmor *et al.*²³⁷⁻²³⁸ and Zitman-Gal *et al.*²⁶⁹, calcitriol increased the expression of eNOS and reduced the expression of IL-6 when cultured endothelial cells were exposed to culture media containing variously, AGE products, hypocalcaemia and elevated PTH. The mechanism by which this occurred was shown to be through the effects of vitamin D on altering intracellular gene expression. Wu-Wong *et al.*¹⁷⁹ and Zitman-Gal *et al.*²⁶⁹ have demonstrated significant alterations in gene expression in coronary VSMC and human endothelial cells exposed to VDRA using RT-PCR and microarray analysis. However, these studies did not evaluate the effect of VDRA on gene expression in a uraemic cellular milieu. Experiments by Dong *et al.*²⁷⁰ in non-uraemic, oestrogen deficient rats (induced by ovariectomy) demonstrated that calcitriol downregulated expression of cyclooxygenase-2 (COX-2) and the thromboxane prostanoid receptor as well as improving endothelial vasodilator function in rat renal arteries. While not evaluating the effect of calcitriol in a model of uraemia, this study provides additional evidence for the effect of calcitriol at a cellular level through the modulation of gene expression.

In vivo studies that have evaluated vitamin D compounds in experimental uraemia have principally used models of advanced uraemia rather than models of the earlier stages of CKD. At the time of experimental design, the effect of nutritional vitamin D in the form of cholecalciferol on endothelial function had been evaluated in SHR¹⁸⁰ but there were no studies examining the effect of ergocalciferol on endothelial function in experimental

uraemia. Therefore, the effect of ergocalciferol on endothelial function in experimental models of uraemia, and particularly mild uraemia reflecting the earlier stages of CKD, remains unclear.

Refining the use of ergocalciferol in CKD and concomitant VDD requires a better understanding of the effect and site of action of ergocalciferol within the endothelium. Given that vitamin D compounds have a range of pleiotropic effects⁹, there is no pecuniary reason for each vitamin D compound to act on similar cellular pathways or to generate the same magnitude of effect. This is supported by the work of Dong *et al.*²⁷⁰, Borges *et al.*¹⁸⁰⁻¹⁸¹ and Wong *et al.*¹⁸⁵ which has alluded to potential differential mechanistic effects of both nutritional and activated vitamin D compounds on endothelial cellular responses, albeit in a variety of experimental models. Specifically, studies in spontaneously hypertensive rats (SHR) and 5/6th nephrectomised animals using both nutritional and VDRA compounds have identified that the mechanism by which these compounds act on the endothelium is multi-faceted and involves changes both in vasodilatory and vasoconstrictor mechanisms^{180-181 239}.

The clinical trial reported in chapter two of this thesis demonstrated that in CKD stage 3-4 and concomitant VDD, treatment with ergocalciferol improved microcirculatory endothelial function in the absence of changes in the structural conformation of the microcirculation, conduit artery function, blood pressure and traditional markers of bone mineral metabolism. These findings suggest that ergocalciferol has a direct effect on the endothelium in a clinical model of mild uraemia, however, the mechanism of this effect remains unclear.

At the time of experimental design, studies investigating the effect of ergocalciferol on endothelial function in uraemia using *in vitro* and *in vivo* models were absent from the scientific literature. Experimental data for the effect of ergocalciferol on endothelial function in uraemia has the potential to provide support for the hypothesis generated in the clinical trial reported in chapter two that ergocalciferol has a direct effect on the endothelium and its function..

The experiments described in this chapter are designed to assess the cellular and functional response of the endothelium after therapy with ergocalciferol in cultured human endothelial cells and in an animal model of mild uraemia. These experiments have a specific focus on the effect of ergocalciferol on eNOS expression and function as well as the effect of ergocalciferol on the functional response of the endothelium.

3.1 Hypothesis

Ergocalciferol upregulates eNOS expression and nitrite production in cultured human endothelial cells and improves endothelial function in an experimental model of uraemia.

Cell culture experiments include an assessment of the effect of ergocalciferol on eNOS expression and nitrite production compared to the effect of $1,25(\text{OH})_2\text{D}_3$. The effect of ergocalciferol on endothelial function in an *in vivo* model of mild uraemia, to evaluate potential mechanistic pathways of ergocalciferol on vasodilatory and vasoconstrictor aspects of endothelial function, was evaluated.

3.2 Methods

3.2.1 Cell culture technique

All reagents were obtained from Sigma Aldrich, Gillingham, UK unless stated. Cell cultures were performed in a MARS ScanLaf Cabinet. Human aortic endothelial cells (HAEC, Promocell, UK), were cultured and passaged on 75 cm² cell culture flasks using Endothelial Cell Growth Medium MV (Promocell) which when prepared contained endothelial cell growth supplement/heparin (ECGS/H) 0.4 %, Fetal Calf Serum 2 %, Epidermal Growth Factor 0.1 ng/mL, Hydrocortisone 1 µg/mL and basic Fibroblast Factor 1 ng/mL. Cells were used within 6 passages. Antibiotics were added to the cell culture medium as follows: 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. The cells were cultured in a humidified incubator (95% air and 5% CO₂) at 37 °C. When 90% confluence was achieved, cells were placed into suspension using 4 mL of trypsin solution and centrifuged at 800 g for 5 min. The cell pellet was resuspended in 1 mL of culture media and then added to 42 mL of cell culture media before being gently inverted 8-10 times. 1 mL of this suspension was added to each of 42 cell culture wells (7 x 6 well cell culture plates (Costar, London, UK). Cells were cultured in these wells until 90-95% confluent assessed visually by light microscopy. Culture media was replaced every 24 h.

Cell viability was assessed with Trypan blue staining. Cells from 1 well from each of 7, 42 well plates was selected at random. When HAEC had reached the desired confluence, cells were gently scraped from each of the selected wells and briefly centrifuges for 5 min at 800 g. The cells were resuspended in fresh culture medium and 25 µL of the suspension were added to 25 µL of 0.4% Trypan blue (Invitrogen, UK). From this suspension, 10 µL was transferred onto a standard counting

haemocytometer slide. Viable (non-stained) cells were counted and the mean percentage of viable and non-viable cells in all 7 wells was calculated.

When approximately 90-95% confluence of cells was achieved, experimental compounds were added to the cultured cells in the cell media. Vitamin D compounds in the form of ergocalciferol and 1,25 (OH)₂ D₃ were diluted in ethanol to the appropriate concentration as per the manufacturer's instructions. Two control experiments were conducted synchronously by the addition of culture media alone and culture media with ethanol, the diluent for the vitamin D compounds, each added at the same volume as the vitamin D compounds to the cell media (10 µL for final dilutions).

Two experimental arms were conducted using either high (300 nmol/L ergocalciferol and 104 pmol/L 1,25 (OH)₂ D₃) or low (30 nmol/L ergocalciferol and 52 pmol/L 1,25 (OH)₂ D₃) concentrations of vitamin D compounds. Incubation with 1,25 (OH)₂ D₃ was undertaken to evaluate the comparative effect on HAEC of an active vitamin D metabolite. Experiments were repeated in triplicate.

Cells were incubated with experimental compounds and experiments were terminated immediately and at 24 h. Twenty four hours after the addition of vitamin D compounds, the cells were lifted using a cell scraper and the resulting cell suspension was aspirated and centrifuged at 1000 g for 5 min in a pre-cooled centrifuge at 4⁰C. The supernatant was aspirated and stored at -20⁰C and the cell pellet immediately frozen at -80⁰ C.

3.2.2 Western blotting of human aortic endothelial cells lysates for endothelial nitric oxide synthase expression

The protein content of cultured HAEC was ascertained using a bicinchoninic acid (BCA) Protein Assay Reagent. HAEC pellets were lysed using 125 μ L of commercial lysate buffer. Standard concentrations of bovine serum albumin were assayed with protein from lysed HAEC according to the instructions by the manufacturer (Thermo Scientific, Slough, UK).

A volume of 30 μ L of cell lysates were denatured after adding 10 μ L of sample buffer (NuPage™, LDS sample buffer, Life Sciences, UK) and heated to 99 °C for 5 min. A 4-12% gradient gel (NuPage™, Tris Bis Precast gel, Invitrogen) was prepared by rinsing the gel in distilled water (dH₂O) and the gel wells with 3 washes of running buffer (NuPage™ MOPS SDS Running Buffer, Invitrogen). The electrophoresis block was assembled and filled with running buffer. A volume of 25 μ L of each protein sample was added to each well in the membrane and 7 μ L of the molecular weight marker (Santa Cruz, UK) was added to well 1. The electrophoresis conditions were 200 V for 1 h 40 min.

The transfer membrane was correctly sized and immersed in 100% methanol for 30 s before rinsing with water for 20 s. The membrane, filter paper and blotting pads, were soaked in NuPage™ Transfer Buffer (Invitrogen). The gel cassette was opened and correctly sized filter paper was placed on top of the gel. The transfer membrane was placed on the gel and a further piece of filter paper was placed on the other side of the gel. The gel was encased in two blotting pads and inserted into the electrophoresis block which was filled with transfer buffer. The outer chamber of the electrophoresis

block was filled with water. The block was attached to a power supply of 30 V for 1 h. The membrane was removed and washed in 20 mL Tris Buffered Saline containing Tween (TBST) for 10 s before the addition of 20 mL of commercial blocking buffer (Startblock, Perbio Sciences, Northumberland, UK). The membrane was agitated in this solution for 30 min. The solution was drained and the membrane agitated in 20 μ L of anti-eNOS antibody diluted to a concentration of 1:1000 in commercial blocking buffer for 1 h. Membrane stripping between the addition of antibodies was achieved by washing the membrane for 15 min in Western blot stripping buffer (Thermo Scientific) followed by 3 washes of 5 min in TBST. Assessment for anti- β actin antibody utilized the same process described above with a 1:4000 dilution of anti- β actin antibody. The membrane was washed a further three times for 5 min in TBST before the addition of goat anti-rabbit antibody (Santa Cruz) bound to horse radish peroxidase at a dilution of 1:2000 for 1 h. A further three washes of 5 min using TBST were performed. Bands were visualized using EZ-ECL Chemiluminescent Reagent (Perbio Sciences) on ECL X-ray film.

3.2.3 Western blotting for phospho-endothelial nitric oxide synthase activity

To establish the effect of vitamin D compounds on the phosphorylation of eNOS, HAEC were lysed with a modified buffer. Freshly made buffer contained 5 mL dH₂O to which the following compounds were added: 100 μ L of 1M Tris, 500 μ L of 1 M NaCl, 3 mL of 0.1 M sodium pyrophosphate (NaPPi) , 100 μ L of 0.2M ethylenediaminetetraacetic acid (EDTA), 1ml of 0.5 M sodium fluoride (NaF) , 100 μ L of 1% Triton X-100, 100 μ L of 0.1 M phenylmethylsulfonyl fluoride (PMSF) , 50 μ L 0.2 M sodium orthovanadate (Na₃VO₄) and 10 μ L of a 1 mg/mL protease inhibitor containing benzamidine, antipain, leupeptin and aprotinin. To ensure maximal cell lysis, the cell pellet and 125 μ L of lysis buffer were repeatedly agitated in an Ependorff vial before freezing at -80⁰C for 10 min.

Western blotting was performed as described previously (section 3.2.2) using 15 μ L of cell lysate. In addition, the membrane was incubated with a 1:1000 concentration of rabbit anti-phospho-eNOS antibody (Ser 1177, Cell Signalling Technology, New England Biolabs, Hertfordshire, UK).

3.2.4 Real time polymerase chain reaction for endothelial nitric oxide synthase gene expression

To establish the fold increase of eNOS expression compared to a control of β actin gene expression, real time polymerase chain reaction (RT-PCR) on cell lysates was performed. Messenger RNA (mRNA) was extracted from cell lysates using commercially available kits (QIAGEN RNeasy mini kit, UK) according to the manufacturer's instructions.

The concentration of mRNA was determined by measuring absorption at 260 nm and assessed for quality by determining the ratio of absorbance between 260 nm and 280 nm (Biotech Photometer, UK). A volume of 0.75 μ g of mRNA was added to 1.2 μ L of Oligo DT¹²⁻¹⁸ primer (0.5 μ g/ μ L)) and 1.2 μ L of 100 nM 2'-deoxynucleoside 5'-triphosphate (dNTP) (all from Invitrogen). Genetic grade water (Purelab Ultra ELGA, Marlow, UK) was added to a total volume of 14 μ L. This mixture was heated to 65^oC for 5 min and then immediately chilled on ice. The contents were then briefly centrifuged before the addition of 4.7 μ L of 5x first standard buffer, 2.3 μ L of 0.1M dithiothreitol (DTT) and 1 μ L superscript reverse transcriptase (all from Invitrogen). The mixture was briefly agitated before incubation at 42^oC for 50 min and then inactivation at 70^oC for 15 min.

A volume of 2 μL of the resulting cDNA mixture was added to 0.4 μL of gene mix (either eNOS (assay ID: Hs01574659_m1) or β actin (assay ID: Hs99999903_m1) Taqman gene expression assay (Applied Biosystems, Warrington, UK)), 4 μL of Master mix (Thermo Scientific) and 3.6 μL of dH_2O to a total volume of 10 μL . TaqMan probes were the reporter probe FAM and the quencher probe VIC. The mixture was centrifuged briefly before RT-PCR was performed on the Applied Biosystems 7900HT Fast Real-Time PCR System. The RT-PCR conditions were one 2 min cycle (50 °C), denaturation for 10 min (95 °C) followed by 40 cycles of denaturation (95 °C for 15 s). Annealing and extension were performed as a single step (60 °C for 1 min). Threshold cycles were determined for eNOS and β actin genes. Data were analyzed with Applied Biosystems (ABI) 7900HT Prism sequence detector software (sequence detection software (SDS) Version 2.3, ABI).

3.2.5 Measurement of cell supernatant nitrite concentrations

Nitrite itself is now considered to be biologically active and a reservoir for $\text{NO}^{150\ 271}$ (section 1.3.1). Nitrite is converted to NO at the haem site of eNOS and therefore, increased cellular expression of eNOS in the presence of nitrite will lead to an increase in NO production, in turn leading to improved endothelial function¹⁵⁰. Nitrite concentrations in supernatant from HAEC cultures were analysed by Dr Alex Milsom using a chemiluminescent technique²⁷². Briefly, the concentrations of nitrite and nitrate were calculated by adding cell supernatant samples and standards to 0.1 M vanadium chloride in 1 M hydrochloric acid refluxing at 90 °C under nitrogen. Nitrite concentrations were then measured using a chemiluminescent technique after the addition of samples to 1.5% potassium iodide in glacial acetic acid under nitrogen at room temperature.

3.2.6 The effect of ergocalciferol in a rat model of mild uraemia

3.2.6.1 Preparation of rat aortic ring tissue for experimental procedures

All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, UK. Mr Julius Kiswich performed animal husbandry, surgery, administration of ergocalciferol or vehicle and, together with Dr T Andrews, animal sacrifice. Dr T Andrews assisted with the preparation of aortic ring tissue. I designed and conducted experimental procedures and performed the data analysis.

Male Wistar rats (approximately 200-250g, Charles River, UK) were fed on a standard rodent diet containing 18.7% protein, 1.0% Ca^{2+} , 0.7% NaCl and 3.6 IU cholecalciferol/g (Lillico Biotechnology, UK) with free access to water. Animals were subjected to a 5/6th nephrectomy (SNx) for the induction of uraemia in a two stage procedure. This model has been shown to develop endothelial dysfunction²¹⁶.

3.2.6.2 Anaesthesia and surgical technique

In the first stage, animals were anaesthetised by an intra-peritoneal injection consisting of 100 mg/mL of ketamine hydrochloride, and 23.3 mg/mL of xylazine hydrochloride made up in a 2:1 ratio (ketamine: xylazine) at a dose of 1.5 mL/kg. The left flank of the animal was shaved and an incision made. The left kidney was exposed, isolated and de-capsulated using small blunt forceps.

Two thirds of the left kidney mass was removed after a flank incision. Upon cessation of bleeding, the renal remnant was replaced in its original anatomical position. Isotonic saline (2 mL) was introduced into the peritoneum to replace fluid lost during surgery. Analgesia was provided pre and post-operatively with Vertegesic, 0.03 mL

(buprenorphine, 0.342 mg/mL, (Alstoe Animal Health, York, UK). In the second stage of the renal mass reduction procedure which followed 10 days later, animals were prepared in the same way and the right kidney was completely removed. Control animals underwent the same procedure without removal of the renal mass however at both stages the renal capsule was removed.

3.2.6.3 Therapeutic intervention with ergocalciferol

Animals received either ergocalciferol 1,000 IU (UCB Pharma, UK), or vehicle (ethyl oleate) by oral gavage at -7 and -2 days before sacrifice. Mr Kiswich designed the randomization protocol for the dosing of either ergocalciferol or vehicle. All other members of the study team were blinded to the allocation. Unblinding was only performed after completion of all study procedures and data collection. This created four experimental groups – sham animals (n=12), of whom half received ergocalciferol and half vehicle and SNx rats (n=12) of whom half received ergocalciferol and half vehicle.

3.2.6.4 Measurement of blood pressure

Arterial blood pressure was measured in separate experiments involving the same four experimental groups, study compound dosing and duration of uraemia. On the day of sacrifice, after anaesthesia, a tracheostomy was performed; and an arterial catheter to monitor pulse and blood pressure was inserted into the right carotid artery. MAP was measured by attaching the catheter to a pressure transducer (Harvard Apparatus, Kent, UK). Blood pressure was recorded for at least 20 min until a stable value was obtained.

3.2.6.5 Sacrifice of animals and preparation of aortic rings

Four weeks following the final stage of the SNx surgery, the animals not used for blood pressure measurement were sacrificed by a guillotine to the neck. The thoracic and abdominal aorta was removed and immediately immersed in a bath of standard Kreb's solution which at x10 strength contained NaCl 69 g/L, KCl 3.5 g/L, MgSO₄·7H₂O 2.9 g/L, KH₂PO₄ 1.6 g/L, NaHCO₃ 21 g/L and D-glucose (anhydrous) 20 g/L. This was diluted to 1/10 strength with dH₂O. 2.5 mL/L of 1 mmol/L CaCl₂ was added to the reconstituted Kreb's solution. Aortic tissue was cleaned of all non-vascular tissue and individual ring segments of approximately 7 mm were prepared. Aortic rings were suspended in Kreb's Ringer's solution containing 2.5 mL/L of 1 mmol/L CaCl₂ in a 10 mL organ bath apparatus which was heated to 37 °C (Organ bath warmer c-85D, Jencons PLS, UK). The aortic rings were suspended on appropriate wires and connected to Lab Chart Reader (ADInstruments, Oxford, UK) by an ADInstruments octabridge at 1 g of resting tension. Carbogen (5% CO₂, 95% O₂) was perfused through the Kreb's solution.

3.2.7 Experimental procedures performed on isolated rat aortic rings

3.2.7.1 Preparation of experimental compounds

All compounds were prepared with fresh components on the day of experimentation. Experimental compounds were initially made up to a 10⁻² M concentration as stock solutions. Specific concentrations in the organ baths were obtained after adding an initial and then cumulative dose of each experimental compound to achieve a log increase in concentration of the compound. Phenylephrine (PE), ACh and Spermine NONOate (SpNO, Cayman Chemical, Cambridge Biosciences, Cambridge, UK) were diluted with 0.9% NaCl. U-46619 (Enzo Life Sciences, Exeter, UK), was dissolved in ethanol and then diluted in 0.9% NaCl.

3.2.7.2 Tissue preparation

Tissue preparation and assessment is shown in Figure 15. Prior to stimulation and experimental procedures, aortic rings were maintained at 1 g of basal resting tension. To ensure reproducibility of results and viability of tissue, aortic rings were pre-contracted with 48 mmol/L KCl on 2 occasions separated by a period of 15 min during which aortic rings were washed with 3 rinses of Krebs solution at 5 minute intervals. Preservation of endothelial function was assessed by contraction of aortic rings with 10 μ M PE and then sequential doses of ACh (1 μ mol/L and 10 μ mol/L). Endothelial function was considered intact if aortic rings demonstrated > 50% relaxation from the contractile state induced by administration of PE.

3.2.7.3 Experimental procedures

In order to determine the potential site of action and magnitude of effect of ergocalciferol on the vasodilatory and vasoconstrictor response of the endothelium, experimental procedures were conducted to assess the pharmacological response of aortic tissue after the addition of compounds known to modify both responses in endothelial tissue. The effect of these compounds was assessed in the 4 experimental groups previously described (section 3.2.6.3). Prior to the addition of these compounds, aortic rings were pre-contracted with PE at a concentration of 10 μ M to achieve a contractile tension of 90% of the response of the second dose of 48 mmol/L KCl in the tissue preparation stage. Cumulative dose response curves were generated for ACh, SpNO, PE, and U-46619 (a thromboxane receptor agonist).

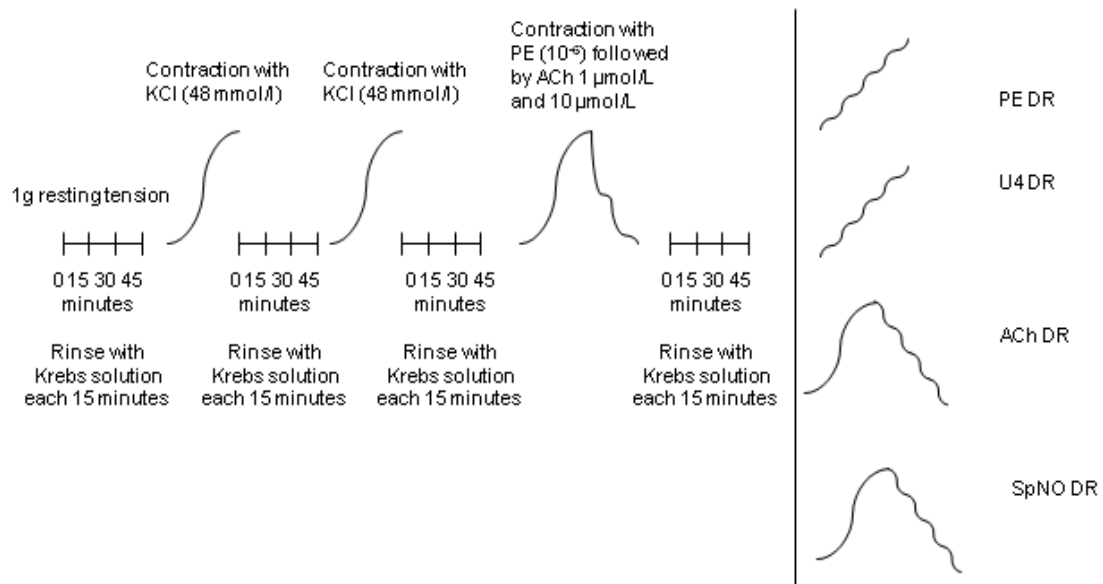


Figure 15 Tissue preparation (before long vertical line) and experimental procedures (after long vertical line). Short vertical hatch lines represent double rinse of aortic ring with Kreb's solution. Experimental procedures were conducted on individual aortic rings after each ring had undergone tissue preparation procedures. Each ring was subjected to one experimental compound only. The number of aortic rings for each experiment is described in Table 11. DR = dose response. PE = phenylephrine, ACh = acetylcholine, SpNO = spermine NONOate, U-4 = U-46619

The vasodilatory endothelial function of aortic rings was assessed by the cumulative addition of ACh (endothelium dependent vasodilator) and an endothelium independent NO donor, SpNO. Endothelial contractile response was assessed by the addition of PE and U-46619. The range of doses used to generate dose response curves was as follows: ACh 0.001 $\mu\text{mol/L}$ – 30 $\mu\text{mol/L}$, SpNO 0.001 $\mu\text{mol/L}$ – 30 $\mu\text{mol/L}$, PE (pre and post nifedipine) 0.001 $\mu\text{mol/L}$ – 30 $\mu\text{mol/L}$, U-46619 0.001 $\mu\text{mol/L}$ – 0.3 $\mu\text{mol/L}$. Experimental procedures are shown in Figure 15.

3.2.8 Measurement of biochemical parameters

Blood was collected on day 0 (sacrifice) and assayed by an automated analyser (IDEXX Laboratories Ltd, Horsham, West Sussex, UK) for urea, creatinine, Ca^{2+} and PO_4 . Serum PTH concentrations were assayed by an ELISA kit (ALPCO Diagnostics, Stratech Scientific Ltd, Newmarket, Suffolk, UK). Serum 25 (OH) D concentrations were assessed by UPLC-MS at the Royal London Hospital (see section 2.2.14.6).

3.2.9 Statistical analysis

3.2.9.1 Human aortic endothelial cell culture analysis

RT-PCR data were analyzed with ABI 7900HT Prism sequence detector software (SDS Version 2.3, Applied Biosystems) using the $\Delta\Delta\text{CT}$ method to determine the differential fold increase of eNOS compared to β actin. Differences in gene expression and nitrite concentrations were assessed by the Student's t test.

3.2.9.2 Aortic ring analysis

Aortic ring tension is expressed in grams (g). The response of aortic rings to KCl to assess reproducibility of contraction is expressed as change in tension in g above resting baseline. Relaxation of aortic rings induced by ACh or SpNO is expressed as

the percentage relaxation after pre-determined contractile tension was achieved. The 2 way ANOVA with repeated measures test was used to assess differences between dose response curves²⁷³. Continuous data were compared as described in section 2.2.15 or using a one-way ANOVA test for comparison between multiple groups. The effect of agonist response is expressed as the $-\log EC_{50}$ (pEC_{50}) and compared with a Student's t test. Data are presented as mean \pm standard error of the mean (SEM). The "n" refers to the number of aortic rings per experiment. Statistical analysis for HAEC and aortic rings was performed using GraphPad Prism software (version 5). A p value of < 0.05 was considered to represent statistical significance.

3.3 Results

3.3.1 Real time polymerase chain reaction results for the effect of ergocalciferol on endothelial nitric oxide synthase gene expression

Cell viability assessed by Trypan blue staining indicated that the mean percentage of non-viable cells from 7 separate wells of cultured HAEC was 7.7%. Compared to the control experiment, there was a 2.4-fold increase in eNOS expression after 24 h of incubation with ergocalciferol 300 nmol/L ($p=0.001$) compared to a 1.6-fold increase with ergocalciferol at a concentration of 30 nmol/L ($p=0.12$ compared to control). The relative fold increase in gene expression between low and high concentration ergocalciferol was significantly different ($p=0.002$). At both high and low concentrations of $1,25(OH)_2 D_3$ there was a slight reduction in fold increase of eNOS gene expression compared to the control experiment (0.8 fold increase ($p=0.59$) and 0.6-fold increase ($p=0.35$) at high and low concentrations respectively). There was no significant difference between gene expression in the control and ethanol experiments ($p=0.79$). The fold increase in eNOS gene expression was significantly higher with high

concentration ergocalciferol compared to high concentration 1,25 (OH)₂ D₃ (p<0.001) and with low concentration ergocalciferol compared to low concentration 1,25 (OH)₂ D₃ (p=0.004, see Figure 16).

3.3.2 Western blotting for endothelial nitric oxide synthase, phospho-endothelial nitric oxide synthase, and β actin

eNOS protein expression was increased after 24 h incubation with high concentration (300 nmol/L) ergocalciferol compared to high concentration 1,25 (OH)₂ D₃ (104 pmol/L). Incubation with high concentration 1,25 (OH)₂ D₃ resulted in lower eNOS protein expression compared to baseline at time 0 (Figure 17). This is in line with the observation that eNOS gene expression by RT-PCR was upregulated after incubation with ergocalciferol but not 1,25 (OH)₂ D₃. There were no differences in β actin expression in all experimental groups. After incubation with low concentration vitamin D compounds, eNOS protein expression at 24 h compared to baseline was increased after incubation with both ergocalciferol (30 nmol/L) and 1,25 (OH)₂ D₃ (52 pmol/L) (Figure 17). Western blotting for the activated phosphorylated form of eNOS comparing incubation with high and low concentrations of vitamin D compounds at 24 h demonstrated reduced expression of phospho-eNOS with both ergocalciferol and 1,25 (OH)₂ D₃ at high compared to low concentration compounds. Phospho eNOS expression was also reduced in the control and ethanol experiments (Figure 18).

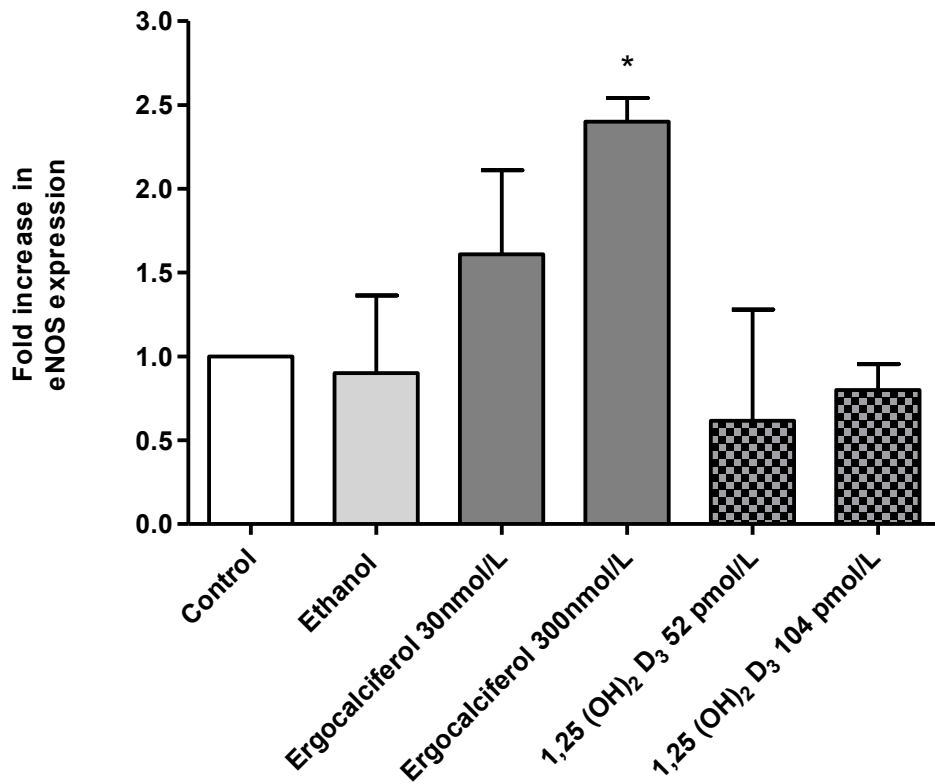
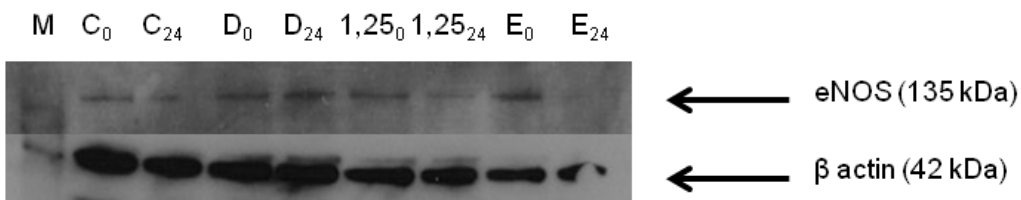


Figure 16 Fold increase in eNOS expression compared to β actin control gene by RT-PCR in cultured HAEC (n=9). Ergocalciferol 300 nmol/L compared to control (p=0.001), ergocalciferol 30 nmol/L compared to control (p=0.12), ergocalciferol 300 nmol/L compared to ergocalciferol 30 nmol/L (p=0.002). 1,25 (OH)₂ D₃ 52 pmol/L and 104 pmol/L compared to control, p=0.35 and p=0.59 respectively. Ergocalciferol 300 nmol/L compared to 1,25 (OH)₂ D₃ 104 pmol/L (p<0.001). Ergocalciferol 30 nmol/L compared to 1,25 (OH)₂ D₃ 52 pmol/L (p=0.004). * = statistically significant compared to control

Panel A – high concentration vitamin D



Panel B – low concentration vitamin D

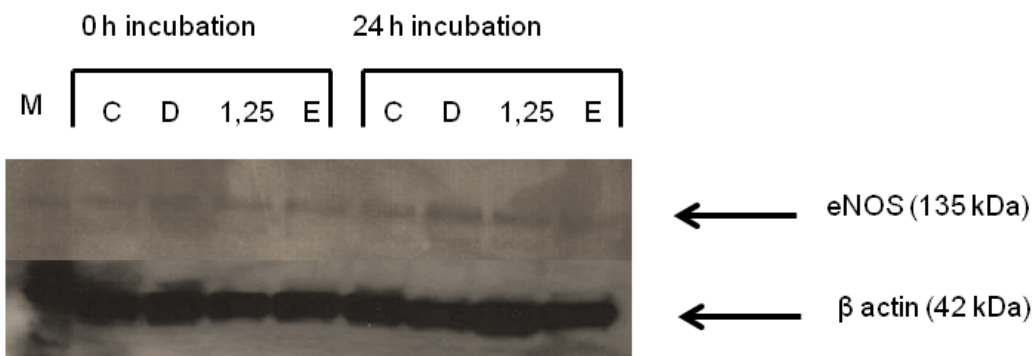


Figure 17 Western blots of eNOS protein expression in cultured human aortic endothelial cells

Panel A - Western blots for eNOS and β actin from HAEC cell lysates incubated with high concentrations of vitamin D compounds. Comparing expression after 0 and 24 h after incubation (ergocalciferol 300 nmol/L, 1,25 (OH)₂ D₃ 104 pmol/L). Key – M=marker for eNOS and β actin antibody. C = control, D= ergocalciferol, 1,25 = 1,25 (OH)₂ D₃, E = ethanol. Therefore D₂₄ refers to cells incubated with ergocalciferol for 24 h and E₀ refers to ethanol at 0 h. kDa = kilodalton

Panel B - Western blots for eNOS and β actin from HAEC cell lysates incubated with low concentrations of vitamin D compounds. Comparing expression after 0 and 24 h after incubation (ergocalciferol 30 nmol/L, 1,25 (OH)₂ D₃ 52 pmol/L). Key – M=marker for eNOS and β actin antibody. C = control, D= ergocalciferol, 1,25 = 1,25 (OH)₂ D₃, E = ethanol. kDa = kilodalton

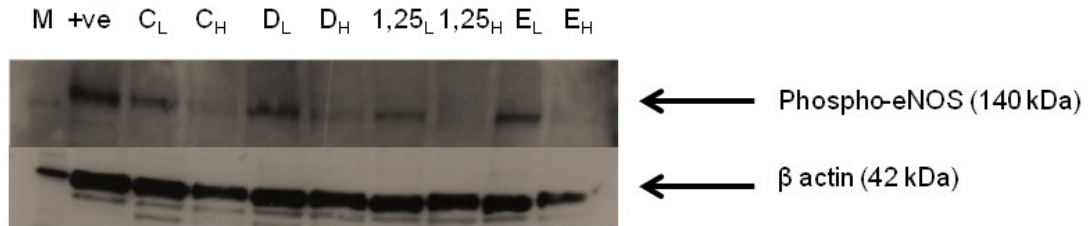


Figure 18 Western blots for phospho-eNOS and β actin using cell lysates from HAEC cultured with high and low concentrations of vitamin D compounds after 24 h incubation. M= marker for phospho eNOS antibody, +ve = Positive control – phospho-eNOS protein from cultured human umbilical vein endothelial cells, C= control experiment, D = ergocalciferol, 1 = 1,25 (OH)₂ D₃, E = ethanol, L = low concentration compounds (30 nmol/L ergocalciferol, 52 pmol/L 1,25 (OH)₂ D₃), H = high concentration compounds (300 nmol/L ergocalciferol, 104 pmol/L 1,25 (OH)₂ D₃). Therefore D_H = cells incubated with high concentration ergocalciferol. kDa = kilodalton

3.3.3 Cell supernatant nitrite concentrations

There was a significant increase in cell supernatant nitrite concentrations after incubation with high compared to low concentration ergocalciferol at 24 h ($p=0.04$, Figure 19). This difference was not observed after incubation with low compared to high concentration $1,25\text{ (OH)}_2\text{ D}_3$ ($p=0.88$). While numerically higher, at 24 h there was no statistically significant increase in nitrite between high concentration ergocalciferol and high concentration $1,25\text{ (OH)}_2\text{ D}_3$ ($p=0.18$, Figure 19).

3.3.4 The effect of ergocalciferol in a 4 week, 5/6th nephrectomy rat model of uraemia - baseline rat physiology and biochemistry

One animal was lost prior to sacrifice in the SNx group. The SNx model effectively induced uraemia. Serum creatinine was higher in SNx compared to sham operated animals ($p<0.0001$) but not different between vehicle and ergocalciferol treated animals in sham ($p=0.18$) or uraemic experimental groups ($p=0.65$) (Figure 20).

Therapeutic intervention with ergocalciferol compared to vehicle increased serum $25\text{ (OH)}\text{ D}$ concentrations in both sham ($44.4 \pm 7.5\text{ nmol/L}$ vs $92.6 \pm 16.8\text{ nmol/L}$ $p=0.0004$) and SNx animals ($53.0 \pm 8.2\text{ nmol/L}$ vs $82.1 \pm 17.4\text{ nmol/L}$, $p=0.005$ Figure 20). There were no differences between serum $25\text{ (OH)}\text{ D}$ concentrations in vehicle ($p=0.12$) or ergocalciferol ($p=0.31$) treated sham and SNx animals. There were no differences between experimental groups in systolic blood pressure (one way ANOVA $p=0.39$) (Figure 21). Two days prior to sacrifice, animal weights differed between sham ($444.4\text{ g SEM} \pm 12.3$) and uraemic rats ($406.3\text{ g SEM} \pm 6.0$) ($p=0.006$) but there were no differences between ergocalciferol and vehicle treated animals in sham ($p=0.17$) and uraemic ($p=0.19$) groups (Figure 21).

Serum calcium (one way ANOVA $p=0.08$), phosphate (one way ANOVA $p=0.64$) and PTH (one way ANOVA $p=0.63$) did not significantly differ between experimental groups (Figure 22).

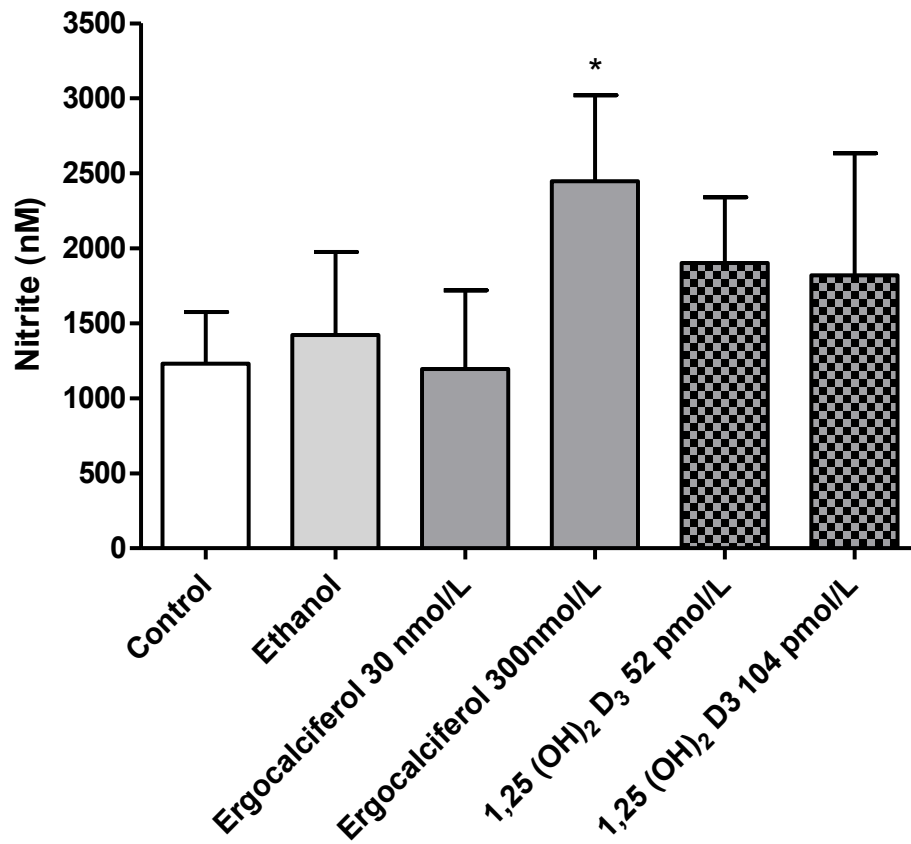


Figure 19 Nitrite concentrations in supernatants of HAEC cultured in high and low concentration vitamin D compounds after 24 h (mean \pm SEM). * = significant compared to ergocalciferol 30 nmol/L (n=9, p=0.04)

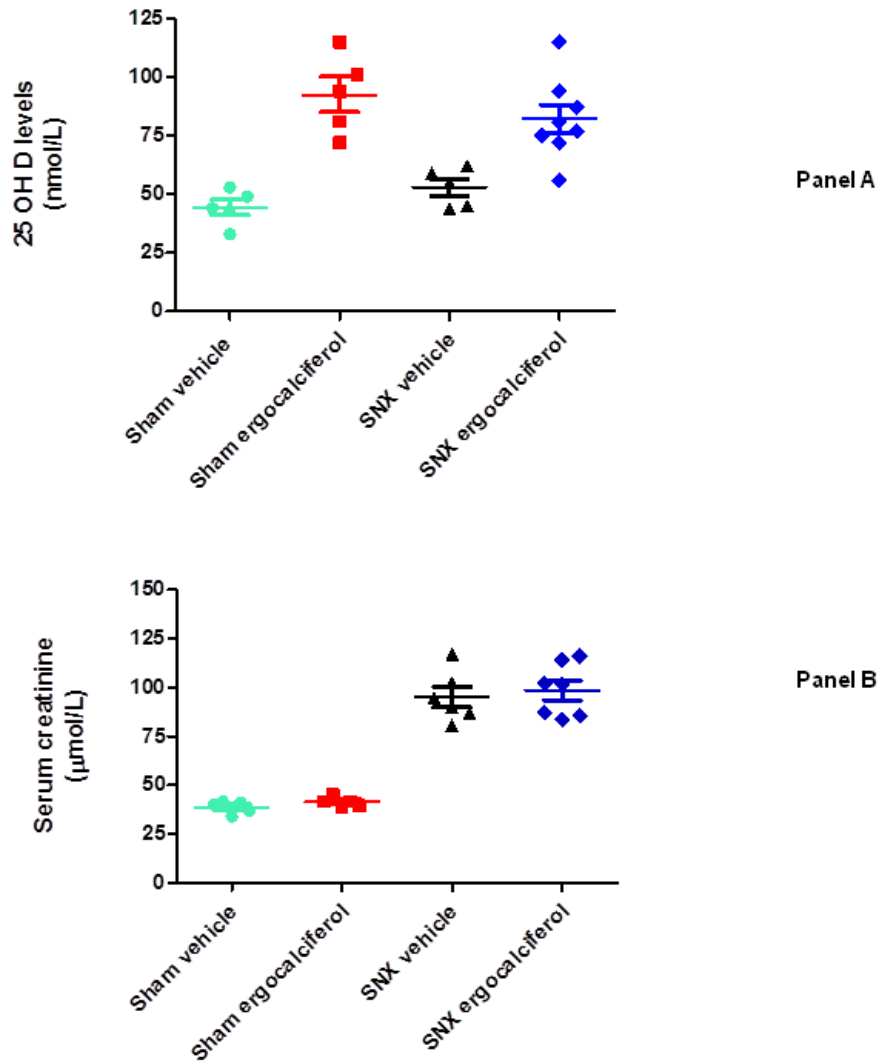


Figure 20 Serum 25 (OH) D (Panel A) and creatinine (Panel B) concentrations at sacrifice.

Panel A - Sham vehicle compared to sham ergocalciferol $p=0.0004$. SNx vehicle compared to SNx ergocalciferol ($p=0.005$). Sham vehicle compared to SNx vehicle ($p=0.12$). Sham ergocalciferol compared to SNx ergocalciferol ($p=0.31$). $n=5-7$

Panel B - Serum creatinine in SNx compared to sham operated animals ($p<0.0001$). Serum creatinine comparing vehicle and ergocalciferol treated sham animals ($p=0.18$). Serum creatinine comparing vehicle and ergocalciferol treated SNx animals ($p=0.65$) SNx = 5/6th nephrectomy model. $n=5-7$

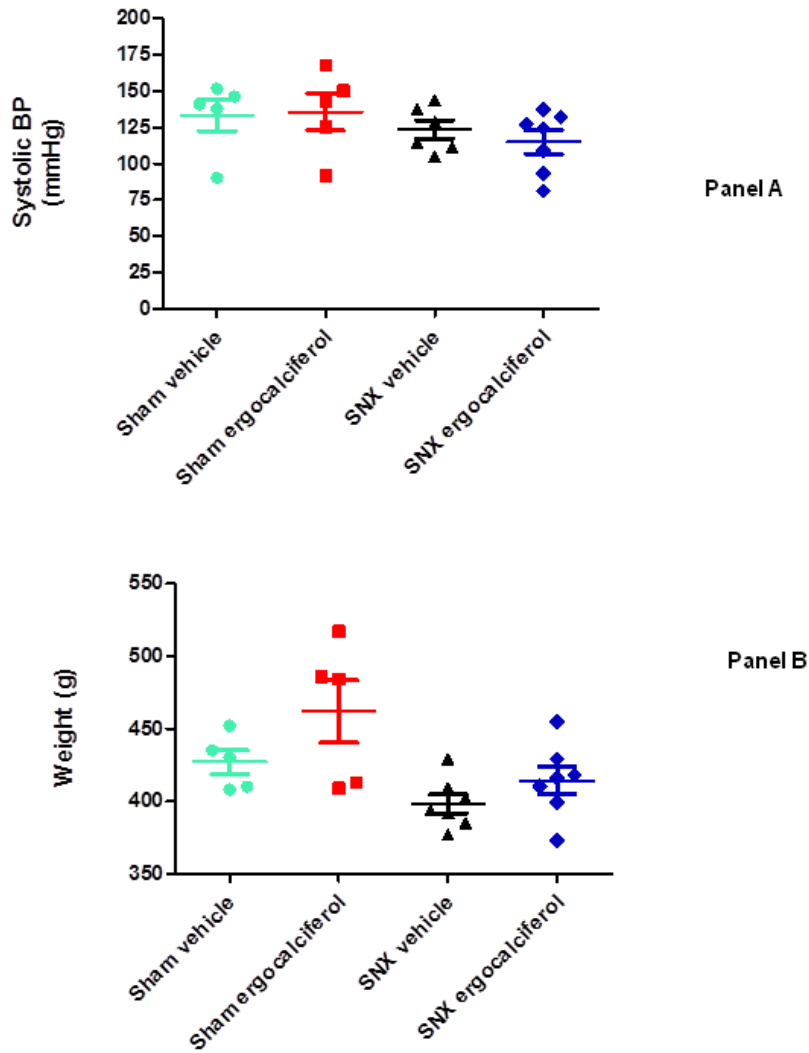


Figure 21 Systolic blood pressure (Panel A) and weight (Panel B) at sacrifice.

Panel A - Systolic blood pressure did not differ between experimental groups (one way ANOVA $p=0.39$). $n=5-7$

Panel B - Weights differed between sham and uraemic rats ($p=0.0006$) but there were no differences between ergocalciferol and vehicle treated animals in sham ($p=0.17$) and SNx ($p=0.19$) groups. $n=5-7$

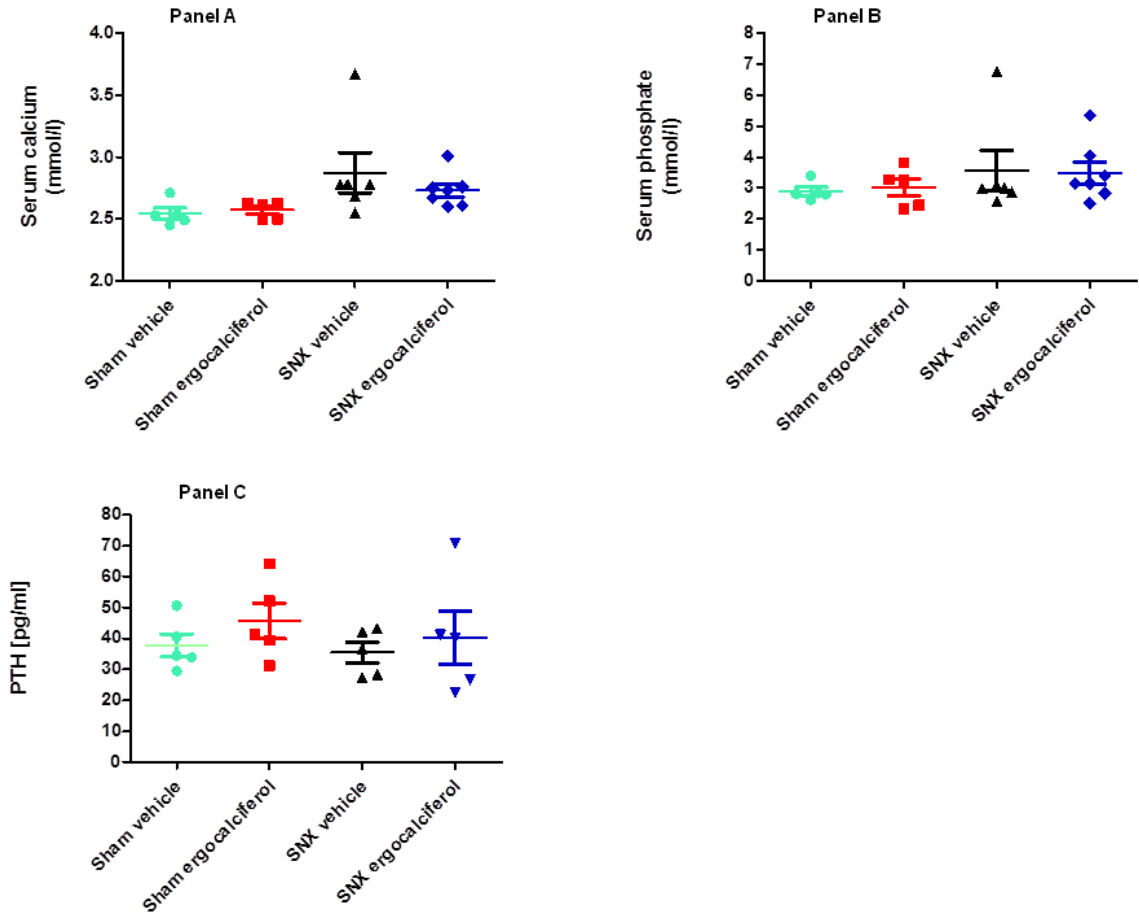


Figure 22 Serum calcium (Panel A), phosphate (Panel B) and PTH (Panel C) concentrations at sacrifice.

Panel A - Serum calcium did not differ between experimental groups (one way ANOVA $p=0.08$). $n=5-7$

Panel B - Serum phosphate did not differ between experimental groups (one way ANOVA $p=0.64$). $n=5-7$

Panel C - Serum PTH did not differ between experimental groups (one way ANOVA $p=0.63$). $n=5-7$

3.3.5 Pre-contraction of aortic rings with 48 mmol/L KCl

There was an equivalent magnitude of contraction above baseline resting tension after the addition of 48 mmol/L KCl in sham animals treated with either vehicle (2.1 ± 0.2 g $n=20$) or ergocalciferol (2.2 ± 0.2 g $n=20$) ($p=0.63$). In uraemic animals, the contraction response to KCl was greater in ergocalciferol (2.4 ± 0.1 g) treated animals compared to vehicle treated animals (1.9 ± 0.1 g) ($p=0.02$).

3.3.6 Endothelial vasodilatory response after the addition of Spermine NONOate and acetylcholine

The addition of SpNO demonstrated that endothelial function was intact and generated almost complete relaxation of aortic tissue after pre contraction with PE. Tissue relaxation did not significantly differ overall between vehicle or ergocalciferol treated animals in either the sham (2 way ANOVA with repeated measures $p=0.33$) or SNx groups (2 way ANOVA with repeated measures $p=0.58$) (Figure 23).

There was a significant overall beneficial effect of ergocalciferol compared to vehicle on endothelial function as evidenced by increased aortic ring vasodilatation after the addition of ACh following pre contraction with PE in both sham (2 way ANOVA with repeated measures $p=0.012$) and SNx animals (2 way ANOVA with repeated measures $p=0.016$) (Figure 23). A summary of the pEC_{50} and maximum achieved aortic ring relaxation is shown in Table 11.

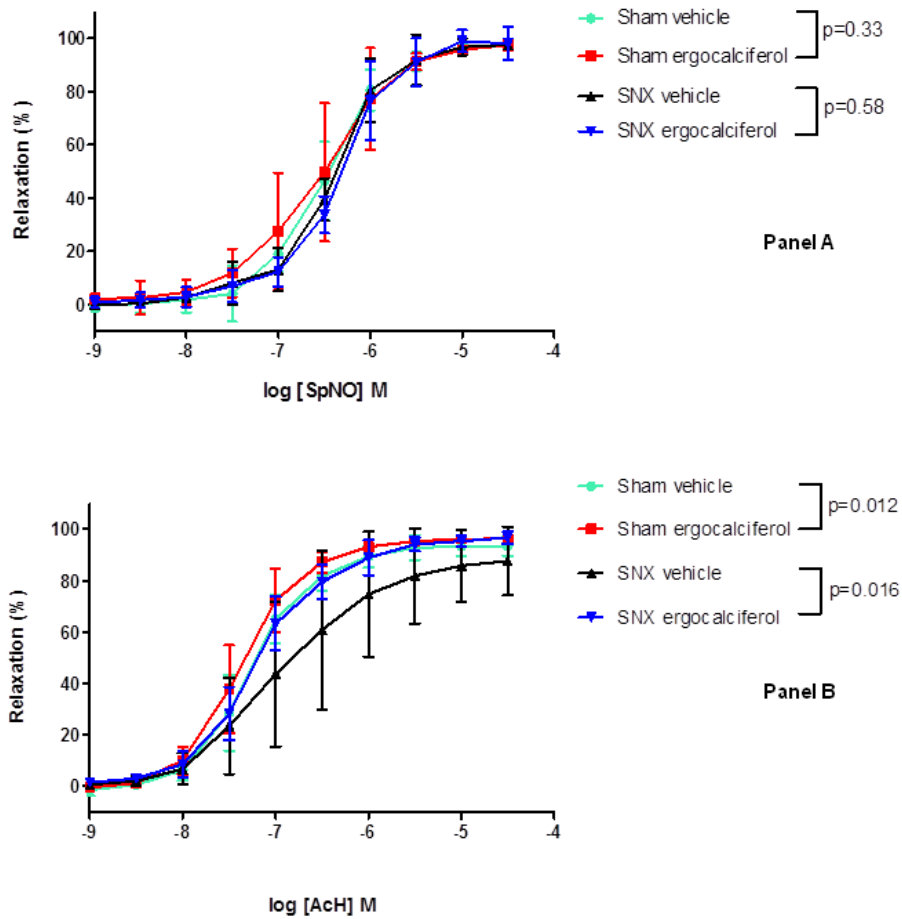


Figure 23 Aortic ring vasodilatory response to SpNO (Panel A) and ACh (Panel B).

Panel A - Tissue relaxation did not significantly differ between vehicle or ergocalciferol treated animals in either the sham (2 way ANOVA with repeated measures $p=0.33$) or SNx groups (2 way ANOVA with repeated measures $p=0.58$). $n=5$ per group

Panel B - Ergocalciferol compared to vehicle improved endothelial relaxation in both sham (2 way ANOVA with repeated measures $p=0.012$) and SNx animals (2 way ANOVA with repeated measures $p=0.016$). $n=5$ per group

Table 11 The effect of ergocalciferol on the response to vasodilators and vasoconstrictors in sham and SNx operated animals.

Treatment	Analysis	Sham				SNx			
		ACh n=5	SpNO n=5	PE n=5	U4 n=5	ACh n=5	SpNO n=5	PE n=5	U4 n=5
Vehicle	pEC ₅₀	7.3 ± 0.06	6.5 ± 0.06	6.8 ± 0.06	6.2 ± 0.2	6.9 ± 0.04	6.4 ± 0.1	6.8 ± 0.07	6.9 ± 0.2
	Max	97.8 ± 2.7%	96.5 ± 2.6 %	4.0 ± 0.07 g	5.3 ± 0.4 g	87.8 ± 1.4%	98.7 ± 5.2%	3.5 ± 0.08 g	6.2 ± 0.5 g
Ergocalciferol	pEC ₅₀	7.4 ±0.07	6.5 ± 0.06	6.7 ± 0.07	6.1 ± 0.2	7.2 ± 0.04	6.2 ± 0.1	6.4 ±0.07	7.0 ± 0.2
	Max	97.9 ± 2.0%	98.3 ± 2.7%	4.3 ± 0.09 g	6.9 ± 0.5 g	95.8 ± 1.3%	96.6 ± 5.4%	4.2 ± 0.08 g [#]	6.2 ± 0.4 g

– significant at p=0.03 compared to corresponding vehicle in the SNx group

ACh – acetylcholine, SpNO = spermine NONOate, PE – phenylephrine, U4 – U-46619, Max – either maximum relaxation as a percentage of relaxation after preconstruction with PE or maximum achieved tension in g. Data are mean ± SEM. pEC₅₀ – negative logarithm of the EC₅₀. n numbers are number of individual aortic ring segments per experiment.

3.3.7 Contractile response of aortic rings to phenylephrine and U-46619

There was no difference in contractile response to PE between vehicle and ergocalciferol treated animals in the sham surgery group (2 way ANOVA with repeated measures $p=0.98$). In the SNx group, ergocalciferol significantly increased overall contractile response to PE compared to vehicle (2 way ANOVA with repeated measures $p<0.0001$) (Figure 24). The maximum achieved tension in g was increased in the SNx group animals treated with ergocalciferol compared to SNx animals treated with vehicle (SNx ergocalciferol $4.2 \pm 0.08g$, SNx vehicle $3.5 \pm 0.08g$, $p=0.03$).

SNx compared to sham operated animals had a significantly different contractile response to U-46619 (2 way ANOVA with repeated measures $p<0.0001$). The pEC_{50} in SNx animals was higher than in sham operated animals (pEC_{50} SNx 6.8 ± 0.3 , pEC_{50} sham 6.2 ± 0.2 , $p<0.0001$). No significant overall differences in contractile response to U-46619 were observed in either sham or SNx animals after treatment with vehicle or ergocalciferol (sham group 2 way ANOVA with repeated measures $p=0.63$, SNx group 2 way ANOVA with repeated measures $p=0.61$) indicating that uraemia rather than ergocalciferol *per se* modulated the response to U=46619 (Figure 24 and Table 11)

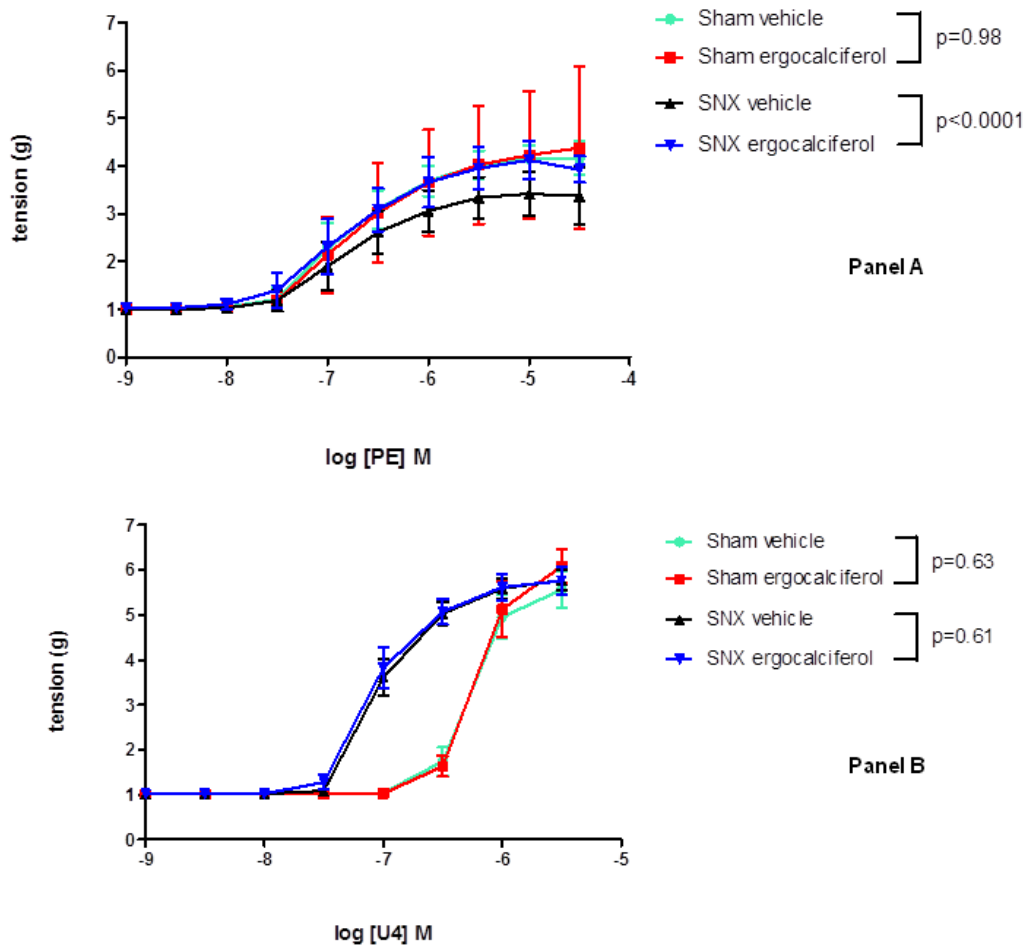


Figure 24 Contractile response to PE (Panel A) and U-46619 (Panel B).

Panel A In the SNx group, ergocalciferol significantly increased overall contractile response to PE compared to vehicle (2 way ANOVA with repeated measures $p < 0.0001$) but there was no difference in contractile response to PE between vehicle and ergocalciferol in the sham group (2 way ANOVA with repeated measures $p = 0.98$). $n = 5$ per group.

Panel B - SNx compared to sham operated animals had a significantly different contractile response to U-46619 (2 way ANOVA with repeated measures $p < 0.0001$). No significant differences in contractile response were observed in either sham or uraemic animals after treatment with vehicle or ergocalciferol (Sham group 2 way ANOVA with repeated measures $p = 0.63$, SNx group 2 way ANOVA with repeated measures $p = 0.61$). $n = 5$ per group.

3.4 Discussion

The principle findings from the experimental work described in this chapter are that in cultured HAEC, eNOS gene expression measured by RT-PCR is increased at high concentrations of ergocalciferol. There was a lower and non-significant increase in expression of eNOS with low concentration ergocalciferol after 24 h incubation. 1,25 (OH)₂ D₃ did not lead to a significant increase in eNOS gene expression. In line with these findings, eNOS protein expression assessed by Western blotting, was increased after 24 h compared to 0 h following incubation with high concentration ergocalciferol compared to high concentration to 1,25 (OH)₂ D₃. eNOS protein expression in Western blots was increased at 24 h compared to 0 h following incubation with both low concentrations of ergocalciferol and 1,25 (OH)₂ D₃. The finding that low concentration 1,25 (OH)₂ D₃ does not affect eNOS gene expression by RT-PCR is discordant with the finding that low concentration 1,25 (OH)₂ D₃ is associated with an increase in eNOS protein expression by Western blotting. The reason for this is currently unclear but may reflect a non genomic effect of low concentration 1,25 (OH)₂ D₃ on eNOS protein expression.

Western blotting for phospho-eNOS at 24 h demonstrated reduced phosphorylation after incubation with high compared to low concentration ergocalciferol and 1,25 (OH)₂ D₃. Phospho-eNOS expression at 24 h, while reduced overall was higher after incubation with high concentration ergocalciferol compared to 1,25 (OH)₂ D₃. However, the reduction in phospho eNOS expression in control and ethanol treated cells makes these results difficult to interpret.

After 24 h, incubation of HAEC with high concentration compared to low concentration ergocalciferol increased cell supernatant nitrite concentrations significantly. However, after 24 h incubation of HAEC with high and low concentrations of $1,25\text{ (OH)}_2\text{ D}_3$, there was no significant change in nitrite concentrations. Nitrite concentrations after incubation with low and high concentration $1,25\text{ (OH)}_2\text{ D}_3$ are similar to those seen with high concentration ergocalciferol, however there was no concomitant increase in eNOS protein production after incubation with $1,25\text{ (OH)}_2\text{ D}_3$. This suggests that nitrite generation by ergocalciferol but not $1,25\text{ (OH)}_2\text{ D}_3$ may be eNOS dependent. However the non-eNOS dependent mechanism by which $1,25\text{ (OH)}_2\text{ D}_3$ produces nitrite is currently unclear. In addition, given that phospho-eNOS expression was inhibited at high concentrations of ergocalciferol, the observed increase in nitrite after incubation with high concentration ergocalciferol may be due to another cellular pathway. Webb *et al.*¹⁵⁰ have identified that this cellular pathway may involve xanthine oxidoreductase however their findings suggest that this pathway becomes important primarily in acidotic conditions.

In an SNx model of mild uraemia, ergocalciferol compared to vehicle improved endothelial vasodilator response to ACh. There were no significant differences in endothelial vasodilator response in either sham or SNx animals after the addition of a direct endothelial NO donor (SpNO) indicating that ergocalciferol improved vasodilator function through a direct effect on the endothelium. Uraemia blunted the contractile response of aortic tissue after stimulation with PE but this was overcome in SNx animals treated with ergocalciferol, returning contractile function to the level seen in sham operated rats. The effect on the endothelium of the thromboxane A_2 mimetic U-46619 was markedly different in SNx compared to sham operated animals and this effect was not changed by ergocalciferol. This effect was most apparent at a

concentration range of $10^{-7.5}$ M to $10^{-6.5}$ M. At the maximum concentration range of 10^{-6} M to 10^{-5} M, there was no difference in contractile response in sham and SNx groups. This suggests that uraemia *per se*, rather than ergocalciferol, modifies the endothelial response to U-46619.

Systolic blood pressure in sham operated and SNx rats was not significantly different. Similarly, systolic blood pressure did not differ in these experimental groups between animals treated with either vehicle or ergocalciferol. In SNx animals treated with vehicle compared to ergocalciferol, both endothelial vasodilatory and contractile responses were reduced compared to animals treated with ergocalciferol. The combined effect of the reduction of vasoconstrictor tone with a concomitant reduction in vasodilatory function may represent a compensatory mechanism in the early stages of uraemia that prevents the development of systolic hypertension. In addition, the reduction in vasoconstrictor tone may represent impaired auto-regulatory function of the arterial tree which could expose end organ capillary beds to variations in systemic systolic blood pressure and consequent tissue damage. These studies have demonstrated that, in SNx rats, ergocalciferol not only improved the endothelial vasodilatory response but also normalised the adrenergic contractile response. The observation that SNx rats treated with ergocalciferol have contractile responses to PE almost normalized to those in sham operated animals implies that uraemia desensitizes the target of PE (α -adrenergic receptor) and that ergocalciferol overcomes this desensitization. This finding suggests that ergocalciferol has a beneficial effect on vascular function both through improving the endothelial vasodilator response and by restoring normal contractile function and therefore vascular auto-regulation. The exact effect by which this process occurs is unclear but may include an increase in alpha adrenergic receptor expression via a genomic effect of ergocalciferol or an

improvement in the function of existing adrenergic receptors through a post translational modification by ergocalciferol.

It would be expected that untreated uraemic animals would go on to develop systolic hypertension as a consequence of progressive kidney failure. The fact that systolic blood pressure was not different between sham operated and SNx rats and that ergocalciferol did not reduce systolic blood pressure in SNx rats may reflect the relatively short duration of uraemia in these studies and the relatively short duration and dose of ergocalciferol. In SNx operated, vehicle treated rats, it is reasonable to hypothesise that with a longer duration of uraemia, the balance of endothelial response will change towards the development of systolic hypertension through an increase in vasoconstrictor tone and a decrease in vasodilator tone. This hypothesis is supported by Wu-Wong *et al.*²³⁹ who demonstrated increased systolic blood pressure in SNx treated rats (181.0 ± 10.0 mmHg) compared to sham operated rats (127.8 ± 3.8 mmHg) with a 6-week duration of uraemia. In addition, a higher dose and longer treatment with ergocalciferol may have altered the vascular response in favour of vasodilatation compared to vasoconstriction which may lead to a reduction in systolic blood pressure in SNx operated rats treated with ergocalciferol.

In contrast to the effect of ergocalciferol on vasoconstrictor tone after stimulation with PE, ergocalciferol did not modulate the response to stimulation of aortic rings with U-46619. Thromboxane A₂ receptor activation by U-46619 in SNx operated animals caused a relatively greater increase in aortic ring tone at lower concentrations than in sham operated animals. This may be due to the enhanced sensitization of thromboxane A₂ receptors to U-46619 in uraemia although the exact mechanism by which uraemia increases sensitization to U-46619 is currently unclear.

The results of the *in vitro* and *in vivo* experiments support the direct and beneficial effect of ergocalciferol on endothelial function. This is mediated by the effect of ergocalciferol on the increased expression and function of eNOS in cultured endothelial cells. The observation that ergocalciferol improves endothelial vasodilatory function after the addition of ACh in an *in vivo* model of mild uraemia implies that ergocalciferol is exerting its effect on the endothelium through a similar mechanism involving eNOS expression and function. Ergocalciferol, through its upregulation of eNOS gene expression, concomitant effect on the increased expression of eNOS protein and changes in nitrite concentrations has the potential to improve endothelial function. Nitrite itself, as well as a functional mediator of endothelial vasodilatation, is also a store for NO¹⁵⁰. Therefore, ergocalciferol may exert its effects through the dual pathways of increased stores and availability of NO.

The findings reported in this chapter support those of the clinical trial described in chapter two of this thesis in which iontophoresis of ACh demonstrated improved function of the endothelium in the ergocalciferol but not placebo group. In the *in vivo* experimental model of endothelial function and in the clinical trial described in chapter two of this thesis, the improvements in endothelial function after treatment with ergocalciferol occurred independently of changes in blood pressure, Ca²⁺, PO₄ and PTH. Jolma *et al.*^{184 274} and Koobi *et al.*²⁷⁵ have demonstrated that endothelial vasodilatory responses are improved by increasing dietary supplementation of calcium in experimental uraemia through effects on the calcium-dependent K⁺ channel. Given that serum calcium did not change in the experiments presented in this thesis, this supports the concept that ergocalciferol is exerting a direct and beneficial effect on endothelial function independently of serum calcium concentrations and that the effect

of ergocalciferol maybe a transcriptional or post–translation effect on the calcium-dependent potassium channel as well as on the expression and function of eNOS.

3.4.1 Summary and conclusions

The experiments described in this chapter provide preliminary evidence for the effect of ergocalciferol on endothelial function and demonstrate its beneficial effect on endothelial responses in an *in vitro* model of cultured HAEC and in an *in vivo* model of mild uraemia. This mechanism involved increased expression and function of eNOS as well as modulation of both endothelial vasodilator and vasoconstrictor tone. The effect of ergocalciferol on endothelial function in an *in vivo* model of mild uraemia was independent of changes in blood pressure and bone mineral parameters.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

4 Summary of principal findings

The experimental procedures in this thesis have demonstrated the following novel findings:

1. In a double blind, randomized controlled trial of ergocalciferol compared to placebo in patients with CKD stages 3-4 and concomitant serum 25 (OH) D concentrations of < 40 nmol/L, ergocalciferol compared to placebo resulted in a rapid increase in serum 25 (OH) D concentrations which after 6 months of therapy was accompanied by a significant improvement in microcirculatory endothelial function. Skin autofluorescence was significantly increased in the placebo group after 6 months of therapy but was unchanged from baseline in the ergocalciferol group. These findings occurred independently of changes in blood pressure, large conduit artery function, bone mineral parameters, left ventricular hypertrophy and the recruitment of new, functionally relevant microcirculatory vessels.

2. In HAEC incubated with high concentration ergocalciferol there was a 2.4 fold increase in eNOS gene expression, increased expression of eNOS protein and an increase in cell supernatant nitrite concentrations. These changes were not induced by 1,25 (OH)₂ D₃. In an *in vivo* model of mild uraemia induced by 5/6th nephrectomy with a shorter period of uraemia compared to other studies, ergocalciferol compared to vehicle improved endothelium dependent vasodilatation after exposure to ACh. Ergocalciferol modulated the contractile response of rat aortic rings to stimulation with phenylephrine in uraemic but not control animals. Uraemia *per se*, rather than ergocalciferol, modulated endothelial responses to U-46619. In line with the findings of the clinical trial presented in chapter two, the modulation of vascular tone by ergocalciferol occurred in the absence of changes in blood pressure, Ca²⁺, PO₄ and PTH.

This chapter discusses the relevance of these findings in the context of the available scientific literature and describes further experimental work which could be conducted to address questions which have arisen from the experiments described in this thesis.

4.1 The effect on the microcirculation of ergocalciferol versus placebo in chronic kidney disease stage 3-4 and vitamin D deficiency: a pilot, double blind, randomized controlled trial

In contrast to other studies in the field^{120 219 243-244}, the clinical trial presented in this thesis has synchronously evaluated macrocirculatory and microcirculatory endothelial changes as well as the structural conformation of the endothelial capillary network using SDF imaging of the sublingual microcirculation. It is interesting to note that the sublingual MFI in patients with CKD at baseline (MFI mean 2.4, range 1.5-3.0) in the clinical trial in this thesis was lower than that reported for normal healthy controls regardless of age (<25 years MFI = 2.85, range 2.75–3.0, > 55 years MFI = 2.81, range 2.66–2.97) and for patients with stage 5 CKD (MFI = 3.0, range 2.78–3.0) by Reynolds *et al.*¹⁷¹. The fact that these two studies were not conducted synchronously combined with the small numbers of patients in each study are the most likely explanations for this finding, rather than a true biological difference as a consequence of CKD stage 3-4.

The findings from our clinical study indicate that the maximum benefit of ergocalciferol on endothelial function occurred at 6 months despite a prompt and sustained rise in serum 25 (OH) D concentrations at 1 month. Therefore, follow-up of the patients in the Marckmann *et al.*¹²⁰ study beyond 8 weeks may have demonstrated improvements in endothelial function, and while this assumption is theoretical, it will have important implications for determining both the optimum duration of therapy of ergocalciferol and

the optimum serum 25 (OH) D concentration to ensure a maximally beneficial effect on the microcirculatory endothelial function.

Nutritional vitamin D therapy in haemodialysis patients in the form of cholecalciferol, reported by Stubbs *et al.*²⁴³, resulted in a reduction of inflammatory markers after only 6 weeks of treatment. Stubbs *et al.*²⁴³ used a replacement regimen for cholecalciferol based on successive serum 25 (OH) D concentrations and it represents one of the first studies to use an incremental dose increase in vitamin D therapy to treat VDD in CKD. In contrast to the study by Marckmann *et al.*¹²⁰, the improvements in markers of inflammation occurred within 6 weeks with the achievement of similar concentrations of serum 25 (OH) D.

Direct comparisons between our study and the studies by Marckmann *et al.*¹²⁰, Assimon *et al.*²¹⁹ Stubbs *et al.*²⁴³ and Chitalia *et al.*²⁴⁴ are difficult due to the heterogeneous populations, study designs and replacement of vitamin D but support further investigation of the effect of nutritional vitamin D on endothelial function in all stages of CKD. The short duration of the clinical study in this thesis and those of Marckmann *et al.*¹²⁰, Assimon *et al.*²¹⁹, Stubbs *et al.*²⁴³ and Chitalia *et al.*²⁴⁴ has meant that the effect of nutritional vitamin D on meaningful clinical outcomes, specifically CV morbidity and mortality, cannot be assessed. However, given the association between endothelial function and future CV events in CKD, it could be inferred that nutritional vitamin D, through its effect on microcirculatory function and reduction of inflammatory mediators, has the potential to reduce CV endpoints where CKD and VDD co-exist although a study in CKD and concomitant VDD powered to detect the effect of nutritional vitamin D compounds on clinically relevant end points including CV events is required to test this assumption.

The strengths of the clinical trial in this thesis are its double blind randomized placebo control design, replacement of vitamin D in line with international guidelines that was standardised for all patients and the use of techniques that specifically and concomitantly assess both macro and microcirculatory endothelial function. At the time of designing this study, microcirculatory endothelial function had not previously been evaluated in patients with CKD and concomitant VDD in a clinical trial setting and thus the current study adds to the existing scientific literature on the subject. Iontophoresis has been used in the setting of clinical trials to evaluate endothelial function. The use of a low current iontophoresis protocol in the current study reduced the direct galvanic effect on the endothelium seen when a higher current is used²⁵⁹. The experimental conditions and iontophoretic protocol were standardised and changes in endothelial function were compared with baseline prior to treatment with ergocalciferol. Therefore, any change seen in LDF after iontophoresis must be due to the direct effect of ergocalciferol itself on microcirculatory endothelial function. Despite some reported limitations of iontophoresis including a lack of standardised protocols, reporting of results as well as the effect of iontophoretic current itself on the skin microcirculation²⁷⁶⁻²⁷⁷, iontophoresis provides a real time, dynamic approach to monitoring the physiological response of the endothelium to a pharmacological challenge which can provide valuable insights into the function of the endothelium in clinical practice.

Limitations of this study include the short follow up time and small sample size. The study duration is insufficient to detect significant differences between treatment groups in key outcome measures including CV events and their relationship to microcirculatory endothelial function. Excluding patients with DM has limited the external validity but improved the internal validity and precision of the present study. The results of this study cannot be applied to patients receiving dialysis for ESKD due to the effect of the

process of dialysis itself on endothelial function. A separate study in patients treated with dialysis for ESKD is required to ascertain if a similar effect of ergocalciferol on endothelial function occurs in this group. The newer equations for kidney function have improved the estimation of kidney function mostly in patients with stage 1-2 CKD and to a lesser extent in patients with stage 3-5 CKD²⁷⁸. Therefore, the use of the MDRD equation, which was in routine use when this study was designed, is unlikely to have materially altered the classification of the stage of CKD in the study population.

4.2 The effect of ergocalciferol on endothelial function in an *in vitro* and *in vivo* experimental model

Existing *in vivo* and *in vitro* studies at the time the experiments in this thesis were designed had not evaluated the effect of ergocalciferol in experimental models of uraemia. In contrast, the majority of studies using *in vitro* and *in vivo* models included assessments of activated vitamin D compounds or did not evaluate the effect of nutritional vitamin D in experimental uraemia. The experiments in this thesis provide preliminary evidence for the effect of ergocalciferol on cultured endothelial cells and in an *in vivo* model which reflects earlier compared to more advanced uraemia.

The studies in this thesis have demonstrated that ergocalciferol has a genomic effect on cultured endothelial cells as evidenced by the increase in fold expression of eNOS after incubation with high concentration ergocalciferol. The results in this thesis suggest that while nitrite generation was increased after incubation with high concentration ergocalciferol but not 1,25 (OH)₂ D₃, the activation of eNOS through phosphorylation was reduced after incubation with high compared to low concentrations of both ergocalciferol and 1,25 (OH)₂ D₃. These results may represent an inhibitory effect of vitamin D at supra-physiological concentrations, and therefore allude to an

alternative cellular source of nitrite production beyond the effect of eNOS. However, the finding that phosphorylation of eNOS was reduced in control and ethanol treated cells means this hypothesis cannot be confirmed.

Andrukhova *et al.*¹⁸⁶ have demonstrated that in wild type compared to VDR knock-out mice, both 25 (OH) D and 1,25 (OH)₂ D₃ increased the expression of eNOS although the increase in eNOS expression was greater with 1,25 (OH)₂ D₃ compared to 25 (OH) D (see section 1.3.4). This is in contrast to the findings reported in this thesis that 1,25 (OH)₂ D₃ did not have a significant effect on expression of eNOS or its phosphorylation. The use of an *in vitro* model in this thesis compared to the *in vivo* model of VDR deficiency used by Andrukhova *et al.*¹⁸⁶ may in part explain this discrepancy.

In an *in vivo* model of experimental mild uraemia, studies in this thesis have demonstrated that ergocalciferol acts both through an endothelium dependent pathway to improve vasodilatory function and that ergocalciferol has an effect on vasoconstrictor function which may reflect restoration of vascular auto-regulation. In line with findings from a study by Wu-Wong *et al.* of the effect of paricalcitol on endothelial function in experimental uraemia²³⁹, the effect of ergocalciferol in a model of mild uraemia was independent of blood pressure, serum Ca²⁺, PO₄ and PTH. The finding that ergocalciferol modulates vasoconstrictor tone has not previously been assessed in existing studies of the effect of vitamin D on endothelial function^{180 239 241 279}. There are several potential mechanisms through which ergocalciferol may affect vasoconstrictor function including genomic and post translational effects. Modifying the dose and duration of ergocalciferol has the potential to move this balance in favour of endothelial vasodilator function. However, additional studies are needed to confirm this hypothesis.

The strengths of these experiments are that they used ergocalciferol rather than activated vitamin D compounds and have thus provided support for the pleiotropic effects of ergocalciferol itself. The *in vivo* experiments used a relatively shorter duration of uraemia (4 weeks compared to 6 weeks) compared to other studies^{239 280} and in keeping with this, the serum creatinine and PTH were lower in the *in vivo* studies in this thesis compared to studies with a longer duration of uraemia^{239 241-242}. This confirms that the model used in the experiments in this thesis reflected less advanced uraemia than achieved in other studies. This has facilitated an examination of the effect of ergocalciferol in an experimental model of mild uraemia when endothelial tissue is more likely to be responsive to therapeutic intervention with nutritional vitamin D compared to more advanced models of CKD. Therefore, the studies in this thesis add to the available scientific literature which has predominantly evaluated activated vitamin D compounds in the later stages of uraemia.

The limitations of the *in vitro* studies are that HAEC were not cultured in media consistent with the degree of CKD in the clinical trial subjects. This was due to the complexity of establishing a culture medium that accurately reflects the earlier rather than more advanced stages of CKD. Consequently, the results from the *in vitro* experiments cannot be directly generalized to the uraemic milieu associated with CKD stage 3-4. A further limitation is that a dose response curve for ergocalciferol and its effects on eNOS expression as well as nitrite production has not been elucidated. These experiments were a proof of concept study for the effect of ergocalciferol on cultured endothelial cells and need to be repeated using a uraemic milieu and include dose response studies the effect of ergocalciferol on eNOS expression and function.

The *in vivo* experiments are limited by the fact that they did not include a contemporary experimental arm in which rats were treated with active vitamin D compounds. This

work has been conducted by other authors and the specific purpose of the studies in this thesis was to evaluate the effect of ergocalciferol. The *in vivo* studies in this thesis used a relatively shorter duration and lower dose of vitamin D therapy compared to other studies^{180 239}, however, serum 25 (OH) D concentrations in both groups increased significantly after therapy with ergocalciferol. We did not measure proteinuria in the animal studies which has been shown by other authors to be related to endothelial function²¹⁶.

The *in vivo* experiments were also limited by the fact that they did not evaluate the specific mediator of endothelial vasodilatation. Measures of nitrite, as used in the *in vitro* experiments could not reliably be applied to the *in vivo* studies due to the effect of uraemic induced anorexia on dietary intake, which has a direct effect on serum nitrite concentrations. This hypothesis is supported by the significant difference in the weight of sham and SNx operated animals. Therefore establishing the exact mechanism through which ergocalciferol causes vasodilatation in a model of mild uraemia and therefore how vasodilatation can be enhanced by manipulating ergocalciferol dose and duration, is an important area of further study.

4.3 The clinical relevance of vitamin D and endothelial function in chronic kidney disease

The current practice of vitamin D therapy to treat VDD in CKD is designed to normalise serum concentrations of 25 (OH) D with the aim of suppressing PTH and normalising calcium homeostasis. The pleiotropic effects of vitamin D are well described as is the beneficial effect of vitamin D therapy in reducing CVD in CKD. At the time of designing this thesis, the association between vitamin D and endothelial function in CKD was

incompletely understood. Several recently published studies of the effect of vitamin D on endothelial function have provided conflicting results^{120 219 243-244}.

The studies presented in this thesis add support to the use of ergocalciferol in terms of its pleiotropic effect on endothelial function in the earlier stages of CKD. The results of the clinical trial in this thesis are supported by the evidence for the direct effect of ergocalciferol on improving endothelial function in the *in vitro* and *in vivo* experiments. These findings require further study but suggest that the use of ergocalciferol in the earlier stages of CKD is likely to have significant beneficial effects beyond the normalization of serum concentrations of 25 (OH) D and suppression of PTH which have been the predominant goals of vitamin D therapy in patients with CKD.

Identifying strategies to refine and improve the use of ergocalciferol in early stage CKD should move beyond serum concentrations of 25 (OH) D and PTH. The results presented in this thesis and supported by the study of Wu-Wong *et al.*²³⁹ suggest that even in the absence of PTH suppression, microcirculatory endothelial function can be significantly improved. Techniques to assess endothelial function in routine clinical practice are becoming more readily available and increasingly operator independent. Given that CV risk in CKD is associated with both endothelial dysfunction and VDD, refining the desired target for serum concentrations of 25 (OH) D, potentially beyond that of > 75 nmol/L suggested for PTH suppression^{49 53} and incorporating parameters of microcirculatory endothelial function in routine clinical practice has the potential to further achieve reductions in CVD risk in CKD and concomitant VDD.

4.4 Further studies

In order to refine and optimise the use of ergocalciferol in the earlier stages of CKD and concomitant VDD as well as addressing the unanswered questions from this thesis, additional studies are required.

The studies that could be conducted include:

1. An evaluation of the clinical relevance of bioavailable vitamin D in patients with CKD in a multi-ethnic population
2. A prospective study of the effect of ethnicity on the replacement of vitamin D in patients from multi-ethnic backgrounds with CKD and concomitant VDD
3. A clinical study evaluating the effect of ergocalciferol on microcirculatory endothelial function and consequent CV morbidity and mortality in patients with CKD and concomitant VDD.
4. Additional *in vitro* and *in vivo* studies of the physiology and metabolism of ergocalciferol and its effect on endothelial function in uraemia

4.4.1 An evaluation of the clinical relevance of bioavailable vitamin D in patients with chronic kidney disease in a multi-ethnic population

The study by Powe *et al.*¹³ did not include patients with significant kidney disease and therefore it is unclear if measures of bioavailable vitamin D and their clinical relevance will differ in patients with CKD compared to patients with no evidence of kidney disease. Additional studies of bioavailable vitamin D in patients with CKD will be critical to ascertain how serum concentrations of bioavailable 25 (OH) D change as CKD progresses and how these measures could refine current clinical practice of the replacement of vitamin D when VDD and CKD coexist. Given the abnormal

metabolism of vitamin D in CKD, this study would offer the potential to elucidate the most relevant vitamin D metabolite to measure. While this should include bioavailable vitamin D, separate measure of serum concentrations of vitamin D₂, vitamin D₃, 25 (OH) D and 1,25 (OH)₂ D₃ could be performed and will enhance the understanding of the most useful measure of vitamin D status for further clinical utility.

4.4.2 A prospective study of the effect of ethnicity on the replacement of vitamin D in patients from multi-ethnic backgrounds with chronic kidney disease and concomitant vitamin D deficiency

Recent data from the Royal London Hospital Kidney Unit has demonstrated that patients from ethnic minority groups have a blunted response to therapy with ergocalciferol in CKD and coexisting VDD (Dreyer *et al.*, manuscript in preparation). This study demonstrated that in 93 patients prescribed an equivalent dose of ergocalciferol for VDD complicating CKD stages 1-5, patients from ethnic minority groups (Black and South Asian) has a significantly higher odds ratio for failing to attain a serum concentration of 25 (OH) D of > 75 nmol/L (adjusted OR 3.65 95% CI 1.25 - 8.13, p=0.01). This was a retrospective, observational study of a relatively small number of patients and while it alludes to the effect of ethnicity on blunting the response of therapy to ergocalciferol, a larger, prospective is now required to confirm these findings.

A prospective study of ergocalciferol therapy in EM groups separately evaluating the effect of White, Black and South Asian ethnicity on serum concentrations of total 25 (OH) D and bioavailable 25 (OH) D after therapy with ergocalciferol is required. This study would ascertain the optimum dose and frequency of ergocalciferol required to

achieve a specific target of serum concentrations of 25 (OH) D across different ethnic groups. This information will be essential when developing dosing strategies of ergocalciferol in different ethnic groups in any future study of the effect of ergocalciferol on microcirculatory endpoints and CV events in patients with CKD and concomitant VDD.

4.4.3 A clinical study evaluating the effect of ergocalciferol on microcirculatory endothelial function and cardiovascular morbidity and mortality in patients with chronic kidney disease and concomitant vitamin D deficiency.

This study has the potential to address the relationship between vitamin D therapy in the form of ergocalciferol, microcirculatory endothelial function and future CV events in patients with kidney disease. The hypothesis would be that achieving a higher serum concentration of 25 (OH) D in patients with CKD and concomitant VDD improves microcirculatory endothelial function and reduces CV events. This study would require a significant follow up period (3-5 years) and need to be adequately powered in order to detect clinically significant CV events such as myocardial infarction, non-ST segment elevation myocardial infarction, sudden death and cerebro-vascular events. In order to achieve sufficient numbers in respective treatment arms, such a study would need to be multi-centred.

A multi-centre study could include important subgroups including dialysis and non-dialysis requiring kidney disease and patients with kidney disease and coexisting DM. A study of this nature has the potential to address the efficacy of vitamin D in the form of ergocalciferol in reducing CV events in CKD in prospective, randomized fashion. Equally, such a study could discern the optimal concentration of serum 25 (OH) D in

order to maximise endothelial function and reduce CV morbidity and mortality. Including a group with a target serum 25 (OH) D concentration of > 75 nmol/L could ascertain if the current guidelines for target serum concentrations of 25 (OH) D need to be re-evaluated. This could be achieved if the randomisation schedule included three distinct study arms, each treated with a variable dose regimen to within a specific range of serum 25 (OH) D concentrations for example, <50 nmol/L, 50-100 nmol/L and 100-150 nmol/L.

This study could also ascertain the clinical utility of microcirculatory endothelial assessments in routine clinical practice as predictors for future CV events. In this study, if microcirculatory endothelial function is shown to predict future CV events and, as has been shown in this thesis, improves with increasing serum concentrations of 25 (OH) D, then the correlation between microcirculatory function and serum concentrations of 25 (OH) D could eventually be used as a clinical tool to determine a target serum concentration of 25 (OH) D that maximally reduces CVD risk in patients with CKD and concomitant VDD.

4.4.4 Additional *in vitro* and *in vivo* studies of the physiology and metabolism of ergocalciferol and its effect on endothelial function in uraemia.

The preliminary *in vitro* experiments reported in this thesis could be repeated with endothelial cells cultured in a uraemic milieu commensurate with the earlier stages of CKD. Dose titration studies have the potential to determine the concentration of 25 (OH) D and 1,25 (OH)₂ D₃ at which eNOS expression and function are optimised. This data could support the proposed clinical trial in determining the optimum concentration of serum 25 (OH) D that maximizes endothelial function.

Additional *in vivo* experiments should focus on the exact mechanism through which endothelial vasodilatation is achieved and how ergocalciferol affects this process. These studies should specifically focus on the measurement of NO and EDHF. Extending the duration of uraemia in an *in vivo* model and modifying the dose of ergocalciferol could determine how the balance between endothelial vasodilator and constrictor activity changes, how this affects vascular auto-regulation and the development of systolic hypertension.

Examination of rat aortic tissue from sham and SNx animals treated with ergocalciferol to examine the tissue level effect of ergocalciferol on eNOS, VDR, α -adrenergic and thromboxane A₂ receptor expression and function are required. These studies could determine if ergocalciferol is exerting its effect at a genomic level or by modifying the efficacy of existing receptors through a post translational mechanism. Further studies to examine the effect of ergocalciferol on calcium-dependent K⁺ channels, will be important to understand the effect of ergocalciferol on this channel and the mechanism by which any effect is achieved.

4.5 Conclusions

This thesis has demonstrated that vitamin D therapy in the form of ergocalciferol for the treatment of VDD in patients with CKD is effective at increasing serum 25 (OH) D concentrations and improves microcirculatory endothelial dysfunction. The effect of ergocalciferol has been shown to be endothelium dependent. This is reflected by *in vitro* and *in vivo* experimental models which have demonstrated that ergocalciferol increased expression and function of eNOS and improves endothelial function in a model of mild uraemia.

The excessive CVD that accompanies CKD is the primary focus of clinical nephrology. This thesis has elucidated the potential beneficial effect of ergocalciferol in reducing this risk through the improvement of endothelial function which is strongly associated with CVD in patients with CKD. However, questions remain as to the optimum strategy for the replacement of vitamin D in CKD and the role of clinical assessments of microcirculatory endothelial function in order to maximally reduce CVD. Further pre-clinical and clinical studies are now required to enhance our understanding of the association between vitamin D and microcirculatory endothelial function in CKD.

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APPENDICES

List of appendices

- I Ethical approval documentation
- II Medicine and Health Care Regulatory Authority (MHRA) approval
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- V Clinical trial study protocol

I. Ethical approval documentation



FINAL R&D APPROVAL

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28th April, 2009

Dear Prof. Yaqoob,

Re: The effect of vitamin D deficiency on the microcirculation of patients with chronic kidney disease and vitamin D deficiency.

ReDA Reference: 006488

Thank you for sending confirmation of your approval from the ethics committee. I am now happy to inform you that the Joint R&D Office of Barts and The London NHS Trust and Queen Mary, University of London has arranged full indemnity cover for your study against any negligence that might occur during the course of your project.

Site list

Site Name	Investigator
Royal London Hospital, Whitechapel Rd	Dr Gavin Dreyer

Please note that all research with an NHS element is subject to the Research Governance Framework for Health and Social Care 2005. If you are unfamiliar with the standards contained in this document, or the BLT and QMUL policies that reinforce them, you can obtain details from the Joint R&D Office, tel 0207 882 7250 or go to <http://www.dh.gov.uk/PolicyAndGuidance/ResearchAndDevelopment/ResearchAndDevelopmentAZ/ResearchGovernance/fs/en>.


You must stay in touch with the Joint R&D Office during the course of the research project, particularly if/when:

- There is a change of Principal Investigator;
- The project finishes;
- Amendments are made, whether minor or substantial;
- Serious Adverse Events have occurred (must be reported within 24 hours of becoming aware of the event).

This is necessary to ensure that your indemnity cover is valid. Should any untoward events occur it is **essential** that you contact the Joint R&D Office immediately. If patients or staff are involved in an incident, you should also contact the Clinical Risk Manager on 0207 480 4132.

I hope the project goes well, and if you need any help or assistance during its course, please do not hesitate to contact the Office.

Yours sincerely,

 The Royal Hospital of St. Bartholomew. The Royal London Hospital.
The London Chest Hospital. The Queen Elizabeth Children's Service.
Head of Research Resources

Head of Research Resources: Gerry Leonard

II. Medicine and Health Care Regulatory Authority (MHRA) approval

Safeguarding public health



Dr G Dreyer
ROYAL LONDON HOSPITAL
DEPARTMENT OF RENAL MEDICINE & TRANSPLANTATION
WHITECHAPEL
LONDON
E1 1BB
UNITED KINGDOM

08/04/2009

Dear Dr G Dreyer

THE MEDICINES FOR HUMAN USE (CLINICAL TRIALS) REGULATIONS 2004 S.I. 2004/1031

Our Reference: 14620/0021/001-0001
Eudract Number: 2008-008745-38
Product: Drisdol (Ergocalciferol)
Protocol number: CKD-VitD-microcirc

NOTICE OF ACCEPTANCE OF AMENDED REQUEST

I am writing to inform you that the Licensing Authority accepts your amended request for a clinical trial authorisation (CTA), received on 03/04/2009.

The authorisation is effective from the date of this letter although your trial may be suspended or terminated at any time by the Licensing Authority in accordance with regulation 31. You must notify the Licensing Authority within 90 days of the trial ending.

Finally, you are reminded that a favourable opinion from the Ethics Committee is also required before this trial can proceed; changes made as part of your amended request may need to be notified to the Ethics Committee.

Yours sincerely,

**Clinical Trials Unit
MHRA**

Medicines and Healthcare products Regulatory Agency
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An executive agency of the Department of Health

III. Patient consent form



CONSENT FORM (Version 1.2) Dated 25/2/10

Title of project: The effect of Vitamin D supplementation on the microcirculation of patients with chronic kidney disease and vitamin D deficiency

Principal Investigator: Dr Gavin Dreyer Chief Investigator: Professor M.M. Yaqoob

Study Number: CKD-VitD-microcirc Eudract number: 2008-008745-38
 Submission reference: 15090/20517/1/897 REC reference: 09/h/073/9

Patient Identification Number for this trial:.....

Please initial box to indicate agreement

1.	I confirm that I have read and understand the information sheet dated 25/2/10 (version 1.2) 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 withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the Barts and the London/ Queen Mary University of London , where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I agree to my GP being informed of my participation in the study.	
5.	I agree to the investigators sending me a text message about taking the study medication	
6.	I agree to take part in the above study.	

 Name of Patient Date Signature

 Name of Person taking consent
 (if different from Investigator) Date Signature

 Investigator Date Signature

1 copy for Patient, 1 for Investigator and original to be kept in medical notes

IV. Patient information sheet

The effect of vitamin D supplementation on the microcirculation of patients with chronic kidney disease and vitamin D deficiency

Invitation to participate:

You have been invited to take part in a post-graduate research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part, and feel free to discuss this issue with anyone else before deciding. If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year.

What is the study all about?

More patients with kidney disease die from heart disease and strokes than end up on dialysis treatment. This is because the microscopic blood vessels in the body can be damaged and fail to function properly. There is growing evidence that giving patients a tablet of vitamin D can improve the function of these blood vessels and therefore reduce the risk of serious heart problems, worsening of kidney disease and generally improve quality of life.

Many people have very low levels of vitamin D in the blood. We would like to understand more about how vitamin D can help patients with chronic kidney disease by comparing 6 months of treatment with vitamin D tablets (called ergocalciferol) to a placebo tablet (a “dummy” pill which has no medical effect). Half the patients in the study will receive vitamin D tablets and the other half will receive the placebo tablet.

Sometimes we don't know which way of treating patients is best. To find out, we need to make comparisons between the different treatments. We put people into groups and give each group a different treatment; the results are compared to see if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly).

Patients will be allocated at random and both you and the study team will not know which tablet you are receiving. This reduces the chance of the study being biased. Certain drugs (such as blood pressure tablets) need to be kept the same during the study and you will be asked to avoid certain over the counter medicines so as not to affect the study results.

Why have I been chosen?

Your doctor has discovered that you have chronic kidney disease (a mild reduction in the function of your kidneys) and also a low level of vitamin D in your blood. We will ask 64 patients like you to participate in this study.

What will happen to my results?

Your results will be strictly confidential. You will be able to see a copy of your results as the study progresses. Your GP and normal kidney specialist will be informed that you are taking part in this trial. They will be able to see your results unless you ask for them not be informed.

We hope to publish this study in a medical journal so we can inform our colleagues about our results.

You will not be identified in this report in any way unless you give your permission.

I am being supervised by Professor M Yaqoob and I plan to use the results of this study to obtain a higher degree (called an MD)

Do I have to take part?

No, you are under no pressure to participate in this study. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected before you withdraw may still be used in our research if you agree. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

What are the alternatives for diagnosis and treatment?

If you do not wish to enter the study, your kidney specialist can prescribe a number of different forms of vitamin D tablets or injections. They will also give some advice about your diet to improve your vitamin D levels.

What will happen if I take part?

The study lasts for 7 months in total. You will be asked to come to a clinic at the Royal London Hospital once a month for 7 months (but twice in the first month). At the end of 7 months, you will be followed up by your normal kidney specialist as before. Some visits will only last 10 minutes but others may take longer (up to one hour). The clinic doctor will address all your usual health needs relating to your kidney problem and in addition will arrange some extra tests (described below relating to the study)

A full summary of the study programme is shown below with explanations of each procedure:

1st visit – meet the doctor, go over what the study involves and agree (or not) to take part. You will have a blood test and an examination. If you agree to be involved, you

may asked to stop certain medicines that contain vitamin D (visit time - about 15 minutes)

2nd visit (2 weeks later) – meet the doctor. You will have some tests to examine the microscopic blood vessels that we are interested in studying. These are explained below. (about 1 hour)

3rd visit (2 weeks later) – meet the doctor. You will have a blood test and be asked for a urine sample. You will be given a form to fill in to ask about your general health. We will book a cardiac (heart) MRI scan, an ultrasound of the neck and arrange for your blood pressure to be recorded over 24 hours. The doctor will examine you and you will be able to discuss any issues that you are concerned about. You will have the tests to examine the microscopic blood vessels as described below. We will also do a heart tracing (ECG). You will also receive your first dose of the study medicine and some capsules to take at home. You will need to take one capsule weekly on the same day of each week. You will need to bring all your medicines with you at each visit

(visit time - about 1 hour)

4th visit (1 month later) – meet the doctor. You will have an examination, give a blood and urine sample and undergo the tests you had previously to look at the microscopic blood vessels. You will

receive your next dose of the study medicine (visit time - about 1 hour)

5th visit (1 month later) - meet the doctor. You will have a blood test and receive your next dose of study medicine (visit time - about 10 minutes)

6th visit (1 month later) - meet the doctor. You will have an examination, give a blood and urine sample and undergo the tests you had previously to look at the microscopic blood vessels. You will receive your next dose of the study medicine (visit time - about 1 hour)

7th visit (1 month later) – meet the doctor. You will give a blood sample and receive your next dose of the study medicine. (visit time - about 10 minutes)

8th visit (1 month later) - meet the doctor. You will give a blood sample and receive your next and last dose of the study medicine. (visit time - about 10 minutes)

9th and last visit (1 month later) - meet the doctor. You will have a blood test and be asked for a urine sample. You will also have the test on the microcirculation. You will be given a form to fill in to ask about your general health. We will book a cardiac (heart) MRI scan, an ultrasound of the neck and arrange for your blood pressure to be recorded over 24 hours. The doctor will examine you and you will be able to discuss any issues that you are concerned about. We will also do a heart tracing (ECG). (visit time - about 1 hour)

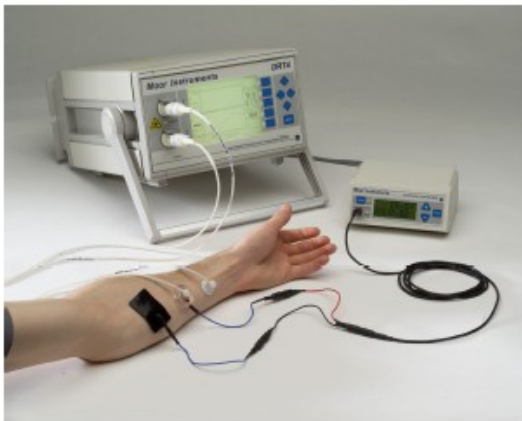
At every visit, the doctor will ask how you are feeling and if you have had any problems or concerns and review your medication. You will be asked to bring your medication with you at each visit. If you have a mobile phone and you are happy to receive messages from us, we will send you a text message to help you remember to take your study medication on the correct day. The message will read “Dear NAME this is a reminder to take your vitamin D study medication today. Thank you”

Tests to look at the microscopic blood vessels

You will have four tests done by the study team.

Pulse wave velocity – this test measures how fast the blood vessels carry the blood in your circulation. This involves having some blood pressure cuffs placed around your arm, leg and neck. They do not inflate as much as a normal blood pressure cuff and you should only experience a mild pressure sensation. A computer records the results. This last about 5 minutes

Iontophoresis – this test looks at how the microscopic blood vessels respond to 2 different drugs. Two plastic discs are taped to your forearm containing a liquid form of the drugs. A small electric current is passed into the discs and a machine records how the blood vessels in your skin responds. This is usually painless but some patients might experience some mild skin irritation that normally passes in a day or so. This lasts about 15 minutes.



Skin auto-fluorescence – this test detects a build up of abnormal deposits in small blood vessels. Patients rest their forearm over a box which emits an ultra-violet light. A computer measures the reflection from your blood vessels and records the results. This is pain free and last about 5 minutes.



Sublingual microvascular flow – this test uses a very small camera placed under the tongue to look at microscopic blood vessels. It is painless and feels like having your temperature taken with a mercury thermometer.



What will happen to any samples I give?

During this trial, we will ask you for a blood and urine sample which will be collected in the usual way when you come to clinic. Your blood and urine tests will be analysed at the Royal London Hospital for

routine tests. Some of the samples will be kept for more specialist testing in laboratories at the William Harvey Research Institute, Charterhouse Square and at the Royal London Hospital. We may ask you if we can use these samples for future research that has not yet been specified. Only staff involved in the study will have access to these blood tests. We will keep your samples anonymous. You will be informed of any abnormal result that requires a change in your treatment. Your samples can be destroyed if you wish.

What do I have to do?

All we ask is that you attend the clinic at the specified times, take the study medicine (which you will receive when you come to clinic), report any problems or changes in your health and attend appointments for scans and other tests that are carried out

elsewhere. You should not normally be involved in another drug study. You can take your regular medication as directed by the study doctor but should avoid any over the counter drugs except for simple pain killers. We would also ask you to let us know if any other doctor has changed your medication.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1998, and the rights you have under this Act.

Information about you will be recorded and stored by the doctor running the trial. It will be kept securely in hospital premises and will be anonymous. In an emergency relating to your health, the doctor running the trial may allow access to other medical staff if this is in your best interest. You will be able to check the data we hold is accurate.

Occasionally, qualified staff from the hospital may access your records for audit purposes. Your records will be kept in this secure manner for 15 years.

Involvement of the General Practitioner/Family doctor (GP)

If you agree, your GP will be informed that you are participating in the study. The study doctor may ask your GP for additional information about any medicines he/she may have prescribed that may contain vitamin D.

Travel expenses

Unfortunately, we will not be able to offer you any money for participating in this trial but if you normally use hospital transport to attend hospital for your appointments, this service will be available.

What are the other possible disadvantages and risks of taking part?

There are very few risks to participating in this study. Apart from the mild discomfort of a blood test and some possible mild skin irritation from one of the tests looking at the microscopic blood vessels, participants should experience no unusual symptoms.

If you have private health insurance, you should inform your company before you decide to participate in the trial.

What happens if there is a problem?

We would not expect you to suffer any harm or injury because of your participation in this study. If you are harmed by taking part in this study, there is no special compensation arrangement. If you are

harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain or

have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Please contact Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please telephone 020 7377 6335, minicom 020 7943 1350, or email pals@bartsandthelondon.nhs.uk, you can also visit PALS by asking at any hospital reception.

What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue as it was before you started the study. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get might help improve the treatment of people with chronic kidney disease and vitamin D deficiency. These conditions are very common and so this study has the potential to improve the lives of many patients similar to you.

What are the side effects of any treatment received when taking part?

The placebo tablet has no medical effect. The vitamin D tablet (ergocalciferol) also has very few side effects. It can occasionally cause your blood calcium levels to rise. Symptoms of high blood calcium include constipation, irritability, drowsiness and thirst. You will be asked if you have any of these problems at each visit. You will also have regular blood tests to monitor your calcium levels. If the levels are found to be high, the trial doctor will be able to offer you treatment and may temporarily stop the study medicine.

What happens when the research study stops?

You will not receive any of the study medicine after the research has ended. You will be followed up by your normal kidney specialist who will determine if you need any form of vitamin D therapy. If the trial shows that vitamin D tablets are effective and you were receiving the placebo medicine, your usual kidney specialist will be able to give you a course of vitamin D therapy after the study has ended

Who can I contact if I have any questions or concerns?

The principal investigator can be contacted by telephone:

0207 377 7000

Ask for Doctor Gavin Dreyer

If you wish to make a formal complaint about the study, please contact:

Jarrard O'Brien, Quality Development, Barts and The London NHS Trust, Healthcare Governance Directorate, 3d floor, Prescot Street, tel 020 7480 4857, email jarrard.obrien@bartsandthelondon.nhs.uk

Who is organising and funding the research and where was it reviewed?

This study is being funded by the Barts and the London Charitable trust. The study team does not receive any funding above the costs of the study.

This study has received a favorable ethical opinion from the East London and the City Research Ethics Committee 1.

V. Study protocol

THE EFFECT OF VITAMIN D ON THE MICROCIRCULATION OF PATIENTS WITH CHRONIC KIDNEY DISEASE AND VITAMIN D DEFICIENCY

A SINGLE CENTRE, DOUBLE BLIND, RANDOMISED CONTROLLED TRIAL
COMPARING ORAL ERGOCALCIFEROL AND PLACEBO

Submission reference: 15090/20517/1/897

Date submitted to R&D Office: 05/03/2009	EudraCT No: 2008-008745-38
Date Submitted to REC: 05/03/2009	Protocol Number. CKD-VitD-microcirc

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Study background

See chapter 2, section 2

Investigational agent

Ergocalciferol - Vitamin D₂, a fat-soluble vitamin important for many biochemical processes including the absorption and metabolism of calcium and phosphorus. In vivo, ergocalciferol is formed after sun (ultraviolet) irradiation of plant-derived ergosterol, another form of vitamin D occurring naturally in human skin.

Ergocalciferol is used to treat chronic hypocalcemia, hypophosphatemia, rickets, and osteodystrophy associated with various medical conditions including chronic renal failure, familial hypophosphatemia, and hypoparathyroidism (postsurgical or idiopathic, or pseudohypoparathyroidism).

Ergocalciferol is indicated for the prevention and treatment of vitamin D deficiency states. Vitamin D deficiency may occur as a result of inadequate nutrition, intestinal malabsorption, or lack of exposure to sunlight, but does not occur in healthy individuals receiving an adequate balanced diet and exposure to sunlight.

Vitamin D is essential for promoting absorption and utilization of calcium and phosphate from the intestine and for normal calcification of bone. Along with parathyroid hormone and calcitonin, it regulates serum calcium concentrations by increasing serum calcium and phosphate concentrations as needed. Vitamin D stimulates calcium and phosphate absorption from the small intestine and mobilizes calcium from bone.

The molecular weight of ergocalciferol is 396.65. It has a plasma half life of 19 to 48 hours (stored in fat deposits in body for prolonged periods).

A dose of 1.25mg (50,000IU) to 5mg daily has been recommended. The dose should be adjusted according to the severity of the condition. This medicine is delivered by oral administration

Adverse events are generally associated with excessive intake of ergocalciferol leading to the development of hypercalcaemia. The symptoms of hypercalcaemia can include; anorexia, nausea, vomiting, diarrhoea, loss of weight, headache, polyuria, thirst, vertigo, constipation, fatigue, bone pain, muscle weakness, abdominal pain, mental disturbances, impaired renal function, kidney stones and cardiac arrhythmias.

A single acute overdose is virtually non-toxic and requires supportive treatment with liberal fluids only. Treatment of chronic overdose with resulting hypercalcaemia consists of immediate withdrawal of the vitamin, a low calcium diet, and generous fluid intake. Severe cases may require hydration with intravenous saline together with symptomatic and supportive treatment as indicated by the patient's clinical condition. Plasma calcium and U&E's should be monitored.

Preclinical Data

See chapter 1, section 1.3 and 1.4.1.1

Clinical Data to Date

See chapter 1, section 1.4.1.2

Dose Rationale and Risk/Benefits

A dose regimen of ergocalciferol 50,000 IU weekly for 12 weeks and then monthly for 3 months has been shown to be effective at both raising serum vitamin D levels and lowering PTH levels in a similar cohort which is the precise effect we hope to achieve in our study group¹³⁰. No patient developed the most common side effect of hypercalcaemia and no other adverse effects were reported. A further study of weekly ergocalciferol (50,000 IU/week for 24 weeks) was also shown to raise serum vitamin D levels with no adverse events reported¹²². We have chosen the dose regimen in the K/DOQI guidelines for the replacement of vitamin D in patients with CKD. This consists of 50,000IU of ergocalciferol weekly for 1 month and then monthly for 5 months. As in the studies above, we anticipate a rise in serum vitamin D levels to therapeutic but well below toxic levels.

Rationale and Risk/Benefits

See chapter 1, section 1.5

Study Aims and Objectives

See section 2.2.4

Study Design

See chapter 2, section 2.2.3

General Design

See chapter 2, section 2.2.3

Primary Study Endpoints

See chapter 2, section 2.2.4.1

Secondary Study Endpoints

See section 2.2.4.2

Primary Safety Endpoints

1. Clinically significant Hypercalcaemia

This is the most likely safety issue to occur during the trial. Previous studies^{122 130} have demonstrated that our proposed dose regimen is very unlikely to cause hypercalcaemia while providing an effective increase in vitamin D levels. Furthermore, it has been reported that vitamin D supplementation as per current guidelines is extremely unlikely to cause hypervitaminosis D and that the toxic effects of vitamin D (hypercalcemia) only start to occur at levels > 200 nmol/L, well above the expected vitamin D level for the dose regimen proposed in this study²⁸¹⁻²⁸².

Subject Selection and Withdrawal

See chapter 2, section 2.2.5 and 2.2.8

Inclusion Criteria

See chapter 2, section 2.2.5

Exclusion Criteria

See chapter 2, section 2.2.5

Females of childbearing potential and males must be willing to use an effective method of contraception (hormonal or barrier method of birth control; abstinence) from the time consent is signed until 6 weeks after treatment discontinuation.

Females of childbearing potential must have a negative pregnancy test within 7 days prior to being registered for protocol therapy.

NOTE: Subjects are considered not of child bearing potential if they are surgically sterile (they have undergone a hysterectomy, bilateral tubal ligation, or bilateral oophorectomy) or they are postmenopausal.

Subject Recruitment and Screening

See chapter 2, section 2.2.5

Patients will be provided with a full explanation of the nature, purpose and requirements of the study including Patient Information Sheets and Consent Forms. They will be invited to participate in a screening evaluation, which will include a medical history and physical examination. The subject's General Practitioners will be informed of an individual's agreement to participate. Results of the screening evaluation will determine eligibility for entry into the study. Patients will have the opportunity to discuss the trial further with an investigator before giving consent.

Withdrawal of Subjects

When and How to Withdraw Subjects

See chapter 2, section 2.2.8

- Intercurrent medical events and prescription of new medication by the patient's general practitioner that are judged by the principal investigator not to interfere with the study protocol will not result in withdrawal. Such events will be recorded in the CRF.
- New patients will be recruited to ensure that the study meets the patient numbers in the power calculation
- If patients choose to withdraw or are withdrawn prior to completion of the study, they will be replaced unless they have withdrawn due to confirmed study drug toxicity.
- All patients who withdraw will be offered an appointment with their usual nephrologist after withdrawal to provide ongoing clinical care
- All withdrawals will be documented on the CRF and the patient's general practitioner and usual nephrologist will be informed.

Data Collection and Follow-up for Withdrawn Subjects

We will use data and urine/blood samples which have already been collected for study analysis providing the patient gives consent for this to happen. Patients will have the opportunity and choice to inform the trial team how their health has been over the study duration even if they withdraw.

Study Drug

Description

See chapter 1, section 1.1.2

Ergocalciferol capsules and matching placebo capsules will be provided by Mawdsley Brooks (UK). Ergocalciferol capsules will contain 50,000 International Unit (IU) (1,25mg) of ergocalciferol. The ergocalciferol capsule will be over-encapsulated into a size 00, red, empty gelatine capsule measuring 20mm and containing no markings. The placebo capsule will physically match the over-encapsulated ergocalciferol exactly.

Product Sourcing Manufacture and Supply

The study drug and placebo will be sourced and supplied by Mawdsley Brooks UK. Manufacture of ergocalciferol is by Sanofi-Aventis Pharmaceuticals, 300, Somerset corporate boulevard, Bridgewater NJ 08807-2454 USA. Sanofi Aventis hold the Marketing Authorisation and necessary licence for product use. Mawdsley Brooks UK are supplied with ergocalciferol from the manufacturer by Pharmaceuticals Trade Services inc, PO Box 561 Gaitier, MS 39553 USA. Manufacture of the placebo is by

Treatment Regimen

See chapter 2, section 2.2.6

Method for Assigning Subjects to Treatment Groups

See chapter 2, section 2.2.7

Preparation and Administration of Study Drug

All study medication will be prepared and logged in the Royal London Pharmacy department. Medication will be dispensed by trained pharmacy personnel or an investigator. Subjects will be observed swallowing the medication at visits 3, 4, 5, 6, 7 and 8 by either pharmacy staff or an investigator.

Subject Compliance Monitoring

During the first month of the trial, patients will receive nine capsules of study drug. These will be provided in a sealed container which will be checked at subsequent study visits to ensure compliance. This will avoid over frequent visits to the trial clinic and will maximize compliance. At subsequent study visits, which require attendance at the hospital, patients will be observed swallowing their medication to ensure compliance. To enhance compliance, a text message will be sent to patients if they have a mobile phone (and if they have indicated they are happy to receive a message from the study team) on the day they are due to take the study medication. The message will read "Dear NAME this is a reminder to take your vitamin D study medication today. Thank you"

Prior and Concomitant Therapies

Prior therapies:

There will be a washout period of 2 weeks for all vitamin D therapies currently being taken by participants. The most commonly prescribed oral vitamin D analogues all have a half life of less than 14 days (Alfacalcidol— 3 hours, Calcitriol—3 to 6 hours, Ergocalciferol—19 to 48 hours). Participants will be screened for use of all over the counter medicines which may contain vitamin D. It is very unlikely patients will suffer any side effects from stopping these medications for this period. Patients will be reassured about the safety of drug withdrawal and the safety and validity of undertaking a randomized, placebo controlled trial. Given that routine supplementation with vitamin D in early CKD is not routine, we anticipate that very few individuals will actually need to stop vitamin D analogues.

The patient's general practitioner will be contacted to ensure no medicines that may contain vitamin D are being taken by participants without their prior knowledge and that no depot vitamin D preparations have been dispensed in the last 6 months.

Concomitant therapies:

Blood pressure medication should be unchanged for study period but additional therapies can be added at the discretion of the investigators if blood pressure requires clinical intervention on safety grounds. General practitioners will be asked to maintain BP medications at a stable dose. Any changes in medication by the study team will be communicated to GP's in writing and included in the CRF. Patients will be asked to

take any concomitant therapies which can affect microvascular function after clinic visits at which these assessments are made (visits 2,3,4,6,9)

Drugs which are likely to have these particular effects include aspirin and vasodilating anti-hypertensives such as calcium channel blockers. There is no evidence to suggest that iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) is directly affected by the presence of any antihypertensive drugs. However, there is a theoretical chance that there may be some affect on vasodilatation particularly by drugs with a known vasodilatory effect eg calcium channel blockers. Hence, patients will be asked to take all their daily medications after all microcirculatory assessments have been made.

Aspirin has been shown to affect microcirculatory response to ACh and SNP but this effect can be corrected by measuring skin resistance²⁸³. However, another group has shown no effect of aspirin on the response to ACh. The maximum half life of aspirin is 20 hours at high doses but is as low as 6 hours when lower doses are used and therefore even a dose taken the day before iontophoresis assessments is very unlikely to affect the results.

Patients will be asked at each visit if they have been prescribed or if they have bought any other medicinal or herbal product which may interfere with the study. If a new drug is being taken, a decision to withdraw the subject will be made by the Principal Investigator based on its pharmacology and pharmacokinetics. All details of concomitant medications will be recorded in the Case Report Form (CRF).

There are no dietary restrictions or restrictions on the use of tobacco or alcohol during this study but patients will be asked to refrain from smoking for 12 hours before undergoing microcirculatory assessments.

Packaging

The packaging and labelling of the investigational medicinal product will be in accordance with applicable local regulatory requirements.

Study medicine kept at the pharmacy in the Royal London Hospital will be in sealed containers with appropriate clinical trial labelling. Each container will contain nine (9) capsules. Patients will receive a labelled container of nine (9) capsules to take home with them after study visit three. These will be labelled in accordance with trial protocol.

Blinding of Study Drug

Both the investigators and patients will be blinded to the study drug. Mawdsley Brooks UK will facilitate randomisation and blinding of the study subjects. A master randomisation list will be held at Mawdsley Brooks UK with a copy kept at the Royal London Hospital Pharmacy department.

Receiving, Storage, Dispensing and Return

Receipt of Drug Supplies

The investigator is responsible for ensuring IMP accountability, including reconciliation of IMP and maintenance of IMP records, throughout the trial in accordance with regulatory requirements. Upon receipt of IMP, the investigator (or Pharmacist) will check for accurate delivery and acknowledge receipt and signing (or initialling) and dating the documentation provided by the sponsor and returning it to the sponsor. A copy will be retained for the investigator file.

Storage of study drug

The IMP should be stored at room temperature (25°C / 77°F) in its original container. On site, all IMP should be stored in a secure location, in a temperature controlled environment, preferably with a temperature log maintained daily, and may be dispensed only by the investigator or by a member of staff specifically authorised by the investigator, or by the pharmacist, as appropriate. Any deviations from the recommended storage conditions should immediately be reported to the sponsor and the use of the IMP interrupted until the Sponsor has given authorisation for its use.

Dispensing of Study Drug

The dispensing of the IMP will be carefully recorded on the appropriate drug accountability forms provided by the sponsor, and accurate accounting will be available for verification by the sponsor, and the sponsors monitor at each visit. Information recorded will include:

Dates, quantities, batch numbers, kit numbers for IMP, expiry dates and trial numbers assigned to the subjects.

Return or Destruction of Study Drug

Any unused IMP must not be discarded or used for any purpose other than the present trial. Subjects should be instructed to return any unused IMP and all empty blisters and packaging.

In summary the IMP accountability records will include:

Confirmation of the IMP delivery to the trial site

The inventory at the site of IMP provided by the Sponsor

The use of each dose by each subject

Any returns or unused product

Dates, quantities, batch numbers, kit numbers for IMP, expiry dates and trial numbers assigned to the subjects.

Laboratory Assays

See chapter 2, section 2.2.14.6

Study Procedures and Schedule of Assessments

See sections 2.2.11, 2.2.12, 2.2.13, 2.2.14

Statistical Plan and Sample Size Determination

See chapter 2, section 2.2.15

Informed consent procedures

It is the responsibility of the Investigator, or a person delegated by the Investigator (the delegation log needs to spell out who is authorised to take consent, only GCP trained individual can take consent) to obtain written informed consent from each subject prior to participation in this study, following adequate explanation of the aims, methods, anticipated benefits and potential hazards of the study.

Ample time must be given for consideration by the patient before taking part. The PI must record when the patient information leaflet (PIL) has been given to the patient. [If the amount of time between the PIL being given and the date of consent is less than 24 hours, the PI needs to explain why this is the case in this study].

The Investigator or designee must explain the subjects are completely free to refuse to enter the study or to withdraw at any time during the study, for any reason.

If new safety information results in significant changes in the risk/benefit assessment, the consent form should be reviewed and updated if necessary. All subjects, including those already being treated, should be informed of the new information, giving a copy of the revised form and give their consent to continue in the study.

Safety and Adverse Events

Expected Adverse Events

1. Hypercalcaemia
2. Starting any form of renal replacement therapy (haemodialysis, peritoneal dialysis, kidney transplant)
3. Mild skin erythema over the site of the iontophoresis ion chambers
4. Undergoing surgery for dialysis access
5. Hospital admission due to sepsis
6. Admission to hospital due to poorly controlled blood pressure

7. Hospital admission for any form of cardiovascular event eg stroke, myocardial infarction etc.
7. Claustrophobia in MRI scanner
8. Mild skin reaction to ECG labels
9. Any elective admission to hospital which is unrelated to the study protocol or admission to hospital as a result of an unrelated medical procedure
10. Allergic reaction or any other medical problems related to contrast media from the cardiac MRI scan.

Definition of Adverse Events

Adverse Event

An AE is any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporarily associated with the use of an Investigational Medicinal Product (IMP), whether or not considered related to the IMP. All such events during this trial will be recorded in the CRF.

Adverse Reaction (AR)

An AR is any untoward and unintended response in a subject to an Investigational Medicinal Product (IMP), which is related to any dose administered to that subject. All adverse events judged by either the reporting investigator or the Sponsor as having a reasonable causal relationship to a medicinal product qualify as adverse reactions. The expression reasonable causal relationship means to convey in general that there is evidence or argument to suggest a causal relationship.

Serious Adverse Event (SAE)

An SAE fulfils at least one of the following criteria:

- Is fatal – results in death (NOTE: death is an outcome, not an event)
- Is life-threatening
- Requires inpatient hospitalisation or prolongation of existing hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect

THE ABOVE IS A BROAD DEFINITION OF AN SAE. HOSPITALISATIONS FOR ELECTIVE PROCEDURES AND CERTAIN EXPECTED ADVERSE EVENTS MAY BE EXEMPT FROM THIS REPORTING PROCESS, IF SPECIFIED IN THE TRIAL PROTOCOL. ADDITIONAL SIGNIFICANT MEDICAL EVENTS MAY BE CLASSED AS SAES.

Suspected Serious Adverse Reaction (SSAR)

An SSAR is an adverse reaction that is classed as serious and which is consistent with the information about the medicinal product as set out in the Summary of Product Characteristics (SmPC) or Investigator's Brochure (IB) for that product.

Suspected Unexpected Serious Adverse Reaction (SUSAR)

The definition of a SUSAR is any suspected unexpected adverse reaction related to an IMP that is both unexpected and serious. In this case the event is not outlined in the Summary of Product Characteristics (SmPC) or Investigator's Brochure (IB) for that product.

Critical Adverse Events

A critical adverse event is an event which may not be classified as serious but is considered to be important to the evaluation of safety. These events may become apparent as the trial progresses and requires close communication between the sponsor and investigators.

Recording of Adverse Events

All events will be recorded on the Adverse Event forms in the patient's record and a copy will be kept in the CRF.

Notification and Reporting of Serious Adverse Events/SUSAR

For UK MAI IMP licensed IMPs only: As the IMP's used in this project are licensed in the UK and used within their marketing authorization, the EXPECTED SARs (outlined in the SmPCs) will be RECORDED in the subjects notes and in the CRF. No SAE forms will be completed and sent to the sponsor.

UNEXPECTED Serious Adverse Event (SAE's) will be recorded in the subjects notes, the CRF and in the sponsor SAE form and reported to the JRO within one working day of the PI or co-investigators becoming aware of the event. The co-investigators listed in this protocol will be authorized to sign the SAE forms in the absence of the PI. Suspected Unexpected Serious Adverse Reactions (SUSAR's) during the trial will be reported to the JRO and the main REC within one working day of the PI or co-investigator becoming aware of the event.

When Adverse Events are Recorded

Events will be recorded at each patient visit. Patient's will be asked specifically if any expected adverse events have occurred or if any other events have occurred in the interval between the last patient visit. Non-leading questions such "How do you feel?" and "have you had any problems since I last saw you?" will be asked at each visit.

Study Stopping Rules

Subjects who are withdrawn because of serious adverse events (including a Grade 3 or 4 toxicity (based on the Common Toxicity Criteria (CTC) version 3.0 – included with this document) will not be replaced. The Ethics Committee will be notified in writing of any study withdrawals that may occur as a result of toxicity.

The study will be stopped if there are any major safety concerns involving the patients involved in the trial as raised by the sponsor or the principal investigator. The study will end when the last patient has received the last dose of study medication and undergone the final assessments.

Unblinding Procedures

Patients and investigators will be unblinded if they are admitted to hospital with hypercalcaemia. Unblinding will be required as there are a number of common medical conditions (myeloma, tuberculosis, sarcoidosis etc) that can cause hypercalcaemia and should be ruled out urgently if a study patient who is taking placebo rather than ergocalciferol presents with symptoms/signs of hypercalcaemia.

Mawdsley Brooks UK will hold a master randomisation list which will be able to identify which arm any subject is in. There is a 24 hour trial hotline which can be contacted by clinical staff at any time should unblinding be required for medical reasons. Furthermore, individual code break envelopes will be available to clinicians at the Royal London Hospital. All renal staff will have access to code break procedures and envelopes which will be locked in a secure office with an entry code on the door.

Code-breaks should only occur when absolutely necessary and beneficial to the patient. In the event of a code break, the sponsor and PI will be informed.

Medical Monitoring

Patients will be reviewed at each visit by an investigator. They will undergo routine evaluations for standard outpatient care as well as more detailed evaluations as required by the trial protocol. This will include a medical history, examination, blood tests for the trial and others as necessary based on clinical evaluation.

The Annual Safety reports (ASR) will be sent by the PI to the sponsor and MHRA (the date of the anniversary is the date on the "notice of acceptance letter" from the MHRA) using the sponsor ASR form. The PI will carry out a risk benefit analysis of the IMPs encompassing all events having arisen on the trial.

The Annual progress report will be sent to the main REC (the anniversary date is the date on the MREC “favourable opinion” letter from the MREC) and to the sponsor.

Data Handling and Record Keeping

Confidentiality

All computers used will be kept on NHS property and password protected. Each patient’s information will be recorded against an individual code which will be stored on a NHS password protected computer. CRF’s will not contain names, rather the study code for that participant and their initials. Every effort will be made to respect patient confidentiality throughout the trial duration.

Study Documents

All relevant documents and correspondence will be kept in the patient’s records. Computerised trial documents will be password protected and held on NHS computers at the Royal London Hospital. The investigator and sponsor will have access to these records.

Case Report Forms

The case report form will contain details of the study name, protocol number, subject initials and trial ID code and other relevant information. It will have a check list for the inclusion and exclusion criteria. It will also contain reporting forms for AE’s, SAE’s and SUSAR’s.

CRF’s for all patients, including those excluded from the study for any reason, will be kept and maintained by the PI. CRF forms are to be completed in black pen and in their entirety with no blank spaces allowed unless data is not available or applicable. This fact must be indicated. Corrections must be struck-through and the correct information entered adjacent to this and initialled and dated by the investigator. Completed CRF’s are to be returned to the sponsor as soon as is practical. Copies are to be retained by the Principal investigator.

Records Retention

All records will be held in the archive system for 20 years following the start of the trial.

Study Monitoring, Auditing, and Inspecting

Study Monitoring Plan

The principal and chief investigator will meet after three months to review AE’s, SAE’s and SUSAR’s. The trial may be subject to routine auditing by Research Governance at Barts and the London NHS Trust.

Phase IV trials:

It is the CI's responsibility to ensure that the BLT monitoring template (designed especially for the BLT/QM sponsored CTIMPs) is completed in a CONTINUOUS fashion throughout the study and kept up to date by the co-investigators (for the first part of the report) and by the MONITORS NAMED ON THE FIRST PAGE OF THIS PROTOCOL (for both the first part and the source data verification part of the template.). This trial is a Phase IV trial, using IMPs licensed in the UK and used within their marketing authorisation, this project is therefore risked as a "low risk (risk B)" project and the monitoring report will be sent to the JRO a year after the first consent has been signed and annually thereafter.

Ethical Considerations

General considerations:

The study will be conducted in accordance with the principles of the Declaration of Helsinki (1997) (Recommendations guiding Medical Doctors in Biomedical Research Involving Human Subjects).

Every effort will be made to minimize discomfort and disruption to the trial participants.

All relevant study documentation will be submitted to a National Research Ethics Service (NRES) and no trial activity will begin until approval from this body has been received. A copy of ethical approval will be forwarded to the sponsor. If ethical approval is suspended or terminated, the sponsor will be informed immediately. Trial progress will be reported to the ethics committee once a year. All SAE's will be reported to the ethics committee as soon as possible and at least within 72 hours.

Study specific ethical considerations:

1. The endpoints of this trial (both in vivo and in vitro) have never been addressed in the setting of a randomized clinical trial. New and relevant information which will change practice to enhance patient care and could influence CKD guidelines can be generated by undertaking this study .
2. The study drug, ergocalciferol, is in routine use for vitamin D deficiency in a number of clinical settings. It has a low side effect profile and is considered to be safe and efficacious.
3. Placebo controlled trials have been in routine use in medical research for years. If a benefit is shown in the ergocalciferol arm, patients who have received placebo can be eligible for ergocalciferol treatment when the trial ends.
4. Secondary hyperparathyroidism (SHPT) is a recognized consequence of CKD and is treated by vitamin D therapy. It is possible that patients with modestly elevated PTH

levels will receive placebo tablets. This means that their SHPT will go untreated for 6 months. However, an analysis of PTH levels in an existing group of patients at the Royal London Hospital with CKD and vitamin D deficiency has shown a low median PTH value (median 11.7, standard deviation 19.44 pmol/L). This level of PTH is unlikely to have any serious clinical consequences if not treated over the trial duration and we do not believe patients are at risk by potentially not receiving ergocalciferol. Patients will be reassured about the safety of the drug and the safety and validity of undertaking a randomized, placebo controlled trial.

5. Patients will be required to attend more frequently than normal and have more blood tests (with the associated discomfort) than they might expect in their routine care. Other tests will be required which patients may not have had. A full explanation and support will be offered to patients at every stage of the trial. We will undertake as many trial procedures in a single visit as possible to minimize disruption to the patients.

6. Blood and urine samples will be stored for further analysis relating to the trial. Patients will be clearly informed of this.

Local Regulations/Declaration of Helsinki

I will ensure that this study is conducted in accordance with the Principles of the “Declaration of Helsinki” (as amended in Tokyo (1975), Venice (1983), Hong Kong (1989), South Africa (1996)). <http://www.wma.net/e/policy/b3.htm> or with the laws of the country in which the research is conducted, whichever accords greater protection to the individual. The study must fully adhere to the principles outlined in the Guidelines for Good Clinical Practice” ICH Tripartite Guideline (January 1997)

Informed Consent (any special conditions i.e. emergency situations?)

All subjects will provide written informed consent before enrolling in this trial. A full explanation of the study aims and procedures will be given in writing and explained face-to-face by the chief investigator. Patients will have at least 24 hours to decide on their participation. It will be made clear that patients can refuse to participate and withdraw from the trial at any stage for any reason. If any new information becomes available about any of the study drugs or techniques, patients will be informed and if this information changes the risk /benefit profile of the trial, a new consent form will be issued and informed consent will be obtained. Patients will be informed that the trial investigators may need to release clinical details of a patient to other health care professionals in an emergency situation relating to the trial. The date of informed consent will be documented in the CRF. The patients will be given a copy of the consent form.

Independent Ethics Committee

All relevant information and forms will be submitted to an external ethics committee by the investigators.

This protocol and the accompanying material given to a potential patient (Patient Information Sheet, Consent form and GP letter) as well as any advertising material will be submitted by the Investigator to an Independent Ethics Committee in the UK. Full approval by the Committee will be obtained prior to starting the study and will be fully documented by letter to the Chief Investigator naming the study site, local PI (who may also be the Chief Investigator) and date the Committee deemed the study as permissible at that site.

Study Finances

Funding Source

All costs associated with this study will be covered wholly by:

Barts and the London Trust

Royal London Hospital

Whitechapel Road

London

E1 1BB

Contact: Dr Alistair Chesser, Department head

Indemnity for the performance of the study

This trial will be covered by standard NHS negligence indemnity

Subject Payments

Subjects will not receive payment for this trial

Sponsorship

Barts and the London NHS trust, Whitechapel Road, London E1 1BB

Publication Plan

The trial results will be published in a peer reviewed journal on completion of the study. Manuscripts for publication (abstract and full text articles) will be reviewed by the principal investigator and be made available to the Sponsor for review prior to submission.