Effects of vitamin D receptor activators on vascular calcification in patients with CKD

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UCL

Declaration

I, Joanne Louise Laycock confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Chronic kidney disease (CKD) patients are at high risk of vascular calcification due to abnormal mineral metabolism, and potentially their treatment with vitamin D receptor activators (VDRAs; calcitriol, alfacalcidol and paricalcitol). The effect of VDRAs on calcification is not fully understood. This thesis compares physiological doses of VDRAs on vascular smooth muscle cell (VSMC) calcification in vessels from children and adults with CKD.

The inferior epigastric artery was harvested during renal transplantation and vessel rings cultured in different calcium (Ca) and phosphorous (P) media with physiological doses of VDRAs. The Ca load and alkaline phosphatase (ALP) activity were quantified and histological analysis performed.

In children's vessel rings, calcitriol increased Ca load by 3.6 fold and ALP activity by 2.2 fold compared to vehicle in high Ca and P medium, but two distinct groups of responders and non-responders to calcitriol were noted. Alfacalcidol increased Ca load by 3.4 fold but had no effect on ALP activity and paricalcitol had no effect on Ca load or ALP activity in any vessel rings. Patient variation was observed: this was independent of dialysis status and renal diagnosis. None of the VDRAs tested affected Ca load or ALP activity in adult vessel rings.

VSMCs were explanted and their dose dependent responses to calcitriol (1, 10 and 100nM) documented. 100nM calcitriol elicited the greatest upregulation in vitamin D dependent gene expression, including the vitamin D receptor (VDR) whose expression was greater in VSMCs explanted from dialysis patients than non-renal controls. Expression of two VDR isoforms (VDR-A and VDR-B) were shown by Western blot analysis. VDR-A expression as a percentage of total VDR was 30$\pm$6.5% in control VSMCs compared to 65$\pm$12% in dialysis VSMCs. VSMCs in which 100nM calcitriol activated the vitamin D response element (VDRE) luciferase, expressed 70$\pm$17% VDR-A compared to 6.8$\pm$2.5% in VSMCs with no VDRE luciferase activation. Patient variation in VDR isoform expression may contribute to the individual patient's responses to VDRAs in both vessel rings and VSMCs.

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Abbreviations

19-nor-1,25$(OH)_2D_2$	19-nor-1α, 25 dihydroxy vitamin D_2 (paricalcitol)
$1OHD_3$	1α hydroxyvitamin D_3 (alfacalcidol)
1,25$(OH)_2D$	1α, 25 dihydroxyvitamin D (calcitriol)
25OHD	25 hydroxyvitamin D
ALP	Alkaline phosphatase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BMD	Bone mineral disorder
BMP-2	Bone morphogenetic protein 2
BSA	Bovine serum albumin
BSP	Bone sialoprotein / Osteopontin
BGP	Bone gla protein / Osteocalcin
Ca	Calcium
CaSR	Calcium sensing receptor
CKD	Chronic kidney disease
cMGP	Carboxylated MGP
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
Dp-ucMGP	Dephosphorylated uncarboxylated MGP
ESKD	End stage kidney disease
FBS	Fetal bovine serum
FGF23	Fibroblast growth factor 23
FGFR	Fibroblast growth factor receptor
GFR	Glomerular filtration rate
H and E	Haematoxylin and eosin
HBSS	Ca free Hanks balanced saline solution
HCl	Hydrochloric acid
HD	Haemodialysis
HGPS	Hutchinson Guilford progeria syndrome
MACS	Myeloid angiogenic cells
MGP	Matrix gla protein
P	Phosphorous
PBS	Phosphate buffer saline
Pi	Inorganic phosphate

PD	Peritoneal dialysis
PFA	Paraformaldehyde
P-Np	Para-nitrophenol
P-Npp	Para-nitrophenol phosphate
PSG	Penicillin, streptomycin and glutamate
PTH	Parathyroid Hormone
RANKL	Receptor activator of nuclear factor kappa-B ligand
RXR	Retinoid X receptor
SE	Standard error
SLE	Systemic lupus erythematosus
SMA	Smooth muscle α actin
SMC	Smooth muscle cell
SMMHC	Smooth muscle myosin heavy chain
TNF-α	Tumour necrosis factor alpha
TRPV6	Transient receptor potential cation channel, subfamily V, member 6,
uMGP	Uncarboxylated MGP
VDR	Vitamin D receptor
VDRA	Vitamin D receptor activator
VDRE	Vitamin D response element
VDR-A	Vitamin D receptor A isoform
VDR-B	Vitamin D receptor B isoform
VSMC	Vascular smooth muscle cell

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Chapter 1 Introduction

1.1 Chronic Kidney Disease is associated with increased cardiovascular risk.

Chronic Kidney Disease (CKD) is a progressive disorder, characterised by a gradual decline in functional nephrons and a reduction in Glomerular Filtration Rate (GFR). CKD is defined by a GFR below 60ml/min (Levey et al., 2005). The incidence and prevalence of kidney failure is an increasing worldwide health problem (Eknoyan et al., 2004). As the GFR deteriorates below 60ml/min, there is a graded increase in the risk of cardiovascular morbidity (Go et al., 2004). CKD culminates in End Stage Kidney Disease (ESKD); by this stage, patients require renal replacement therapy in the form of dialysis.

Cardiovascular disease is the most common cause of death in CKD patients receiving dialysis, the rate of cardiovascular mortality in dialysis patients in their 20s is comparable to octogenarians (Foley et al., 1998). In ESKD there are disturbances in mineral metabolism that lead to hypercalcaemia and hyperphosphatemia, predisposing patients to vascular calcification (Shroff et al., 2012). The high risk of cardiovascular mortality in CKD patients is largely due to vascular calcification.

1.2 Mineral metabolism

1.2.1 The role of the kidney, FGF23 and PTH in Ca and P homeostasis.

The kidney is a major regulator of serum phosphorous (P); it is important for both P excretion and regulating the resorption of P and Calcium (Ca) to accommodate bone turnover. The kidney relies on autocrine signalling from fibroblast growth factor 23 (FGF23) to maintain P homeostasis (Kuro-o, 2010b) (Shimada et al., 2004b). When serum P levels are high, FGF23 is synthesised by osteocytes and osteoblasts to raise circulating levels of FGF23. FGF23 binds to the fibroblast growth factor receptor (FGFR) on the basolateral membrane of the kidney tubules.

As shown in Figure 1-1 binding of FGF23 to the FGFR has 2 downstream effects to reduce serum P towards homeostatic levels (Mazzaferro et al., 2010):

1. FGF23 blocks the synthesis of 1α hydroxylase (required for activation of vitamin D) and upregulates the synthesis of 24 hydroxylase (an enzyme that deactivates vitamin D). The combined effect is reduced levels of active vitamin D (Shimada et al., 2004a). This prevents further increase of serum P as vitamin D promotes bone turnover and resorption of Ca and P.

2. FGF23 blocks the synthesis of, and increases endocytosis of the Na/P cotransporter on the apical membrane of the kidney tubule (Shimada et al., 2004a). The reduced number of P transporters decreases P reabsorption from the filtrate therefore increasing P excretion.

In order for FGF23 to bind to its receptor, the co-receptor klotho is required; in the presence of klotho the FGFR is conferred specific to FGF23 (Urakawa et al., 2006) (Kurosu et al., 2006). Klotho was originally identified in mice as a gene that was mutated in a premature aging phenotype, this included arteriosclerosis, growth retardation and skin atrophy (Kuro-o et al., 1997). Many studies have shown that defects in the FGF23-klotho axis cause premature aging syndrome due to increased P, Ca and vitamin D (Kuro-o, 2010c). A low P diet in FGF23 deficient mice, rescued many of the premature aging phenotypes despite further raising Ca and vitamin D levels. This suggests that P is primarily responsible for the premature aging phenotype (Kuro-o, 2010c).

Figure 1-1: Dual mechanism FGF23 to reduce serum P.

Vitamin D increases the transcription of fibroblast growth factor 23 (FGF23) in osteocytes by binding to the vitamin D receptor (VDR) which interacts with the retinoid x receptor (RXR). Phosphorous (P) also increases transcription of FGF23, the mechanism of this has not yet been determined. Klotho confers the fibroblast growth factor receptor (FGFR) specific to FGF23 and the FGF23 secreted by osteocytes interacts with the klotho-FGFR complex on the basal membrane of kidney tubules. FGF23 blocks the synthesis of and increases endocytosis of the NaPi-2a cotransporter on the apical membrane of the kidney tubule. This decreases P reabsorption and increases P excretion via the urine to complete the negative feedback loop and maintain P homeostasis. FGF23 also maintains vitamin D homeostasis, it increases Cyp24 and decreases Cyp27b1 transcription to promote inactivation and suppress synthesis of 1,25 dihydroxyvitamin D. Thereby reducing serum vitamin D levels and the vitamin D induced resorption of Ca and P from bone (Kuro-o, 2010a).

Hyperphosphatemia also up-regulates parathyroid hormone (PTH) secretion. PTH promotes P and Ca resorption from bone raising serum levels further, however PTH also increases P excretion therefore its overall effect is to decrease serum P (Koeppen. B. M, 2010). Excessive PTH secretion is prevented by FGF23 which acts as a negative feedback mechanism and binds to the FGFRs in the parathyroid gland to downregulate PTH secretion. As in the kidney tubules, these FGFRs require klotho to confer them specific to FGF23 (Kurosu et al., 2006).

The parathyroid gland is well known to contain Calcium Sensing Receptors (CaSR) and PTH is the primary regulator of serum Ca and is secreted when serum Ca levels are low (Souberbielle et al., 2010). PTH has 3 mechanisms of action to increase serum Ca as shown in Figure 1-2.

1. PTH increases Ca reabsorption from the kidney tubule.

2. PTH upregulates transcription of Cyp27b1 in the kidney to increase 1α hydroxylase activation of vitamin D. This leads to increased serum levels of 1,25 dihydroxyvitamin D which increases Ca absorption from the small intestine.

3. PTH and 1,25 dihydroxyvitamin D both increase bone turnover by upregulating the expression of RANKL in osteoblasts, RANKL drives the maturation of pre-osteoclasts to mature osteoclasts. Osteoclasts resorb the bone matrix and increase resorption of both Ca and P into the serum.

Once normal serum Ca levels are restored the stimuli to upregulate PTH secretion is removed therefore acting as a negative feedback mechanism.

Recent data has shown that the kidney itself also expresses CaSR predominantly in the thick ascending limb of the loop of Henle and has a role in the regulation of Ca homeostasis independent of PTH. This was shown in thyroparathyroidectomized, PTH supplemented rats where renal tubular Ca reabsorption was increased by chronic inhibition of CaSR independently of the PTH (Loupy et al., 2012).

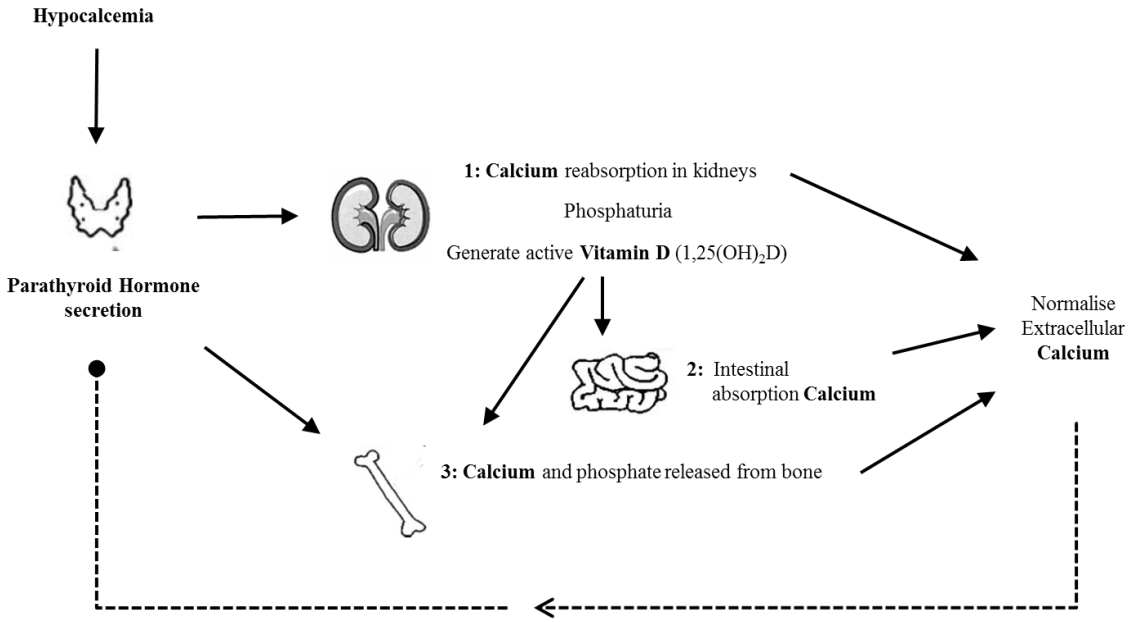


Figure 1-2: Role of PTH in Ca homeostasis.

Hypocalcaemia is detected by Ca sensing receptors (CaSRs) in the parathyroid gland and stimulates parathyroid hormone (PTH) secretion. PTH restores normal serum Ca by; increasing Ca reabsorption from the kidneys, upregulates Cyp27b1 transcription for the activation of vitamin D which increases Ca absorption in the intestine and increases Ca and P resorption from bone.

1.2.2 The impact of CKD on FGF-23, PTH and Ca and P homeostasis.

In CKD, P excretion by the kidney is impaired therefore raised serum P is associated with CKD. In the early stages of CKD, impaired kidney function is compensated for by increased FGF23 production normalising serum P. As GFR declines further serum P is increased despite high serum FGF23 (John et al., 2011). CKD is associated with klotho deficiency, in the absence of klotho the FGFR is not conferred specific to FGF23 and FGF23 is unable to act on the kidney tubule to reduce serum P towards normal levels (Hu et al., 2012). This P retention leads to persistent hyperphosphatemia and stimulates a further increase in FGF23 levels however homeostatic mechanisms can no longer restore the P balance.

Chronic hyperphosphatemia results in parathyroid cell proliferation, parathyroid gland hyperplasia and secondary hyperparathyroidism (Koizumi et al., 2013b). Excessive PTH secretion contributes to excessive bone turnover, reducing bone density and leading to CKD-Bone Mineral Disorder (CKD-BMD) (Moe et al., 2009), as well as chronic hypercalcemia and chronic hyperphosphatemia which is a direct stimulus for vascular calcification (Hruska et al., 2008).

1.3 Vascular Calcification

1.3.1 What is vascular calcification?

Vascular calcification is the deposition of hydroxyapatite crystals in the extracellular matrix of the vessel wall. Hydroxyapatite is formed from the crystallisation of Ca ions and inorganic P (Pi) ions, it has a mineral composition similar to that found in bone (Schlieper et al., 2010). At physiological pH of 7.4 Pi exists predominantly as $H_2PO_4^-$ and HPO_4^{2-} in a 1:4 ratio and is neutralised by Ca^{2+} ions to produce hydroxyapatite $Ca_{10}(PO_4)6(OH)_2$ (Villa-Bellosta and Egido, 2015)

Once considered a passive degenerative process that occurs in ageing, vascular calcification has now been recognised as a highly regulated, cell mediated process similar to bone ossification (Shroff et al., 2013). A nuclear magnetic resonance study showed that the molecular structure of Ca and P bio-minerals in ectopic calcified human plaque closely resembles that of bone, including a glycosaminoglycan scaffold, suggesting that similar mechanisms regulate physiological and pathological calcification (Duer et al., 2008). Both processes require a microenvironment that enables extracellular crystal growth; this is formed by the release of matrix vesicles into the extracellular matrix, where mineral nucleation and calcification can then occur. To initiate ectopic vascular calcification several molecular processes must occur simultaneously, this includes; apoptosis, osteochondrogenic differentiation, down regulation of mineralisation inhibitors and the release of pro calcific matrix vesicles (Shanahan et al., 2011).

1.3.2 Characteristics of arteriosclerosis are distinct from atherosclerosis.

There are two distinct types of vascular calcification, atherosclerosis and arteriosclerosis. In atherosclerosis, calcification occurs in lipid rich plaques at damaged patches of the tunica intima. Atherosclerosis is associated with traditional cardiovascular risk factors including; age, obesity, dyslipidaemia and smoking (Shroff et al., 2013). Arteriosclerosis (also known as Monckeberg's sclerosis) is associated

with CKD and diabetes, it is characterised by calcification of the vascular smooth muscle cells (VSMCs) in the tunica media. Sheet like calcification forms in the tunica media layer resulting in a concentric thickening of the vessel wall and increased vascular stiffness, that leads to systolic hypertension and left ventricular hypertrophy (Blacher et al., 1999). Although distinct diseases, atherosclerosis and arteriosclerosis can coexist in various combinations particularly in older diabetics and adults with CKD. These patients have been exposed to traditional cardiovascular risk factors for atherosclerosis and disease specific risk factors of arteriosclerosis (McIntyre, 2008). Coronary autopsy samples from renal patients had comparable tunica intima calcification to non-renal patients (atherosclerosis), but a higher proportion of tunica media calcification (arteriosclerosis) (Gross et al., 2007). In young dialysis patients and those without comorbidity, calcification is exclusively in the tunica media (Shroff et al., 2008b). From here onwards vascular calcification will refer to arteriosclerosis.

Figure 1-3: Arteriosclerosis and atherosclerosis.
Von kossa stained vessel rings showing calcified areas. A) Medial calcification observed in arteriosclerosis. B) Intimal calcification of intimal plaque observed in atherosclerosis. **A** – Adventitia. **M** – Tunica media. **MC** – Medial calcification. **IP** – Intimal plaque. **IC** – Intimal calcification. Histology accessed 28/03/2016 from http://library.med.utah.edu/WebPath/CVHTML/CV007.html and http://library.med.utah.edu/WebPath/CVHTML/CV168.html.

1.4 The highly regulated process of vascular calcification and its risk factors in CKD.

Ectopic vascular calcification is a highly regulated process that occurs in the matrix surrounding VSMCs. Dysregulated mineral metabolism in CKD plays a key role in driving calcification, however, high levels of Ca and P alone do not result in the passive deposition of hydroxyapatite crystals in the vasculature (Shroff et al., 2012). Key processes involved in VSMC calcification include: apoptosis, osteo/chondrocytic differentiation, release of matrix vesicles and reduced expression of calcification inhibitors. A combination of risk factors found in the uremic milieu are required to enable this pathological calcification to develop (Shroff et al., 2012).

1.4.1 Apoptosis of VSMCs

Apoptosis of VSMCs is associated with and plays a key role in promoting vascular calcification. High intracellular Ca has been shown to drive apoptotic cell death, and the culture of either VSMCs or vessel rings in high Ca and P (as observed in CKD) reduced VSMC density by 30% (Proudfoot et al., 2000). Apoptotic bodies in the extracellular matrix provide a nidus for the accumulation of hydroxyapatite crystals and the initiation of calcification, it is in part accountable for the increased ectopic calcification observed in CKD as reviewed by (Shroff and Shanahan, 2007). VSMCs in culture undergo apoptosis, in these cells apoptosis has been shown to precede calcification. Inhibition of apoptosis reduced calcification by 40% demonstrating its importance in driving calcification (Proudfoot et al., 2000).

1.4.2 Matrix vesicle release.

The release of mineralisation competent matrix vesicles into the extracellular matrix provides a nucleation site for hydroxyapatite crystals to form, it is important in both physiological and ectopic calcification (Kapustin et al., 2011). Healthy VSMCs release small matrix vesicles into the extracellular matrix, however they do not support

mineralisation as they contain limited hydroxyapatite and are loaded with mineralisation inhibitors; matrix gla protein (MGP), osteopontin and fetuin A that prevent the spontaneous precipitation of Ca and P in the serum (Kapustin et al., 2011).

High Ca and P conditions increased the rate of both vesicle release and apoptosis in human VSMCs (Reynolds et al., 2004). Initially, this may be a defence mechanism to extrude excess hydroxyapatite, however long term increased matrix vesicle release can be detrimental to calcification. VSMCs persistently exposed to the high Ca and P levels observed in CKD released matrix vesicles that contained preformed calcium phosphate $Ca(H_2PO_4)_2$, were depleted of MGP and enabled hydroxyapatite crystal growth (Reynolds et al., 2004). It was later shown by (Kapustin et al., 2011) that raised extracellular Ca was required for release of calcifiable matrix vesicles, these vesicles shared properties with chondrocyte matrix vesicles. This includes expression of Ca binding annexins and exposed phosphatidylserine on the surface of matrix vesicles providing a site for hydroxyapatite nucleation therefore supporting the early stages of ectopic calcification.

1.4.3 Perturbation in the level of physiological calcification inhibitors

Expression of calcification inhibitors MGP, pyrophosphate, fetuin A and osteopontin prevent ectopic calcification in healthy soft tissue.

MGP is endogenously expressed in both VSMCs and chondrocytes. Local expression of MGP in the vessel wall is required for inhibition of vascular calcification, this was shown in an experiment on MGP knockout mice where expression of MGP in VSMCs prevented calcification but high circulating levels of MGP did not (Krueger et al., 2009). MGP is expressed in its inactive form as dephosphorylated uncarboxylated MGP (dp-ucMGP) and requires two post translational modifications, γ-carboxylation and serine phosphorylation to form fully active p-cMGP as reviewed by (Epstein, 2016). Phosphorylation of 3 serum residues seem to be important for MGP secretion (Fusaro et al., 2011). UcMGP has 5 glutamic acid residues which require vitamin K for their γ-carboxylation to form 5 γ-carboxyglutamate (GLA) residues and produce

carboxylated MGP (cMGP). Defects in the vessel wall increase the local expression of ucMGP in VSMCs, however in a state of vitamin K deficiency which often occurs in CKD, ucMGP cannot be activated and accumulates at sites where vascular calcification is able to proceed (Cranenburg et al., 2008). The prevalence and severity of vitamin K deficiency is higher in CKD than the general population for 2 reasons. The first, dietary restrictions in CKD that limit P intake also reduce vitamin K consumption. The second, during γ-carboxylation vitamin K is oxidised and has to be recycled by reduction for subsequent carboxylase activity, CKD patients are often prescribed warfarin (a vitamin k antagonist) which blocks the reductase pathway and prevents vitamin K recycling (Wuyts and Dhondt, 2016). In addition, uremia was shown to reduce vitamin K γ-carboxylase activity in a rat model leading to accumulation of ucMGP and calcification that was reversed by vitamin K treatment (Kaesler et al., 2014).

Pyrophosphate is a potent endogenous inhibitor of calcification, the main source of pyrophosphate in VSMCs is from the hydrolysis of adenosine triphosphate (ATP) to generate AMP and pyrophosphate (Villa-Bellosta and Egido, 2015). Pyrophosphate can be released into extracellular fluid and inhibit mineralisation as shown in Figure 1-4, however it can also be dephosphorylated and inactivated by alkaline phosphatase (ALP). The inhibitory effect of pyrophosphate on calcification has been shown both *in-vitro* and *in-vivo*, physiological levels of 3-5μM pyrophosphate completely inhibited calcification in rat aortic VSMCs (Villa-Bellosta and Sorribas, 2011). Pyrophosphate treatment also inhibited vascular calcification in Hutchinson Guilford Progeria Syndrome (HGPS) mice. This phenotype was associated with increased ALP activity and reduced ATP synthesis therefore increased degradation and defective production of pyrophosphate resulting in calcification prone mice (Villa-Bellosta et al., 2013).

Plasma levels of calcification inhibitor pyrophosphate were found to be lower in haemodialysis patients (2.26 + 0.19 uM) compared to healthy patients (3.26 + 0.17 uM). In the haemodialysis patient's pyrophosphate was reduced by a further 32% immediately post dialysis (Lomashvili et al., 2005). In addition, the upregulation of ALP observed in CKD (discussed below) would upregulate the de-phosphorylation and inactivation of pyrophosphate.

Fetuin A is a circulating calcification inhibitor that is taken up by VSMCs and concentrated in matrix vesicles, it reduces apoptosis and promotes phagocytosis of matrix vesicles by VSMCs therefore reducing their ability to nucleate P and Ca (Reynolds et al., 2005). Fetuin A levels are correlated with GFR and there was a graded decline in fetuin A from CKD stage 3-5 (Zhan et al., 2013).

Osteopontin is normally expressed in mineralised tissue such as bone and teeth (Nemcsik et al., 2012). Post translational phosphorylation is required for osteopontin to have an inhibitory effect on calcification, this was shown in human VSMCs where only the phosphorylated osteopontin was able to dose dependently inhibit calcification (Jono et al., 2000). Osteopontin was also shown to accumulate in calcified vessels and reduce the extent of calcification *in-vivo,* as double knock out MGP/osteopontin mice had more extensive calcification than MGP only knockout mice (Speer et al., 2002), demonstrating the calcification inhibitory properties of osteopontin. As with pyrophosphate, the upregulated ALP activity observed in CKD leads to dephosphorylation and inactivation of osteopontin, reducing its effect on inhibiting calcification.

1.4.4 Osteo / chondrocytic differentiation

VSMCs, osteocytes, chondrocytes and adipocytes are all derived from mesenchymal stem cells. The terminal differentiation of these stem cells lines is dependent on the paracrine and autocrine factors in the microenvironment (Oreffo et al., 2005).

VSMCs have great phenotypic plasticity and are able to dedifferentiate into mesenchymal-like stem cells this is important during cell stress and for vascular repair, they may either reach senescence or undergo an osteo/chondrocyte phenotypic change (Opitz et al., 2007). This is evidenced by changes in the expression of VSMC and osteo / chondrocyte cell markers.

A cell linage study in mice lacking the calcification inhibitor MGP, found that 97% of calcifiable cells in the tunica media were derived from VSMCs which had early up

regulation of Runx2 (an osteoblast transcription factor) and down regulation of myocardin (Speer et al., 2009). Notably the osteo/chondrocytic differentiation preceded calcification.

In human arteries with Monckeberg's sclerosis that undergo osteo/chondrocytic differentiation, up regulation of ALP has been observed in the vessel wall along with other osteogenic markers including bone gla protein (BGP), bone sialoprotein (BSP) and collagen II (Shanahan et al., 1999). There expression is regulated by osteogenic transcription factors, such as Runx2 and Sox9 which are also up-regulated in calcified vessels and in VSMCs that spontaneously calcify (Tyson et al., 2003).

In-vitro co expression of ALP and collagen I was sufficient to induce mineralisation in high P medium (Murshed et al., 2005). This suggests that increased ALP activity alone would enable extracellular matrix mineralisation in the vasculature as the tunica media has a collagen rich matrix. ALP is a hydrolase enzyme normally expressed in bone but not normally expressed in VSMCs as shown in Figure 1-4. ALP dephosphorylates the calcification inhibitor pyrophosphate which renders it inactive and releases free inorganic phosphate (Pi) (Villa-Bellosta and Egido, 2015). Also, in an *in-vitro* study on human VSMCs ALP treatment dephosphorylated the calcification inhibitor osteopontin and promoted calcification (Jono et al., 2000).

Excessive P levels observed in CKD is thought induce cellular stress and drive the oseto / chondrocytic differentiation of contractile VSMCs to a synthetic osteoblast like phenotype. High serum P results in the downregulation of VSMC markers, SMα actin, SM22α and myocardin along with upregulation of osteo/chondrocytic genes including; Runx2, osterix, ALP, osteopontin, type 1 collagen and osteocalcin (Shanahan et al., 2011). Culture of human VSMCs in uremic conditions found in CKD changed the VSMC phenotype by decreasing expression of VSMC contractile marker genes by 50-80% (Monroy et al., 2015). In addition, osteoblast marker ALP is thought to be upregulated in CKD, a circulating factor present in uremic serum increased ALP activity by two fold which in turn increased pyrophosphate hydrolysis enabling calcification (Lomashvili et al., 2008).

Figure 1-4: Schematic diagram showing effect of ALP activity on pyrophosphate in VSMCs and osteoblasts.

In healthy VSMCs, pyrophosphate generated from the hydrolysis of ATP inhibits mineralization, there is no ALP activity. In osteoblasts, pyrophosphate is dephosphorylated and inactivated by ALP enabling mineralization. In uremia, pathological upregulation of ALP contributes to calcification. Image adapted from (Persy and McKee, 2011)

1.5 Vitamin D

1.5.1 Vitamin D and the systemic regulation of Ca

In the late 1800s and early 1900s, rickets was a disease of epidemic proportions, the healing properties of both cod liver oil and exposure of the skin to UV light led to the discovery of vitamin D between 1919 and 1924 as reviewed by (DeLuca, 1988). Vitamin D was produced as a pharmacologic treatment by the 1940s and used in the treatment of bone mineral disorders such as rickets.

The role of vitamin D in bone mineralisation is through the elevation of plasma Ca and P via 3 key mechanisms.

1. **Increase Ca absorption from the small intestine**. Vitamin D increases transcription of the Ca channel TRPV6 and calbindin in the small intestine to increase the efficiency of Ca absorption from 10 to 40% (Holick 2007).

2. **Increase resorption of Ca and P from bone.** Vitamin D up regulates RANKL expression in osteoblasts and drives the maturation of pre-osteoclasts to osteoclasts which resorb Ca and P from bone and release it into the circulation (Turner, Hanrath et al. 2013).

3. **Reduce PTH secretion.** Vitamin D increases expression of the vitamin D receptor (VDR) and the CaSR in the parathyroid glands to increase its sensitivity to both Ca and vitamin D. The effect of vitamin D on the parathyroid gland is to downregulate PTH expression in order to prevent excessive Ca and P resorption and extensive bone turnover (Brown, Dusso et al. 2002).

1.5.2 Vitamin D metabolism

Vitamin D is a term used to describe several related compounds including calciferol, calcidiol and calcitriol. Calciferol is pre-vitamin D and can refer to either pre-vitamin D_2 (ergocalciferol) or pre-vitamin D_3 (colecalciferol), these are the 2 major forms of vitamin D in their biologically inactive forms. To be activated calciferol undergoes 2 hydroxylation stages to produce calcidiol (25OHD) and then calcitriol ($1,25(OH)_2D$).

The majority (>90%) of the body's required vitamin D is obtained from sunlight (Holick, 2007). Exposure to UV-B radiation converts the skin pigment 7-dehydro cholesterol to pre-vitamin D_3. Low levels of pre-vitamin D_2 and D_3 can also be obtained from some foods such as oily fish. Both pre-vitamin D_2 and D_3 have to undergo two hydroxylation stages in order to activate them as shown in Figure 1-5. The first stage is catalysed by 25 hydroxylase and converts pre-vitamin D_2 and D_3 to 25-hydroxylated vitamin D (25OHD). The 2^{nd} stage is catalysed by 1α hydroxylase and converts 25OHD to the active form 1α, 25 dihydroxyvitamin D ($1,25(OH)_2D$). The majority of 1α and 25 hydroxylases are expressed in the kidney and liver respectively, however both hydroxylase enzymes are expressed locally in VSMCs and other tissues enabling an autocrine/paracrine regulation of the cells vitamin D metabolism.

The series of hydroxylation stages required for the metabolism of vitamin D are regulated by a group of cytochrome P450 enzymes (CYPs). CYPs capable of 25 hydroxylation include the low affinity CYP27A1 and the high affinity CYP2R1 as well as potentially the less well defined CYP2J3 and CYP3A4 (Jones et al., 2014). The biological importance of CYP2R1 was confirmed by analysis of a patient with a homozygous Leu99Pro mutation in CYP2R1 which eliminated 25 hydroxylase enzyme activity and correlated with a form of hereditary rickets (Cheng et al., 2004). 25 hydroxylation occurs in a substrate dependent manner therefore 25OHD is the main form of vitamin D found in circulation and clinically vitamin D status is determined by measuring 25OHD in the serum.

The activation of 25OHD to $1,25(OH)_2D$ is highly regulated, this is important as it has a key role in maintaining homeostatic levels of Ca and P in the serum as discussed on

page 31. So far only one gene has been identified encoding 1α-hydroxylase, that is CYP27B1 discovered by (St-Arnaud et al., 1997) who mapped it to the same locus as mutations that resulted in vitamin D dependency rickets. 1α-hydroxylase is expressed when serum Ca levels are low and $1,25(OH)_2D$ exerts its biological effects via the VDR to increase serum Ca. In order to maintain homeostasis, 1α hydroxylation is highly regulated by multiple negative feedback mechanisms including raised serum levels of; Ca, FGF23 and $1,25(OH)_2D$ itself, as well as positive feedback from PTH, these are all shown in Figure 1-5. Once in its active form, the half-life of $1,25(OH)_2D$ is 10-20 hours (Levine et al., 1985) however it can be inactivated by the 24 hydroxylase enzyme.

The CYP24A1 gene transcribes 24 hydroxylase which drives the hydroxylation of $1,25(OH)_2D$ to calcitroic acid which is water soluble and easily excreted, CYP24A1 also hydroxylates the precursor 25OHD to the inactive metabolite $24,25(OH)_2D$ (Reddy and Tserng, 1989). The clinical importance of CYP24A1 in the 24 hydroxylase degradation of $1,25(OH)_2D$ was shown by (Schlingmann et al., 2011) who identified recessive mutations in CYP24A1 in 6 children with idiopathic infantile hypercalcemia due to increased vitamin D sensitivity.

Figure 1-5: Vitamin D metabolism

Activation of pre-vitamin D requires 25-hydroxylation to 25 hydroxyvitamin D (25-OH-D_3) and 1α hydroxylation to form active 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). 1, 25-$(OH)_2D_3$ binds to the VDR to exert its biological effects, then it is catabolized by 24 hydroxylase to eventually form cacitroic acid which is water soluble. Activity of CYP27B1 and CYP24A1 are closely regulated by 1, 25-$(OH)_2D_3$, serum Ca ions (sCa^{2+}) and parathyroid hormone (PTH). There is an additional negative feedback mechanism on CYP27B1 through the 1, 25-$(OH)_2D_3$, fibroblast growth factor 23 (FGF23), klotho axis. sPO4 – serum phosphate. Figure from (Schlingmann et al., 2011).

1.5.3 Vitamin D Receptor (VDR): Mechanism of action and different VDR isoforms

$1,25(OH)_2D$ mediates its effects via the VDR which is expressed in almost all tissues throughout the body. The VDR is part of the Nuclear Hormone Receptor superfamily and shares a similar structure with them enabling it to regulate the transcription of multiple genes (Haussler et al., 1998). The VDR contains a zinc finger DNA binding domain at the N terminal (Haussler et al., 1995). The ligand binding domain is located at the C terminal along with 2 conserved retinoid x receptor (RXR) binding domains. At the extreme end of the C terminal there is also an activation function (AF2) which is a ligand dependent activation domain that is essential for transcriptional activation.

Ligand binding to the VDR leads to a conformational change in the C terminal that facilitates strong dimerization with the RXR. As shown in Figure 1-6, the ligand bound VDR-RXR heterodimer trans-locates from the cytoplasm to the nucleus. Here it regulates transcription by interacting with a specific sequence in the promoter region of target genes, known as the vitamin D response element (VDRE) (Haussler et al., 1998). Binding of the VDR to the VDRE triggers the recruitment of multiple protein cofactors and transcriptional machinery leading to either transactivation or repression of genes (Crofts et al., 1998).

The human VDR gene is complex, 14 alternative 5' variants of the VDR transcript have been identified by 5' rapid amplification of cDNA ends (RACE). This gives rise to 3 different isoforms of the VDR protein shown in Figure 1-7, transcripts 1-5 originate from exon 1a and are translated from the initiation codon in exon 2 and encode the 427 amino acid VDR-A protein (Crofts et al., 1998), this corresponds to the originally identified cDNA sequence for human VDR (Baker et al., 1988). Transcripts 6-10 originate from exon 1d where there is a second initiation codon, therefore have the potential to encode N terminal variant proteins. Transcript 6 was identified as transcribing VDR-B1 with a 50 amino acid extension and transcript 9 encoded VDR-B2 with a 23 amino acid extension (Crofts et al., 1998). VDR transcripts 1, 2 and 6 shown in Figure 1-7 are confirmed on the NCBI reference sequence database as transcript 1, 2 and 3 respectively (Pruitt et al., 2014).

VDR-B2 could not be detected in any tissue tested, however presence of the 54KDa VDR-B1 isoform was confirmed in human kidney tissue, 786 kidney cell line and three human intestine cell lines where it was expressed at comparable levels to VDR-A, as well as in MG63 osteosarcoma cells where VDR-B1 was detected at a third the level of VDR-A (Sunn et al., 2001). The additional 50 amino acids at the N terminal of VDR-B1 extends the A/B domain from 23 to 73 amino acids, this A/B region of nuclear receptors often contain a ligand independent activation function domain therefore the VDR-B1 isoform may have different functional properties to the VDR-A. From here on VDR-B will refer to the 477 amino acid VDR-B1 isoform.

Many genes contain a VDRE in their promoter and approximately 3% of the human genome is regulated either directly or indirectly by vitamin D (Bouillon et al., 2008). Vitamin D has a wide range of physiological functions, this includes both its role in the regulation of systemic Ca described in 1.5.1 and non-calcaemic effects to be discussed in 0.

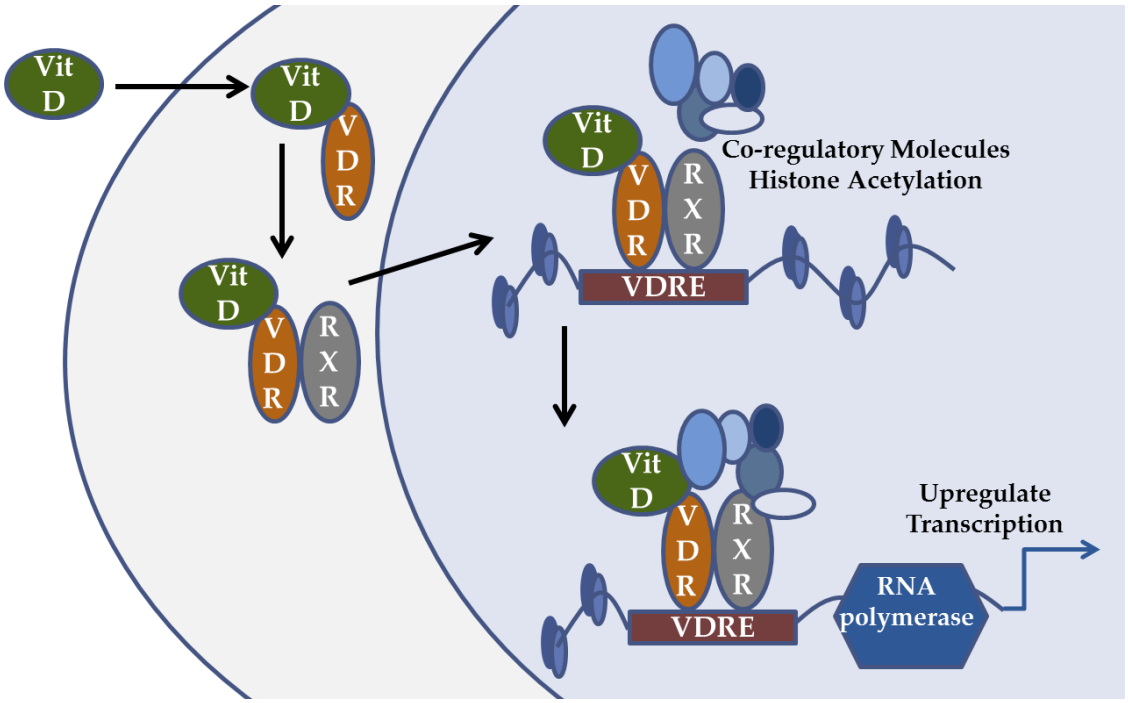


Figure 1-6: Schematic diagram of vitamin D activation of the VDR

The active form of vitamin D, $1,25(OH)_2D$ binds to VDR and facilitates its dimerization with the RXR. This complex interacts with the VDRE in the promoter region of multiple genes and triggers the recruitment of coregulatory proteins which regulate histone acetylation and consequently transcription. VDR – Vitamin D Receptor. RXR – Retinoid X Receptor. VDRE – Vitamin D Response Element.

Figure 1-7: Human VDR gene

Adapted from (Crofts et al., 1998). A) Exons in the human VDR gene. B) Structure of the human VDR transcripts, transcripts 1-5 originate from exon 1a, transcripts 6-10 from 1d and transcripts 11-14 originate from exon 1f. Boxed numbers (1, 6 and 10) indicate transcripts with highest expression. All transcripts have a translation initiation codon in exon 2 and 1d transcripts have an additional upstream initiation codon. Starred transcripts encode different proteins, shown in C. C) Transcript 1 (NM_000376) encodes the original 427 amino acid protein, the 23 amino acid of its A/B domain are shown in bold, transcript 6 and 9 encode proteins with a 50 amino acid and 23 amino acid N terminal extension respectively. Transcripts in green box are confirmed in the NCBI reference sequence database (Pruitt et al., 2014).

1.5.4 Vitamin D plethora!

In recent years there has been a great scientific interest in the therapeutic benefits of vitamin D, outside its regulation of Ca homeostasis and bone mineralisation. There are many studies suggesting that vitamin D is associated with an array of beneficial health outcomes on; cardiovascular health, cancer, immune system, muscle function, chronic pain and other disorders. Despite the numerous association studies, an umbrella review that considered the relationship between vitamin D and 137 different health outcomes could not identify a clear role for vitamin D. In fact, the only consistently significant outcome was the relation between maternal vitamin D status and birth weight (Theodoratou et al., 2014).

A negative relationship between low vitamin D levels and high plasma renin activity that leads to hypertension and cardiac hypertrophy has been observed clinically for over 2 decades (Burgess et al., 1990). A role for $1,25(OH)_2D$ as a negative regulator of the renin – angiotensin – aldosterone system was supported in both VDR null mice which had increased renin expression, and in wild type mice injected with $1,25(OH)_2D$ which had suppressed renin expression. Furthermore, in cell culture $1,25(OH)_2D$ was shown to suppress renin transcription (Li et al., 2002). $1,25(OH)_2D$ is thought to suppress renin in order to maintain cardiovascular health and reduce blood pressure, improving left ventricular function and decreasing the risk of left ventricular hypertrophy. However, patients with CKD given 2µg/day oral paricalcitol for 48 weeks showed no difference in left ventricular mass index relative to the placebo group (Thadhani et al., 2012).

Many observational or epidemiological studies have found a strong association between vitamin D and cancer prevention or survival for 15 different cancer types including colorectal, skin and breast cancers as reviewed by (Grant, 2016). In a study of 99 colorectal cancer patients which found that CYP24A1 (which inactivated $1,25(OH)_2D$) expression was higher in cancer than adjacent regions and patients with high CYP24A1 mRNA expression had reduced overall survival (Sun et al., 2016). Despite these associations the mechanism has not been confirmed, $1,25(OH)_2D$ is thought to inhibit tumour progression through the inhibition of cell proliferation, increased cell differentiation and induction of apoptosis.

Vitamin D deficiency is associated with increased risk of autoimmune diseases such as arthritis, multiple sclerosis and diabetes mellitus type 1 (Peelen et al., 2011). The VDR is expressed in resting monocytes and dendritic cells as well as in activated T and B lymphocytes (Provvedini et al., 1983), many immune cells also express enzymes involved in vitamin D metabolism. Following a pathogen challenge macrophages increased expression of VDR and CYP27A1 (vitamin D activating enzyme), 100s of $1,25(OH)_2D$ target genes have been identified in immune cells. The overall effect of these target genes is to suppress inflammation as reviewed by (Chun et al., 2014), therefore it is thought that vitamin D serves as an immune regulating agent to prevent autoimmune disorders. For example, there is a high incidence of vitamin D deficiency in patients with the autoimmune disorder systemic lupus erythematosus (SLE) (Mandal et al., 2014). SLE affects endothelial repair regulated by myeloid angiogenic cells (MACs). It has been shown in SLE MACS in-vitro that calcitriol reduced their IL-6 secretion, as well as increasing SLE MACs angiogenic activity and normalising MAC phenotype (Reynolds et al., 2016).

Several clinical studies have shown an association between vitamin D deficiency and chronic pain, for example in white middle aged British females (but not males) there was an association between 25(OH)D and reduced incidence of widespread chronic pain (Atherton et al., 2009). However, a review of 10 random double blind trials found that there was no consistent effect of vitamin D supplements in the relief of chronic pain compared to placebo (Straube et al., 2015).

Many countries have a widespread deficiency in vitamin D. As vitamin D supplements are readily available over the counter and health care professionals are inundated with the benefits of vitamin D, it is not surprising that instances of overzealous vitamin D treatment leading to intoxication are increasingly common. Despite these risks, toxic doses of vitamin D have not yet been established.

1.5.5 CKD patients are deficient in Vitamin D

Vitamin D status is determined by measuring serum 25OHD, levels less than 50nM are considered as vitamin D deficiency by expert consensus (Norman et al., 2007) and up to 75nM 25OHD is insufficient for optimal Ca absorption (Heaney et al., 2003).

Patients with CKD are often deficient in 25OHD measured in the serum, there are multiple reasons for this:

- CKD patients are less active therefore they have less exposure to sunlight.

- The skins ability to synthesise pre-vitamin D in response to UV light may be impaired in CKD. Despite CKD patients having normal epidermal content of 7-dehydrocholesterol, on exposure to UV-B irradiation their photo-production of cholecalciferol was less than in normal subjects (Jacob et al., 1984).

- Reduced apatite and dietary restrictions to limit Na and K intake may reduce ingestion of natural sources of pre-vitamin D. A dietary assessment in which 91 haemodialysis (HD) patients and 85 controls completed a 7-day food journal, found that total energy and nutrient intake was significantly lower in HD patients. This included pre-vitamin D intake which was on average 1.1µg per day in HD patients and 1.6µg per day in controls (Fusaro et al., 2016).

- Proteinuria in CKD leads to loss of vitamin D binding protein hence increased loss of all vitamin D compounds, particularly 25OHD which has the highest binding affinity (Sato et al., 1982).

- Patients on peritoneal dialysis were found to have reduced levels of vitamin D binding protein due to their loss, along with bound vitamin D compounds in the peritoneal dialysis fluid (Koenig et al., 1992). Vitamin D binding proteins are important for increasing the half-life of vitamin D compounds.

In addition to 25OHD deficiency, CKD patients lose the ability to convert 25OHD to the active $1,25(OH)_2D$. There are numerous possible explanations for this.

- As explained above CKD patients are likely to have chronic hyperphosphatemia and high levels of FGF-23, this increases CYP24A1 expression and promotes 24 hydroxylation of $1,25(OH)_2D$ to inactive metabolites (Koizumi et al., 2013a).

- Renal production of the 1α hydroxylase enzyme is reduced with the decline in renal mass (Pitts et al., 1988).

- The activity of 1α hydroxylase may be suppressed in the acidic and uremic milieu found in CKD, furthermore the increased secretion of FGF-23 suppresses CYP27B1 expression reducing 1α hydroxylase activity (Bouillon et al., 2014).

- In health, 25OHD and D binding protein from the glomerular ultra-filtrate are taken up by endocytosis by the renal megalin and delivered to 1α hydroxylase in the proximal tubule. In CKD, expression of renal megalin is reduced therefore limiting the delivery of 25OHD to 1α hydroxylase (Nykjaer et al., 1999).

A combination of these factors lead to 25OHD and further $1,25(OH)_2D$ deficiency in CKD patients, this has a detrimental effect on mineral metabolism and bone development particularly in children who could develop rickets. As discussed above $1,25(OH)_2D$ plays a key role in mineral homeostasis including the negative regulation of PTH.

Vitamin D receptor activators (VDRAs) are often prescribed for control of secondary hyperparathyroidism in CKD patients who are deficient. Excessive use of VDRAs could have toxic effects due to its direct action on other tissues, a greater understanding of the risks involved is required.

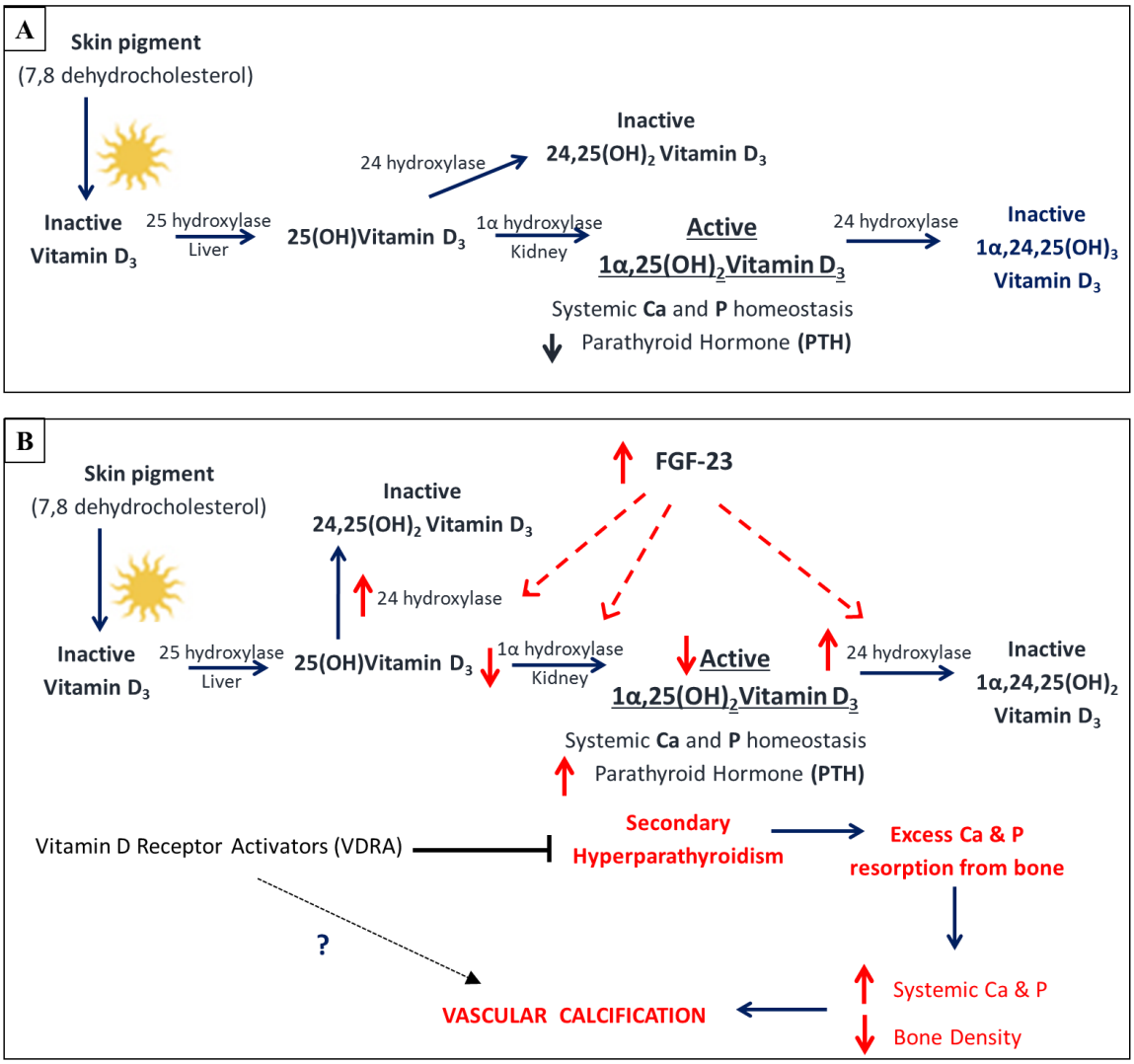


Figure 1-8: Effect of vitamin D deficiency on mineral homeostasis in CKD

Schematic diagram representing A) A state of vitamin D sufficiency, 7,8 dehydrocholesterol is converted to active $1\alpha,25(OH)_2D_3$ for the systemic regulation of Ca and P homeostasis including the negative regulation of PTH secretion. B) A state of vitamin D deficiency in CKD. The increased circulating level of FGF-23 upregulates 24 hydroxylase for inactivation of vitamin D compounds. 1α hydroxylase production in the kidney is reduced due to decreased renal mass and it is further down regulated by FGF-23, hence restricting the activation of $25OHD_3$. The reduced circulating level of $1\alpha,25(OH)_2D_3$ is unable to regulate PTH, leading to increased PTH secretion and secondary hyperparathyroidism. Excessive, unregulated PTH levels in the serum accelerate the resorption of bone, leading to a reduced bone density and increased systemic Ca and P levels increasing the risk of vascular calcification. Pharmacological intervention with VDRAs prevents secondary hyperparathyroidism, however the direct effect of VDRAs on VSMCs and vascular calcification is not known.

1.5.6 Vitamin D receptor activators

CKD is a state of vitamin D deficiency, this exacerbates the disrupted mineral metabolism that occurs early in the progression of CKD, giving rise to hyperphosphatemia, secondary hyperparathyroidism and bone mineral disorder. In these patients VDRAs are often prescribed, particularly in children to prevent secondary hyperparathyroidism (Brown et al., 2002). There are numerous analogues of VDRA, this study will concentrate on 3 of them that are most commonly used:

Alfacalcidol (1α hydroxyvitamin D_3 ($1OHD_3$))

Alfacalcidol is a synthetic prohormone that requires activation by 25 hydroxylase to produce the active form of $1,25(OH)_2D_3$. 25 hydroxylase is mainly expressed in the liver but is also expressed locally in some cell types. Alfacalcidol is often prescribed to children with CKD who have vitamin D deficiency in order to treat secondary hyperparathyroidism.

Calcitriol ($1,25(OH)_2D_3$)

Calcitriol is the natural vitamin D hormone in its active form (Slatopolsky et al., 1995). It is a non-selective VDRA with a similar affinity for the VDR in the parathyroid glands as the VDRs in the bone and intestinal lumen (Christakos et al., 2003), therefore as well as suppressing PTH calcitriol can increase Ca and P resorption and absorption leading to hypercalcemia and hyperphosphatemia.

Paricalcitol ($19nor,1,25(OH)_2D_2$)

Paricalcitol is a synthetic analogue of vitamin D_2 that lacks a carbon-19 methylene group found in all natural vitamin D metabolites (Slatopolsky et al., 1995). Paricalcitol is a selective VDRA that was developed to reduce the risk of hypercalcemia and hyperphosphatemia, it has a higher affinity for VDRs in the parathyroid gland than in the bone and intestinal mucosa. In a study on 20 haemodialysis patients, calcitriol and paricalcitol were equally efficient at reducing PTH but paricalcitol had a less calcemic effect (Veceric-Haler et al., 2016).

Figure 1-9: Chemical structure of VDRAs
Chemical structure of the 3 different VDRAs studied. **A)** $1,25(OH)_2D_3$ (Calcitriol) is the natural active form of vitamin D. **B)** 19-nor-$1,25(OH)_2D_2$ (Paricalcitol) is a synthetic analogue, it has the carbon 28 and double bond at carbon 22 characteristic of vitamin D_2, however it lacks carbon 19 and the exocyclic double bond therefore differs from all natural forms of vitamin D. **C)** $1OHD_3$ (Alfacalcidol) is an inactive synthetic analogue of vitamin D that requires activation by the 25 hydroxylase enzyme. Adapted from (Slatopolsky et al., 1995).

1.6 Direct effects of 1,25$(OH)_2$D on VSMCs

1,25$(OH)_2$D is able to have direct effects on VSMCs. VSMCs express both the 1α and 25 hydroxylase enzymes as well as the VDR, therefore are able to act as an autocrine system (Somjen, Weisman et al. 2005). VDRAs may either reduce or promote ectopic calcification and there is controversial literature surrounding the benefits and detriments of VDRAs on vascular calcification.

A review of the benefits of oral VDRA supplements in double-blind, placebo- and randomized-controlled trials found that VDRAs could have cardio protective effects however this was not consistent (Ku et al., 2013). In excess 1,25$(OH)_2$D may increase the risk of hypercalcemia and vascular calcification. It is important to maintain levels of 1,25$(OH)_2$D within the normal physiological range. Despite daily oral alfacalcidol supplements given to children on dialysis 36% had low levels and 11% had high 1,25$(OH)_2$D outside the normal range of 40-150pMol/litre (Shroff et al., 2008a). In this study both high and low levels were associated with increased carotid intima media thickness and coronary artery calcification. This suggests that there is a narrow physiological range where vitamin D is beneficial to vascular calcification in CKD patients, currently vitamin D is routinely prescribed to children with CKD therefore it is important to understand the pro and non-calcaemic mechanisms of different VDRAs.

There is evidence that VDRAs have a bimodal effect on calcification with both high and low levels being associated with an increased risk. In a cross sectional study of 126 haemodialysis patients, those with 25OHD deficiency had a higher incidence of vascular calcification than those with normal 25OHD levels (Wang et al., 2015). This bimodal effect of vitamin D on vascular calcification suggests that both the extreme low and high doses of vitamin D are causative of calcification.

There are a number of ways that vitamin D may impact on vascular calcification, either as a protective agent or by promoting vascular calcification. Some of these are discussed below.

1.6.1 Vitamin D: Protective mechanisms against calcification

1.6.1.1 Regulate calcification inhibitors

Calcitriol has been shown to up-regulate the inhibitory proteins, osteopontin (Lau et al., 2012) and MGP. In rat VSMCs transiently transfected with luciferase constructs regulated by an MGP promoter, physiological concentrations of calcitriol were shown to upregulate transcription (Farzaneh-Far et al., 2001). These calcification inhibitors are important in the suppression of ectopic calcification.

1.6.1.2 Upregulate CaSR expression

Increased expression of the CaSR in VSMCs can increase their sensitivity to Ca exposure. At a narrow concentration range, calcitriol was shown to increase expression of CaSR and protect against calcification in human VSMCs (Mary et al., 2015).

1.6.1.3 Anti – inflammatory effect

The VDR is expressed in immune cells (Kassi, Adamopoulos et al. 2013) and vitamin D is known to suppress inflammatory cytokines including TNF-α, IL6 and IL 10 (Ku, Liu et al. 2013). Inflammation is a key component in atherosclerotic plaque calcification and also has a role in arteriosclerosis associated with CKD.

1.6.2 Vitamin D: Causative mechanisms of calcification

1.6.2.1 Stimulate proliferation

Vitamin D stimulates VSMC proliferation through upregulation of vascular endothelial growth factors (VEGF) (Cardus, Parisi et al. 2006) and increased VSMC migration (Rebsamen, Sun et al. 2002) which both promote calcification.

1.6.2.2 Osteo/chondrocytic differentiation

VDRAs also promote osteoblast differentiation of VSMCs including the up-regulation of Runx2 and osteocalcin which favours ectopic calcification in soft tissue (Shroff and Shanahan 2007).

It is essential to understand the molecular mechanisms by which vitamin D effects vascular calcification in CKD. In recent years there has been numerous studies in this controversial area, they utilise different models, induce calcification by different mechanism and administer different doses of VDRAs. This has led to very varied outcomes in terms of the effect if VDRAs on vascular calcification.

1.7 Models to study the effects of vitamin D on VSMC calcification in CKD

1.7.1 *In-vivo*: Animal Models

In-vivo studies enable the systemic effects of Vitamin D on mineral metabolism to be studied alongside the direct effects of vitamin D on VSMCs. The chronic nature of CKD cannot be modelled in animals, however the reduced kidney function can be mimicked in mice and rats by 5/6 nephrectomy to produce an *in-vivo* CKD model. To produce this CKD model, animals undergo right total nephrectomy and 1 week later 2/3 left nephrectomy by ligation (Perez-Ruiz et al., 2006). These CKD animals have a good survival rate enabling mid to long term studies of several months. There is no animal model to replicate dialysis however to some extent the mid to long term physiological effects of VDRAs can be explored in this model.

Numerous groups have studied vascular calcification using this *in-vivo* CKD model, in these CKD animals a high P diet could induce vascular calcification. For example, in CKD DBA/2J mice fed a high, 1.5% P diet, calcified regions were observed in the aorta's medial area and Ca load increased 8.5 fold compared to in mice fed a normal, 0.5% P diet (Lau et al., 2012). In this model, both calcitriol (30ng/kg) and paricalcitol (100ng/kg or 300ng/kg) reduced vascular calcification, however even the highest doses were not able to lower PTH due to the excessively high P diet overstimulating PTH secretion (Lau et al., 2012). This did enable the study to reveal protective effects of VDRAs on calcification that are independent of PTH suppression; paricalcitol but not calcitriol increased expression of osteopontin in VSMCs, and both of the VDRAs increased serum klotho (which was reduced by the CKD model), leading to increased P excretion to correct hyperphosphatemia, hence reduce the elevated level of FGF-23 (Lau et al., 2012). The excessively raised P levels in this study do not reflect P levels observed in CKD and a more modest increase in P consumption would improve the physiological relevance.

In contrast, a study on 5/6 nephrectomy Sprague Dawley rats found that a high, 1.2% P diet did not induce calcification but it did elevate PTH levels (Mizobuchi et al., 2007). As there was no calcification in the control group any protective effects of the VDRAs in this model could not be observed. In the CKD Sprague Dawley rats, 40ng/kg calcitriol suppressed PTH but caused extensive calcification whereas paricalcitol was able to suppress PTH without affecting Ca load in the aortic arch even thou administered at doses of 160ng/kg and 240ng/kg. Calcitriol increased serum Ca and P levels to a greater extent than paricalcitol, calcitriol but not paricalcitol also had a direct effect on VSMCs and increased their expression of osteoblast transcription factor Runx2 and upregulated its target gene osteocalcin (Mizobuchi et al., 2007). In a similar study also on CKD Sprague Dawley rats, in this instance fed a standard P diet showed that a clinically relevant dose of calcitriol (30ng/kg) increased vascular calcification, as shown by increased Ca deposits in the tunica media. Again, calcitriol had a direct effect on VSMCs and increased Runx2 expression, it also reduced MGP expression and increasing endothelial cell proliferation (Koleganova et al., 2009). It is not possible to determine from either of these in-vivo studies, if the increased calcification observed in response to calcitriol was due to the elevated levels of

systemic Ca and P or direct effects of calcitriol on VSMCs such as the upregulation of osteoblast genes.

The CKD model in Wistar rats fed a high, 1.2% P diet showed that calcitriol (80ng/kg) increased aortic calcification, as did paricalcitol (240ng/kg) to a lesser extent. Both VDRAs partially reduced PTH levels, despite the high doses of calcitriol and paricalcitol neither were able to suppress PTH to normal levels, to do so would require even higher and less physiologically relevant doses (Lopez et al., 2008).

In the studies discussed above the differential effects of calcitriol and paricalcitol on vascular calcification range between protective, null and causative as summarized in Table 1-1. This could be a consequence of differences in study design including; extent of high P diet, dose of VDRAs and species used. A high P diet was used in many studies to induce calcification however it is also important to maintain P levels within those physiologically relevant to CKD. 30ng/kg calcitriol and 100ng/kg paricalcitol reflect the current clinical doses (Lau et al., 2012), however many studies used much higher doses of VDRAs which could reduce the clinical relevance of their results. These studies all used the 5/6 nephrectomy CKD model however it was applied to different species (DBA/2J mice, Sprague Dawley rats and Wistar rats), this could contribute to the differential effects observed of calcitriol and paricalcitol on vascular calcification, it highlights the importance of using human tissue as a clinically relevant model to observe the effects of VDRAs on calcification.

1.7.2 *Ex-Vivo:* Organ culture of aorta

Culture of the thoracic aorta from mice in elevated inorganic P has been shown to induce vascular calcification of the tunica media (Akiyoshi et al., 2015). This enables the phenotypic changes that occur during calcification to be studied in an inducible model where the vessel architecture is still intact. Aging and diabetic mice are known to be at high risk of calcification, aortas from these groups cultured in high iP had more calcification than controls therefore this model reflects the calcification susceptibility observed *in-vivo* (Akiyoshi et al., 2015).

It would be of interest to study the effects of VDRAs on calcification in CKD vessels using this *ex-vivo* model, as it would enable the direct effects VDRAs on VSMCs within their whole vessel architecture to be studied independently of changes in systemic Ca and P levels. However, as discussed above an ideal animal model that reflects the chronic nature of CKD and the impact of dialysis treatment has not yet been developed.

Table 1-1: *In-vivo* studies on the effects of VDRAs on VSMC calcification.
Comparison of in-vivo 5/6 nephrectomy CKD models including animal type and diet used to induce calcification or not. For each model the effect on calcification of the control condition (nil or vehicle) is shown. The effect on calcification of the VDRAs is in relation to the control condition. Red shading indicates increased calcification. Green shading indicates no or reduced calcification. ↑ - Increased. ↓ - Decreased. BGP – Bone gla protein. Ca – Calcium. FGF-23 – Fibroblast growth factor 23. MGP – Matric gla protein. P – Phosphate. PTH – Parathyroid hormone.

***In-Vivo* Method** 5/6 nephrectomy	**VDRA**	**Effect on Calcification**
DBA/2J Mice High P diet (1.5% P) Controls (0.5% P) (Lau et al., 2012)	Nil	Calcification, ↑P, ↑PTH, ↑FGF-23, ↓Klotho
	30ng/kg Calcitriol 100ng/kg Paricalcitol	↓Calcification Normalise P, FGF23 & klotho. PTH remains high
Sprague Dawley Rats High P diet (1.2%) (Mizobuchi et al., 2007)	Vehicle	No Calcification, ↑PTH
	40ng/kg Calcitriol	Calcification, ↑serum Ca and P, ↓PTH, ↑Runx2, ↑BGP
	160ng/kg Paricalcitol	No Calcification, ↑serum Ca ↓PTH
Sprague Dawley Rats Normal diet (0.9% Ca and 0.8% P) (Cardus et al., 2007)	Vehicle	No Calcification, ↑PTH
	100ng/kg Calcitriol	Calcification, ↑ serum Ca and P, ↓PTH
	300ng/kg Paricalcitol	No calcification, ↑ serum Ca, ↓PTH
Wistar Rats High P (1.2%), low Ca (0.6%) diet (Lopez et al., 2008)	Vehicle	No Calcification, ↑P
	80ng/kg Calcitriol	↑ ↑ Calcification, ↑P
	240ng/kg Paricalcitol	↑ Calcification, No change P
Sprague Dawley Rats Normal diet (Koleganova et al., 2009)	Nil	No calcification
	30ng/kg Calcitriol	↑ Calcification, ↑Runx2, ↓MGP

1.7.3 *In -Vitro*: VSMC explants

VSMCs can be explanted from human vessels therefore have the benefit of being species specific. Furthermore, the Ca and P levels in the culture media can be controlled so that the direct effects of vitamin D on VSMCs can be studied independently of changes in systemic Ca and P levels. Vascular calcification can be studied in-vitro in explanted VSMCs by increasing the Ca and/or P content of the culture medium to induce VSMCs to calcify (Proudfoot et al., 2000).

Calcification was induced in human VSMCs by increasing mineral content of the medium more than 3.5 fold from 0.9mM P to 3.3mM P. This dose of P did indeed increase Ca deposition in the VSMCs, it also increased expression of osteoblast genes; Runx2, Msx2 and bone morphogenetic protein 2 (BMP-2), a transforming growth factor involved in the regulation of bone development (Martinez-Moreno et al., 2012). In this study, 10nM calcitriol further increased expression of osteoblast genes BMP-2, Runx2, Msx2 and BGP, whereas 30nM paricalcitol reduced expression of BMP-2 in VSMCs. In the high P medium, calcitriol elevated Ca deposition whereas paricalcitol reduced Ca deposition. This suggests that both VDRAs have direct but opposing effects on VSMC calcification (Martinez-Moreno et al., 2012). A major limitation of this study is that there was no vehicle control for either of the VDRAs which are both only soluble in ethanol, ethanol is likely to be toxic to cells and could be influencing the observed responses. In addition, raising the P content of the medium to 3.3mM could be considered excessive, it is a toxic dose and does not reflect mineral levels observed physiologically.

The causative effect of calcitriol on calcification is not consistent across all *in-vitro* studies on human VSMCs. In VSMCs induced to calcify with 2.5mM P and TNF-α (known to accelerate osteogenic processes), those treated with vehicle had extensive calcification and increased mRNA expression of Runx2 and BGP. Relative to vehicle, 1nM and 10nM calcitriol had no effect on Ca deposition whereas, 100nM calcitriol significantly reduced; Ca deposition, expression of osteogenic BGP and expression and secretion of MMP-2 which promotes elastin degradation (Aoshima et al., 2012). This protective effect of calcitriol was only observed at the supra-physiological dose of 100nM, most studies use 10nM calcitriol to represent physiological doses.

Furthermore, the same study showed that in the absence of TNF-α, even 100nM calcitriol had no effect on Ca deposition in VSMCs (Aoshima et al., 2012). TNF-α is upregulated by chronic inflammation as observed in atherosclerosis therefore this study is of physiological relevance to atherosclerosis however has distinct characteristics from arteriosclerosis observed in CKD.

In a different experimental set up, much lower doses of calcitriol were shown to have a protective effect on calcification in VSMCs. It was shown that 1nM calcitriol upregulated expression of total and cell surface CaSR via the VDR in both normal and high Ca media. VSMC calcification induced by high Ca (5mM) medium was prevented by 1nM calcitriol treatment (Mary et al., 2015). This response was observed at a narrow concentration range and neither 0.1 or 10nM calcitriol had a significant effect on calcification. The protective effect of calcitriol observed in this study through the upregulation of CaSR expression may only be effective in models induced to calcify in high Ca media. Although raised serum Ca is observed in CKD patients it is preceded by and accompanied with raised serum P. In addition, 5mM Ca is almost 3 fold that observed in normal medium and would be a toxic dose not observed clinically, therefore the physiological significance of this model is questionable.

As summarised in Table 1-2 a range of protective and calcification promoting effects of VDRAs were observed in different studies on VSMCs. Despite that each study was species specific and used VSMCs from humans, these VSMCs were not disease specific to CKD. They varied in their mode of inducing vascular calcification, with some using high mineral conditions that are not clinically relevant.

Table 1-2: *In-vitro* studies on the effects of VDRAs on VSMC calcification.
Comparison of *in-vitro* studies in human VSMCs, tissue culture medium was supplemented to induce calcification as indicated for each study. For each study the effect on calcification of the control condition (nil or vehicle) is shown. The effect on calcification of the VDRAs is in relation to the control condition. Red shading indicates increased calcification. Green shading indicates no or reduced calcification. ↑ - Increased. ↓ - Decreased. BGP – Bone gla protein. BMP-2 – Bone morphogenic protein-2. Ca – Calcium. CaSR – Calcium sensing receptor. P – Phosphate.

***In-Vitro* Method** human VSMCs	**VDRA**	**Effect on Calcification**
Control 0.9mM P or **High P 3.3mM P** (Martinez-Moreno et al., 2012)	Nil	Calcification, ↑BMP-2, ↑Runx2, ↑Msx2
	10nM calcitriol	↑ Calcification, ↑BMP-2, ↑Rumx2, ↑Msx2
	30nM paricalcitol	↓Calcification, ↓BMP-2, No change Msx2 or Runx2
Control 0.9mM P or **High P 2.5mM P & TNF-α** (Aoshima et al., 2012)	Vehicle	Calcification, ↑Runx2, ↑BGP
	1nM, 10nM, 100nM calcitriol	↓Calcification (dose dependently), ↓BGP (100nM only)
Control 0.9mM P or **High P 3.3mM P & TNF-α** (Guerrero et al., 2012)	Nil	Calcification, ↑BMP-2
	10nM calcitriol	↑ Calcification, ↑ Runx2, ↓BMP-2
	30nM paricalcitol	↓ Calcification, ↓BMP-2
Control 1.8mM Ca or **High Ca 5mM Ca** (Mary et al., 2015)	Vehicle	Calcification
	1.01nM, 0.1nM, 10nM calcitriol	No change in calcification
	1nM calcitriol	↓Calcification, ↑CaSR

1.7.4 *Ex-Vivo*: Intact human arterial vessel rings

Ex-vivo studies of arterial rings from medium sized human muscular arteries enable the whole tissue architecture of the vessel wall to be studied including the lamellae, tunica intima, tunica media and adventitia. Vessel rings maintain their contractile phenotype for several weeks in culture enabling extended experiments to take place. This model was established to study calcification in vessels of children with CKD, (Shroff et al., 2008b) and enabled vessels from pre-dialysis and dialysis patients to be compared under experimental conditions. Vessels from children are particularly important to study vascular calcification as they have been exposed to minimal traditional cardiovascular risk factors such as dyslipidemia, hypertension, smoking and aging itself therefore the risk of atherosclerosis is low and effects of the uremic milieu and the impact of dialysis on the development of arteriosclerosis can be studied in isolation. This vessel ring model showed that culture of vessel rings in high Ca-P medium (2.7mM Ca and 2mM P) led to Ca deposition in the tunica-media of dialysis vessel rings and to a lesser extent in pre-dialysis vessel rings but not in control vessels (Shroff et al., 2010). In this model, many of the processes that occur *in-vivo* in calcification were observed in the calcified dialysis vessel rings, this included; apoptosis of VSMCs, increased number of matrix vesicles in the extracellular matrix and increased expression of osteoblast marker Runx2. ALP activity was also increased by high P media but not by high P and Ca media (Shroff et al., 2010)

This *ex-vivo* model of human vessel rings from patients with CKD has not previously been used to test the effects of VDRAs on vascular calcification, this model formed the basis of this thesis. The effects of VDRAs on vascular calcification in explanted human VSMCs from both healthy control and CKD patients was also studied. In both models, calcification was induced by a modest increase in the media's Ca and P content (1.5 and 2 fold respectively) to mimic raised mineral content observed in CKD.

1.8 Hypothesis and Project Strategy

Cardiovascular disease, in particular vascular calcification is the leading cause of death in people with CKD. Children with CKD are routinely prescribed VDRAs to compensate for their vitamin D deficiency, prevent secondary hyperparathyroidism and to reduce the risk of bone disease. The direct effect of these VDRAs on VSMC calcification is not fully understood and current literature is confounding.

The hypothesis examined in this PhD thesis was; VDRAs play a key role in driving vascular calcification in vessels from CKD patients and the differential expression pattern of the VDR can determine the extent of mineralisation in an *in vitro* and *ex vivo* model.

Chapter 3:

- **Aim 1:** To establish whether different VDRAs, calcitriol, alfacalcidol and paricalcitol accelerate the progression of vascular calcification in arterial rings isolated from children with CKD.

The effects of three different VDRAs on vascular calcification in pre-dialysis and dialysis children were tested by treating intact paediatric arterial rings in pro calcaemic conditions with calcitriol, alfacalcidol or paricalcitol. The Ca content was quantified using a colourimetric cresolphthalein assay and the level of calcification was also visualised by histology.

Chapter 4:

- **Aim 1:** To determine whether VDRAs alfacalcidol and calcitriol act via different mechanisms to promote vascular calcification in vessel rings from children with CKD.

- **Aim 2:** To elucidate the different expression profiles in vessel rings that have an increased Ca load in response to calcitriol (responders) and vessel rings whose Ca load does not respond to calcitriol (non-responders).

In order to examine the mechanistic effects of VDRAs on arterial rings, extensive histological analysis was performed to visualise any structural differences and changes in protein expression. In addition, activity of the osteoblast enzyme ALP in the arterial rings was determined by a colorimetric assay.

Chapter 5:

- **Aim 1:** To establish whether the effect of calcitriol on calcification in VSMCs is dose dependent.

- **Aim 2:** To determine whether paricalcitol is less calcaemic than calcitriol in VSMCs.

- **Aim 3:** To elucidate whether dialysis VSMCs are more susceptible to VDRA induced calcification than control VSMCs.

For these studies VSMCs were explanted from the arteries of paediatric patients, both healthy controls and CKD dialysis patients. VSMCs were treated in pro calcaemic medium with either physiological dose calcitriol and paricalcitol or a range of calcitriol doses from a sub physiological to a supraphysiological dose. Ca content of treated VSMCs was quantified by colourimetric cresolphthalein assay. ALP activity was also determined by a colourimetric assay and changes in RNA or protein expression were analysed.

Chapter 6:

- **Aim 1:** To determine whether VDR expression in vessels varies between individuals giving rise to the different responses to calcitriol treatment in both arterial vessel rings and explanted VSMCs.

Arterial vessel rings were grouped dependent on calcification in response to calcitriol and their VDR expression was visualised by immunohistochemistry and quantified. In control and dialysis VSMCs treated with VDRAs, the total VDR mRNA expression was determined by qPCR and protein expression of VDR isoforms analysed by western blot. To investigate differences in VDR transactivation capacities, VSMCs were transfected with a luciferase construct under a VDRE promoter.

Chapter 7:

- **Aim 1:** To elucidate whether VDRAs accelerate the progression of vascular calcification in arterial rings isolated from adults with CKD.

Arterial vessel rings from adults with CKD, either pre dialysis or on dialysis were treated in pro calcaemic conditions with VDRAs calcitriol and paricalcitol. Their Ca content was quantified by a colourimetric cresolphthalein assay and level of calcification visualised by histology.

Chapter 2 Methods

2.1 Collection and processing of patient vessels

2.1.1 Ethics and patient selection

This study was approved by the NHS Health Research Authority, National Research Ethics Service (HRA NRES) committee London – Bloomsbury. Informed written consent was obtained from all patients or their parents/guardians and children if appropriate. For the children's vessel study all patients were aged 1 to 17 years and for the adult vessel study the patient age range was 19 to 71 years. All patients were free from confounding pro-atherosclerotic risk factors. To ensure this, patients who had underlying inflammatory disorders, vasculitis, diabetes, or dyslipidaemia were excluded from the study. Patient details were recorded at the time of vessel collection including; age, gender, primary renal diagnosis, type of and time on dialysis (if applicable) and any vitamin D supplements prescribed. All vessels were allocated a unique vessel ID to fully anonymise all samples, this is shown in Table 2-1 along with the patient information. There were 3 categorise of vessels:

- Controls: Non-renal patients undergoing routine abdominal surgery, who were free from inflammatory disease.

- Pre-dialysis: CKD patients, normally CKD stage V (GFR < 15ml/min) who were undergoing renal transplant or catheter insertion prior to dialysis.

- Dialysis: CKD patients at ESKD who were receiving renal replacement therapy in the form of peritoneal dialysis (PD) or haemodialysis (HD) prior to renal transplant.

Table 2-1: Patient information for children's vessel rings.
Vessel ID format: Year. Age and gender. Unique number. Vessel type.
IE – Inferior epigastric artery. ME – Mesenteric artery. CAKUT – Congenital anomalies of the kidneys and urinary tract. ATN – Acute Tubular Necrosis. HD – Haemodialysis. PD – Peritoneal dialysis.

Vessel ID	Age (Years)	Gender	Primary renal Diagnosis	Type of & time on dialysis (months)	Alfacalcidol supplements
07.6M.44.IE	6	Male	CAKUT	Pre-dialysis	Yes
07.16F.46.IE	16	Female	Unknown	Pre-dialysis	Not known
07.10M.52.IE	10	Male	Neonatal ischemia and ATN	Pre-dialysis	Not known
07.9M.54.IE	9	Male	Glomerulopathy	Pre-dialysis	Yes
13.14M.105.IE	14	Male	Cystic kidney disease	Pre-dialysis	Yes
13.5M.110.IE	5	Male	CAKUT	Pre-dialysis	Yes
13.8F.111.IE	8	Female	CAKUT	Pre-dialysis	Yes
13.12F.113.IE	12	Female	Glomerulopathy	Pre-dialysis	Yes
14.15M.115.IE	15	Male	CAKUT	Pre-dialysis	Yes
14.11M.118.IE	11	Male	CAKUT	Pre-dialysis	Yes
14.8F.122.IE	8	Female	Bilat Wilms Tumour	Pre-dialysis	Yes
07.15F.45.IE	15	Female	CAKUT	88 HD	Yes
07.2M.48.IE	2	Male	CAKUT	0.25 PD	Yes
17.15F.49.IE	15	Female	Cystic kidney disease	30 PD	Yes
07.2M.50.IE	2	Male	CAKUT	21 HD	Yes
07.1M.51.IE	1	Male	Glomerulopathy	0.02 PD	Yes
07.15F.53.IE	15	Female	Glomerulopathy	0.6 PD	Yes
13.9M.104.IE	9	Male	CAKUT	12 HD	Yes
14.8M.114.IE	8	Male	CAKUT	14 HD	Yes
14.17M.116.IE	17	Male	CAKUT	5 HD	Yes
14.13M.119.IE	13	Male	CAKUT	8 HD	Yes
05.1.5M.04.IE	1.5	Male	Glomerulopathy	17 HD	Yes
12.13M.16.IE	13	Male	CAKUT	32 HD	No
13.15F.24.IE	15	Female	CAKUT	23 PD	Yes
06.9M.34.IE	9	Male	CAKUT	10 PD	Not known
06.8F.36.IE	8	Female	Cystic kidney disease	13 HD	Not known
13.16M.101.IE	16	Male	Cystic kidney disease	12 HD	Yes
13.15F.112.IE	15	Female	Glomerulopathy	25 HD	Yes
14.8F.123.ME	8	Female	N/A - Laparotomy	Control	No
15.2F.127.ME	2	Female	N/A – Closure of Stroma	Control	No
15.5M.128.IE	5	Male	N/A - Ileostomy	Control	No
13.0.5F.109.ME	0.5	Female	N/A – Anorectal reconstruction	Control	No

2.1.2 Vessel types

All vessels used in these studies were medium sized muscular arteries, this included inferior epigastric, mesenteric and omental arteries.

The inferior epigastric artery is a medium sized muscular artery that arises from the external iliac artery. It runs in the subcutaneous fat perpendicular to the surgical incision for a renal transplant. During a renal transplant the inferior epigastric is routinely dissected out and discarded to enable the surgeon deeper access into the abdomen. In this study the inferior epigastric arteries were collected during renal transplant from pre-dialysis and dialysis patients. They were utilised for *ex-vivo* studies of vessel rings and to explant VSMCs for *in-vitro* studies

The omentum and the mesentery are folds of intra-abdominal tissue that are highly vascular. The omentum is a membrane layer that lines the abdominal cavity and organs within. The mesentery is a double layer of peritoneum that connects the small intestine to the wall of the abdominal cavity. A small piece of either omentum or mesentery is often removed from patients having abdominal surgery in order to access the viscera. For example, omentectomy is routinely performed when a catheter is inserted for peritoneal dialysis. Medium sized muscular arteries in the omentum or mesentery were dissected out of CKD and non-renal control patients for *ex-vivo* studies of vessel rings and VSMCs were explanted for *in-vitro* studies.

2.1.3 Dissecting and cutting vessel rings.

Vessels were collected and transported in M199 tissue culture medium (Sigma) and stored at 4˚C for a maximum of 24 hours before dissection in a sterile tissue culture hood. The surrounding fat and adventitia were gently stripped from the arteries. Vessels were dissected into 1mm-thick rings to enable perfusion in culture; between 12 and 20 rings were obtained from each vessel.

2.2 *Ex-vivo* methods

2.2.1 Treatment of vessel rings

Depending on the length of vessel available, up to three rings were cultured per condition for analysis of Ca content, ALP activity and for histology. Three baseline rings were also taken prior to culture. The vessel rings were cultured in FBS free M199 with PSG for 14 days. FBS promotes VSMC growth therefore was not used in the culture of vessel rings. Rings were treated in either normal Ca-P medium as a control and high Ca-P medium to promote mineralisation. Normal Ca-P medium consisted of M199 alone which has 1mM phosphate (P) and 1.8mM calcium (Ca). In high Ca-P medium the mineral content was increased to a final concentration of 2mM P and 2.7mM Ca with the addition of NaH_2PO_4 and $CaCl_2$. Vitamin D treatment with either paricalcitol (Abbvie), calcitriol (Sigma D1530) or alfacalcidol (Selleckchem S1468) was added to a final concentration 10nM. In each case ethanol was used as the vehicle and its concentration was adjusted to 3.4mM in each condition (Figure 1). Medium was refreshed every three days.

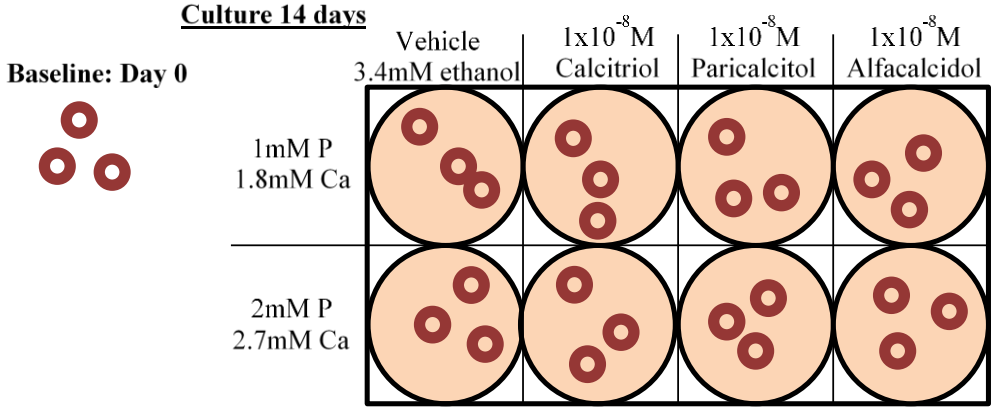


Figure 2-1: Schematic of treatment conditions for *ex-vivo* culture of vessel rings. For each condition a ring was analysed for Ca content, ALP activity and histology.

2.2.2 Vessel ring Ca load analysis

Vessel rings were washed in HBSS (Ca free Hanks Balanced Saline Solution) and stored at -80°C until analysis. To extract Ca, vessel rings were placed in a Dounce Glass Tissue Homogenizer with 200µl 0.1M HCL and homogenised every 30 minutes for 2 hours. The tissue and solution were transferred to an Eppendorf and centrifuged at 13000g for 2 minutes. The supernatant was removed and the Ca content of the supernatant was determined by o-cresolphthalein assay. The pellet was washed in HBSS and homogenised every 30 minutes for an hour in 0.1M NaOH / 0.1% SDS. Again the tissue and solution were transferred to an Eppendorf and centrifuged at 13000g for 2 minutes, the supernatant was retained for BioRad protein assay.

2.2.2.1 O-Cresolphthalein assay

A colorimetric assay to determine Ca concentration, in which Ca reacts with o-cresolphthalein in an alkaline solution to form a violet complex that has maximum absorbance at 577nm. A standard curve was obtained by 1:2 serial dilutions of 55µl 1mg/ml $CaCl_2$ in 55µl 0.1M HCl in a 96 well plate. Samples were diluted in 0.1M HCl and a total of 55µl added to the plate for comparison to the standard. To each of the standard and samples, 25µl dH_2O, 200µl ammonia buffer and 10µl o-cresolphthalein solution were added and the absorbance was read immediately at 560nm by using a TECAS Genios Pro Multifunction Microplate Reader. Ca content was normalised to protein.

2.2.2.2 BioRad Protein Assay

Protein concentrations were measured to normalise Ca concentrations. 5µl supernatant in 0.1M NaOH / 0.1% SDS was pipetted into a 96-well plate and the standard was prepared from 1:2 serial dilutions of 1mg/ml bovine serum albumin (BSA). BioRad DC protein assay kit was used according to the manufacturer's instruction and absorbance was read at 710nm.

2.2.3 Vessel ring Alkaline Phosphatase activity

Vessel rings were washed in HBSS and stored at -80°C for a maximum of 7 days before analysis. To extract ALP, vessel rings were homogenised in a Dounce Glass Tissue Homogenizer in 120µl 10% SDS every 10 minutes for an hour, during this process homogenisers were kept on ice to avoid enzyme degradation. The supernatant was retained for both a colorimetric ALP assay to determine enzyme activity and a protein assay for normalisation as above.

2.2.3.1 Alkaline Phosphatase Assay

To determine ALP activity 100µl of 1mg/ml para-Nitrophenol phosphate (p-Npp) in ALP buffer was added to 50µl of sample (25µl supernatant diluted in 25µl 10% SDS) in a 96-well plate and incubated at 37˚C in the dark for 30 minutes. P-Npp is an ALP substrate, and activity of ALP is determined by the decay of colourless p-Npp to yellow para-nitrophenol (p-Np). A standard was prepared immediately prior to reading the absorbance. 1:2 serial dilutions of 50µl p-Np in H_2O were made and 100µl 1mg/ml p-Npp added to each well. The absorbance was read at 405nm using a TECAS Genios Pro Multifunction Microplate Reader and p-Np formation in samples calculated from the standard. ALP activity calculated as enzyme units (U, defined as product formed (µmol) over reaction time (min)) and standardised to protein content (mg), as determined by BioRad protein assay.

2.2.4 Sample preparation for histology

2.2.4.1 Embedding and sectioning of vessel rings

Vessel rings were fixed in 4% paraformaldehyde (PFA) for 24 hours, then dehydrated in increasing concentrations of ethanol and then xylene in the Shandon Hypercenter XP processor and subsequently embedded in paraffin wax blocks using a Sakura Tissue-TEK

TEC tissue embedding console. Sections of 7μm were cut using a rotary microtome (Leica RM2125RTF).

2.2.4.2 Dewax and rehydration

Prior to staining, slides were dewaxed in xylene (2x 5 minutes) and rehydrated in graded concentrations of ethanol (1 minute 100% ethanol, 1 minute 100% ethanol, 1 minute 70% ethanol, 1 minute 50% ethanol) and washed in H_2O (3 minutes).

2.2.4.3 Dehydrate and mount

After staining slides were dehydrated in graded concentrations of ethanol (1 minute 50% ethanol, 1 minute 70% ethanol, 1 minute 100% ethanol, 1 minute 100% ethanol) and cleared in xylene (2x 5 minutes). Then a coverslip was mounted with DPX mounting medium. Slides were visualised on a Zeiss Axioplan scope and images taken with a Zeiss colour camera using Axiovision software.

2.2.5 Haematoxylin and eosin (H&E) staining

Slides were dewaxed and rehydrated then stained in Harris haematoxylin (Sigma) for 5 minutes and rinsed in H_2O. Slides were differentiated in 0.3% acid alcohol (HCL in 70% ethanol) then rinsed in H_2O again. Finally, slides were stained in stained in eosin (Sigma) for 3 minutes and then dehydrated as above.

2.2.6 Von Kossa staining

Slides were dewaxed and rehydrated then incubated in 2% silver nitrate in dH_2O under a 100-watt light for 2 hours. During this process Ca deposits are replaced by silver, which can be observed as metallic silver and is black in colour. To remove any unreacted silver, slides were washed 3x 2 minutes in H_2O, 2x5 minutes in 3% sodium thiosulphate in dH_2O

and a further 2x5 minutes in H_2O. Slides were counterstained in haematoxylin and eosin as described above.

2.2.7 Immunohistochemistry

Slides were dewaxed and rehydrated then incubated in citrate buffer (Antigen unmasking solution, Vector laboratories) at sub-boiling temperatures for 15 minutes before being allowed to cool to room temperature. This step unmasks antigen binding sites in the tissue, as formalin fixing can induce molecular crosslinks in proteins changing the 3D structure of epitopes. Slides were washed (3x 5 minutes in phosphate buffer saline PBS) then incubated for 10 minutes in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Slides were washed again and then non-specific antigen binding was blocked by incubating slides for 30 minutes in blocking serum diluted in PBS as per Table **2-2**. Blocking serum was made from a species that neither the primary nor secondary antibodies were raised in. Primary antibody was diluted in blocking serum as required (shown in Table **2-2**) and sections were incubated in the primary antibody overnight at 4˚C in a humidifying chamber. The next day they were washed (3x5 minutes in PBS-T) and incubated for 1 hour in biotinylated secondary antibody linked to horseradish peroxidase (1:200 in 1% blocking serum), then washed again (3x5 minutes in PBS-T). To amplify the secondary antibody signal, slides were incubated with the avidin/biotinylated enzyme complex (ABC reagent) for 30 minutes at room temperature, and then washed (3x5 minutes PBS). Depending on sensitivity of primary antibody, slides were incubated between 30 seconds and 4 minutes with DAB (3, 3- diaminobenzadine), a horseradish peroxidase substrate that produces a dark brown product. Slides were counterstained with haematoxylin, dehydrated and mounted as above. Blocking serum, DAB, secondary antibodies and ABC reagent were obtained from Vectastain ABC kit, Vector Laboratories.

Table 2-2: Immunohistochemistry antibodies.

Primary Antibody	Dilution	Blocking solution	Secondary Antibody	DAB time
mAb VDR (D-6) (Santa Cruz sc-13133)	1:500	1% Horse serum	Mouse 1:200	3 min
Smooth Muscle α actin (Sigma A2547)	1:10 000	1% Horse serum	Mouse 1:200	1 min
CYP24A1 (Abcam ab54594)	1:100	1% Horse serum	Mouse 1:200	50 sec
Alkaline Phosphatase (Abcam ab108337)	1:500	1% Horse serum	Rabbit 1:200	2-3 min
Runx2 M70 (Santa Cruz SC10758)	1:200	10% Horse serum in 3% BSA	Rabbit 1:100	3 min

2.2.8 Cell counts in vessel rings

The total numbers of VSMCs per unit area of the tunica media of vessel rings were calculated using H&E stained sections. For each vessel ring, 3 different frames were chosen at random and imaged at 40x magnification. An outline of the tunica media region was selected manually and its area was calculated using Image J software. This software was also used to keep a tally of the number of nuclei counted. The mean number of nuclei per area of tunica media was calculated using Microsoft Excel.

The number of VSMCs in the tunica media that were expressing VDR were also counted by applying this method to sections that were positive for VDR expression after immuno-staining.

2.3 *In-Vitro* Methods

2.3.1 Explanting VSMCs from vessels

To explant VSMCs, a vessel ring was cut into tiny pieces and cultured in a 6-well plate in M199 with 20% foetal bovine serum (FBS) as well as 100 U/ml penicillin, 100 U/ml streptomycin and 0.29 mg/ml glutamine (PSG). Plates were incubated in a 4% CO_2 and 37°C incubator (Thermo Haraeus HERAcell 150), to ensure tissue adhered to the plate they were not disturbed for the first 7 days, then the medium was refreshed every 3-4 days. Patient information for explanted control and dialysis VSMCs are shown in Table 2-3 and Table 2-4 respectively.

Table 2-3: Patient information for healthy control VSMC explants.
VSMC ID format: Year. Age and gender. Unique number. Vessel type. OM – Omental artery. ME – Mesenteric artery.

VSMC ID	Age (Years)	Gender	Operation vessel obtained from	Vessel Type	Vitamin D supplements
13.0.1F.106.OM	0.1	Female	Laparoscopy	Omental Artery	None
13.10F.108.OM	10	Female	Laparoscopy	Omental Artery	None
06.1M.39.ME	1	Male	Ileostomy	Mesenteric artery	None
13.1F.28.OM	0.5	Female	Anorectal reconstruction	Omental Artery	None
13.3M.31.OM	3	Male	Splenectomy	Omental Artery	None
13.5F.32.OM	5	Female	Lap ACE	Omental Artery	None
13.5M.33.OM	5	Male	LAP ACE	Omental Artery	None

Table 2-4: Patient information for dialysis VSMCs.
Vessel ID format: Year. Age and gender. Unique number. Vessel type.
IE – Inferior epigastric artery. OM – Omental artery. HD – Haemodialysis. PD – Peritoneal dialysis.

VSMC ID	Age (Years)	Gender	Primary renal Diagnosis	Type of and time on dialysis (months)	Vessel Type	Vitamin D supplements
05.1.5M.04.OM	1.5	Male	Glomerulopathy	17 HD	Omental Artery	Alfacalcidol
12.13M.16.IE	13	Male	Dysplasia	32 HD	Inferior Epigastric	Alfacalcidol
13.15F.24.IE	15	Female	Dysplasia	23 PD	Inferior Epigastric	Alfacalcidol
06.9M.34.IE	9	Male	Dysplasia	10 PD	Inferior Epigastric	Alfacalcidol
06.8F.36.IE	8	Female	Cystic Kidneys	13 HD	Inferior Epigastric	Alfacalcidol
13.16M.101.IE	16	Male	Cystic Kidneys	12 HD	Inferior Epigastric	Alfacalcidol
13.15F.112.IE	15	Female	Glomerulopathy	25 HD	Inferior Epigastric	Alfacalcidol
14.13M.119.IE	13	Male	Unknown	8 PD	Inferior Epigastric	Alfacalcidol
14.14M.124.IE	14	Male	Dysplasia	10 HD	Inferior Epigastric	Alfacalcidol
4.17M.125.IE	17	Male	Dysplasia	18 PD	Inferior Epigastric	Alfacalcidol

2.3.2 Passaging and freezing VSMCs

VSMCs were grown in M199 supplemented with 20% FBS and PSG. At 80% confluence VSMCs were passaged by splitting 1:2. Cells were washed with HBSS then incubated with 0.25% Trypsin-EDTA (Sigma) for 5 minutes at 37°C to dislodge the cells. Trypsin was neutralised with M199 with 20% FBS and PSG and cell split 1:2.

For freezing, VSMCs were trypsinized as above and cells were centrifuged at 700g for 5 minutes at 4°C. The pellet was re-suspended in freezing medium (FBS with 10% dimethyl sulfoxide (DMSO)), transferred to a cryo vial and frozen gradually in a Mr. Frosty Freezing Container (Thermo Scientific) containing isopropanol in a -80°C freezer. After 24 hours frozen cells were transferred to liquid nitrogen.

2.3.3 Calcification of VSMCs

VSMCs were seeded in 6-well or 48-well plates with M199 supplemented with 20% FBS and PSG. At 80% confluence treatment began, cells were cultured in M199 with PSG and reduced serum (5% FBS). It is necessary to reduce serum content as FBS contains calcification inhibitors.

Calcemic conditions were created by increasing the mineral content of M199 from 1mM P and 1.8mM Ca (normal Ca-P medium) to a final concentration of 2mM P and 2.7mM Ca (high Ca-P medium) by the addition of NaH_2PO_4 and $CaCl_2$. Cells were treated with VDRAs, either 10nM paricalcitol (Abbot) or calcitriol (Sigma) at 1nM, 10nM or 100nM. In each case ethanol was used as the vehicle to a final concentration of 3.4mM and 3.4mM ethanol was included as a vehicle control. Experiments were performed in pairs with VSMCs explanted from one control and one dialysis patient treated simultaneously. Media was changed every 3 days and cells were cultured until the onset of calcification was observed by light microscopy (between 4 and 6 days).

Ca deposition in treated VSMCs was quantified by o-cresolphthalein assay. In addition, also ALP activity was determined, RNA was isolated for qPCR analysis and protein was harvested for western blot analysis.

2.3.4 VSMC Ca load analysis

Cells were washed in HBSS and incubated at room temperature with 0.1M HCL for 2 hours to dissolve the Ca deposits. The supernatant was analysed for Ca content using the o-cresolphthalein assay described above. Ca content was analysed in triplicate wells of a 48-well plate and normalised to protein content of triplicate neighbouring wells. Protein was harvested in 0.1M NaOH in 1% SDS and analysed with the BioRad protein assay described above.

2.3.5 VSMC Alkaline Phosphatase Activity

VSMCs in a 6well plate were washed in HBSS and each well was harvested in 100µl 1% Triton X-100 in PBS. Samples were kept on ice throughout as ALP is a heat labile enzyme. Samples were freeze-thawed twice from -80˚C to room temperature then centrifuged at 13000g for 5 minutes at 4˚C. The supernatant was retained for the ALP activity assay and BioRad protein assay as described above.

2.3.6 RNA isolation and Reverse Transcription

Cells were lysed in RNA STAT-60 (Amsbio) and the RNA in the aqueous phase was separated out using chloroform, and then precipitated with isopropanol. The RNA pellet

was washed in 75% ethanol and re-suspended in Diethylpyrocarbonate (DEPC) treated H_2O.

Reverse transcription of RNA was carried out with Random primers (Promega), Oligo dT primers (Promega), dNTP mix (Eurogentec), RNAsin RNase inhibitor (Promega), 5x Mu-MLV buffer (Eurogentec), Mu-MLV reverse transcriptase (Eurogentec) and DEPC H_2O. The RNA and primers were incubated at 65˚C for 5 minutes prior to the reaction to denature the RNA. The reverse transcription reaction was carried out at 25˚C for 10 minutes for primers to anneal and then 37˚C for 50 minutes for the reverse transcription to produce cDNA. A final incubation at 95°C for 5 minutes terminated the reaction and inactivated reverse transcriptase. The reaction was then cooled to 4˚C in an Applied Biosystems 2720 Thermal Cycler. The resulting cDNA was diluted to 2µg/100µl in DEPC H_2O.

2.3.7 Primer validation: Polymerase Chain Reaction

Primers were validated to confirm that they were specific in only amplifying the expected product. PCR was carried out in 50µl reaction volume, with 2x PCR mastermix, 0.125µM of each forward and reverse (primer sequences shown in Table 2-5) and 0.1µg cDNA. 40 cycles of 94˚C for 30 seconds, 60˚C for 30 seconds and 72° for 30 seconds were carried out in an Eppendorf vapo protect PCR machine

PCR product was resolved on a 2% agarose gel (agarose in TAE) next to a Quick load purple 100 bp DNA ladder (New England Biolabs) at 120V for 30 minutes then visualised under ultraviolet light to estimate length of PCR products.

2.3.8 Quantitative real time PCR

QPCR was carried out in 20µl reaction volume, with MESA GREEN qPCR Master Mix (Eurogentec) with 0.125µM of each forward and reverse primer Table 2-5 and 0.04µg cDNA. The cDNA was heated to 94°C to activate the polymerase. Then cDNA underwent 45 cycles of 94˚C for 15seconds (denaturation) and 60˚C for 60 seconds (primer anneals to single strand and polymerase synthesises a complimentary sequence). This was carried out in a Corbette RotorGene 3000. A melt curve was also obtained by 1˚C inclines at 65-99˚C.

Ct (Cycle threshold) values were determined and gene expression was quantified using a standard curve. To obtain standards, PCR product was purified using the QIAquick PCR purification kit (Qiagen) and diluted to 0.05pM, 0.005pM, 0.0005pM and 0.00005pM. Gene expression was normalised to 18S rRNA expression.

Table 2-5: **Primer sequences**.

Gene	Primer sequence (5' to 3')	Annealing temperature	Amplicon length
18S rRNA	F: CCCAGTAAGTGCGGGTCATAA R: CCGAGGGCCTCACTAAACC	60°C	101bp
ALP	F: ACGAGCTGAACAGGAACAACGT R: CACCAGCAAGAAGAAGCCTTTG	55°C	104bp
BSP	F: AGTTTCGCAGACCTGACATCC R: TTCATAACTGTCCTTCCCACG	55°C	161bp
CYP24A1 (24 hydroxylase)	Qiagen QT00015428	60°C	119bp
CYP27A1 (25 hydroxylase)	Qiagen QT00088536	60°C	105bp
CYP27B1 1α hydroxylase	Qiagen QT01678012	57°C	184bp
CYP2R1 (25 hydroxylase)	F: GAAAGCAGAGCCAGGTGTACG R: TCATGAATAAAGGAAGGCATGG	58°C	150bp
MGP	Qiagen QT01004423	60°C	99bp
Runx2	Qiagen QT00020517	60°C	102bp
SM22	F:TTGAAGGCAAAGACATGGCAGCAG R: TCCACGGTAGTGCCCATCATTCTT	60°C	89bp
SMA	F: TGACAATGGCTCTGGGCTCTGTAA R: TTCGTCACCCACGTAGCTGTCTTT	60°C	142bp
SMMHC	F: AGAAGCCAGGGAGAAGGAAACCAA R: TGGAGCTGACCAGGTCTTCCATTT	60°C	131bp
Total VDR	F:GCCCACCATAAGACCTACGA R:AGATTGGAGAAGCTGGACGA	60°C	203bp

2.3.9 Western Blotting

Untreated cells in T25 flasks (baseline conditions) were harvested in 200µl of immunoprecipitation (IP) protein lysis buffer. The protein concentration was determined by BioRad protein assay. Protein lysates were mixed with 2x sample buffer. Protein from cells cultured and treated in 6-well plates were harvested directly into 100µl of 2x sample buffer per well, in order to avoid diluting samples.

All samples were sonicated twice for 5 seconds and heated to 95°C for 5 minutes to denature the tertiary structure, enabling samples to be separated by electrophoresis in a size dependent manner.

20µg of baseline protein or 20µl of protein harvested from treated cell lines were loaded onto a 10% polyacrylamide gel alongside a Precision Plus protein standard (Bio-Rad) and subject to sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) at 120V for approx. 90 minutes in a Bio-Rad Mini-Protean Tetra System.

Semi-dry transfer was used to transfer proteins onto a methanol charged PVDF Immobilon-P transfer membrane, proteins were transferred at 25V for 60 minutes in a Bio-Rad Trans-Blot Semi-Dry Transfer Cell. Membranes were blocked in 5% milk (Marvel) in TBS-T buffer for 60 minutes, then probed overnight at 4˚C with primary antibody in 5% milk in TBS-T (dilutions shown in Table 2-6). Membranes were washed 3x 10 minutes in TBS-T, then probed with the corresponding horse radish peroxidase linked secondary antibody for 60 minutes and washed again 3x 10 minutes. An enhanced chemiluminescence (ECL) kit (ECL Prime Western Blotting Detection Reagent – GE Healthcare) and Fujifilm – Fuji Medical X-Ray Film were used to visualise proteins. Membranes were re-probed for β-actin as a gel loading control. The optical density of each band was quantified using Image J and normalised to the corresponding optical density of β-actin.

Table 2-6: Antibodies for western blot analysis.

Primary Antibody	Dilution	Secondary Antibody	Dilution
mAb VDR (D-6), Santa Cruz sc-13133	1:500	Anti-Mouse IgG, GE Healthcare NA931V	1:5000
β-actin, Sigma A2228	1:10 000	Anti-Mouse IgG, GE Healthcare NA931V	1:5000

2.3.10 Luciferase assay – VDRE

The Cignal Reporter Assay Kit VDRE (Qiagen CCS-2029L) was used to determine VDRE activation.

VSMCs were seeded at a density of 20 000 cells per well in a 24-well plate in M199 with 20% FBS. At 90% confluence cells were serum starved for 24 hours in M199 supplemented with 0.5% BSA and PSG only. Transfection reagents were prepared using Lipofectamine LTX and Plus Reagent (Invitrogen). Lipofectamine LTX was diluted 1:9 in OptiMEM (Gibco) and Plus reagent was diluted 1:18 in OptiMEM with 400ng construct per well. These transfection mixtures were combined (1:1) and incubated at room temperature for 5 minutes. The transfection mixture was added 1:10 to serum free M199 and cells were transfected for 6 hours with either VDRE reporter construct, positive control or negative control. The constructs specifications are shown in Table 2-7.

After 6 hours, the transfection media was removed and replaced with serum free media containing treatment conditions. Triplicate wells of VSMCs were treated for 24 hours with either 100nM Calcitriol or 3.4mM ethanol as a vehicle control. Cells were washed in PBS and the Dual Glo Luciferase Assay system (Promega) was used to determine luciferase activity in each well, this was normalised to renilla activity as a control for transfection

efficiency. Luminescence was read using a Berthold Technologies, Mithras LB 940 multimode microplate reader.

A duplicate plate was seeded (80 000 cells/well in a 6-well plate), these cells were not transfected, however they were simultaneously treated with 100nM calcitriol or 3.4mM ethanol and harvested for either RNA or protein as described above.

Table 2-7: Components of Cignal Reporter Assay Kit.

Component	Specification
VDRE Reporter	Inducible Vitamin D Responsive Firefly luciferase construct
	Constitutively expressing Renilla luciferase construct
Positive Control	Constitutively expressing GFP
	Constitutively expressing Firefly luciferase construct
	Constitutively expressing Renilla luciferase construct
Negative Control	Non inducible Firefly luciferase construct
	Constitutively expressing Renilla luciferase construct

2.3.11 Immunocytochemistry

VSMCs were seeded on coverslips in a 24-well plate at a density of 15 000 cells/well. Cells were fixed in 4% PFA and washed 3 x PBS. Cells were permeabilised in 0.5% NP40 for 3 minutes and washed again 3x in PBS. To block non-specific antigen binding, cells were submerged in 3% BSA in PBS for 1 hour, then incubated at 4°C overnight in a humidifying chamber with primary antibody 1:500 mAb VDR (D-6) (Santa Cruz sc-13133) in 3% BSA in PBS. Cells were washed 3x in PBS, then incubated with secondary

antibody (Donkey anti-Mouse Alexa Fluor 488, Invitrogen) diluted 1:500 in 3% BSA in PBS for 1 hour at room temperature in the dark. Cells were washed again in PBS and incubated for 5 seconds with DAPI (Sigma) 1:7000 in 3% BSA in PBS, then immediately washed again 3x in PBS. Coverslips were mounted onto slides using Mowiol mounting medium for imaging with an Olympus IX81 fluorescence microscope.

2.4 Statistics

2.4.1 Gaussian D'Agostino and Pearson Omnibus Normality Test

Statistical analysis was performed in Graph Pad Prism 5. The Gaussian D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution of data. When $P>0.05$ samples were considered to have passed the normality test and it could be assumed that the sample was from a normally distributed population. $P<0.05$ indicated that the probability of that sample being chosen from a normally distributed population was less than 5%, therefore the sample distribution was considered to deviate significantly from the Gaussian ideal, the sample failed the normality test and the population was assumed not to be normally distributed. This normality test can generate false negatives and false positives, therefore it was used as a guide only and common sense was also applied.

2.4.2 Parametric and Non-Parametric T-Tests

Statistical tests were performed in Graph Pad Prism 5 to determine if two samples were significantly different from each other. The parametric, student's t-test was applied when both sets of data were considered to be normally distributed. A non-parametric, Mann-Whitney U test was applied when either or both of the samples were thought to be from a

population that did not follow Gaussian distribution, this test is based on rank and does not assume the population's distribution.

All tests were unpaired as each treatment condition was tested on individual vessel rings or populations of cells. Tests were always two-tailed to determine the significance of a change that could be either an increase or decrease.

For both the unpaired two-tailed t-test and the two-tailed Mann-Whitney U test a $P<0.05$ was considered statistically significant. This indicated that there was less than a 5% probability that the 2 samples were chosen at random from the same population, therefore the 2 populations were statistically different from each other.

2.4.3 Correlation coefficient

The correlation coefficient was calculated using Graph Pad Prism 5 to analyse the relation between two measured variables. Pearson correlation coefficient (R) assumes an approximate Gaussian distribution and was used as appropriate. When either or both of the variables did not follow Gaussian distribution the Spearman correlation coefficient (RS) was calculated, this is based on rank and does not assume Gaussian distribution. The correlation coefficient (R or RS) has a range of -1 (perfect negative correlation) to 1 (perfect positive correlation) with 0 representing no correlation.

Two-tailed P values were calculated for both R and RS. The P value shows the probability that the correlation coefficient (R or RS) could have been calculated from random samples with no correlation. R (or RS) would be considered statistically significant if $P<0.05$, i.e. there was less than a 5% probability that R (or RS) was calculated from a random sample.

2.5 Buffers and solutions

4% PFA (Paraformaldehyde)

- 4% PFA
- PBS
- pH 7.5

ALP buffer

- 0.1M Glycine
- 1mM MgCl2
- 1mM ZnCl2
- pH 10.4

Ammonia buffer

- 0.24% NH4Cl
- 5% NH4OH
- pH 10.5

Immunoprecipitation (IP) protein lysis buffer

- 0.1M Tris-HCl pH 8.1
- 1% Triton X-100
- 0.15M NaCl
- 10µl protease inhibitor (Sigma)

Mowiol mounting medium for immunocytochemistry

- 10% Mowiol
- 25% glycerol
- 0.1M Tris
- pH 8.5

O-Cresolphthalein solution

- 1mg/ml o-cresolphthalein
- 0.0672% NH4Cl
- 1.4% NH4OH
- pH 10.5

PBS-T

- 100ml H_2O per PBS tablets
- 0.1% (v/v) Tween 20

SDS-PAGE 2x sample buffer

- 62.5mM Tris pH6.8

- 10% Glycerol
- 2% SDS
- 0.075% bromophenol blue
- 5% (v/v) β-mercaptoethanol (added fresh prior to use)

SDS-PAGE resolving gel

- 10% acrylamide
- 375mM Tris pH 8.8
- 0.1% SDS
- 0.13% APS (stock 10% ammonium persulfate (Sigma) in H_2O)
- 0.13% TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine, Sigma)

SDS-PAGE stacking gel

- 5% acrylamide
- 125mM Tris pH 6.8
- 0.1% SDS
- 0.1% APS (10% ammonium persulfate (Sigma) in H_2O)
- 0.2% TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine, Sigma)

SDS-PAGE running buffer

- 25 mM Tris
- 250 mM glycine
- 0.1% SDS
- pH 8.3

TBS-T

- 150mM NaCl
- 10mM Tris
- pH 8
- 0.2% (v/v) Tween 20

TAE (Tris-acetic acid-EDTA)

- 40mM Tris
- 20mM acetic acid
- 1mM EDTA

Western blot transfer buffer

- 20% (v/v) methanol
- 25mM Tris
- 0.2M glycine
- 1% SDS
- pH 8.3

Chapter 3 The effects of VDRAs on vascular calcification in vessel rings isolated from children with CKD.

3.1 Introduction

Vessel rings from children with CKD enable the effects of VDRAs on arteriosclerosis to be studied without the confounding factors of atherosclerosis associated with aging and traditional cardiovascular risk factors. Data in this chapter were collected from paediatric patients with CKD who were undergoing renal transplant at Great Ormond Street Hospital. It is representative of the general paediatric CKD population including patients with different underlying renal diagnoses, varying ages, different genders, and patients on dialysis (including haemodialysis or peritoneal dialysis and with varying dialysis vintage) or in pre-dialysis CKD stage 5. To compensate for this patient variation, numerous rings were obtained from each patient's vessel and the effects of different treatment conditions will be compared against the baseline vessel characteristics, such that each vessel can be its own internal control. Children's vessels provide an ideal model to study uremic changes on the vessel wall as children are free of pre-existing cardiovascular risk factors like diabetes, dyslipidaemia or heart disease and patients are non-smokers.

It has previously been demonstrated that these rings can remain viable for 2 weeks in serum free tissue culture medium (M199) supplemented with Ca and P (Shroff et al., 2010), this method was used throughout this thesis.

3.1.1 Aim

1. To establish whether different VDRAs, calcitriol, alfacalcidol and paricalcitol, accelerate the progression of vascular calcification in arterial rings isolated from children with CKD.

This will be addressed using the following key questions;

1. What effect do physiological doses of the VDRAs, calcitriol, alfacalcidol and paricalcitol, have on vascular calcification in vessels from children with CKD?

2. Are there any differences in susceptibility to VDRA-induced vascular calcification between vessel rings from pre-dialysis and dialysis patients?

3. Do any patient related factors affect the response of children's vessel rings to VDRA-induced vascular calcification, including gender, age and primary renal diagnosis?

3.2 Methods

For this study, the inferior epigastric artery was collected from patients listed the methods. Vessel rings were cultured in either normal Ca-P medium (normal M199 tissue culture medium which contains 1mM P and 1.8mM Ca M199) or high Ca-P medium (M199 with added P and Ca to a final concentration of 2mM P and 2.7mM Ca) for 14 days, with the medium changed every 3 days. Vessels were exposed to physiological doses (10nM) of the different VDRAs, alfacalcidol, calcitriol or paricalcitol. Ethanol was used as the vehicle for each VDRA at a final concentration of 3.4mM; therefore 3.4mM ethanol was used as the vehicle control for vessels cultured without VDRAs. After 14 days of culture, vessel rings were either homogenised and their Ca content quantified by a colorimetric σ-cresolphthalein assay, or they were fixed in formalin, paraffin embedded for sectioning and the Ca content visualised by von Kossa staining as described in the methods chapter. All data shown as mean $\pm$ SE.

Table 3-1: Vessel rings and the treatment conditions applied.
Vessel ID format: Year. Age and gender. Unique number. Vessel type.

	Vessel ID	Calcitriol	Alfacalcidol	Paricalcitol
Pre-dialysis	07.6M.44.IE*	✓	✓	
	07.16F.46.IE*	✓	✓	
	07.10M.52.IE*	✓	✓	
	07.9M.54.IE*	✓	✓	
	13.14M.105.IE	✓		✓
	13.5M.110.IE	✓		✓
	13.8F.111.IE	✓		✓
	13.12F.113.IE	✓		✓
	14.15M.115.IE	✓		✓
	14.11M.118.IE	✓		
	14.8F.122.IE	✓		✓
Dialysis	07.15F.45.IE*	✓	✓	
	07.2M.48.IE*	✓	✓	
	07.15F.49.IE*	✓	✓	
	07.2M.50.IE*	✓	✓	
	07.1M.51.RA*	✓	✓	
	07.15F.53.IE*	✓	✓	
	13.9M.104.IE	✓		✓
	14.8M.114.IE	✓		✓
	14.17M.116.IE	✓		✓
	14.13M.119.IE	✓		✓
Controls	14.8F.123.ME	✓		✓
	15.2F.127.ME	✓	✓	✓
	15.5M.128.IE	✓		
	13.0.5F.109.ME	✓		✓

*Vessels collected and cultured by Rukshana Shroff in 2007. All analysis and histology for these vessels performed by myself, Joanne Laycock.

3.3 Ca load in children's CKD vessels.

3.3.1 At baseline children's CKD vessels showed no hydroxyapatite deposition.

For this study it was important to consider the condition of each vessel prior to culture, therefore for each vessel, two rings were obtained on the day the vessel was harvested. One ring was analysed for its Ca content by σ-cresolphthalein assay and the other ring was formalin fixed for histology, von Kossa staining was performed to show the presence of hydroxyapatite deposition.

The σ-cresolphthalein assay showed a wide range of baseline Ca load both in pre-dialysis and dialysis vessels, this ranged from 16 to 243 ng/(ng/ul) and 9 to 318 ng/(ng/ul) respectively. Individual values for vessel Ca load obtained from the σ-cresolphthalein assay are shown in Table 3-2 and Table 3-3.

Despite some vessel rings having a high σ-cresolphthalein score at baseline (shown in Table 3-2 and Table 3-3), none of the baseline vessel rings were positive for von Kossa, examples shown in Figure 3-3. This suggests that quantification of the vessel Ca load by σ-cresolphthalein assay is a more sensitive measure of vascular calcification than histology for visualising hydroxyapatite deposition.

3.3.2 Culture of vessel rings in high Ca-P medium increased their Ca load.

The effect of VDRAs on vascular calcification was considered in both normal Ca-P and high Ca-P media which is representative of the high circulating levels of Ca and P found in the serum of some CKD patients

When vessel rings were cultured in normal Ca-P medium, there was no change in Ca load relative to baseline. Culture of vessel rings in high Ca-P medium significantly increased the Ca load from 134.3$\pm$27.3 in normal Ca-P medium to 495.1$\pm$73.0 in high Ca-P medium ($p<0.0001$), as shown in Figure 3-1. In some vessel rings from both pre-dialysis and dialysis patients cultured in high Ca-P medium, calcified regions of the tunica media could be identified by von Kossa, examples of this are shown in Figure 3-3.

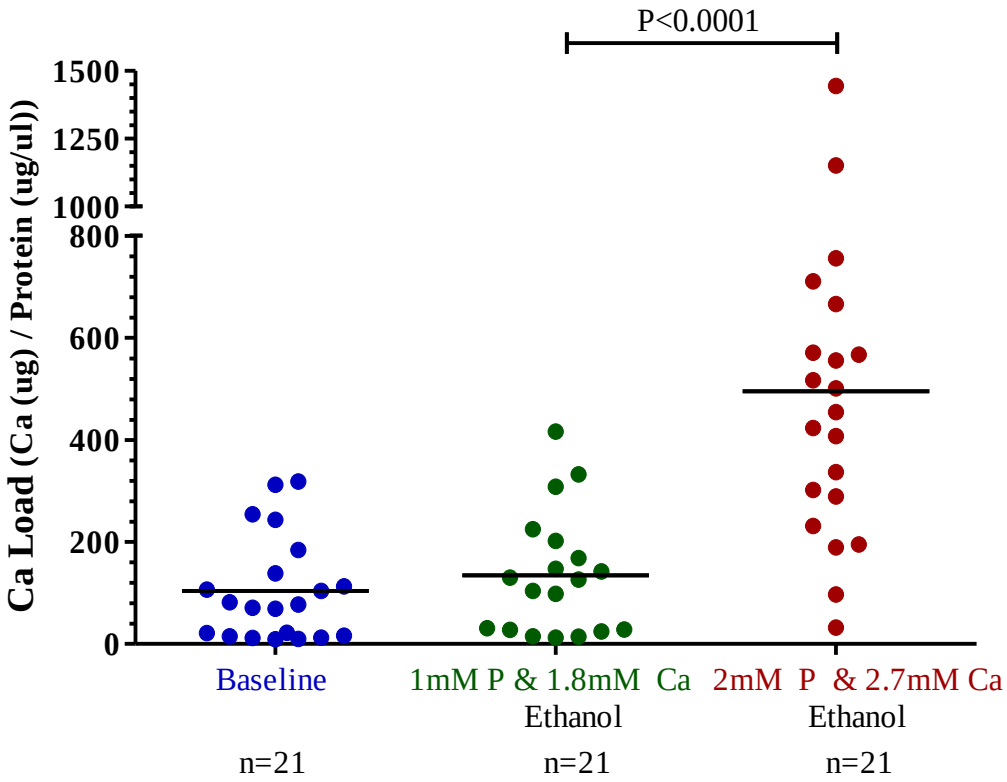


Figure 3-1: High Ca-P medium induced calcification in CKD children's vessel rings.

Vessel rings harvested from children with CKD were isolated at baseline or cultured for 14 days in either normal Ca-P medium (1mM P and 1.8mM Ca M199) or high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification. Both were in the presence of the vehicle 3.4mM ethanol. Ca load in the vessel wall analysed by σ-cresolphthalein assay**.** D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined unpaired two-tailed t-test.

3.4 The effect of VDRAs on Ca load in vessel rings from children with CKD.

3.4.1 Calcitriol and alfacalcidol, but not paricalcitol, promoted vascular calcification in vessel rings isolated from children with CKD.

The previous section showed that culture of CKD vessel rings in normal Ca-P did not affect Ca load, however culture in high Ca-P medium increased their Ca load. The next section will show that the three different VDRAs studied affect Ca load to different extents.

The effects of calcitriol, paricalcitol and alfacalcidol on vascular calcification in vessels from children with CKD were assessed using this *ex-vivo* model. Each VDRA was used at a final concentration of 10nM, this dose represents normal physiological levels of vitamin D in healthy individuals. Vessel rings were cultured for 14 days with one of the VDRAs in either normal Ca-P medium or with high Ca-P medium.

Either with or without any of the VDRAs, increasing the mineral content in the medium significantly increased the Ca load in the vessel wall as represented in Figure 3-2.

In normal Ca-P medium 10nM calcitriol did not affect Ca load, however in high Ca-P medium 10nM calcitriol further increased mean Ca load in the vessel wall (Figure 3-2A). Despite the increase in mean Ca load observed between high Ca-P medium and high Ca-P medium with calcitriol, there was a subset of vessel rings that did not respond to calcitriol and showed no change in Ca load relative to high Ca-P only. Responders and non-responders to calcitriol were investigated further in Chapter 4.

10nM Alfacalcidol significantly increased Ca load in the vessel rings cultured in high Ca-P medium, however alfacalcidol had no effect on Ca load in normal Ca-P medium; Figure 3-2B. The effect of paricalcitol, (a synthetic analogue of calcitriol that is thought to be less calcaemic), on calcification in the *ex-vivo* vessel rings was also tested. Paricalcitol had no effect on Ca load in either normal or high Ca-P media (Figure 3-2C).

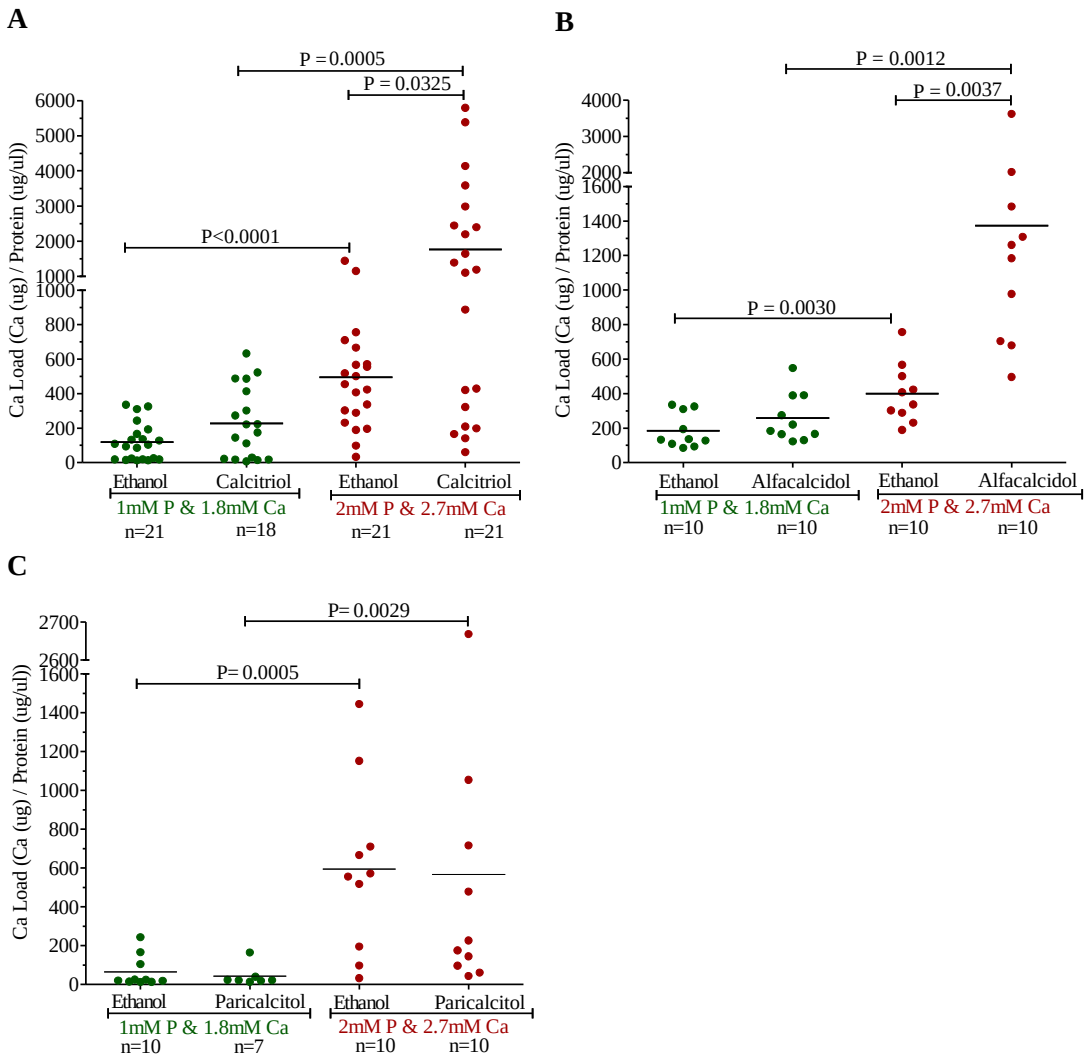


Figure 3-2: Ca load in vessel rings from CKD children cultured with VDRAs.

Vessel rings isolated from children with CKD were cultured in either normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca), as well as either the vehicle of 3.4mM ethanol or **A)** 10nM calcitriol, **B)** 10nM alfacalcidol or **C)** 10nM paricalcitol. The different y-axis on all 3 graphs accommodate the different calcification potentials of the 3 VDRAs. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by A&C) two-tailed Mann Whitney test and B) unpaired two-tailed t-test.

3.4.2 Ca deposition was specific to the tunica media.

The data shown above was determined by σ-cresolphthalein assay which quantifies Ca load in the vessel wall. Ca deposition in each vessel ring was also visualised by von Kossa stain. This demonstrated that Ca deposition was localised to the tunica media, as shown in Figure 3-3 E and F. At baseline, all vessel rings were negative for von Kossa staining, examples of von Kossa positive and negative rings are shown in Figure 3-3.

The von Kossa staining was semi-quantitatively scored as 0, +, ++, +++ or ++++ (whilst blinded to the σ-cresolphthalein score) dependent on the estimated percentage of tunica media positive for von Kossa, and therefore Ca deposition. Each score was assigned a colour and this gives rise to the key in Figure 3-4.

The Ca load determined by σ-cresolphthalein assay, and its corresponding colour for von Kossa positive areas are represented graphically in Figure 3-4. After culture in normal Ca-P medium all vessel rings were negative for von Kossa. There was a wide range of von Kossa positive densities in vessel rings cultured in high Ca-P medium as demonstrated in Figure 3-3. Figure 3-4A shows that, for vessel rings cultured in high Ca-P with or without calcitriol, the rings with a higher Ca load tended to have a higher von Kossa density and be grouped as ++, +++ or ++++. However, some vessel rings with a high Ca load determined by the σ-cresolphthalein assay were negative for von Kossa, this suggests that histological analysis of Ca load is not as sensitive as the quantitative σ-cresolphthalein assay.

A summary of all von Kossa scores and Ca load in high Ca-P medium for pre-dialysis and dialysis vessels are shown in Table 3-2 and Table 3-3 respectively.

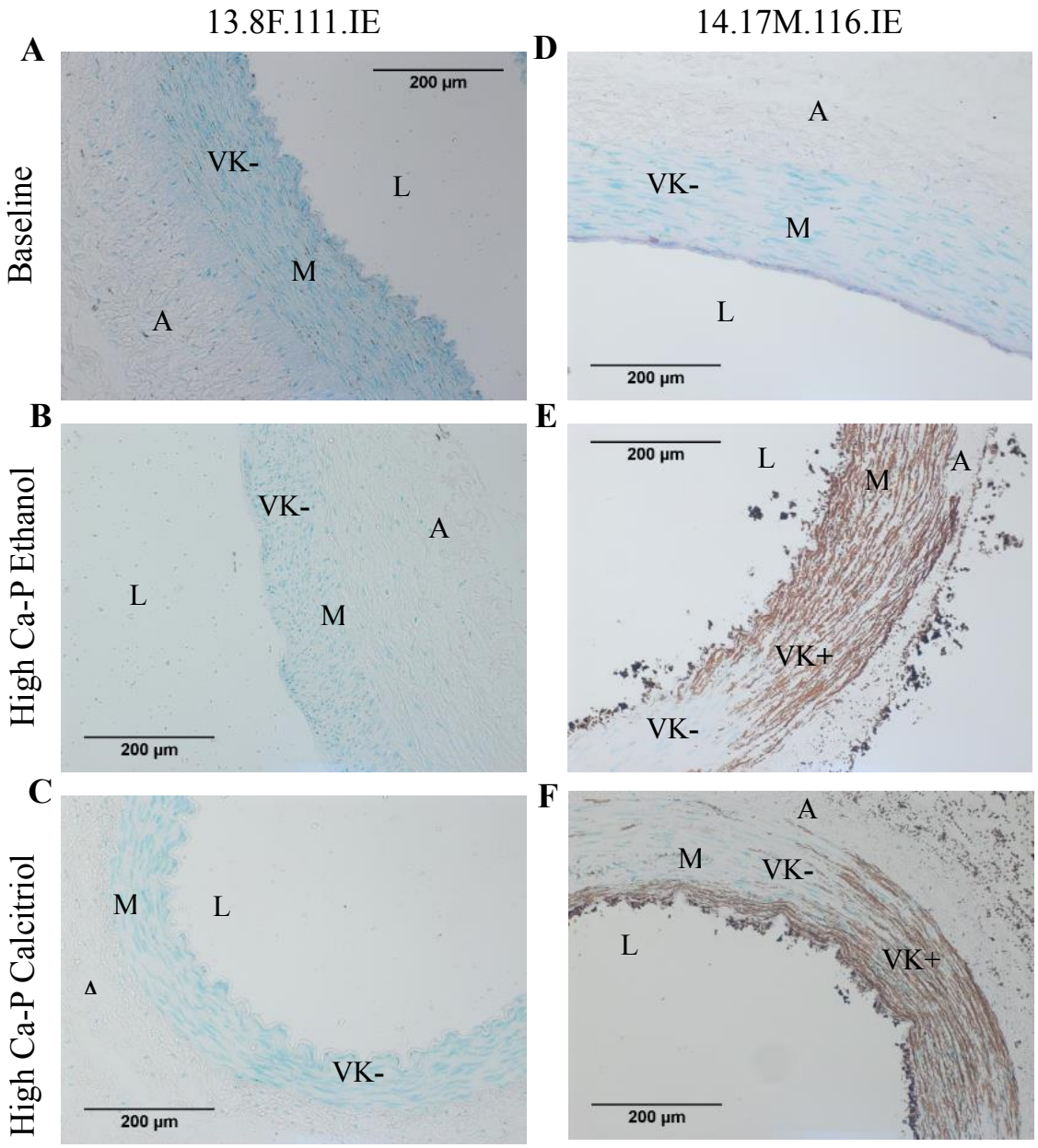


Figure 3-3: Von Kossa.

Vessel rings harvested from children with CKD were isolated at baseline (A&D) or cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification, in the presence of 3.4mM ethanol (B&E) or calcitriol (C&F). Vessel rings were fixed in PFA and paraffin sections von Kossa stained for hydroxyapatite deposition (brown) and counterstained by methyl green. A,B,C) Vessel 13.8F.111.IE (8 year old female, pre-dialysis). D,E,F) Vessel 14.17M.116.IE (17 year old male, 5 months haemodialysis). Images taken at 20 x magnification

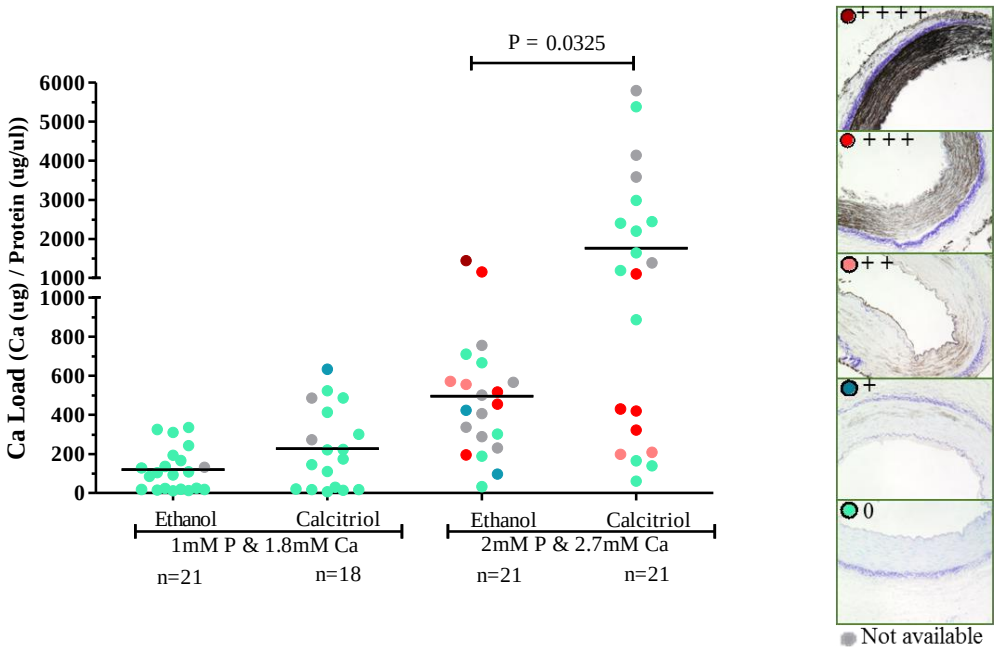


Figure 3-4: Ca load and von Kossa density in vessel rings.

Vessel rings isolated from children with CKD were cultured for 14 days with either 3.4mM ethanol or 10nM calcitriol, in either normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca). Vessel rings were harvested and their Ca load analysed by σ-cresolphthalein assay and the density of Ca deposition visualised by von Kossa stain. Ca loads were colour coded dependent on the density of the vessels von Kossa positive area (black), a representative key is shown above. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution of Ca load scores and statistical significance was determined by two-tailed Mann Whitney test.

Table 3-2: Summary of pre-dialysis children's Ca load

Vessel IDs with their corresponding Ca load and von Kossa score in each treatment condition. Ca load (Ca (µg) / protein (µg/µl)) as determined by the σ-cresolphthalein assay and von Kossa score determined by the density of von Kossa positive area as shown in the key.

	Vessel ID	Method		1mM P and 1.8mM Ca				2mM P and 2.7mM Ca			
			Baseline	Ethanol	Calcitriol	Alfacal	Parical	Ethanol	Calcitriol	Alfacal	Parical
Pre-dialysis	14.11M.118.IE	Von kossa	0	0	0			+++	+++		
		Ca load	12	25	17			455	430		
	14.8F.122.IE	Von Kossa	0	0	0		0	++++	+++		++++
		Ca load	13	13	87		18	1445	322		2669
	13.12F.113.IE	Von Kossa	0	0				++	0		0
		Ca load	15					556	140		43
	13.14M.105.IE	Von Kossa	0	0	0		0	0	0		0
		Ca load	16	31	21		23	32	60		61
	14.15M.115.IE	Von Kossa	0	0				+++	+++		+++
		Ca load	22	28			39	195	1102		716
	07.16F.46.IE	Von Kossa	0	0	0	0			0	0	
		Ca load	69	147	221	183		756	2197	1184	
	07.10M.52.IE	Von Kossa	0	0	0	0		0	0	0	
		Ca load	71	98	301	129		189	1645	679	
	07.6M.44.IE	Von Kossa	0	0	0	0		0	0	0	
		Ca load	82	104	487	122		302	2443	977	
	13.8F.111.IE	Von Kossa	0	0	0			0	0		0
		Ca load	106	225	111			666	887		1054
	07.9M.54.IE	Von Kossa									
		Ca load	138	126	273	220		231	1388	704	
	13.5M.110.IE	Von Kossa	0	0				0	0		0
		Ca load	243					711	1191		226

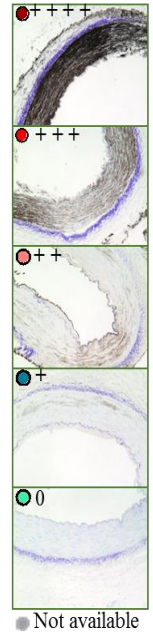
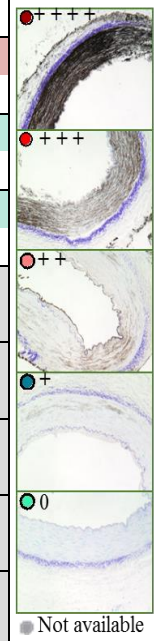


Table 3-3: Summary of dialysis children's Ca load.
Vessel IDs with their corresponding Ca load and von Kossa score in each treatment condition. Ca load (Ca (µg) / protein (µg/µl)) as determined by the σ-cresolphthalein assay and von Kossa score determined by the density of von Kossa positive area as shown in the key.

	Vessel ID	Method		1mM P and 1.8mM Ca				2mM P and 2.7mM Ca			
			Baseline	Ethanol	Calcitriol	Alfacal	Parical	Ethanol	Calcitriol	Alfacal	Parical
Dialysis	14.17M.116.IE	Von Kossa	0	0	0		0	+++	+++		+++
		Ca load	9	15	7		13	517	420		478
	14.13M.119.IE	Von Kossa	0	0	0		0	++	++		++
		Ca load	10	28	29		21	571	209		96
	13.9M.104.IE	Von Kossa	0	0	0		0	+	0		0
		Ca load	21	14	17		21	97	165		175
	14.8M.114.IE	Von Kossa	0	0	0		0	+++	++		0
		Ca load	77	130	145		164	1151	198		144
	07.2M.48.IE	Von kossa	0	0	0	0			0	0	
		Ca load	104	168	174	166		289	2984	1308	
	07.2M.50.IE	Von Kossa									
		Ca load	113	142	486	164		337	4138	496	
	07.1M.51.RA	Von Kossa	0	0	0	0			0		
		Ca load	184	202	223	274		407	2396	1484	
	07.15F.53.IE	Von Kossa	0	0	0						
		Ca load	254	416	523	390		567	5794	3622	
	07.15F.45.IE	Von Kossa	0	0	0	0			0	0	
		Ca load	312	308	413	389		501	5380	1262	
	07.15F.49.IE	Von Kossa			+	0		+		+	
		Ca load	318	332	633	547		423	3582	2017	



3.4.3 Calcitriol increased Ca load more so than alfacalcidol in CKD children's vessel rings.

Having established that calcitriol and alfacalcidol, but not paricalcitol significantly increased Ca load in *ex-vivo* vessel rings from CKD children cultured in high Ca-P media. The length of vessels obtained was insufficient to study all 3 VDRAs in the same patient, however in the next section the relative effects of either alfacalcidol or paricalcitol were compared to the effect of calcitriol on Ca load in vessel rings from the same patients.

Figure 3-5 compares the Ca load between vessel rings from the same patient treated with calcitriol and either alfacalcidol or paricalcitol. In normal Ca-P medium there was no difference in the Ca load between vessel rings treated with alfacalcidol and calcitriol. In high Ca-P medium calcitriol induced significantly more calcification than alfacalcidol. The Ca load in vessel rings treated with paricalcitol was not significantly different to calcitriol in either normal or high Ca-P media. Variable responses to paricalcitol were observed in high Ca-P medium with paricalcitol increasing Ca load in 4/10 vessels and decreasing or not effecting Ca load in 6/10 vessels.

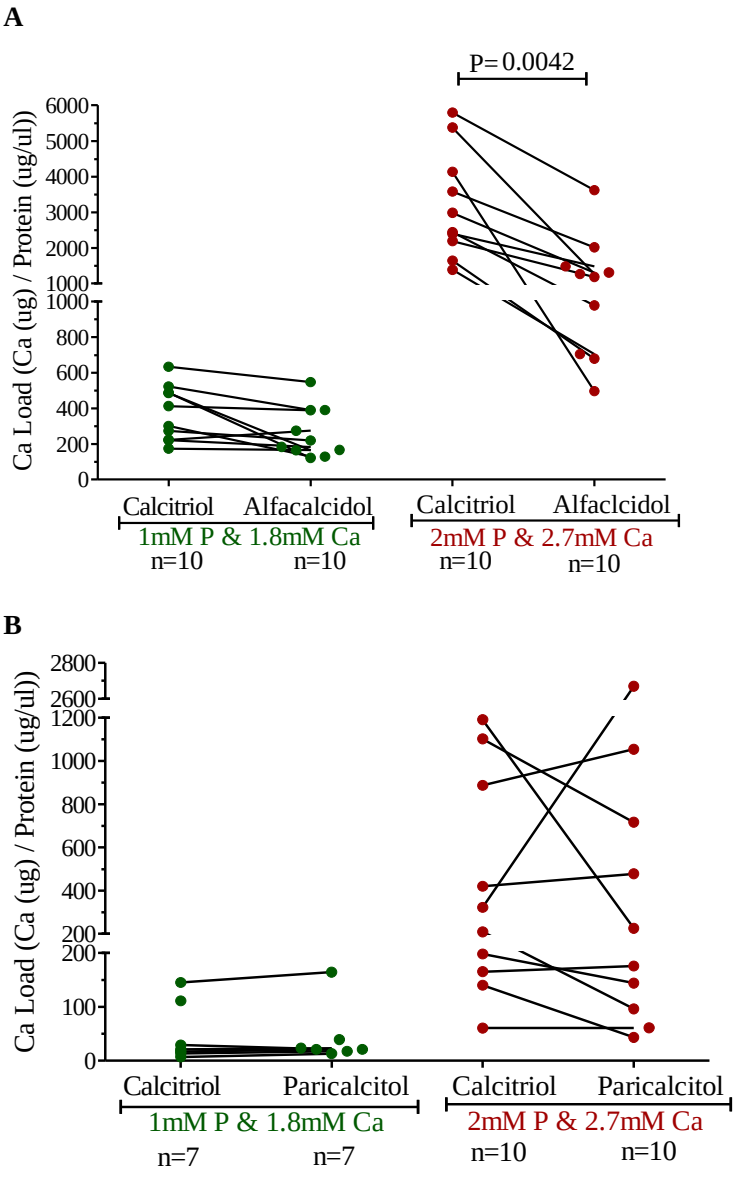


Figure 3-5: Comparison of Ca load in CKD children's vessel rings treated with different VDRAs.

Vessel rings isolated from children with CKD were cultured in both normal Ca-P medium (1mM P and 1.8mM Ca) and high Ca-P medium (2mM P and 2.7mM Ca), as well as either **A)** 10nM calcitriol and 10nM alfacalcidol, or **B)** 10nM calcitriol and 10nM paricalcitol. After 14 days vessel rings were harvested and analysed by σ-cresolphthalein assay for Ca load. Ca load in vessel rings from the same patient treated in different conditions are linked by lines. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by A) unpaired two-tailed t-test.

3.4.4 There was positive correlation between the vessel's Ca load at baseline and after culture in high Ca-P medium with calcitriol.

There was a wide range of baseline Ca load in the vessel rings from 9 to 318 µg/ (µg/µl), this suggests a high variance in susceptibility to calcification between patients. To determine if baseline Ca load could be used as a predictor of the patient's Ca load after treatment with any of the VDRAs in high Ca-P their correlation was considered.

As shown in Figure 3-6A there was a strong positive correlation between Ca load at baseline and after treatment in high Ca-P medium with calcitriol (Spearman r = 0.76, P<0.0001). There was no significant correlation between baseline Ca load and the Ca load in vessel rings cultured in high Ca-P medium only, with alfacalcidol or with paricalcitol. Therefore, in the presence of pre-existing vessel calcification calcitriol, but not the other VDRAs, induces significantly greater calcification.

There was no correlation between Ca load in vessel rings cultured in high Ca-P medium only to Ca load after culture in high Ca-P medium with calcitriol, alfacalcidol or paricalcitol (data not shown). Therefore, high Ca load in response to high Ca-P medium does not indicate that there will be a greater Ca load in that vessel in response to any of the VDRAs. This suggests that increase in Ca load in response to VDRAs may be driven by mechanisms other than Ca-P deposition alone.

It was considered whether vessel ring Ca load in response to treatment with calcitriol in high Ca-P was a predictor of Ca load in response to treatment with other VDRAs. Figure 3-7 shows that there was no significant correlation in Ca load after treatment in high Ca-P medium with calcitriol and alfacalcidol. The Spearman correlation coefficient showed that there was significant positive correlation (RS=0.72 P=0.02) between the Ca load in vessel rings after treatment with calcitriol and paricalcitol in high Ca-P. This suggests that paricalcitol affects calcification via a similar mechanism as calcitriol whereas alfacalcidol acts via a different mechanism.

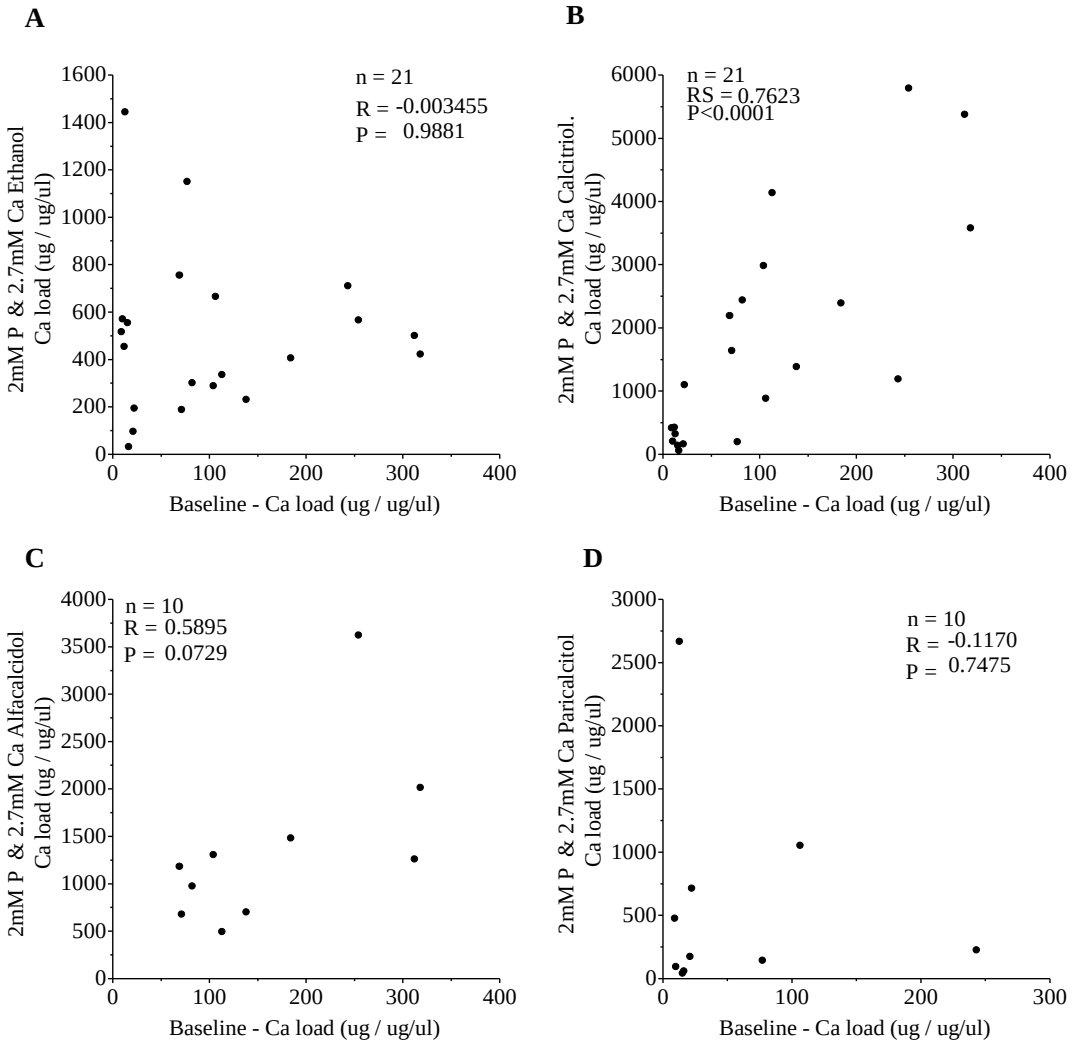


Figure 3-6: Correlation between Ca load in vessel rings at baseline and cultured in high Ca-P medium with different VDRAs.

Vessel rings isolated from children with CKD were taken at baseline and cultured in high Ca-P medium (M199 with 2mM P and 2.7mM Ca) with A) vehicle ethanol, B) calcitriol, C) alfacalcidol or D) paricalcitol. After 14 days Ca load was analysed by σ-cresolphthalein assay and correlation to Ca load at baseline determined. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution. The correlation was determined by calculating A, C and D) the Pearson correlation coefficient or B) the Spearman correlation coefficient and their statistical significance determined by two-tailed P values.

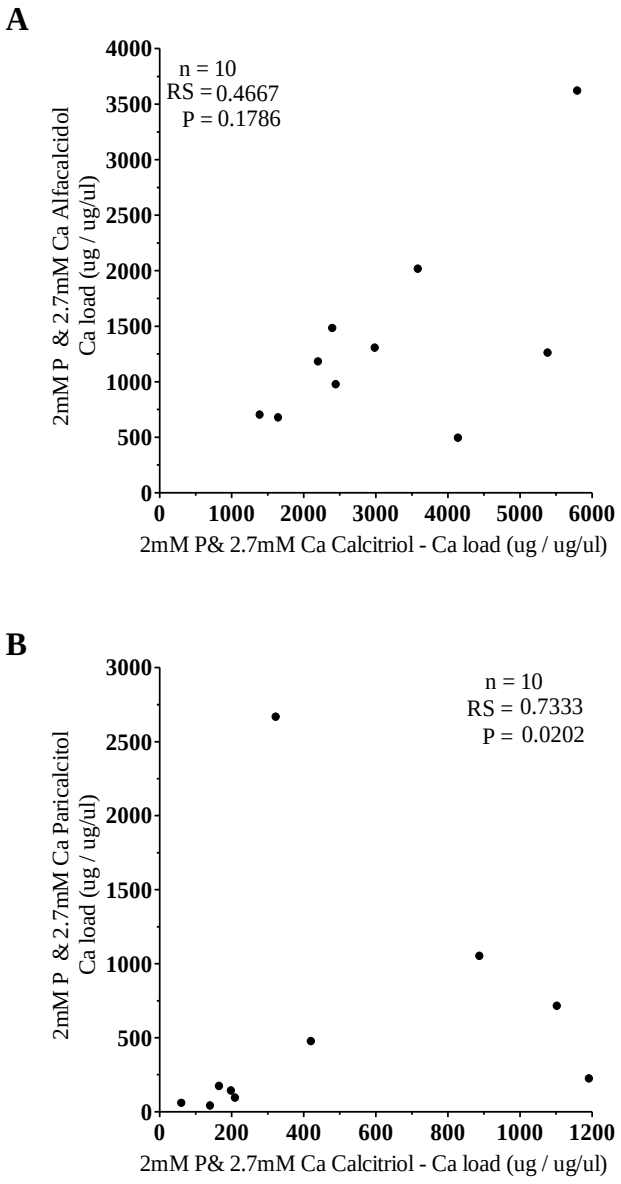


Figure 3-7: Correlation between Ca load in vessel rings cultured in high Ca-P medium with different VDRAs.

Vessel rings isolated from children with CKD were cultured in high Ca-P medium (M199 with 2mM P and 2.7mM Ca), as well as calcitriol and either A) alfacalcidol or B) paricalcitol. After 14 days vessel rings were harvested and their Ca load analysed by σ-cresolphthalein assay. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution. The correlation and its statistical significance were determined by calculating the Spearman correlation coefficient and two-tailed P values.

3.5 There was no difference in Ca load between vessel rings from pre-dialysis and dialysis patients.

Vessels were collected at the time of renal transplant from patients who were either in pre-dialysis CKD or on dialysis. Pre-dialysis patients were in CKD stage 5 and do not require renal replacement therapy. Dialysis patients received renal replacement therapy in the form of either heamodialysis or peritoneal dialysis. Dialysis is thought to have detrimental effects on the vasculature, as it can replace only a fraction of normal renal function, and may *per se* induce inflammation and haemodynamic stresses. Therefore, it was important to consider whether vessel rings from dialysis patients differed to pre-dialysis patients in their Ca load at baseline, in response to high Ca-P conditions and in response to different VDRAs.

3.5.1 There was no difference in Ca load in pre-dialysis and dialysis vessels at baseline or after culture.

Initially Ca load in pre-dialysis and dialysis vessel rings were compared at baseline and after culture in normal Ca-P as well as in high Ca-P media. As shown in Figure 3-8, there was no difference in the vessel Ca load between pre-dialysis and dialysis vessel rings at baseline or after culture in normal or high Ca-P medium.

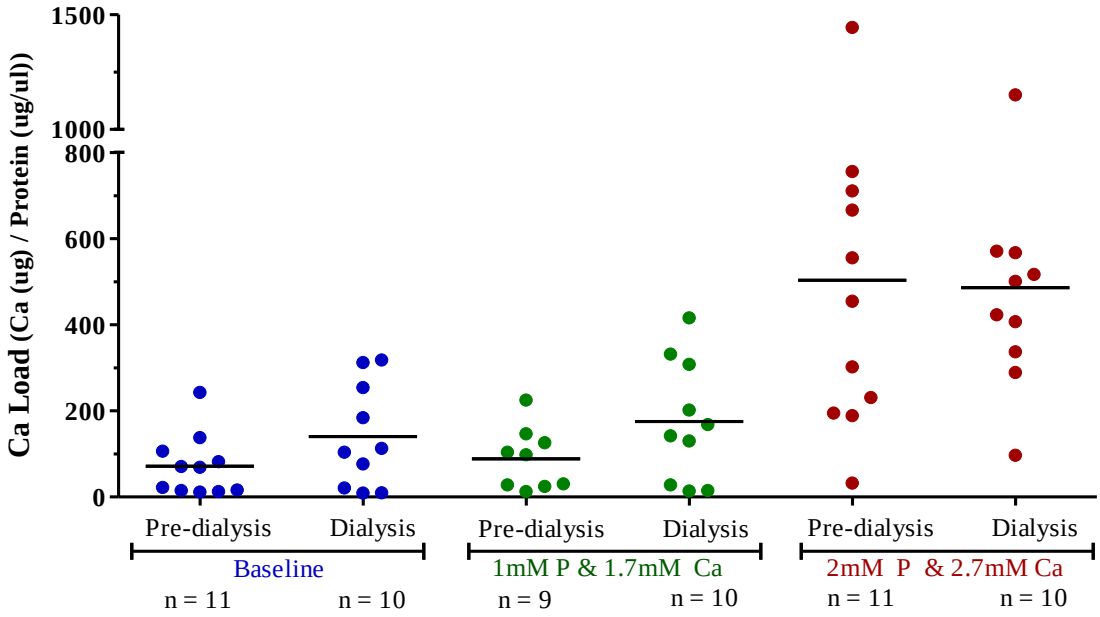


Figure 3-8: Ca load in pre-dialysis and dialysis vessel rings.

Vessel rings were isolated from either pre-dialysis or dialysis CKD children, rings were either taken at baseline or cultured for 14 days in either normal Ca-P medium (M199 with 1mM P and 1.8mM Ca) or high Ca-P medium (M199 with 2mM P and 2.7mM Ca) to induce calcification. Both were in the presence of 3.4mM ethanol, which will be used as the vehicle in this project. Ca load in the vessel wall analysed by σ-cresolphthalein assay. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by unpaired two-tailed t-test.

3.5.2 VDRAs affected Ca load in pre-dialysis and dialysis vessel rings to the same extent.

The previous section described experiments that showed that calcitriol and alfacalcidol both increase Ca load in CKD vessel rings.

Next, it was considered whether any of the VDRAs had a greater effect on the Ca load in vessel rings from dialysis than pre-dialysis patients. In vessel rings treated with calcitriol and alfacalcidol (Figure 3-9 A and B), there was a trend for a higher Ca load in dialysis than pre-dialysis vessel rings in both normal Ca-P as well as high Ca-P media. There was a significant increase in Ca load in both pre-dialysis and dialysis vessel rings treated with either calcitriol or alfacalcidol when the mineral content was increased from normal to high Ca-P media (Figure 3-9A and B). Vessel rings from dialysis patients treated with paricalcitol in high Ca-P medium tended to have a lower Ca load (not significant) than pre-dialysis vessel rings; shown in Figure 3-9 C. In both pre-dialysis and dialysis vessel rings treated with calcitriol in high Ca-P medium each had a group of vessel rings that responded to calcitriol with an increased Ca load and a group that showed no response to change in Ca load with calcitriol treatment, possible causes for these differences are discussed in Chapter 4.

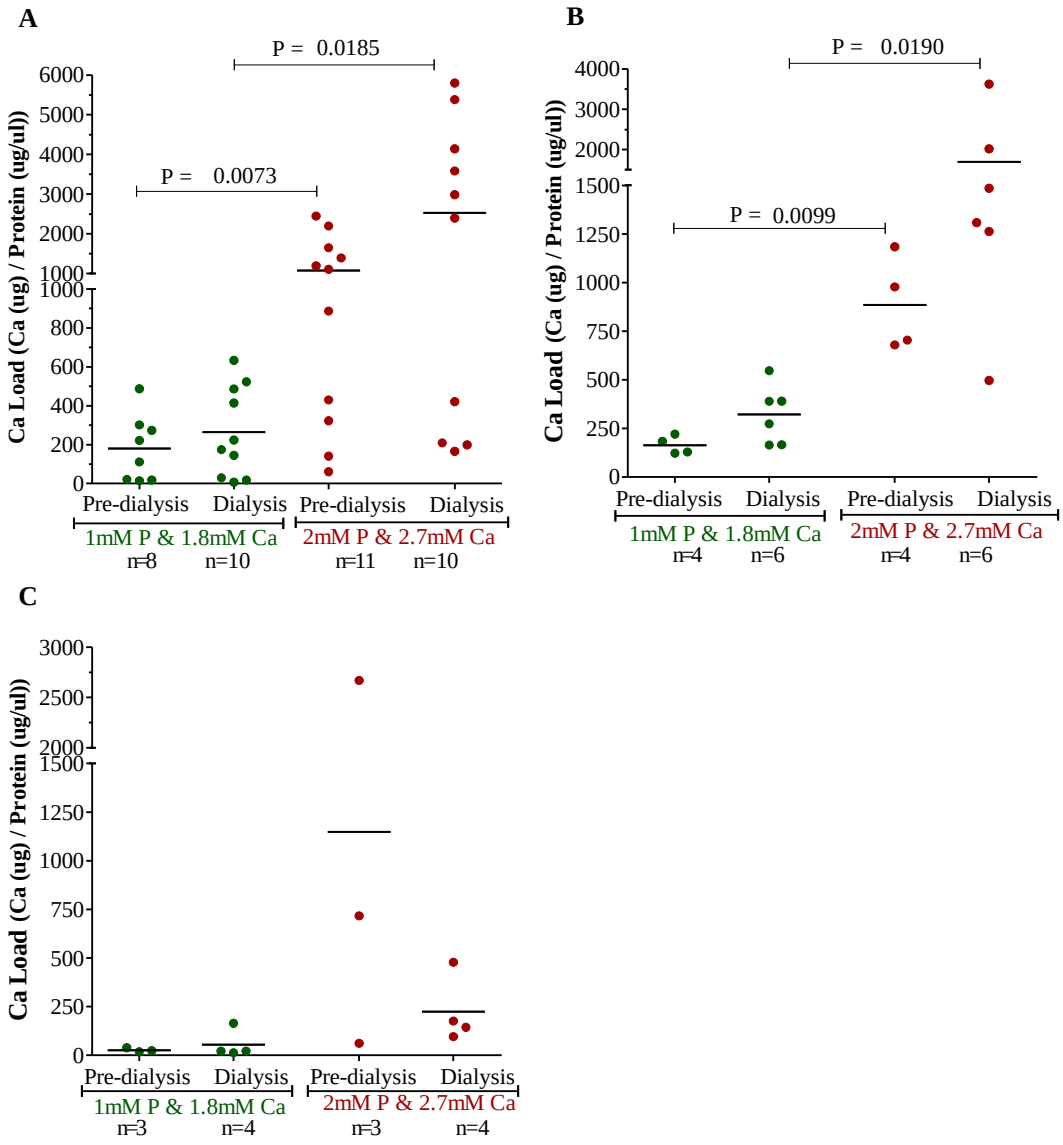


Figure 3-9: Comparison of the effect of VDRAs on Ca load in vessel rings from pre-dialysis and dialysis patients.

Vessel rings were isolated from either pre-dialysis or dialysis CKD children and cultured for 14 days in either normal Ca-P medium (M199 with 1mM P and 1.8mM Ca) or high Ca-P medium (M199 with 2mM P and 2.7mM Ca) to induce calcification. Vessel rings were treated with A) 10nM calcitriol, B) 10nM alfacalcidol or C) 10nM paricalcitol. Ca load in the vessel wall analysed by σ-cresolphthalein assay. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by; A) two-tailed Mann Whitney test and B and C) two-tailed t-test.

3.5.3 The type of and length of time on dialysis did not affect Ca load in vessel rings.

Both pre-dialysis and the dialysis patient groups had a large variation in Ca load with in each condition. The effect of dialysis on Ca load in each treatment condition was analysed further taking into consideration type of and time on dialysis. There was no significant difference between the Ca load in vessel rings from patients on haemodialysis and peritoneal dialysis at baseline or cultured in any of the treatment conditions with different VDRAs (data not shown).

There was no significant correlation between baseline Ca and time on dialysis, as determined by Pearsons correlation coefficient R=0.60 and P=0.065. In addition, there was no significant correlation between time on dialysis and Ca load; after treatment in high Ca-P medium only, with calcitriol, alfacalcidol or paricalcitol.

The type of and time on dialysis did not affect Ca load in response to VDRAs. As there were two distinct groups; those that responded and those that did not respond to calcitriol treatment with an increased Ca load, it were investigated whether other patient variables including gender, age and underlying renal diagnosis affected the Ca load in response to treatment.

3.6 The effect of other patient related factors on Ca load.

3.6.1 Ca load was higher in vessel rings from females than males.

This study included vessel rings from both male and female CKD patients therefore it was important to consider whether gender affects Ca load in the vessel wall. In this group of patient's, females tended to have a higher Ca load than males at baseline. As shown in Figure 3-10A, mean Ca load was significantly higher in females than males after 2 weeks of culture in either normal or high Ca-P media.

Next, the effect of different VDRAs in males and females were considered. Figure 3-10B, C and D show that the trend for a higher Ca load in females compared to males persisted after treatment with each of the VDRAs, calcitriol, paricalcitol and alfacalcidol. Ca load in females was significantly greater than males after culture for 14 days in normal medium with alfacalcidol.

There was no difference between the male and female groups in their underlying kidney disease or dialysis status including length of time on and type of dialysis, however there was a trend for females to be older than males, data shown in the appendix Figure-10-1 (NS p=0.07). Males ranged from 1 to 17 years with a mean of 8.6$\pm$1.4 years whereas females ranged from 8 to 16 years with a mean of 12.7$\pm$1.3 years.

The females in this study tended to be older and had a higher Ca load in each treatment condition than males; therefore, it was tested whether age was a predictor of Ca load in the vessels from children with CKD. Linear regression analysis showed that age was not a predictor of baseline Ca load or Ca load in vessel rings cultured in high Ca-P medium only, with calcitriol, alfacalcidol or paricalcitol (data not shown).

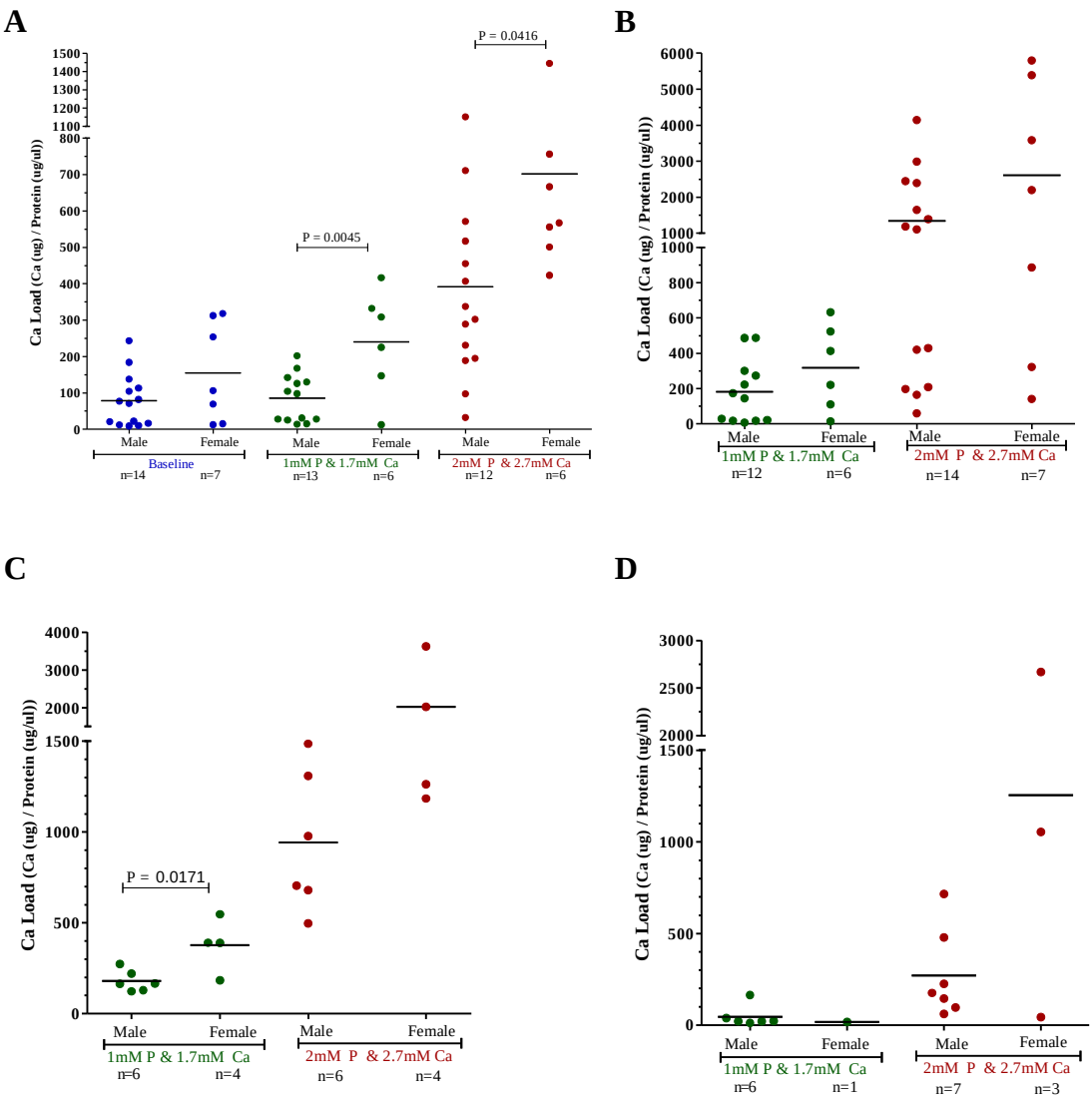


Figure 3-10: Ca load in vessel rings from male and female CKD patients.

Vessel rings were isolated from male and female CKD children and the Ca load in the vessel walls were analysed by σ-cresolphthalein assay. A) At baseline and after culture for 14 days in either normal Ca-P medium (M199 with 1mM P and 1.8mM Ca) or high Ca-P medium (M199 with 2mM P and 2.7mM Ca) to induce calcification. In addition, rings were cultured in normal and high Ca-P media with B) calcitriol, C) alfacalcidol and D) paricalcitol. D'Agostino and Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by; A, C, D) two-tailed t-test, and B) Mann Whitney test.

3.6.2 Primary renal diagnosis did not affect Ca load in the vessel rings.

All children with CKD were eligible for this study, CKD includes many forms of renal disease that lead to a chronic loss in renal function, and therefore it was important to consider the effect of primary renal diagnosis on Ca load in the vessel rings. Patients were grouped as having CAKUT (congenital anomalies of the kidney and urinary tract), glomerulopathy or other. One-way ANOVA tests showed that there was no significant difference between the groups in the Ca load at baseline or after culture in high Ca-P medium with any of the VDRAs (data shown in appendix Figure -10-2).

3.7 Control vessels

3.7.1 Harvesting control vessels

It was important to compare Ca load in CKD vessels to healthy non-renal control patients both at baseline and in response to culture in high Ca-P with and without different VDRAs. Age matched control vessels were obtained from non-renal patients who had no inflammatory complications and were undergoing routine abdominal surgery at Great Ormond Street Hospital. The inferior epigastric artery obtained from CKD patients is a medium sized muscular artery, ideally this same vessel would be obtained from control vessels for comparison, however, vessel collection is limited to those that can be easily accessed during surgery and are dispensable without any risk to the child. The inferior epigastric artery can be accessed only via some surgical approaches, so for this study only one inferior epigastric artery was harvested from a control patient for *ex-vivo* analysis. As alternative, medium sized muscular arteries from a different vascular bed were obtained. The mesentery, which is easily accessible during routine abdominal surgery, were harvested and the mesenteric arteries dissected out. Control vessels used in this study are shown in Table 3-1 and the patient information is shown in the methods.

3.7.2 Ca load in control vessel rings

Similar to CKD vessels, in control vessel rings increasing mineral content from normal to high Ca-P significantly increased Ca load as shown in Figure 3-11. Calcitriol had no effect on Ca load in control vessels in either normal or high Ca-P media, this differs to CKD vessel rings that had an increased Ca load in response to calcitriol.

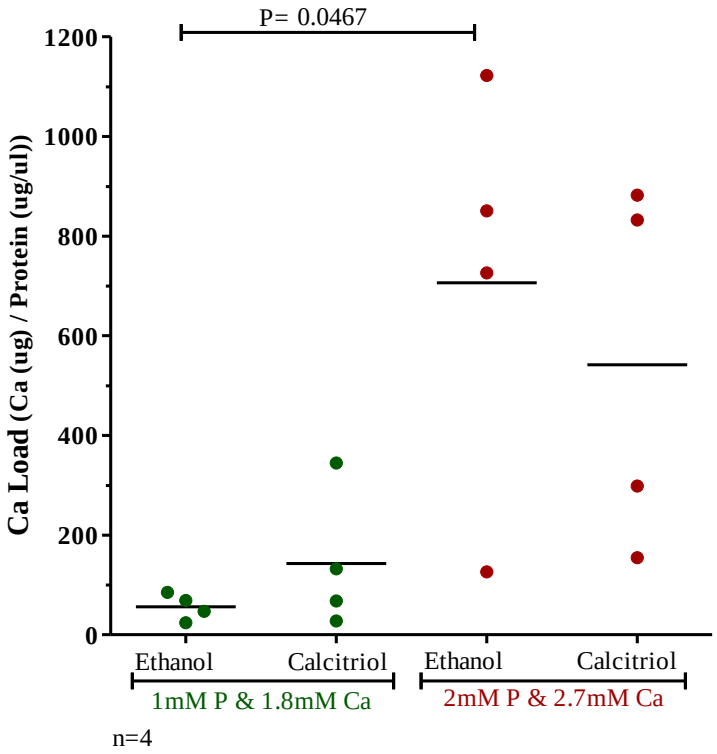


Figure 3-11: Ca load in control vessel rings.

Vessel rings isolated from healthy control children were cultured in either normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca), as well as either the vehicle of 3.4mM ethanol or 10nM calcitriol. Statistical significance was determined by unpaired two-tailed t-test.

3.8 Discussion

3.8.1 Summary

Data in this chapter established that the different VDRAs, calcitriol, alfacalcidol and paricalcitol, have different effects on accelerating the progression of vascular calcification in arterial rings isolated from children with CKD.

The key findings of this chapter were:

1. Calcitriol, and to a lesser extent alfacalcidol increased Ca load in vessel rings from children with CKD. Whereas paricalcitol had no effect on Ca load in vessel rings. Vessel rings treated with calcitriol formed two groups, responders whose Ca load increased and non-responders whose Ca load did not change.

2. Vessel rings from pre-dialysis and dialysis patients were equally susceptible to VDRA-induced vascular calcification.

3. Susceptibility to VDRA-induced vascular calcification in children's vessel rings was independent of age, gender, dialysis vintage and primary renal diagnosis. Ca load was higher in females then males in both normal and high Ca-P without VDRA treatment.

3.8.2 Vessel Histology

Histological analysis by haematoxylin and eosin of each arterial vessel ring at baseline showed that the paediatric vessels studied here were free from atherosclerosis, furthermore von Kossa staining showed no evidence of intimal calcification. Calcification in cultured vessel rings occurred in the tunica media layer, indicative of Monckberg's sclerosis which is associated with CKD. However, von Kossa staining showed that there was no evidence

of Ca or hydroxyapatite deposition at baseline in any of the vessel rings despite some having high Ca loads quantified by the σ-cresolphthalein assay. This coincides with data collected previously in our group where vessels from children with CKD had high Ca loads at baseline, yet 0% of the pre-dialysis and only 25% of the dialysis vessels had diffuse speckled calcification in the tunica-media detected by von Kossa. Both data suggest that Ca accumulation occurs before calcification can be detected by von Kossa.

3.8.3 Von Kossa Vs σ-cresolphthalein assay

The summarised Ca loads in pre-dialysis (Table 3-2) and dialysis vessel rings (Table 3-3) show the Ca load in arterial rings after treatment. Rings with the highest Ca load quantified by σ-cresolphthalein tended to have von Kossa positive areas within the tunica media, with no evidence of intimal calcification in any vessels. However, some arterial rings showed no von Kossa positivity in any treatment condition regardless of the high Ca load determined by σ-cresolphthalein assay. Discrepancies between Ca load determined by σ-cresolphthalein assay and by von Kossa analysis are likely to arise as analysis is performed on a single neighbouring ring for each methodology, unfortunately it is not possible to apply both techniques to the same ring and there was insufficient tissue for repeated analysis. Furthermore, there is the technical limitation of von Kossa positivity being determined from a single 7μm vessel ring section, the lateral or central location of this section within the 1mm vessel ring may affect its degree of exposure to treatment conditions.

Both methods have their advantages, von Kossa staining enables the regional deposition of Ca to be analysed whereas the σ-cresolphthalein assay is unable to distinguish between medial and intimal calcification. However, von Kossa staining is not a sensitive method to identify or quantify calcium load, our previous work showed that von Kossa positivity in only 25% of vessels that exhibited the highest Ca load (Shroff et al., 2008b). The σ-cresolphthalein assay is a sensitive assay that is highly accurate, as described by (Zak et al., 1975) it involves a direct reaction between Ca and the σ-cresolphthalein complex

therefore there is minimal risk of contamination. The data in this chapter focuses on quantification of the Ca load from each arterial ring by the σ-cresolphthalein assay. The accompanied histological analysis of Ca deposition in the vessel rings was important to show the localisation of hydroxyapatite deposition, in each case this was specific to the tunica media and is representative of Monckberg's sclerosis associated with CKD (Lanzer et al., 2014).

3.8.4 VDRAs had differential effects on vascular calcification in children's vessel rings.

The effect of 10nM calcitriol on the Ca load in vessel rings split the vessel rings into two populations; responders and non-responders, this is observed in Figure 3-2A. Responders to calcitriol showed an increase in Ca load in high Ca-P with calcitriol, whereas the Ca load in non-responders remained below 600 µg/(µg/µl) and was comparable to that of rings cultured in high Ca-P. Previous literature is confounding, for example (Guerrero et al., 2012) showed that 10nM calcitriol had no effect on calcification in either the thoracic aorta from Westar rats or in human VSMCs, whereas (Martinez-Moreno et al., 2012) found that 10nM calcitriol increased Ca load in human VSMCs.

The data in this chapter suggests that there is variation between individual vessels in their susceptibility to calcitriol induced calcification, this could account for the varied results observed in the current literature. Experiments to determine the possible mechanisms for this differential response between individuals were described in Chapter 4. This will include the effect of calcitriol on responder and non-responder vessel ALP activity, expression of proteins involved in osteoblast conversion and expression of the vitamin D inactivating enzyme 24 hydroxylase. To further investigate individual patient responses to vitamin D, Chapter 6 examines the expression of the vitamin D receptor in both vessel rings and explanted VSMCs.

Paricalcitol had no effect on Ca load in the vessel rings whereas calcitriol increased Ca load as shown in Figure 3-2. Never the less comparison of vessels treated with both calcitriol and paricalcitol as shown in Figure 3-5B found that there was no difference in the effect of calcitriol and paricalcitol in high Ca-P medium on Ca load in the vessel rings. In addition, there was a positive correlation (RS=0.73, P=0.02) between the Ca loads in vessel rings subject to these treatment conditions (Figure 3-7B). In contrast current literature showed that *in-vitro* 30nM paricalcitol but not calcitriol had a protective effect on calcification in rat aortic rings and human VSMCs by reducing inflammatory cytokines (Guerrero et al., 2012). Paricalcitol also reduced expression of BMP2 and other osteoblast markers in human VSMCs in high P medium whereas calcitriol further increased their expression (Martinez-Moreno et al., 2012).

The Ca load after treatment with alfacalcidol in high Ca-P medium did not correlate significantly to the Ca load after treatment with calcitriol (Figure 3-7A). In addition, the Ca load was significantly lower with alfalcalcidol than with calcitriol in high Ca-P medium as shown in Figure 3-5A. Alfacalcidol is thought to be less calcaemic than other VDRAs, for example in a randomised crossover study (Hansen et al., 2014) found that alfacalcidol but not paricalcitol significantly increased the expression of the calcification inhibitor Fetuin A, this is a circulating inhibitor therefore its expression would not affect calcification in the isolated vessel ring model. All children were treated with alfacalcidol, therefore all vessels would have had exposure to alfacalcidol *in-vivo*.

The data presented here suggests that calcitriol and paricalcitol may be acting on vascular calcification via similar mechanisms, whereas alfacalcidol could be acting via an alternative mechanism. The mechanism of action of different VDRAs on calcification, for example their effect on the activity of the osteoblast related enzyme ALP will be explored further in both the arterial vessel rings (Chapter 4) and in VSMCs (Chapter 5).

Calcitriol and paricalcitol are both active forms of vitamin D_3, respectively they are the naturally occurring 1α, 25 dihydroxyvitamin D_3 and the synthetic analogue 19 nor 1α, 25 dihydroxyvitamin D_2 developed for its reduced calcaemic effect on the intestine.

Alfacalcidol on the other hand, is an inactive form of vitamin D, 1α hydroxyvitamin D_3 as discussed in the introduction.

Alfacalcidol requires autocrine activation by 25 hydroxylase in the isolated vessel rings. In Chapter 4 it was shown that 25 hydroxylase is locally expressed in VSMCs therefore alfacalcidol can be activated in isolated vessel rings, however the expression and activity of the 25 hydroxylase enzyme may be a regulatory, limiting factor for alfacalcidol. Alfacalcidol is routinely prescribed to children with CKD in Europe, and therefore its effect on calcification in cultured *ex-vivo* vessel rings from this cohort is of great interest.

The mechanisms of action by which different VDRAs affect vascular calcification in vessel rings and the distinctly different effects of calcitriol on vessel rings are explored in chapter 4.

3.8.5 There was no difference in susceptibility to VDRA-induced vascular calcification between vessel rings from pre-dialysis and dialysis patients.

Data collected here suggests that there is no difference between the Ca load in vessel rings from pre-dialysis CKD stage 5 and dialysis patients, neither at baseline, after culture in normal or high Ca-P media (Figure 3-8). In addition, there was no significant difference between the Ca load in dialysis and pre-dialysis vessel rings after treatment with any of the VDRAs. This differs from previous findings where dialysis vessels had a higher baseline Ca load than pre-dialysis vessels (Shroff et al., 2008b) (Todd et al., 2015). In this chapter, a trend was observed of a higher Ca load in dialysis vessels than pre-dialysis after treatment with calcitriol or alfacalcidol however this was not significant. Interestingly, both pre-dialysis and dialysis vessel rings treated with calcitriol have responders and non-responders shown in Figure 3-9.

In addition, the data presented here showed no difference between susceptibility to calcification between haemodialysis and peritoneal dialysis patients. This differs to

(Srivaths et al., 2014) who found in a cohort of paediatric patient's cardiac calcifications were more prevalent in haemodialysis (9/21) than peritoneal dialysis (2/17) patients. This study's focus is cardiac rather than vascular calcification, which could account for the differences observed. Both studies have low patient numbers and a higher sample number is required to determine any difference in calcification prevalence between the modes of dialysis.

Interestingly the average age in females (12.7+1.3) tended to be greater than males (8.6+1.4) (NS), this coincided with a tendency for a greater Ca load in females than males in each of the conditions. The Ca load was significantly greater in vessel rings from females than males cultured in normal or high Ca-P media as well as normal Ca-P medium with alfacalcidol. A Doppler ultrasound was used to analyse vascular compliance in 600 volunteers aged 3 days to 65 years, they found vascular compliance increased rapidly in the first decade and declined rapidly in the 2nd decade before reaching a steady decline into later life (Laogun and Gosling, 1982). Vascular compliance therefore susceptibility to calcification may decline in the first decade and increase again in the 2nd decade (Toussaint and Kerr, 2007). Similarly, in the male cohort (IQR 4.3–13.3 years), a significant negative correlation between age and Ca load was observed, whereas in the older female cohort (IQR 8-15 years) there was a trend (NS) for a positive correlation between age and Ca load. Together this suggests the possibility that the changes in vascular compliance with age during the paediatric years could be closely linked to their susceptibility to vascular calcification observed here in *ex-vivo* culture (Doyon et al., 2013). To confirm whether the different trends observed between Ca loads in males and females are a consequence of gender difference or the staggered age range within these cohorts it would be of interest to study vessels from more male and female children of closely matched age.

3.8.6 Control Vessels

Previous data from our lab showed that dialysis vessels but not control vessels calcified after exposure to either high P or high Ca-P media (Shroff et al., 2010) (Todd et al., 2015), whereas the data presented here indicates that both control and dialysis vessels calcify on exposure to high Ca-P medium. A number of factors may have contributed to calcification of the control vessels in this study including the toxicity of the vehicle ethanol and the vascular bed from which controls were obtained.

A key difference between this study and previous ones is that in this study, the high Ca-P medium which increased Ca load, also contained 3.4mM ethanol. Ethanol is known to be toxic to cells, for example in Saos -2 cells, doses of ethanol from 1mM to 300mM reduced cell proliferation and affected mineralisation (Vignesh et al., 2006).

There are a limited number of control vessels available from routine abdominal surgery, unfortunately these tend to be from a different vascular bed to the CKD vessels. The mesenteric arteries dissected from control patients may have different susceptibility to calcification than the inferior epigastric. Arterial calcification was induced in a rat model, interestingly the mesenteric arteries had more calcification than any other vascular bed identified by a striking uptake of 3 different radiotracers used to identify calcification by CT scan (Bordoloi et al., 2015). This suggests that mesenteric vessels may have increased susceptibility to calcification relative to other vascular beds and demonstrates the importance of obtaining inferior epigastric arteries from the non-renal controls for comparison. Furthermore, at least one control child (13.0.5F.109.ME) is known to have had previous operations in the same abdominal area, their vessels may have been structurally damaged during this process predisposing the vessel to calcification when exposed to the high Ca-P *ex-vivo*.

3.8.7 Limitations

The work in this chapter was conducted on whole vessel rings from children with CKD, as discussed in the introduction, this is an extremely valuable and clinically relevant model for studying the effects of VDRAs. Human vessels are a scarce resource, therefore the vessel numbers in this chapter are limited by the number of transplant operations performed and the number of consenting patients that fit the eligibility criteria. The length of the vessel obtained determined how many 1mm vessel rings were dissected from it for treatment in different conditions. It was not possible to obtain enough rings from the same vessel to compare all three VDRAs (calcitriol, alfacalcidol and paricalcitol) at the same time, therefore the effects of alfacalcidol and paricalcitol were not compared. In each vessel the effects of calcitriol were compared to the vehicle control (ethanol) and where possible either alfacalcidol or paricalcitol. Calcitriol is the natural active form of vitamin D_3 therefore an important reference point with which to compare other VDRAs.

The experiments in this chapter consider the effects of different VDRAs at a concentration of 10nM, this is equivalent to the physiological level therefore is the most clinically relevant concentration. In previous literature the effect of VDRAs on calcification, has been shown to be dose dependent therefore the ideal would be to compare a range of concentrations, for example from a sub physiological dose of 1nM to a supra physiological dose of 100nM. The limited vessel availability restricted the study to consider the effects of physiological dose VDRAs only in the vessel rings.

Ideally, multiple experimental repeats would be performed on each vessel to show that the data was reproducible for each patient's vessel rings, unfortunately this was not possible with the limited length of the vessels obtained. In addition, the tissue size was insufficient to extract RNA, as vitamin D is a known transcriptional regulator of multiple genes it is important to investigate its effect on mRNA expression of genes contributing to mineralisation. As there were many more avenues to explore and human vessels are a very valuable but limited resource it was essential to maximise their utilisation, therefore VSMCs were explanted from the human vessels. This enabled a wider range of

experimental conditions and techniques to be applied and is discussed in chapters 5 and 6.

All children were treated with alfacalcidol at the time of harvesting their vessels. This may have some influence on their response to *ex-vivo* exposure to VDRAs, such as by upregulating the VDR after prolonged exposure to vitamin D therapy. Effects of VDRAs on expression of the VDR in vessel rings and VSMCs is discussed in chapter 6.

Throughout these studies ethanol was used as a vehicle, this was necessary as VDRAs are only partly soluble in some alcohols. All 3 VDRAs were solubilised in ethanol to the same final concentration of 3.4mM regardless of the final VDRA concentration. For cell culture, ethanol is recommended as the vehicle. In human VSMCs cultured in high inorganic P, only concentrations of ethanol ≥60mM significantly increased expression of Runx2 and osteocalcin as well as upregulating ALP activity (Oros et al., 2012). These mechanisms contributed to an increased calcium deposition in VSMCs exposed to ≥60mM ethanol (Oros et al., 2012). In this study, it was important to use ethanol as a vehicle for the VDRAs at a concentration that would not influence calcification, and therefore the final concentration of ethanol was 3.4mM, considerably below the range shown to affect calcium deposition. Culture of vessel rings in normal Ca-P medium with 3.4mM ethanol as a vehicle control had no effect on Ca load relative to baseline, the mean $\pm$ SE was 134.3 $\pm$ 27.3 in comparison to 104.3 $\pm$ 22.1 at baseline. However, the effect of ethanol on Ca load in high Ca-P medium has not been studied relative to high Ca-P medium only, in future studies an important additional control would be high Ca-P only.

Chapter 4 By what mechanisms do VDRAs induce vascular calcification in children's vessel rings?

4.1 Introduction

The previous chapter showed that each of the VDRAs, calcitriol, paricalcitol and alfacalcidol elicited a different pattern of effects on calcification in the vessel rings. This indicates that each VDRA may act on vascular calcification via a different mechanism or combination of mechanisms.

Paricalcitol had no significant effect on Ca load in the vessel rings therefore its mechanism of action on calcification will not be investigated further.

Alfacalcidol consistently increased Ca load in the vessel rings, however to a lesser extent than calcitriol. To understand the causative mechanisms of both calcitriol and alfacalcidol on calcification, here I compare the effect of both these VDRAs in two of the highly regulated processes of calcification discussed in the introduction, cell viability and the activity of osteoblast enzyme ALP which is a marker of osteogenic conversion.

In Chapter 3, the Ca load of children's vessels treated with calcitriol separated them into two distinct populations, non-responders and responders. In high Ca-P medium, calcitriol had no effect on Ca load in 8 children's vessels (non-responders), whereas it considerably increased calcification in the other 13 children's vessels (responders). The mechanistic basis of these two different responses is not yet understood. This chapter will explore the expression profiles of SMC and osteoblast markers in non-responder and responder vessels to determine any differences in osteogenic conversion. In addition, the expression of 24 hydroxylase, a downstream target of vitamin D that is also involved in vitamin D inactivation will be investigated to determine any difference between non-responders and responders' ability to upregulate the expression of vitamin D target genes.

4.1.1 Aims

1. To determine whether VDRAs alfacalcidol and calcitriol act via different mechanisms to promote vascular calcification in vessel rings from children with CKD.

2. To elucidate the different expression profiles in vessel rings that have an increased Ca load in response to calcitriol (responders) and vessel rings whose Ca load does not respond to calcitriol (non-responders).

These will be addressed using the following key questions;

1. Can alfacalcidol be activated locally in VSMCs?

2. Do alfacalcidol or calcitriol affect ALP activity in vessel rings?

3. Do alfacalcidol or calcitriol affect the cell viability of VSMCs in vessel rings?

4. Are markers of osteogenic conversion at baseline or after calcitriol treatment different in responders and non-responders?

5. Do responders and non-responders differ in their expression of 24 hydroxylase, a downstream target of vitamin D?

4.2 Method

To determine the expression of vitamin D activation enzymes in VSMCs, VSMCs were explanted from children's vessel rings. RNA was harvested from the VSMCs and expression of CYP27A1 and CYP27B1 determined by qPCR analysis. In this experiment VSMCs explanted from both dialysis patients and control patients were used, the VSMC

ID for explants utilised are shown on the axis and the relevant patient information is in the Methods.

To investigate the mechanistic effects of calcitriol and alfacalcidol on vascular calcification in vessels of children with CKD, vessel rings were taken at baseline and cultured in both normal Ca-P medium (1mM Ca and 1.8mM P) and high Ca-P media (2mM Ca and 2.7mM P) with either the vehicle of 3.4mM ethanol, 10nM calcitriol or 10nM alfacalcidol. After 14 days of culture, 1 vessel ring per condition was homogenised and their ALP activity quantified by a colorimetric assay. All data shown as mean $\pm$ SE. The other vessel ring was fixed in formalin, paraffin embedded for sectioning and stained. Staining included haematoxylin and eosin (H and E) for VSMC cell counts and immunostaining of SMA, Runx2, ALP as well as 24 hydroxylase. Vessels utilised in each experiment are shown in Table 4-1 and the corresponding patient information is shown in Methods.

Table 4-1: Vessels utilised in each experiment.
Patients' vessels utilised in the viability assay, ALP activity assay and comparison of responder and non-responder vessel rings including immunohistochemistry staining of SMA, Runx2, ALP and 24 hydroxylase. R – Responder. NR – Non-responder.
Vessel ID format: Year. Age and gender. Unique number. Vessel type.

	Vessel ID	Viability	ALP	Responders Vs Non-Responders
Pre-dialysis	07.6M.44.IE*	✓	✓	✓ (R)
	07.16F.46.IE*	✓	✓	✓ (R)
	07.10M.52.IE*	✓		
	07.9M.54.IE*		✓	
	13.14M.105.IE			✓ (N-R)
	13.5M.110.IE			✓ (R)
	14.15M.115.IE		✓	
	14.8F.122.IE		✓	
Dialysis	07.15F.45.IE*	✓	✓	
	07.2M.48.IE*	✓	✓	
	07.15F.49.IE*		✓	
	07.2M.50.IE*		✓	
	07.1M.51.RA	✓	✓	
	14.8M.114.IE		✓	✓ (N-R)
	14.13M.119.IE			✓ (N-R)

*Vessels collected and cultured by Rukshana Shroff in 2007. All analysis and histology for these vessels performed by myself, Joanne Laycock.

4.3 Differential effects of calcitriol and alfacalcidol on vascular calcification.

It was shown in Chapter 3, using a model of intact vessel rings from children with CKD cultured in high Ca-P medium that both alfacalcidol and calcitriol increased Ca load. The Ca load was significantly higher in vessel rings treated with calcitriol than with alfacalcidol. In this section the potential mechanisms for the differential effects of calcitriol and alfacalcidol were explored, this included expression of alfacalcidol activation enzymes, effects on cell viability and effects on ALP expression.

4.3.1 VSMCs express 25 hydroxylase for the activation of alfacalcidol.

As discussed in the introduction inactive vitamin D requires 2 hydroxylation enzymes for its activation. 1α hydroxylase which is mainly expressed in the kidney and is encoded by the CYP27B1 gene, and 25 hydroxylase which is encoded by both the CYP2R1 and the CYP27A1 genes and is mainly produced by the liver. However, many organ systems in the body produce low levels of both of the activation enzymes for autocrine / paracrine function.

VDRAs can either be administered in their active di-hydroxylated form ($1,25(OH)_2D$) or in their inactive form requiring hydroxylation to activate them. Calcitriol is the natural active form of vitamin D, whereas alfacalcidol (1α-OHD) is inactive and requires 25 hydroxylase for its activation as described in the introduction.

It was important to determine if 25 hydroxylase was expressed in the vessel rings to enable alfacalcidol to be activated hence function in its active form. MRNA expression of the vitamin D activation genes CYP27B1, CYP2R1 and CYP27A1 could not be determined in the vessel rings as there was insufficient tissue to extract RNA. Alternatively, VSMCs were explanted from the vessel rings and the mRNA expression of the vitamin D activation enzymes in explanted VSMCs from both healthy controls and dialysis patients

was examined. As shown in Figure 4-1, all control (n=4) and dialysis (n=6) VSMC explants expressed both CYP27A1 and CYP27B1 enabling autocrine activation of vitamin D in VSMCs. Interestingly, mRNA expression of CYP2R1 was not detectable in any of the VSMCs.

This data shows that the 25 hydroxylase enzyme required to activate alfacalcidol and encoded by CYP27A1 is expressed in VSMCs from control and dialysis vessels, therefore it can be assumed that the VSMCs in *ex-vivo* vessel rings were also able to express 25 hydroxylase for the local activation of alfacalcidol. There was no significant difference between control and dialysis VSMCs in their expression of either CYP27A1 or CYP27B1, there was some variation in expression of up to 3 fold between different VSMCs within each group.

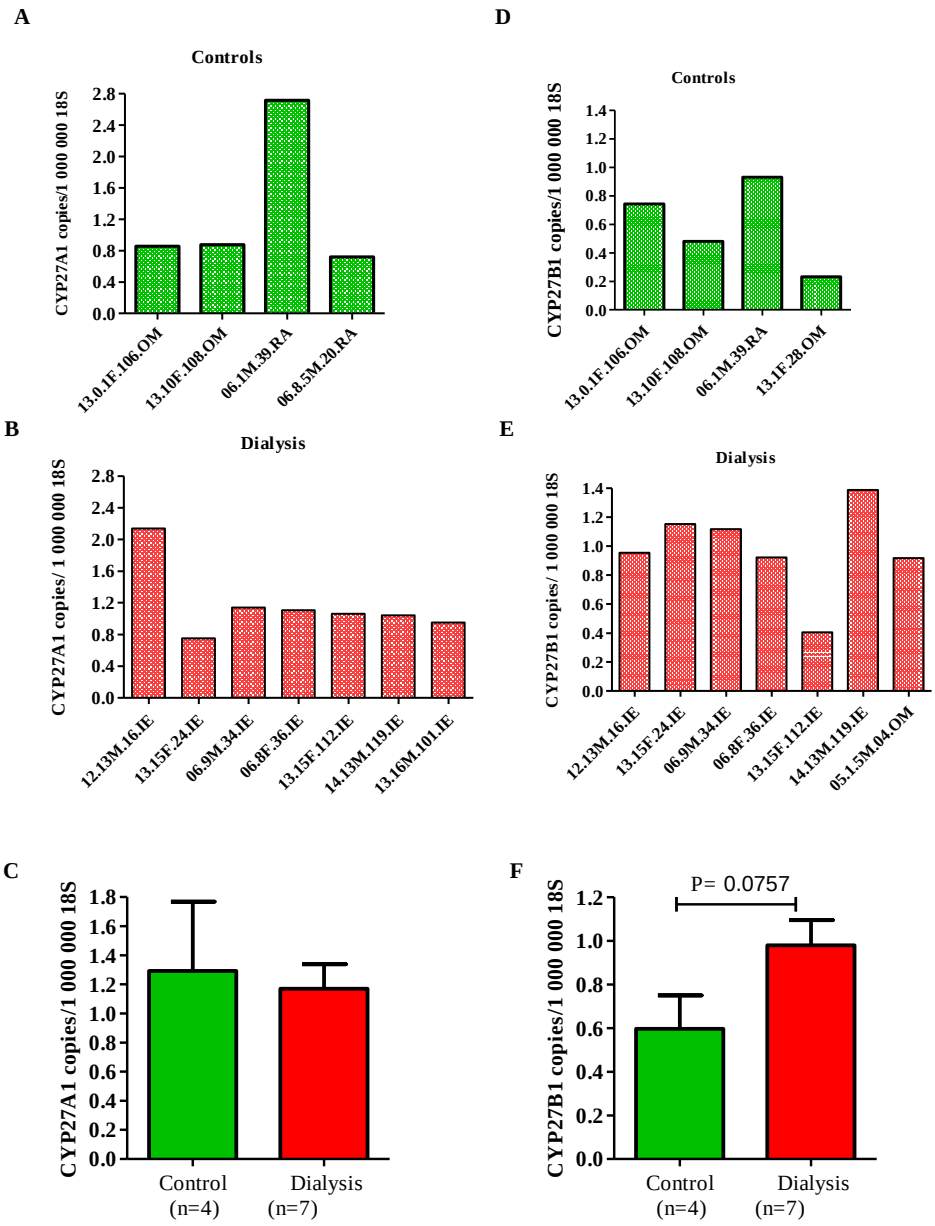


Figure 4-1: Expression of vitamin D activation enzymes in explanted VSMCs.

VSMCs were explanted from control (A and D) or dialysis (B and E) vessel rings. The mRNA expression of vitamin D hydroxylation enzymes was determined by qPCR for A, B, C) CYP27A1 and D, E, F) CYP27B1.

4.3.2 Calcitriol but not alfacalcidol increased ALP activity in vessel rings from children with CKD.

The previous section showed that alfacalcidol can be activated locally in VSMCs, it was shown in Chapter 3 that both alfacalcidol and to a greater extent calcitriol increased Ca load in the vessel rings. In this section, I consider the effect of both alfacalcidol and calcitriol on ALP activity in the vessel rings to determine if this mechanism contributed to the increased calcification observed.

Culture of vessel rings in either normal Ca-P or high Ca-P medium did not affect ALP activity relative to baseline as shown in Figure 4-2A. ALP activity in the vessel wall was increased by calcitriol in both normal Ca-P medium (from 9.6$\pm$1.0 to 13.0$\pm$1.1 U/mg ($p=0.043$)) and even more so in high Ca-P medium (from 11.4$\pm$1.3 to 24.5$\pm$2.5 U/mg ($p=0.0004$)) as shown in Figure 4-2B. There was also a trend for alfacalcidol to increase ALP activity in high Ca-P medium only however this was not significant.

In the presence of either calcitriol or alfacalcidol, raising the mineral content from normal to high Ca-P medium increased the ALP activity in vessel walls, whereas in the absence of a VDRA high Ca-P medium had no effect on ALP activity; as shown in Figure 4-2A, B and C.

In order to confirm if the VDRAs have differential effects on ALP activity, a direct comparison was made between the ALP activities in vessel rings from the same patients treated with different VDRAs. As shown in Figure 4-2D calcitriol increased ALP activity significantly more than alfacalcidol in both normal Ca-P and high Ca-P media.

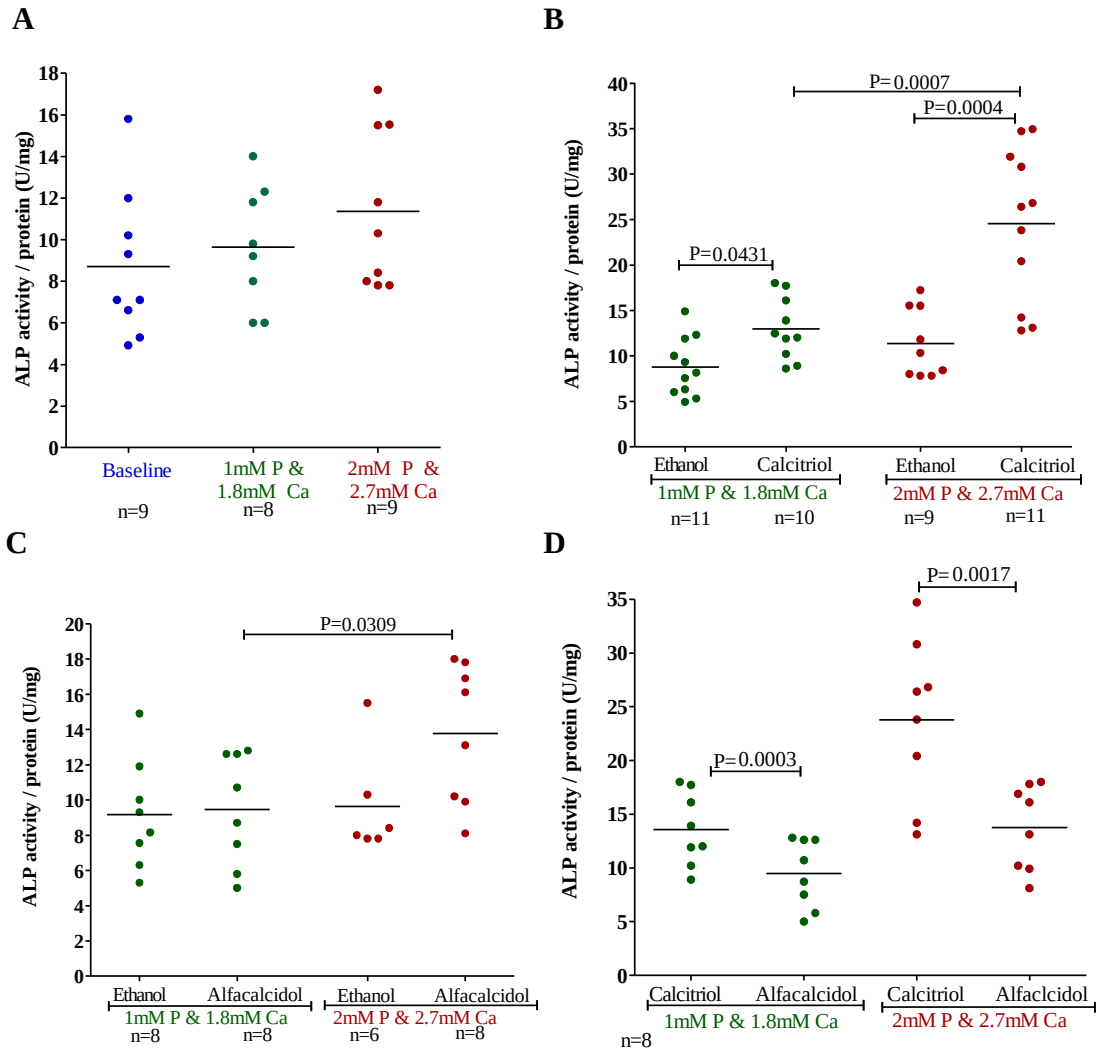


Figure 4-2: Calcitriol increased ALP activity in CKD children's vessel rings.

Vessel rings were isolated from children with CKD. A) A ring was taken at baseline and others cultured for 14 days in either normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P (2mM P and 2.7mM Ca) to induce calcification. In addition, rings were cultured with B) calcitriol, C) alfacalcidol or D) a comparison of calcitriol and alfacalcidol. Vessel rings were homogenised and the ALP activity in the vessel wall was determined by a colourimetric assay. Statistical significance was analysed by unpaired two-tailed t-test.

4.3.3 Calcitriol increased ALP activity to a greater level in dialysis than pre-dialysis vessel rings.

As demonstrated in chapter 3, there was a trend (NS) for vessel rings from dialysis patients cultured in high Ca-P medium with either calcitriol or alfacalcidol to have a higher Ca load than vessel rings from pre-dialysis patients cultured in the same conditions. Here I compare the ALP activity in vessel rings from pre-dialysis and dialysis patients. ALP activity was analysed in vessels which were of sufficient length to culture an additional ring per treatment condition. ALP activity was determined by a colorimetric assay and normalised to protein content. Some treatment conditions have lower n numbers due to, limited tissue availability or insufficient protein extracted for accurate protein measurement.

There was no difference in ALP activity between pre-dialysis and dialysis vessel rings at baseline or after culture in normal Ca-P or high Ca-P media. As shown in Figure 4-3 in vessel rings treated with calcitriol in high Ca-P medium, ALP activity was greater in dialysis (29.6$\pm$1.9 U/mg) than pre-dialysis (18.5$\pm$3.6 U/mg) vessel rings ($p=0.019$). In vessel rings treated with alfacalcidol there was no significant difference in ALP activity between pre-dialysis and dialysis vessel rings.

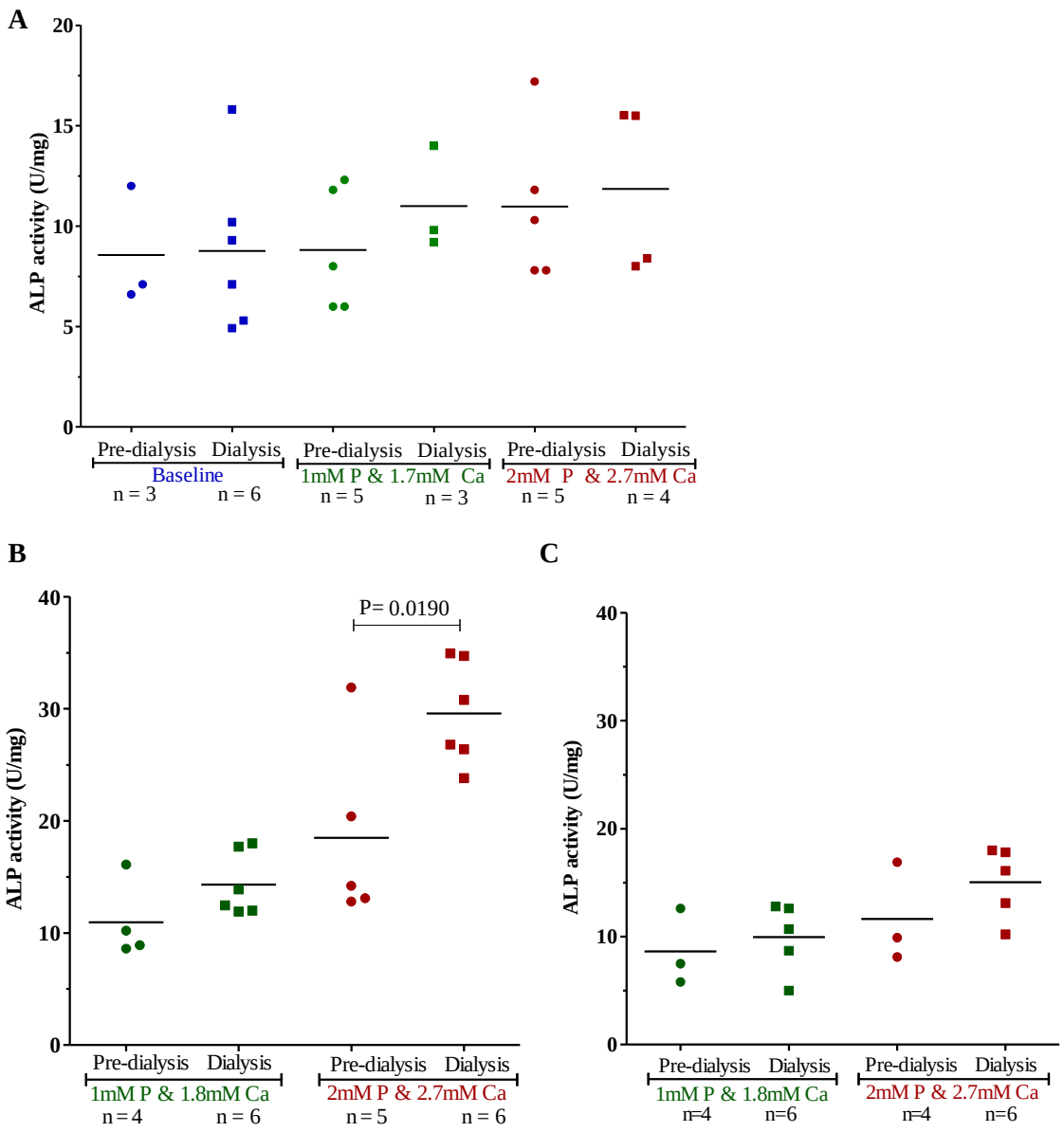


Figure 4-3: Comparison of ALP activity in pre-dialysis and dialysis vessel rings.

Vessel rings were isolated from children with CKD who were either pre-dialysis or receiving dialysis. A) A ring was taken at baseline and others cultured for 14 days in either normal Ca-P (1mM P and 1.8mM Ca) or high Ca-P (2mM P and 2.7mM Ca) to induce calcification. In addition, rings were cultured with B) calcitriol or C) alfacalcidol. Vessel rings were homogenised and the ALP activity in the vessel wall was determined by a colorimetric assay. Statistical significance was analysed by unpaired two-tailed t-test.

4.3.4 ALP activity was not a significant predictor of Ca load in children's vessel rings in the presence of calcitriol or alfacalcidol.

It was shown in Chapter 3 that alfacalcidol and to a greater extent calcitriol increased Ca load in vessel rings cultured in high Ca-P medium. A similar pattern was observed with ALP activity in this chapter where there was a trend for alfacalcidol to increase ALP activity in high Ca-P medium and calcitriol significantly increased ALP activity in the vessel rings in high Ca-P medium. Next, I considered whether ALP activity was a significant predictor of Ca load in vessel rings from the same patient treated in high Ca-P alone, with alfacalcidol or calcitriol.

In high Ca-P only, ALP activity was a significant predictor of Ca load (R^2 = 0.5386, P=0.0244) as shown in Figure 4-4A. Despite that in high Ca-P medium calcitriol increased both Ca load and ALP activity in the vessel rings, ALP activity was not a significant predictor of Ca load in the presence of calcitriol, nor was it with alfacalcidol (Figure 4-4B and C). Although increased ALP activity may contribute to calcification, the extent to which these VDRAs upregulate ALP activity is independent of their effects on VSMC calcification.

This suggests that in vessel rings from CKD patients treated with calcitriol or alfacalcidol the Ca load may be affected by other mechanisms in addition to changes in ALP activity.

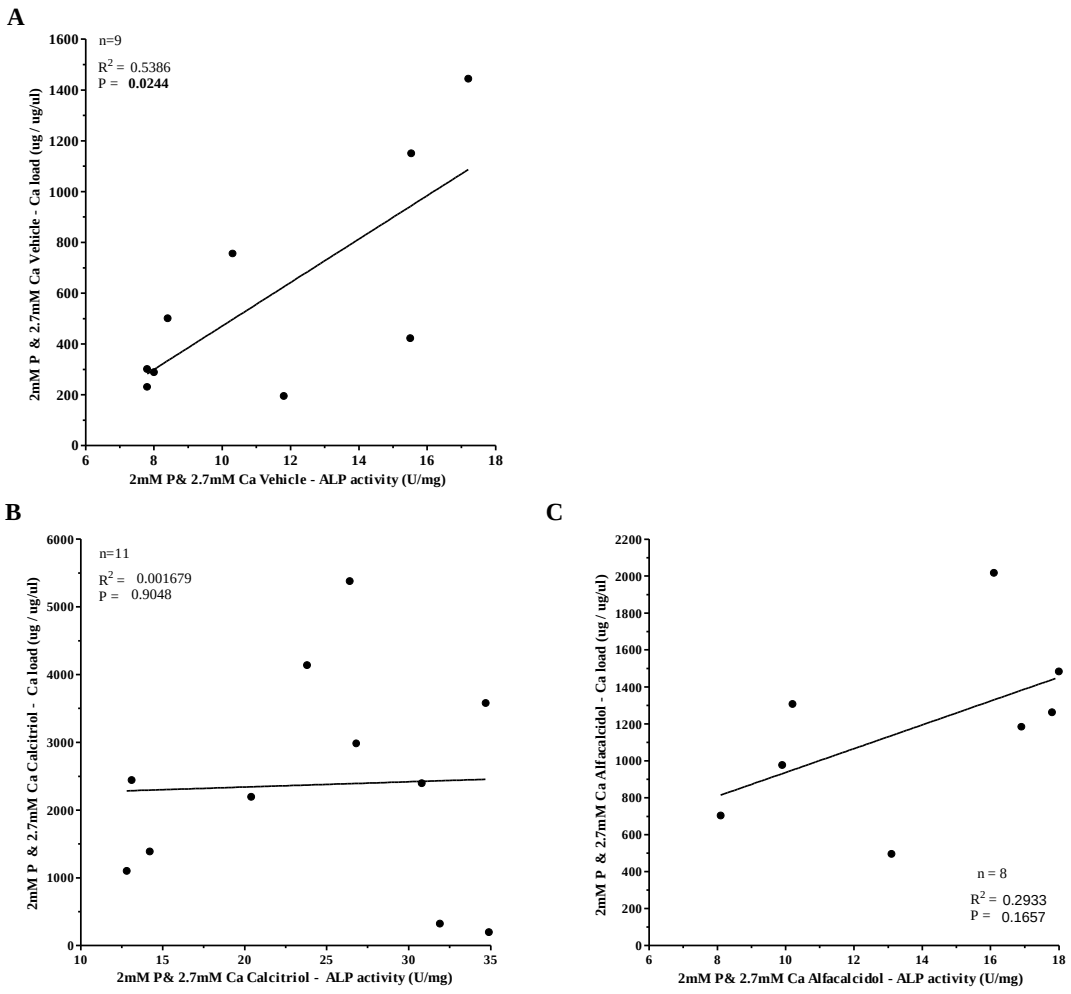


Figure 4-4: Is ALP activity a predictor of Ca load in vessel rings?

Vessel rings were isolated from CKD children and cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification. Vessel rings were treated with A) Ethanol, B) Calcitriol or C) Alfacalcidol Ca load and ALP activity in the vessel walls were determined by colourimetric assays and linear regression analysis was performed to determine if ALP activity was a significant predictor of Ca load.

4.3.5 Calcitriol but not alfacalcidol reduced VSMC viability in children's vessel rings.

The previous section showed that calcitriol but not alfacalcidol increased ALP activity in vessel rings cultured in high Ca-P medium. However, ALP activity was not a significant predictor of Ca load in vessel rings treated with either calcitriol or alfacalcidol, therefore they may be acting by additional mechanisms contributing to vascular calcification. In this next section I consider whether changes in VSMC viability could be a mechanism that contributes to the increased Ca load (as observed in Chapter 3) in children's vessel rings after treatment with alfacalcidol or calcitriol.

The effect of calcitriol and alfacalcidol on cell viability in the vessel rings from children with CKD, was determined by counting the smooth muscle cell density in the tunica media layer in sections stained with H and E. A representative example of a vessel ring in each treatment condition is shown in Figure 4-5. The structural components of the vessel rings remained intact including the tunica adventitia consisting of connective tissue, the tunica media containing VSMCs and the internal elastic lamella making up the tunica intima. Cystic areas in the tunica media were observed in some vessel rings cultured in high Ca-P with alfacalcidol, artificial damage can occur during processing and sectioning of the vessel rings, however the central location of these damaged regions indicates that they are cystic areas with VSMC cell death.

VSMC cell counts showed that alfacalcidol did not affect cell viability in either normal Ca-P or high Ca-P medium. High Ca-P medium did not affect cell number, however in high Ca-P medium, calcitriol reduced cell number from 26.5$\pm$2.4 to 15.5$\pm$3.2 cells per 100μm^2 ($p=0.02$) as shown in Figure 4-6.

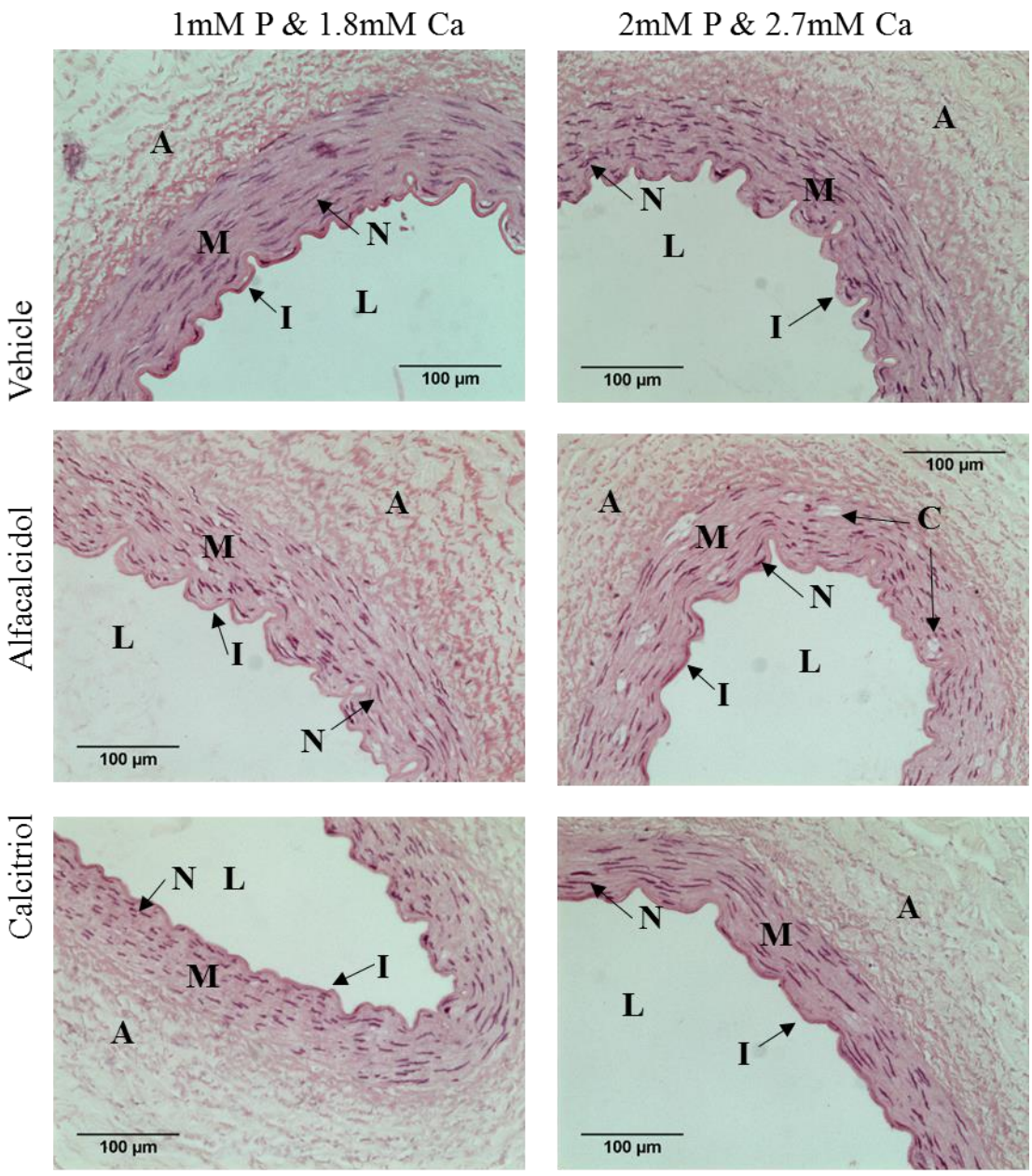


Figure 4-5 Cell viability shown by Haematoxylin and Eosin of treated vessel.

Haematoxylin and Eosin stain of pre dialysis vessel 07.6M.44.IE after 14 days culture in either normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P M199 medium (2mM P and 2.7mM Ca). With the addition of either the vehicle (3.4mM ethanol), alfacalcidol or calcitriol. Images taken at 20x magnification. **A-** Adventitia. **M** – Tunica Media. **L** – Lumen. **I** – Internal elastic lamella. N – VSMC Nuclei. C – Cystic area.

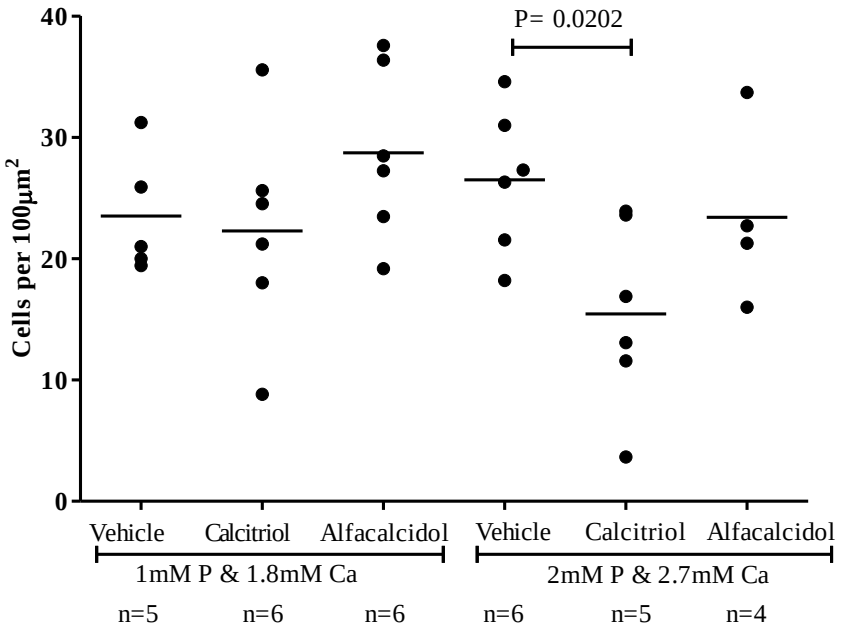


Figure 4-6: Calcitriol reduced cell number in high Ca-P medium.

Vessel rings were isolated from children with CKD. Vessel rings were cultured for 14 days in either normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca). With the addition of either the vehicle (3.4mM ethanol), alfacalcidol or calcitriol. Vessel rings were stained with H and E and the number of cells per 100µm^2 of tunica media were counted in 3 different areas per vessel, the mean of these is shown for n = 4-6 patients' vessels per condition. Statistical significance was analysed by unpaired two-tailed t-test.

4.4 Comparison between children's vessel rings that responded and did not respond to calcitriol.

In chapter 3 it was shown that the mean effect of calcitriol in high Ca-P medium was to significantly increase Ca load. It was also observed that the vessel rings' Ca loads separated into 2 groups, responders whose Ca load had increased in response to calcitriol and non-responders whose Ca loads remained low. In this next section non-responder and responder vessel rings were compared for their expression of osteogenic markers as well as their expression of 24 hydroxylase a sensitively regulated downstream gene of vitamin D.

4.4.1 There was no difference between responders and non-responders to calcitriol in their expression of osteogenic markers.

During VSMC calcification, smooth muscle cells undergo osteogenic conversion and develop an osteoblast like phenotype as discussed in the introduction. This is characterised by a downregulation of smooth muscle cell markers and an upregulation of osteogenic markers. It was considered whether non-responders and responders to calcitriol had differential expression of either smooth muscle or osteogenic markers at baseline and after culture in high Ca-P with or without calcitriol treatment. The expression of SMA, Runx2, and ALP were compared in 3 non responder and 3 responder vessel rings.

There was no observed difference between responders and non-responder vessel rings in their expression of SMA, Runx2 or ALP. SMA was expressed consistently throughout the tunica media in both non-responder and responder vessels an example is shown in Figure 4-7. There were no Runx2 positive nuclei in baseline vessel rings, however they could be observed in both non-responder and responder vessel rings cultured in high Ca-P with or without calcitriol, and example of this is shown in Figure 4-8 B and C. ALP was expressed in large areas throughout the media of vessel rings both at baseline and after culture, an example of this is shown in Figure 4-9.

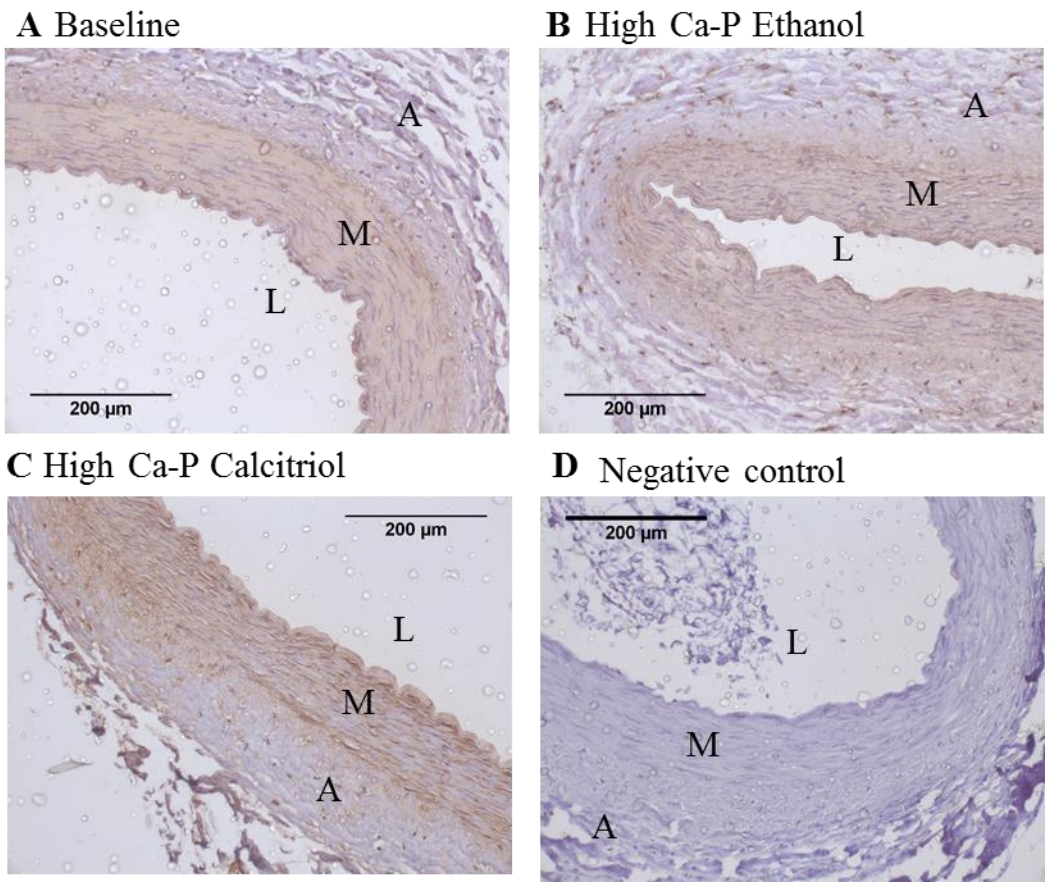


Figure 4-7: SMA expression in vessel rings.

Vessel rings that were harvested from children with CKD were isolated at baseline (A) or cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification, in the presence of 3.4mM ethanol (B) or calcitriol (C). Vessel rings were immune-stained for SMA (Brown) and counter stained with haematoxylin. D) Negative control with no primary antibody. Example shown: Non responder vessel 14.134M.119.IE. Images taken at 20 x magnification. **A**- Adventitia. **M** – Tunica Media. **L** – Lumen.

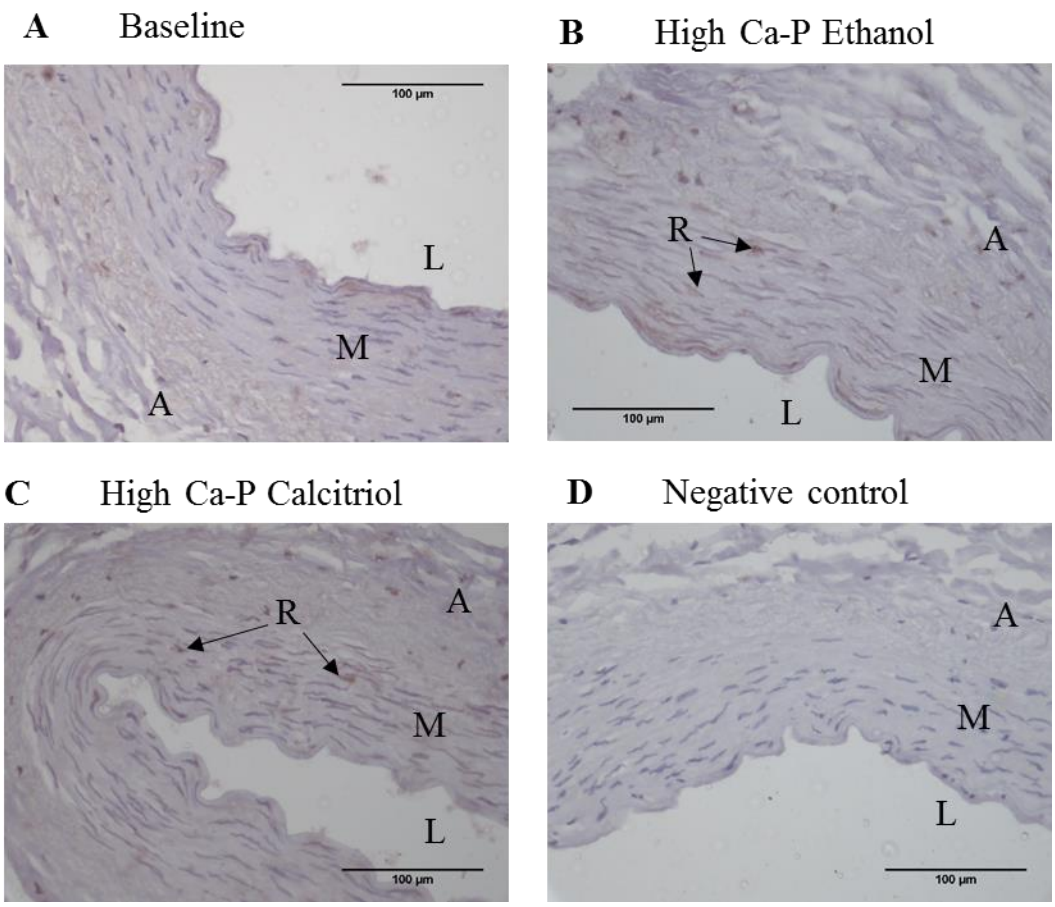


Figure 4-8: Runx2 expression in vessel rings.

Vessel rings that were harvested from children with CKD were isolated at baseline (A) or cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification, in the presence of 3.4mM ethanol (B) or calcitriol (C). D) negative control with no primary antibody. Vessel rings were immune-stained (Brown) for Runx2 and counter stained with haematoxylin. Example vessel ring 14.134M.119.IE is shown. Images taken at 40 x magnification. **A**- Adventitia. **M** – Tunica Media. **L** – Lumen. **R**- Runx2 positive nuclei.

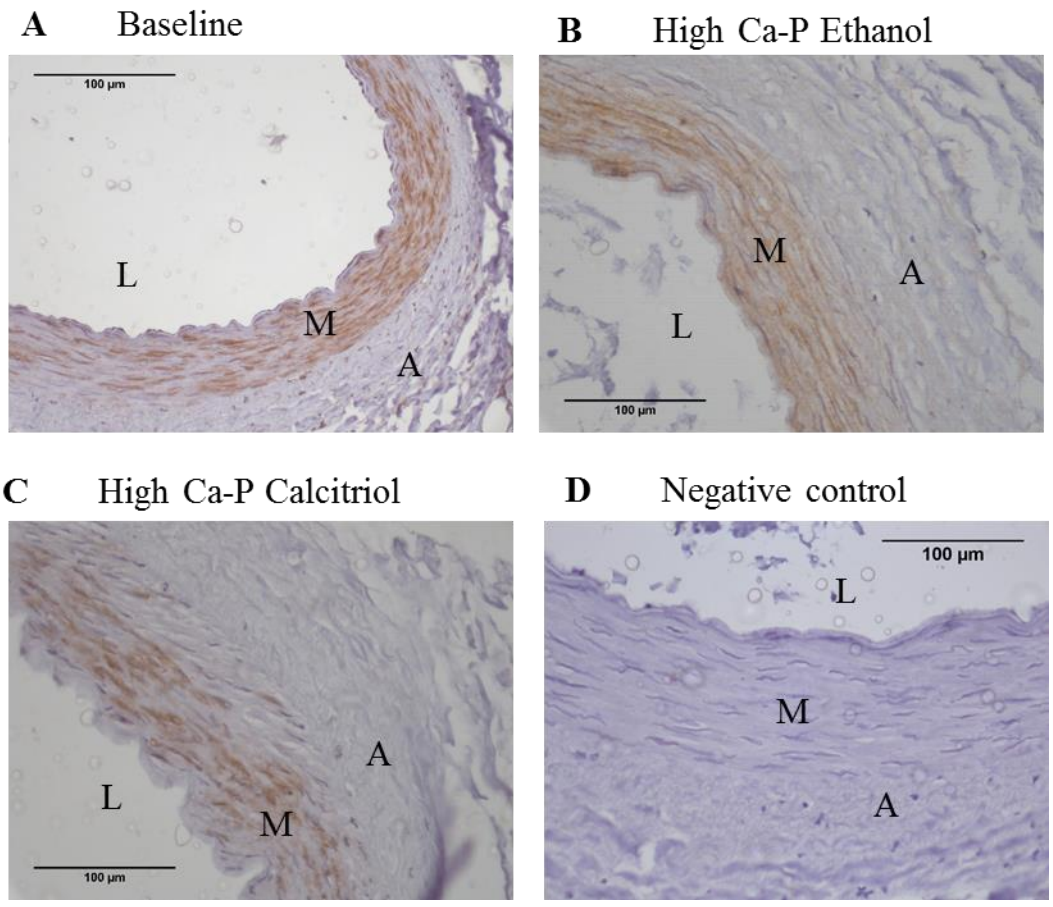


Figure 4-9: ALP expression in vessel rings

Vessel rings that were harvested from children with CKD were isolated at baseline (A) or cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification, in the presence of 3.4mM ethanol (B) or calcitriol (C). D) Negative control with no primary antibody. Vessel rings were immune-stained (Brown) for ALP and counter stained with haematoxylin. Example vessel ring 14.8M.114.IE. Images taken at 40x magnification. **A**- Adventitia. **M** – Tunica Media. **L** – Lumen.

4.4.2 Vessel rings that responded and did not respond to calcitriol differ in their expression profile of 24 hydroxylase.

It was shown in the previous section that there was no difference in osteogenic conversion between non-responder and responder vessel rings before or after culture. In the next section the expression of 24 hydroxylase, which is sensitively upregulated by VDRAs was compared between responder and non-responder vessel rings. The CYP24A1 gene transcribes the 24 hydroxylase enzyme for the inactivation and degradation of vitamin D, as discussed in the introduction its expression is regulated by VDRAs forming a negative feedback loop.

24 hydroxylase was highly expressed in the tunica media of both non-responder and responder vessel rings at baseline. After 14-day culture in high Ca-P medium only or high Ca-P with calcitriol, 24 hydroxylase was consistently expressed in the responder vessel rings tunica media at similar levels to baseline as shown in the example in Figure 4-10 D, E, F. 24 hydroxylase expression was reduced in non-responder vessel rings cultured in high Ca-P only. Whereas its expression was greatly increased in the tunica media of non-responder vessel rings cultured in high Ca-P media with calcitriol, as shown in Figure 4-10 B and C. This differential 24 hydroxylase expression in the high Ca-P medium only was observed in 3 responder and 3 non-responder vessel rings, the semi quantitative summary is shown in Table 4-2.

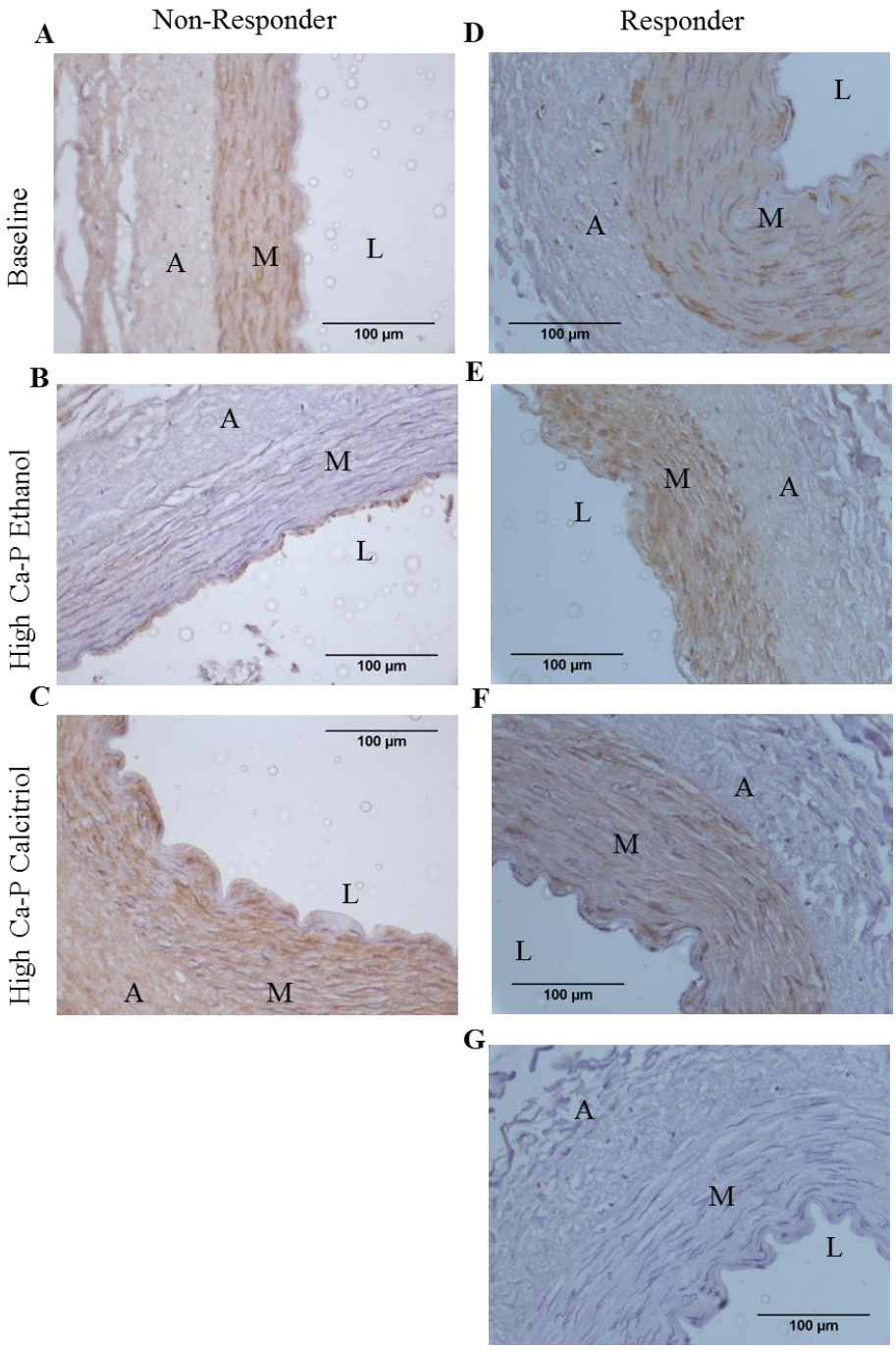


Figure 4-10: 24 hydroxylase expression in non-responder and responder vessels.
Vessel rings that were harvested from children with CKD were isolated at baseline (A and D) or cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification, in the presence of 3.4mM ethanol (B and E) or calcitriol (C and F). Vessel rings were immune-stained for 24 hydroxylase (Brown) and counter stained with haematoxylin. A,B,C,G) Non responder vessel 14.8M.114.IE. D,E,F) Responder vessel 07.16F.46.IE. G) negative control with no primary antibody. Images taken at 40x magnification. **A**- Adventitia. **M** – Tunica Media. **L** – Lumen.

Table 4-2: Summary of 24 hydroxylase expression in responder and non-responder vessel rings.

Vessel rings that were harvested from children with CKD were isolated at baseline or cultured for 14 days in high Ca-P medium to induce calcification, in the presence of 3.4mM ethanol as vehicle or calcitriol. Vessel rings from 3 responder and 3 non-responder vessels were immune-stained for 24 hydroxylase. The area of tunica media positive for 24 hydroxylase was semi-quantitatively quantified.

0 0% positive
\+ 1-25% positive
++ 26-50% positive
+++ 51-75% positive
++++ 76%-100% positive

		Baseline	High Ca-P Ethanol	High Ca-P Calcitriol
Responders	**07.16F.46.IE**	+ + + +	+ + + +	+ + + +
	07.6M.44.IE	+ + + +	+ + + +	+ + + +
	13.5M.110.IE	+ + + +	+ + + +	+ + + +
Non-responders	**14.8M.114.IE**	+ + + +	0	+ + + +
	13.14M.105.IE	+ + +	+ +	+ + + +
	14.13M.119.IE	+ +	+ +	+ + + +

4.5 Discussion

4.5.1 Summary

Data presented in this chapter showed that calcitriol and alfacalcidol act via different mechanisms to promote vascular calcification in vessel rings from children with CKD. Furthermore, it was determined that non-responder and responder vessel rings differ in their expression profiles of the vitamin D target 24 hydroxylase.

The key findings were:

1. Alfacalcidol can be activated locally in VSMCs as demonstrated by the expression of 25 hydroxylase (CYP27A1) as well as 1α hydroxylase (CYP27B1) in explanted VSMCs.

2. Calcitriol but not alfacalcidol increased ALP activity in vessel rings cultured in either normal Ca-P or high Ca-P media.

3. Calcitriol, but not alfacalcidol reduced VSMC viability in vessel rings cultured in high Ca-P media, this is known to precede VSMC calcification and was demonstrated by a reduction in VSMC number.

4. Non responder and responder vessel rings expressed comparable levels of markers of osteogenic conversion (SMA, Runx2 and ALP).

5. Responder vessel rings had persistently high expression of 24 hydroxylase in all conditions, where as non-responders had reduced 24 hydroxylase expression after culture with high Ca-P medium only.

4.5.2 Expression of vitamin D activation enzymes

When comparing the mechanistic actions of calcitriol and alfacalcidol in vessel rings it was essential to determine if alfacalcidol was functioning in its active or inactive form. Autocrine activation of alfacalcidol would require expression of the 25 hydroxylase enzyme.

Expression of CYP27B1 encoding the 1α-hydroxylase was evident in each of the explanted VSMCs from both control and dialysis vessels as shown in Figure 4-1. This agrees with previous literature showing 1α-hydroxylase expression and activity in human VSMCs (Somjen et al., 2005). CYP27B1 expression in dialysis VSMCs was approximately 150% that of control VSMCs (NS), this increased expression in CKD coincides with findings that human inferior epigastric arteries from CKD patients had approximately 200% of the 1α hydroxylase protein expression found in control vessels (Torremade et al., 2016).

CYP2R1 could not be detected in explanted VSMCs from either control or dialysis patients, however each VSMC explant expressed CYP27A1 gene encoding 25 hydroxylase. Hence, it can be assumed that VSMCs in the vessel rings also expressed the 25 hydroxylase enzyme, enabling alfacalcidol to be locally activated in this *ex-vivo* model and to mimic the effect it would have on vessels *in-vivo*. CYP27A1 and CYP2R1 are less well studied than CYP27B1, both are expressed in the human endometrium and in thyroid cells (Bergada et al., 2014) and (Bennett et al., 2012), there is also evidence for the expression of CYP2R1 in human brain pericytes (El-Atifi et al., 2015) and in renal cells (Urbschat et al., 2013) indicating the ability of 25 hydroxylase to have autocrine function in specific tissue types. There is no literature to suggest that CYP2R1 nor CYP27A1 are expressed in VSMCs and this is the first time CYP27A1 expression in human VSMCs has been shown.

Due to the small amount of human tissue harvested from patients, expression of vitamin D activation enzymes, 1α hydroxylase and 25 hydroxylase were not investigated in vessel rings. Given the expression in VSMCs, it may be suggested that vessel rings also express

vitamin D activation enzymes. In future studies it would be of interest to confirm expression of vitamin D activation enzymes in the vessel rings.

4.5.3 Calcitriol but not alfacalcidol increased ALP activity in children's vessel rings.

In Chapter 3 it was shown that high Ca-P medium increased Ca load in vessel rings from children with CKD, this was further increased by alfacalcidol and to a greater extent by calcitriol. Here I considered if calcitriol or alfacalcidol had an effect on ALP activity that could contribute to increased calcification in the vessel rings.

ALP is an osteogenic enzyme normally expressed in bone and not in healthy VSMCs therefore an increase in its expression or activity is a marker of osteo / chondrocytic differentiation. ALP is known to dephosphorylate and inactivate the calcification inhibitor pyrophosphate (Villa-Bellosta and Sorribas, 2011), as discussed in the introduction. Therefore, as well as being an osteoblast marker, an increase in ALP activity further promotes vascular calcification.

Previous findings from our group showed that high P only medium increased ALP activity in CKD vessel rings by approximately 2 fold whereas high Ca-P medium reduced ALP activity (Shroff et al., 2010). This differed from findings in this thesis, high Ca-P medium had no effect on ALP activity in the children's CKD vessel rings despite the increase in Ca load observed in the vessel rings cultured with high Ca-P in Chapter 3. In this study, ALP activity was investigated in a small number of vessels only (n=3-6) and more n numbers are required to confirm the null effect of high Ca-P on ALP activity. Furthermore, in this study each of the vessel rings were cultured in the presence of 3.4mM ethanol as the vehicle control, ethanol may interfere with ALP activity and it would be of interest to include a nil control along with a vehicle control in future studies. Interestingly data presented here showed that in the presence of either alfacalcidol or calcitriol, increasing the mineral content to high Ca-P medium did increase ALP activity by approximately 1.5 and 2 fold respectively.

In this study, 10nM calcitriol significantly increased ALP activity in children's CKD vessel rings both in normal Ca-P medium and by a greater extent in high Ca-P medium. Previously, it was shown that 1nM calcitriol increased ALP activity by 301% in bovine VSMCs (Wang et al., 2002). In human aortic SMCs, both 10nM and 100nM calcitriol dose dependently increased ALP activity. Furthermore, patients' serum collected directly after the administration of calcitriol and applied to VSMCs for 5 days led to a significant increase in ALP activity (Becs et al., 2016).

Vessel rings cultured in high Ca-P medium with calcitriol had both the highest Ca load shown in Chapter 3 and the highest ALP activity shown in this chapter. However, ALP activity was not a significant predictor of Ca load in vessel rings treated with either calcitriol or alfacalcidol. This suggests calcitriol and alfacalcidol triggered other pro - calcification mechanisms.

4.5.4 Calcitriol but not alfacalcidol reduced cell viability in children's vessel rings.

Previous studies have shown that reduced cell viability caused by high P levels precedes calcification, for example incremental increases in P concentration from 1mM to 10mM dose dependently reduced cell viability in a rat aortic cell line cultured for 10 days, this was consistent with and may have caused increased Ca and P deposition (Shin and Kwun, 2013). Therefore, the effect of calcitriol and alfacalcidol on cell viability were studied.

In this chapter high Ca-P alone did not affect cell viability, this may be explained as the 2mM P was a modest increase in P relative to the study above. However, in previous work by our group the same high Ca-P medium reduced VSMC density in CKD vessel rings (Shroff et al., 2010). Interestingly, in high Ca-P medium calcitriol but not alfacalcidol did reduce cell viability as shown in Figure 4-6. This agrees with previous literature as calcitriol has previously been shown to reduce cell viability, 0.1 and 1nM calcitriol increased apoptosis and reduced proliferation in human VSMCs treated for 24 hours (Sato et al., 2016).

It can be concluded that alfacalcidol and calcitriol act on vascular calcification by different mechanisms. In high Ca-P medium calcitriol both increased ALP activity and reduced cell viability leading to a significant increase in Ca load. Alfacalcidol also increased Ca load in high Ca-P medium, however it did not increase ALP activity or reduce cell viability therefore the mechanism by which alfacalcidol increased calcification has yet to be defined.

4.5.5 Comparable expression of, SMA, Runx2 and ALP in vessel rings that responded and did not respond to calcitriol.

As discussed in the introduction, past literature shows contradictory responses to calcitriol in different experimental set ups, in some models calcitriol increased Ca load where as in others it reduced Ca load. Therefore, it is understandable that calcitriol had different effects on calcification in vessel rings from different individuals. I observed in Chapter 3 that the Ca load after treatment with high Ca-P separated vessel rings into 'responders' whose Ca load increased in response to calcitriol, and 'non-responders' whose Ca load was not effected by calcitriol. Here I compared the expression level of osteogenic markers in responder and non-responder vessel rings to explore any differences in osteogenic conversion.

A previous study in our group observed that SMA expression was patchy in dialysis relative to control vessel rings which may be an early indication of osteogenic conversion (Shroff et al., 2010). In this chapter I compared SMA expression at baseline and after culture in high Ca-P medium with or without calcitriol in CKD vessel rings. There was no downregulation of SMA protein expression in either the non-responder or responder vessel rings to indicate osteogenic conversion.

Vascular calcification is associated with the transition of VSMCs to an osteogenic phenotype, in fact Runx2 expression in VSMCs was found to be essential to precede arterial medial calcification in mice, as SMC specific Runx2 knock out mice did not develop vascular calcification (Lin et al., 2015). There was no baseline expression of

Runx2 in vessel rings, these baseline rings did not show evidence of calcification as shown by von Kossa staining in Chapter 3. Therefore, this agrees with previous literature showing that Runx2 was only expressed in calcified and not healthy inferior epigastric arteries from dialysis patients (Moe et al., 2003). This chapter showed that Runx2 was expressed in vessel rings cultured in high Ca-P medium this agrees with previous work in our group which also showed that high Ca-P medium increased expression of Runx2 in children's dialysis vessel rings (Shroff et al., 2010). The addition of calcitriol to high Ca-P did not further increase the expression of Runx2 in either responder or non-responder vessel rings. Previous literature shows mixed evidence for the effects of calcitriol on Runx2 expression in human VSMCs, when induced to calcify with high P and TNFα, 1nM, 10nM and 100nM calcitriol had a trend to reduce Runx2 mRNA expression with no significant effect (Aoshima et al., 2012). Whereas in human VSMCs induced to calcify in high P only, 10nM calcitriol increased Runx2 mRNA expression (Martinez-Moreno et al., 2012).

In the CKD vessel rings shown here, ALP was expressed at baseline as well as after culture in high Ca-P medium. This agrees with previous literature as CKD is associated with the upregulation of tissue nonspecific ALP in the vasculature, for example rats made uremic by either 5/6 nephrectomy or by feeding adenine showed increased ALP protein expression and activity (Lomashvili et al., 2008). A circulating factor in uremic serum is thought to increase ALP activity, aortic rings from normal rats cultured in uremic serum also had increased ALP activity (Lomashvili et al., 2008). As discussed above multiple studies have shown that calcitriol increased ALP activity, however there is little evidence for the effect of calcitriol on ALP protein expression. In this study calcitriol had no effect on ALP expression in either responder or non-responder vessels. Ideally ALP activity rather than expression would be compared as this represents the functional form of ALP. Only 2 of the non-responder vessel rings had sufficient tissue in order to analyse ALP activity therefore it was not possible to compare ALP activity between non-responder and responder vessel rings in this study.

Expression levels of SMA, Runx2 and ALP were comparable between non-responder and responder vessel rings in each treatment condition. High Ca-P medium with or without

calcitriol did not affect expression of SMA or ALP, however Runx2 expression was increased by high Ca-P medium.

4.5.6 Differential expression of 24 hydroxylase in vessel rings that responded and did not respond to calcitriol.

To further explore the mechanistic differences between calcitriol responder and non-responder vessel rings, expression of 24 hydroxylase was compared. 24 hydroxylase is transcribed by the CYP24A1 gene which is strongly induced by calcitriol, when bound to the VDR it interacts with the 2 VDRE in the promoter region of CYP24A1 (Jones et al., 1998). CYP24A1 expression is highly regulated by vitamin D, at the time of vessel collection all CKD patients were receiving alfacalcidol supplements, therefore the expression of 24 hydroxylase evident in baseline vessel rings is likely to be in response to these *in-vivo* supplements.

CYP24A1 mRNA is downregulated by PTH (Shinki et al., 1992). In culture most cells lose the expression of their PTH receptors, in AOK B50 cells which are stably transfected with PTH receptors, PTH was shown to destabilise CYP24A1 mRNA and reduced its half-life by 4.2 fold from 5.7$\pm$0.7 hours to 1.4$\pm$0.4 hours (Zierold et al., 2003). In this study of *ex-vivo* vessel rings, expression of the PTH receptor is unknown and PTH was not present in the culture medium therefore a CYP24A1 mRNA half-life of approximately 6 hours can be assumed. After 14 days in culture in the absence of a VDRA it was anticipated that 24 hydroxylase would not be expressed at detectable levels. As expected 24 hydroxylase was not expressed in non-responder vessel rings cultured in high Ca-P only and the addition of calcitriol increased expression of 24 hydroxylase. Interestingly the high levels of 24 hydroxylase did persist in responder vessel rings after 2 weeks in culture without exposure to a VDRA. Consequently, when these vessel rings were exposed to calcitriol expression of 24 hydroxylase remained persistently high and there was no upregulation of 24 hydroxylase expression when exposed to calcitriol.

The ability of non-responder vessel rings to upregulate 24 hydroxylase expression in response to calcitriol, leading to the inactivation of calcitriol may have prevented calcitriol from causing an increase in Ca load. Vessel rings that expressed 24 hydroxylase after culture in high Ca-P medium only, were unable to further upregulate their already high 24 hydroxylase expression in response to calcitriol treatment. Vessel rings inability to regulate 24 hydroxylase for the inactivation of calcitriol may have contributed to the increased Ca load by calcitriol observed in the responder but not in the non-responder vessel rings.

4.5.7 Limitations

As discussed in Chapter 3, experiments on whole vessel rings from patients were limited by tissue availability. In the following chapter, work on VSMCs explanted from both healthy control and dialysis patients enable wider analysis of the effects of VDRAs on vascular calcification. This includes analysis of the dose dependent effect of calcitriol on calcification as well as extracting RNA to determine the response to VDRAs at the transcriptional level.

Chapter 5 The effects of VDRAs on calcification in VSMCs explanted from children with CKD.

5.1 Introduction

The effect of vitamin D on vascular calcification is thought to be dose dependent with both high and low doses leading to an increased risk, as described in the introduction. It would be of great interest to compare the effect of different doses of calcitriol on vascular calcification in CKD. Studies on whole vessel rings shown in Chapter 3 and 4, were limited by tissue availability and size of the vessels obtained. To overcome these limitations VSMCs were explanted from the vessels and this chapter explores the dose dependent effect of calcitriol on calcification in explanted VSMCs from control and dialysis patients.

In the vessel rings no effect of paricalcitol on vascular calcification was observed, which differs from previous literature as discussed in the introduction and chapter 3. This chapter compares the effects of physiological dose paricalcitol to calcitriol in explanted VSMCs.

Due to the small size of vessel rings, there was insufficient tissue from which to extract RNA and to examine the mechanistic effects of VDRAs at the transcriptional level. This is an important aspect as ligand bound VDR acts as a transcriptional regulator for many genes. Experiments on explanted VSMCs discussed in this chapter enabled the expression of vitamin D target genes to be examined at the transcriptional level.

Patients receiving dialysis represent the extreme cases with end stage kidney disease, therefore, VSMCs from these patients were compared to those from healthy control, non-renal patients.

5.1.1 Aims

1. To establish whether the effect of calcitriol on calcification in VSMCs is dose dependent.

2. To determine whether paricalcitol is less calcaemic than calcitriol in VSMCs.

3. To elucidate whether dialysis VSMCs are more susceptible to VDRA induced calcification than control VSMCs.

These will be addressed using the following key questions;

1. Do control and dialysis VSMCs express different levels of smooth muscle and osteogenic markers at baseline?

2. What effects do physiological dose paricalcitol and calcitriol at a range of doses have on calcification? Does this differ between VSMCs from control and dialysis patients?

3. Does calcitriol increase CYP24A1 expression in a dose dependant manner? Are 10nM calcitriol and 10nM paricalcitol equally effective at upregulating CYP24A1 expression?

4. Do physiological dose paricalcitol or calcitriol at a range of doses effect calcification of VSMCs by regulating the expression or activity of osteoblast enzyme ALP? Does this differ between VSMCs from control and dialysis patients?

5. Do physiological dose paricalcitol or calcitriol at a range of doses protect against VSMC calcification by regulating expression of the calcification inhibitor MGP or the osteoblast transcription factor Runx2? Does this differ between VSMCs from control and dialysis patients?

5.2 Method

For this study the omental or mesenteric artery was collected from healthy control patients and either the inferior epigastric or omental artery was collected from dialysis patients, all patient information is shown in the Methods. The VSMCs used in each experiment are shown in Table 5-1. VSMCs were explanted from these vessels and used for all experiments in this chapter.

VSMCs were cultured in either normal Ca-P medium (normal M199 tissue culture medium which contains 1mM P and 1.8mM Ca M199) or high Ca-P medium (M199 with added P and Ca to a final concentration of 2mM P and 2.7mM Ca) to induce calcification. VSMCs were cultured for between 4 and 6 days until the onset of calcification, with the medium replaced every 3 days.

VSMCs were cultured with either the vehicle as 3.4mM ethanol or VDRA calcitriol at one of three doses from sub physiological (1nM) to physiological (10nM) and supra physiological (100nM), as well as with a physiological dose of paricalcitol (10nM). VSMCs from each patient were analysed in triplicate for their Ca content, the Ca load in VSMCs was determined by o-cresolphthalein assay and was normalised to protein content. Experiments with insufficient calcification (Ca load < 5µg/ µg in all treatment groups) were excluded from analysis.

Treated VSMCs were also analysed for their ALP activity and their mRNA extracted for qPCR analysis. All data is shown as mean $\pm$ SE.

Table 5-1: VSMCs utilised in each experiment.

VSMC ID format: Year. Age and gender. Unique number. Vessel type.	**Baseline mRNA**	**Ca load**	**CYP24A1 mRNA**	**ALP mRNA**	**ALP activity**	**Runx2 mRNA**	**MGP mRNA**
Controls VSMC ID							
13.0.1F.106.OM	✓	✓	✓	✓	✓	✓	✓
13.10F.108.OM	✓	✓	✓	✓	✓	✓	✓
13.1F.28.OM*	✓			✓	✓	✓	✓
13.5M.33.OM*	✓				✓#		
13.3M.31.OM*	✓				✓#		
13.5F.32.OM*	✓		✓				
Dialysis VSMC ID							
13.16M.101.IE	✓	✓	✓	✓	✓	✓	✓
12.13M.16.IE*	✓	✓	✓	✓	✓	✓	✓
13.15F.24.IE*	✓	✓	✓	✓	✓	✓	✓
14.17M.125.IE	✓	✓#	✓	✓	✓#		
13.15F.112.IE	✓				✓#		
14.13M.119.IE	✓				✓#		
14.14M.124.IE	✓				✓#		
06.8F.36.IE**	✓						
06.9M.34.IE**	✓						
05.1.5M.04.OM**	✓						

*VSMCs explanted by Alex Todd. ** VSMCs explanted by Rukshana Shroff. All other VSMCs explanted by myself, and experiments on all explants performed by myself, Joanne Laycock unless indicated by #.

Experimental repeat on this VSMC isolate performed under my supervision by Annelies DeMare, a master's student that I, Joanne Laycock trained, planned experiments for and assisted in the lab. All data interpretation and analysis was performed by myself.

5.3 Control VSMCs have higher expression of smooth muscle cell markers at baseline than dialysis VSMCs.

All experiments in this chapter were performed on VSMCs that were explanted from vessels from children who were either CKD patients on dialysis or healthy control patients. It was important to characterise the phenotype of these explanted cells to confirm they were of VSMC origin. VSMCs are contractile cells that regulate vascular tone, as such fully differentiated VSMCs express a unique repertoire of contractile proteins including smooth muscle α-actin (SMA), smooth muscle myosin heavy chain (SMMHC), h1 calponin, SM22α and smoothelin (Gomez and Owens, 2012). These VSMC markers may also be expressed in other cell types therefore expression of only one of these cell markers is not sufficient.

The phenotype of explanted control and dialysis cells was confirmed by qPCR. MRNA expression of smooth muscle cell markers SMA, SM22α and SMMHC were confirmed in each cell type (Figure 5-2 A-I).

As an early indication of calcification, VSMCs dedifferentiate towards their mesenteric stem cell origin and they develop an osteoblast like phenotype, including the expression of some osteogenic markers. Therefore, the mRNA expression at baseline of osteogenic markers Runx2 and BSP were determined in each of the isolated control and dialysis VSMCs.

Each of the cell lines isolated from both control and dialysis patients expressed SMA, SMMHC, SM22 alpha, BSP and Runx2 at varying levels. As shown in Figure 5-2, control cell lines expressed higher levels of SMA than dialysis cell lines. The dialysis cell line, 05.1.5M.04.OM showed particularly high levels of both BSP and Runx2 mRNA expression as shown in Figure 5-2 K and N. Across all cell lines tested there was no significant difference in Runx2 and BSP expression between control and dialysis VSMCs as shown in Figure 5-2 L and M.

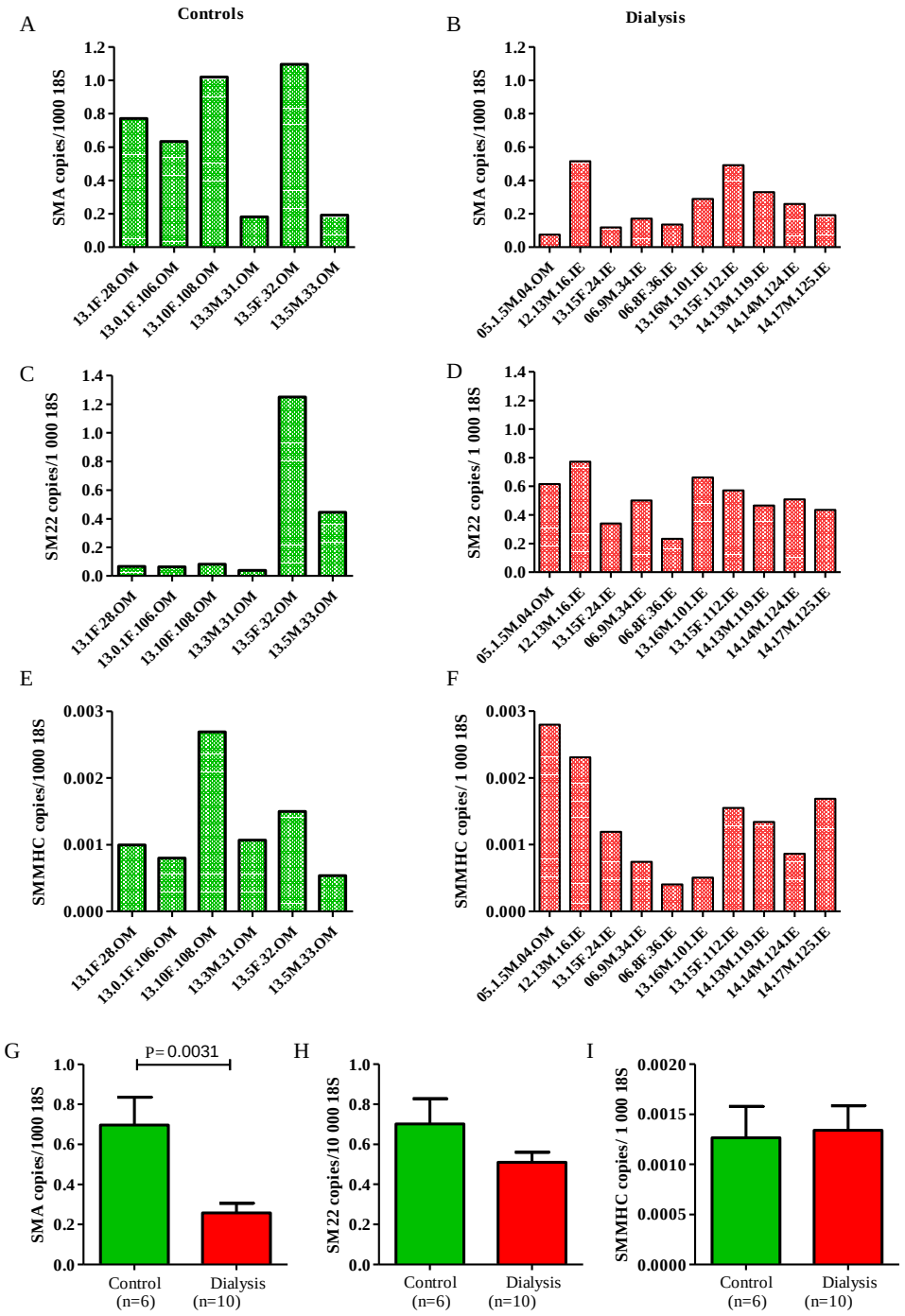


Figure 5-1: Baseline mRNA expression of SMC markers.
QPCR analysis was performed to determine the mRNA expression of SMC markers in VSMCs explanted from children's vessels, expression was normalised to that of reference gene 18s. VSMCs were from 6 non-renal control patients (A, C and E) and 10 dialysis patients (B, D, F). Cell marker expression between these 2 groups was compared (G, H, I), data is shown as mean ± SE and statistical significance determined by an unpaired two-tailed t-test.

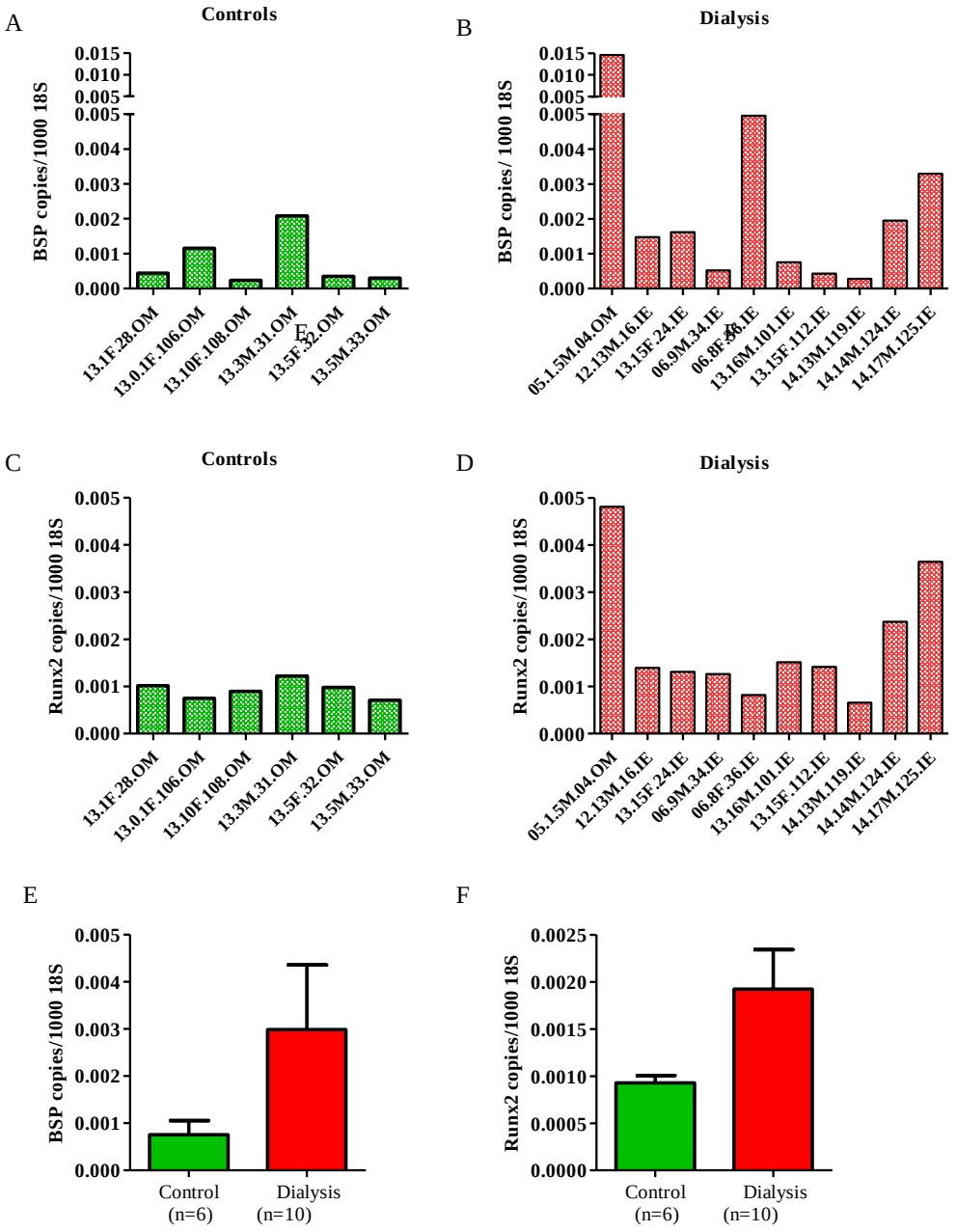


Figure 5-2: Baseline mRNA expression of osteoblast cell markers.
QPCR analysis was performed to determine the mRNA expression of osteoblast markers in VSMCs explanted from children's vessels, expression was normalised to that of reference gene 18s. VSMCs were from 6 non-renal control patients (A and C) and 10 dialysis patients (B and D). Cell marker expression between these 2 groups was compared (E and F) data is shown as mean ± SE and statistical significance determined by an unpaired two-tailed t-test.

5.4 Ca load in VSMCs explanted from control and dialysis children.

5.4.1 VDRAs reduced Ca load in VSMCs explanted from children.

The previous section showed that the explanted cells expressed several VSMC markers therefore could be identified as such. Next I considered the effect of VDRAs on calcification in these VSMCs.

Initially VSMCs were cultured in normal Ca-P medium as control, or high Ca-P medium to induce calcification. As shown in Figure 5-3A, high Ca-P medium significantly increased Ca load in VSMCs from 0.59±0.17µg/µg to 10.16±0.49µg/µg ($p<0.0001$). The addition of a vehicle did not affect the Ca load of VSMCs in high Ca-P medium (9.29±0.69µg/µg).

The physiological dose of 10nM calcitriol reduced Ca load from 9.29±0.69µg/µg to 5.57±0.85µg/µg ($p=0.007$), whereas 1nM and 100nM calcitriol had no effect on Ca load (Figure 5-3B). Similarly10nM paricalcitol reduced Ca load in children's VSMCs to 4.28±0.83 ($p=0.001$) as shown in Figure 5-3C.

These data consider the effect of high Ca-P medium and VDRAs on VSMCs from children regardless of their CKD status. Following this it was important to consider whether VSMCs from CKD patients on dialysis were more susceptible to calcification than those from healthy controls, and whether they responded differently to VDRA treatment. For this, VSMCs from patients on dialysis were compared to VSMCs from healthy controls.

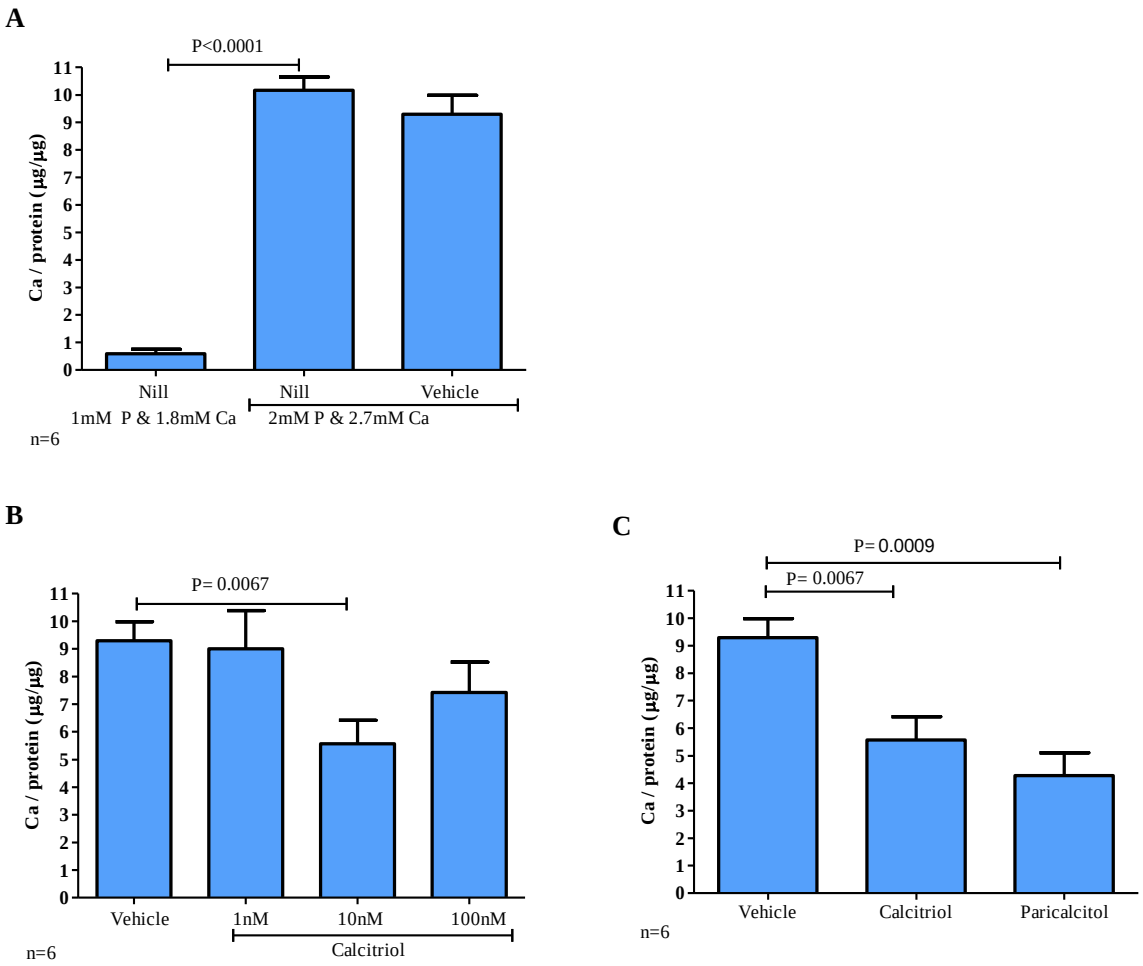


Figure 5-3: Ca load in VSMCs:
VSMCs (P9 or P10) explanted from 7 children were cultured for between 4 and 6 days, until the onset of calcification. Each experiment was done in triplicate and the mean for each VSMC type is shown. Experiments with insufficient calcification (Ca load < 5µg/µg in all treatment groups) were excluded from analysis: VSMCs were treated with: A) Normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca) with and without the vehicle control of 3.4mM ethanol. B) High Ca-P medium with the vehicle, 1nM, 10nM or 100nM calcitriol. C) High Ca-P medium with the vehicle, 10nM calcitriol or 10nM paricalcitol. Data is shown as mean $\pm$ SE and statistical significance determined by an unpaired two-tailed t-test.

5.4.2 There was no difference between control and dialysis VSMCs in their susceptibility to calcification.

The cultured VSMCs were explanted from 2 healthy control and 4 dialysis children. A comparison of control and dialysis VSMCs showed that culture in high Ca-P medium increased calcification comparably in both cell types, as shown in Figure 5-4A. The addition of the vehicle to high Ca-P medium had no effect on Ca load in either control or dialysis VSMCs.

In dialysis VSMCs treated in high Ca-P medium, the physiological dose of calcitriol (10nM) significantly reduced Ca load from 9.17$\pm$0.25µg/µg to 5.41$\pm$1.33µg/µg ($p=0.032$) whereas the extreme doses had no effect, as shown in Figure 5-4B. A similar pattern was observed in control VSMCs in high Ca-P medium, 10nM calcitriol reduced Ca load from 9.53$\pm$2.58µg/µg to 5.87$\pm$0.16µg/µg, ($n=2$ therefore the statistical significance was not calculated).

The physiological dose of paricalcitol (10nM) significantly reduced Ca load in dialysis VSMCs from 9.17$\pm$0.25µg/µg to 3.01$\pm$0.54µg/µg ($p<0.001$), as shown in Figure 5-4C. Again a similar effect was observed in control VSMCs where Ca load was reduced 9.53$\pm$2.58µg/µg to 5.86$\pm$1.36µg/µg ($n=2$) with 10nM paricalcitol.

Large variations were observed within both control and dialysis VSMCs in their susceptibility to calcify in the high Ca-P medium alone or with any of the treatment conditions observed. These experiments were performed on VSMCs explanted from different patients; cells from different individuals may calcify to different extents in response to the treatment conditions and give rise to the observed variations, for this reason it is important to consider the effect of VDRA treatment on individual cell lines.

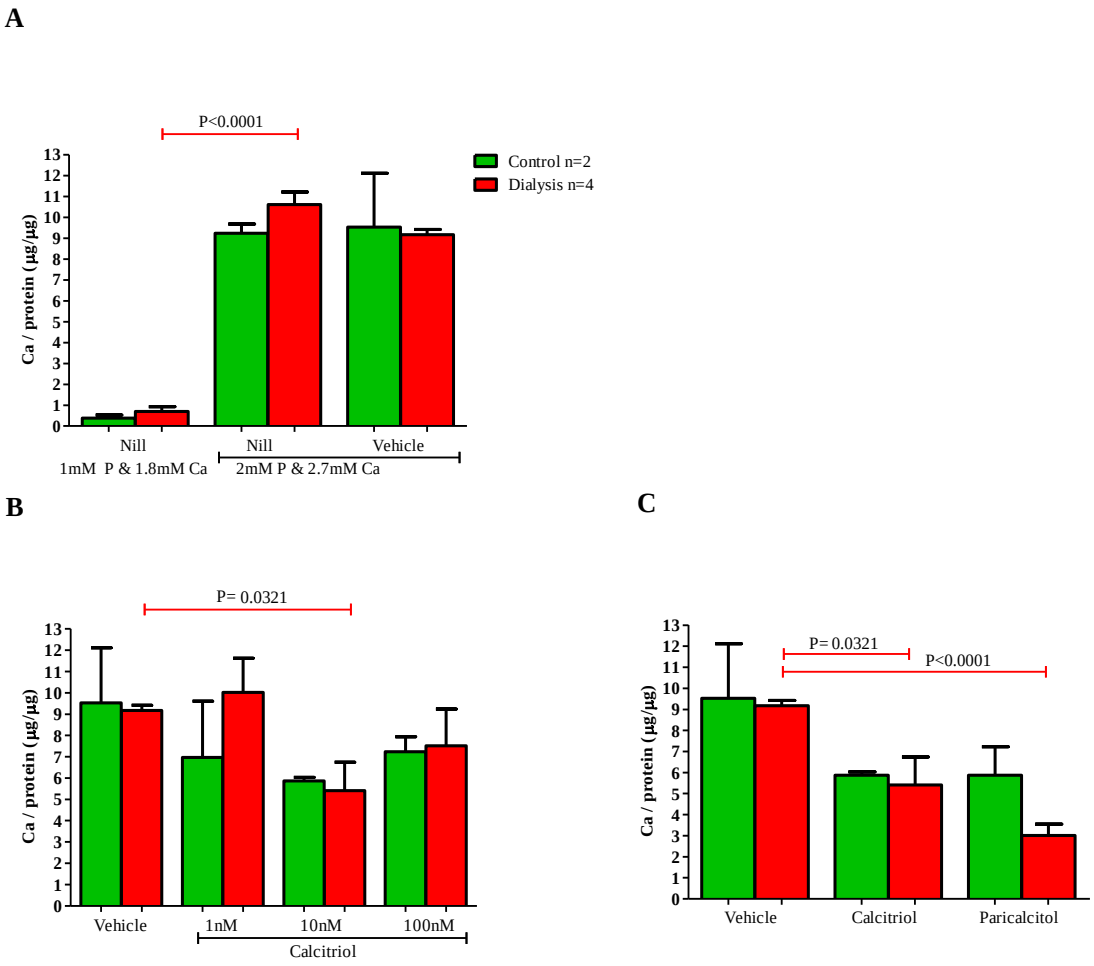


Figure 5-4: Ca load in control and dialysis children's VSMCs.
VSMCs (P9-P10) explanted from 2 healthy control and 4 dialysis children were cultured for between 4 and 6 days, until the onset of calcification. Each experiment was done in triplicate and the mean for each VSMC type is shown. Experiments with insufficient calcification (Ca load < 5µg/µg in all treatment groups) were excluded from analysis: VSMCs were treated with: **A**) Normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca) with and without the vehicle of 3.4mM ethanol. **B**) High Ca-P medium with the vehicle, 1nM, 10nM or 100nM calcitriol. **C**) High Ca-P medium with the vehicle, 10mM calcitriol or 10nM paricalcitol. Data is shown as mean ± SE, statistical significance between treatments in dialysis VSMCs was determined by an unpaired two-tailed t-test.

5.4.3 The effect of calcitriol on Ca load formed a U-shaped dose response curve, the 'U's troth shifted in VSMCs from different individuals.

The mean VSMC Ca loads presented in Figure 5-4B showed that 10nM calcitriol reduced Ca load in VSMCs, whereas both 1nM and 100nM calcitriol had no effect on Ca load in either control or dialysis VSMCs.

The large standard errors suggest that there was wide variation between VSMCs from individual patients in their level of Ca deposition in response to different VDRA treatment conditions. The extreme doses of calcitriol may have had an effect on Ca load in VSMCs from some individuals, however this information was lost when only the mean response of all control or dialysis VSMCs was presented, therefore it is important to consider the effect of VDRA treatment on VSMCs from each individual.

In the 2 different control VSMCs, both 1nM and 10nM calcitriol significantly reduced calcification in high Ca-P medium (Figure 5-5A and B), 100nM calcitriol also reduced calcium deposition in one of these control VSMCs but had no effect in the other. The effect of 1nM and 10nM calcitriol on reducing Ca load was most noticeable in control VSMCs 13.10F.108.OM (Figure 5-5A). In these cells, Ca deposition was reduced from 7.54$\pm$0.21µg/µg to 1.27$\pm$0.04 µg/µg and 1.43$\pm$0.21µg/µg with 1nM and 10nM calcitriol respectively; 100nM calcitriol had no effect on Ca load in these VSMCs. In control VSMCs 13.0.1F.106.OM, 1nM, 10nM and 100nM calcitriol all significantly reduced Ca deposition. The greatest reduction in Ca deposition was observed by 10nM calcitriol which reduced Ca load from 19.35$\pm$0.59µg/µg to 9.11$\pm$0.22µg/µg, this was significantly lower than after treatment with either 1nM or 100nM calcitriol, as shown in Figure 5-5B.

Unlike in control VSMCs, 1nM calcitriol had no significant effect on Ca load in any of the 4 dialysis VSMCs. Both 10nM and 100nM calcitriol significantly reduced Ca load in some of the dialysis VSMCs. In the dialysis VSMCs 13.16M.101.IE, there was a trend for 1nM calcitriol to reduce Ca load in high Ca-P medium from 9.87$\pm$1.65µg/µg to 5.37$\pm$1.66µg/µg (NS). In these VSMCs 10nM calcitriol significantly reduced Ca load from 9.87$\pm$1.65µg/µg to 2.85$\pm$0.71µg/µg ($p=0.003$) whereas 100nM calcitriol had no

effect on Ca load, as shown in Figure 5-5C. This differed from dialysis VSMCs 12.13M.16.IE, in which 1nM calcitriol had no effect on Ca load in high Ca-P medium. There was a trend for 10nM calcitriol to reduce Ca load (NS) however it was 100nM calcitriol that significantly reduced Ca load from 18.73±2.21 µg/µg to 8.40±2.62 µg/µg ($p=0.039$) (Figure 5-5D). In VSMCs 14.17M.125.IE there was a trend for 10nM calcitriol to reduce Ca load in high Ca-P (NS). Neither 1nM nor 100nM calcitriol had an effect on Ca load, however both of these extreme concentrations had a significantly higher Ca load than VSMCs treated with 10nM calcitriol (Figure 5-5F). In dialysis VSMCs 13.15F.24.IE, calcitriol at 1nM, 10nM or 100nM had no effect on Ca deposition (Figure 5-5E).

In 4 of the 6 VSMCs from different individuals, a U-shaped pattern was observed between the concentration of calcitriol and VSMC Ca load. Both low and high dose calcitriol were associated with higher Ca loads, and the physiological dose of 10nM calcitriol had the greatest protective effect on Ca load. In the remaining 2 VSMCs, both of which were dialysis, the lower dose of 1nM calcitriol was again associated with a higher Ca load than 10nM calcitriol, however the highest dose tested of 100nM calcitriol continued to have a protective effect on Ca load. Overall the individual cell population data shows that 10nM calcitriol is the dose most consistently protective of calcification in VSMCs; it significantly reduced Ca load in both of the control cells and 1 of the 4 cells from dialysis patients. 1nM and 100nM doses of calcitriol both reduced Ca load in 2 of the cell lines, however the extreme doses of calcitriol did not consistently reduce Ca load.

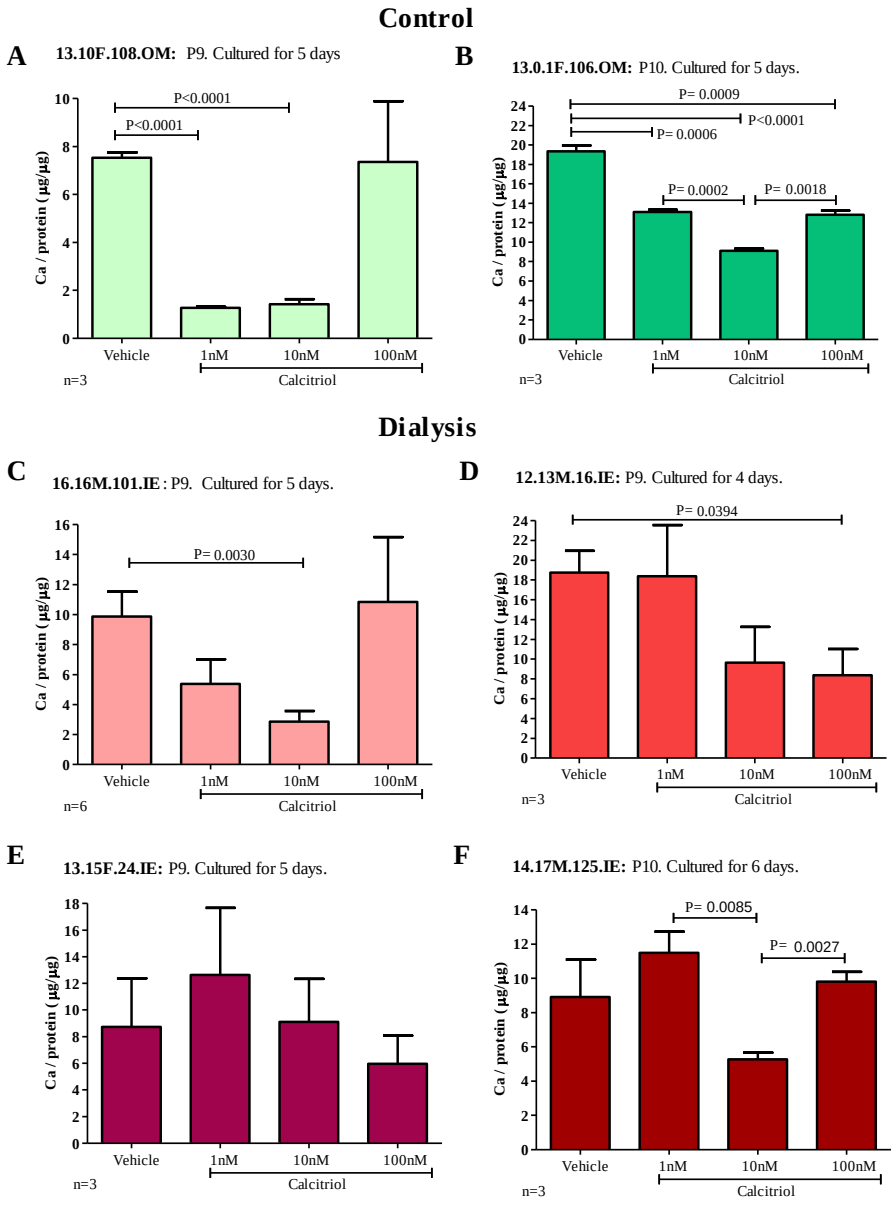


Figure 5-5: Ca load in individual control and dialysis children's VSMCs treated with calcitriol.

VSMCs (P9 or P10 as stated) were treated in high Ca-P medium with the vehicle, 1nM, 10nM or 100nM calcitriol for between 4 and 6 days (as stated), until the onset of calcification. Experiments with insufficient calcification (Ca load < 5µg/µg in all treatment groups) were excluded from analysis. VSMCs used were healthy controls A) 13.10F.108.OM B) 13.0.1F.106.OM or dialysis C) 16.16M.101.IE D) 12.13M.16.IE E) 13.15F.24.IE F) 14.17M.125.IE. Data is shown as mean ± SE and statistical significance was determined by an unpaired two-tailed t-test.

5.4.4 Paricalcitol reduced Ca load in VSMCs from some individuals.

The mean VSMC Ca loads that are presented in Figure 5-3C and Figure 5-4C show that in high Ca-P, 10nM paricalcitol reduced the Ca load. There was no significant difference in the mean Ca load between VSMCs treated with 10nM calcitriol and 10nM paricalcitol.

As discussed above VSMCs from individual patients may vary in their Ca deposition in response to different VDRA treatment conditions. This information can be lost when considering only mean values, which combine the data from different individual patients' VSMCs. Here I compare the effect of 10nM calcitriol and 10nM paricalcitol on Ca load in VSMCs from each of the 6 individual patients.

In 2 individual control patients' VSMCs, 10nM paricalcitol significantly reduced Ca load in high Ca-P medium. In both of these VSMCs the Ca load was significantly higher in cells treated with 10nM paricalcitol compared to 10nM calcitriol (Figure 5-6A and B).

Similarly there was a trend for 10nM paricalcitol to reduce the Ca load in high Ca-P medium in each of the 4 different dialysis VSMCs, which was statistically significant in 13.16M.101.IE and 12.13M.16.IE (Figure 5-6C and D). Ca load was reduced by 10nM paricalcitol from 9.87$\pm$1.65 µg/µg to 3.78$\pm$0.54 µg/µg and from 18.73$\pm$2.21 µg/µg to 3.79$\pm$0.86 µg/µg respectively. There was no significant difference in Ca load after treatment with 10nM calcitriol or 10nM paricalcitol in any of the dialysis VSMCs.

The above data show that both 10nM calcitriol and 10nM paricalcitol have a protective effect on VSMCs from control and dialysis patients. The amplitude of this effect varies between VSMCs from different individuals.

To understand the mechanism by which VDRAs affect Ca load, their effect on other parameters in control and dialysis VSMCs was analysed.

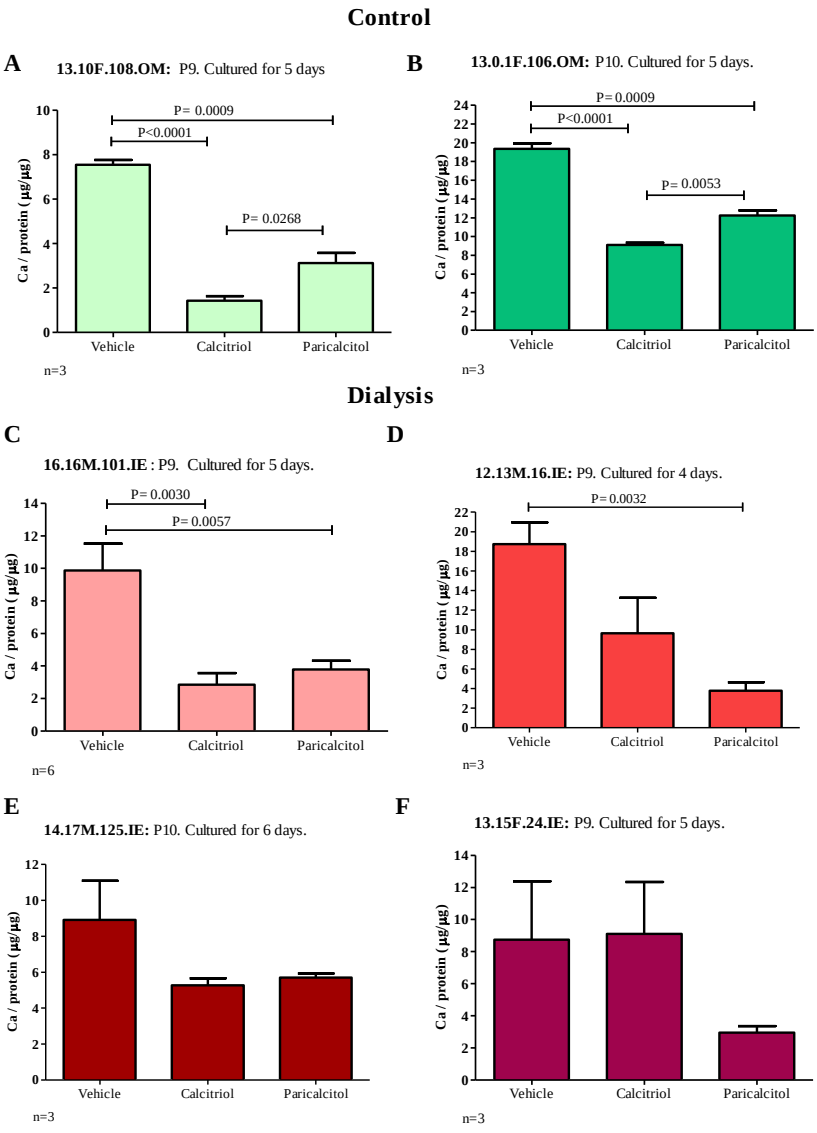


Figure 5-6: Ca load in individual control and dialysis children's VSMCs treated with calcitriol and paricalcitol.

VSMCs (P9 or P10 as stated) were treated in high Ca-P medium with the vehicle, 10nM calcitriol or 10nM paricalcitol for between 4 and 6 days (as stated), until the onset of calcification. Experiments with insufficient calcification (Ca load < 5µg/µg in all treatment groups) were excluded from analysis. VSMCs used were healthy controls A) 13.10F.108.OM B) 13.0.1F.106.OM or dialysis VSMCs C) 13.16M.101.IE D) 12.13M.16.IE E) 13.15F.24.IE F) 14.17M.125.IE. Data shown as mean $\pm$ SE and statistical significance was determined by an unpaired two-tailed t-test.

5.5 By what mechanisms do VDRAs affect vascular calcification in VSMCs from control and dialysis children?

5.5.1 Dialysis VSMCs upregulated CYP24A1 mRNA expression to a greater extent than control VSMCs.

The dose dependent effect of calcitriol on Ca load in VSMCs formed a U-shaped curve with both high and low doses associated with a higher Ca load in some of the VSMCs. This is an atypical dose response pattern; therefore, it is of interest to consider the effect of the calcitriol dose on the transcriptional regulation of a gene that is well established as being regulated by the VDR.

It is widely accepted that CYP24A1 is sensitive to upregulation by VDRAs. CYP24A1 transcribes the 24 hydroxylase enzyme which converts 25(OH)D and $1,25(OH)_2D$ to 24 hydroxylated metabolites for degradation as discussed in the introduction, it is a key enzyme in the vitamin D metabolism pathway. Here I compare the mRNA expression of CYP24A1 in control and dialysis VSMCs treated with VDRAs, as determined by qPCR analysis. All data is shown as copies/1000 18s.

CYP24A1 was expressed at low levels (0.04 ± 0.03) in VSMCs cultured in normal Ca-P medium. Culture of VSMCs in high Ca-P medium with or without the vehicle did not affect expression of CYP24A1, as shown in Figure 5-7A. There was a trend for calcitriol to increase CYP24A1 expression in a dose dependent manner with from 0.02 ± 0.01 to 0.12 ± 0.08 and 3.52 ± 2.57 with 1nM and 10nM calcitriol (NS). In high Ca-P medium 100nM calcitriol increased CYP24A1 expression to 21.72 ± 7.06; this was significantly higher than the vehicle ($p=0.0096$, $n=7$) as well as 1nM and 10nM calcitriol, as shown in Figure 5-7C. 10nM paricalcitol also increased CYP24A1 expression in VSMCs to 3.68 ± 1.45 ($p=0.027$), as shown in Figure 5-7E.

CYP24A1 expression was analysed in both control and dialysis VSMCs. The trend for a dose dependent increase in CYP24A1 expression with 1nM and 10nM calcitriol was evident in both control and dialysis VSMCs. In both groups CYP24A1 expression was

significantly greater in VSMCs treated with 100nM calcitriol than either the vehicle, 1nM or 10nM calcitriol (Figure 5-5 D). Similarly, there was a significant increase in CYP24A1 expression with 10nM paricalcitol in both control and dialysis VSMCs (Figure 5-5F).

There was a trend for the expression of CYP24A1 to be higher in dialysis than control VSMCs in each of the treatment conditions. In VSMCs treated with 100nM calcitriol in high Ca-P medium, the CYP24A1 expression was significantly greater in dialysis (34.5 ± 6.9) than control VSMCs (4.76 ± 1.38) ($p=0.0156$).

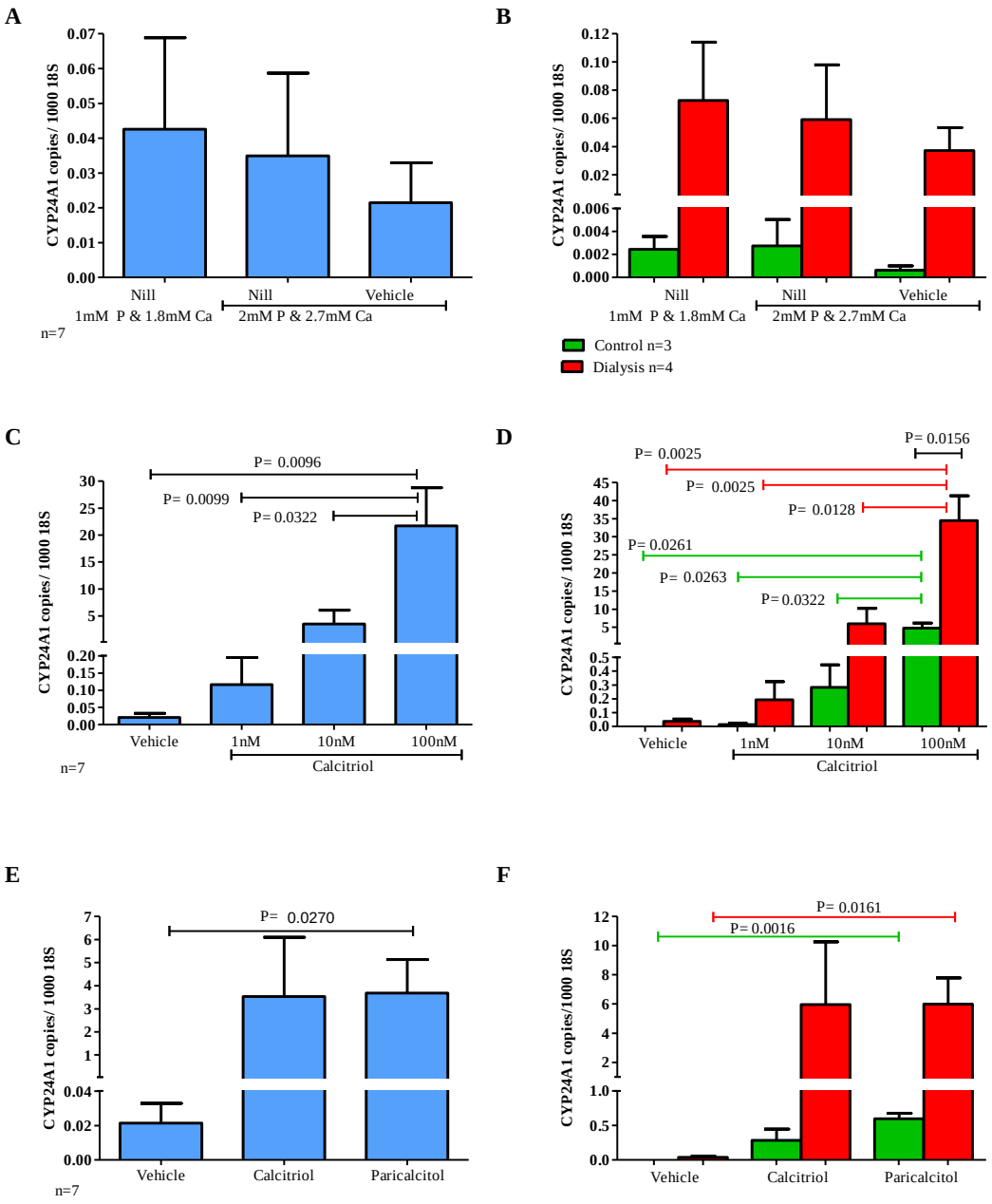


Figure 5-7: CYP24A1 mRNA expression in VSMCs.
VSMCs were cultured in A and D) normal Ca-P medium, high Ca-P or high Ca-P and the vehicle (3.4mM ethanol), B and E) high Ca-P medium with either the vehicle, 1nM, 10nM or 100nM calcitriol or C and F) high Ca-P medium with either the vehicle, 10nM calcitriol or 10nM paricalcitol. RNA was extracted and CYP24A1 expression determined by qPCR analysis and normalised to expression of 18S. A, C, E) CYP24A1 expression in control and dialysis VSMCs combined. B, D, F) Comparison of CYP24A1 expression in control and dialysis VSMCs. Data shown as mean $\pm$ SE and statistical significance was determined by an unpaired two-tailed t-test.

5.5.2 Calcitriol increased ALP expression in VSMCs explanted from control and dialysis children.

ALP is normally expressed in osteoblasts but not expressed in VSMCs, as discussed in the introduction, it is both a marker of osteo / chondrocytic differentiation and an enzyme that breaks down the calcification inhibitor pyrophosphate (Villa-Bellosta and Sorribas, 2011). Data presented in chapter 4 showed that 10nM calcitriol upregulated ALP activity and increased Ca load in vessel rings from CKD children, in addition ALP activity in the presence of calcitriol was significantly greater in vessel rings from dialysis than pre-dialysis patients. VSMCs explanted from dialysis vessel rings represent the extreme CKD phenotype. Here I compare ALP expression and activity in dialysis and control VSMCs. I will also consider the dose dependent effect of calcitriol as well as 10nM paricalcitol on both ALP mRNA expression and ALP activity in VSMCs.

ALP was expressed in VSMCs cultured in normal Ca-P medium; the level of expression was not affected by high Ca-P medium or the vehicle. Neither the lower doses of calcitriol nor 10nM Paricalcitol had an effect on ALP expression in VSMCs, as shown in Figure 5-8B and C. The highest concentration of calcitriol (100nM) increased ALP expression from 0.0008$\pm$0.0003 to 0.0022$\pm$0.0004 copies/1000 18s ($p=0.011$, $n=7$) (Figure 5-8B).

Comparison of ALP expression in control and dialysis VSMCs showed that there was a trend for dialysis VSMCs to express higher levels of ALP in most of the treatment conditions (Figure 5-8D, E, F). In VSMCs treated with 10nM calcitriol, ALP expression was significantly higher ($p=0.019$) in dialysis VSMCs (0.0012$\pm$0.0001 copies/1000 18S) compared to control VSMCs (0.0005$\pm$0.0002 copies/1000 18S).

This trend of higher ALP expression in dialysis VSMCs did not continue in VSMCs treated with 100nM calcitriol. In control VSMCs, 100nM calcitriol significantly increased ALP expression in high Ca-P medium from 0.0005$\pm$0.0002 to 0.0027$\pm$0.0007 copies/1000 18S ($p=0.035$). Whereas in dialysis VSMCs, 100nM calcitriol caused a modest increase in ALP expression (NS) as shown in Figure 5-8E.

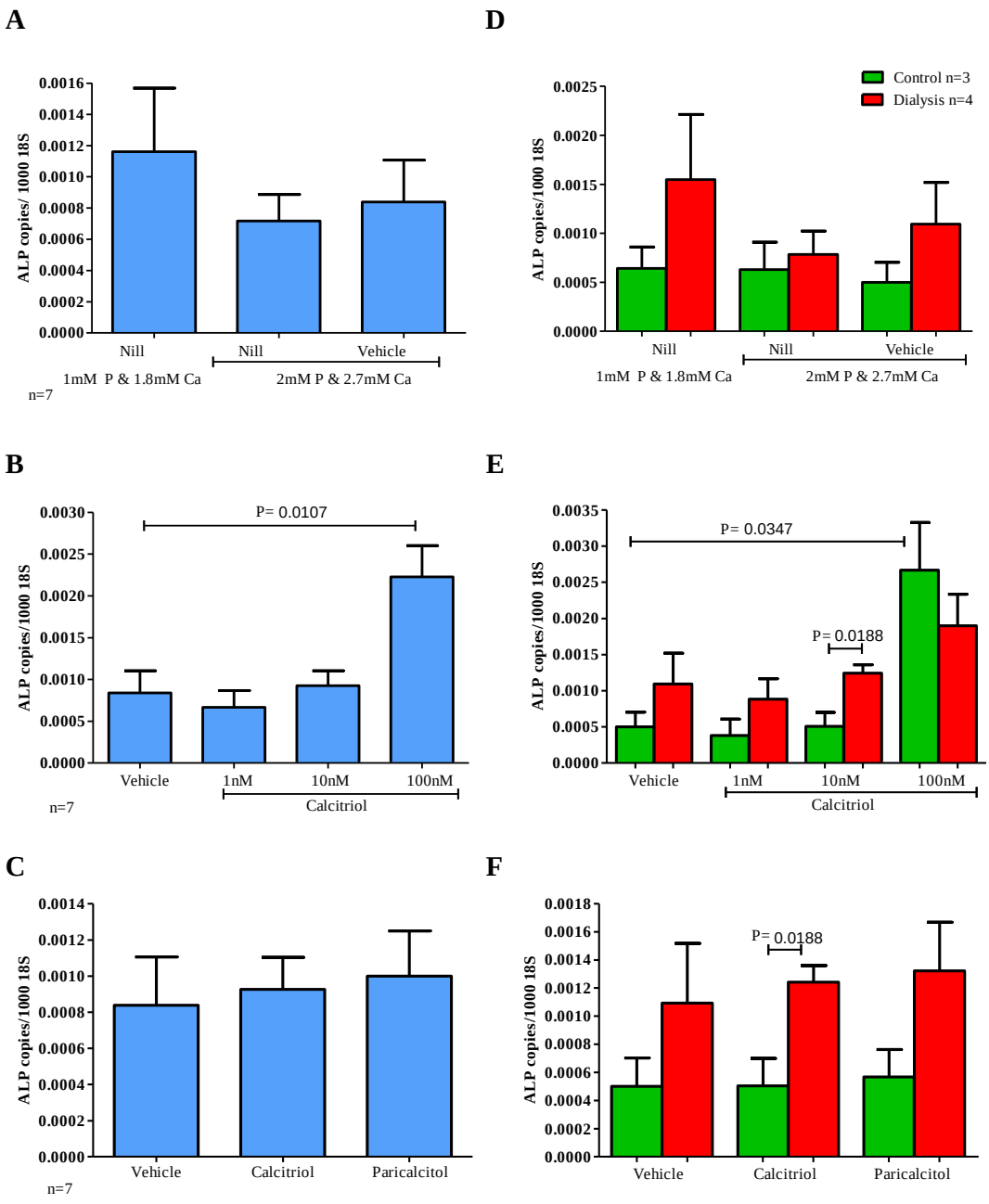


Figure 5-8: ALP mRNA expression in VSMCs.
VSMCs were cultured in A and D) normal Ca-P medium, high Ca-P or high Ca-P and the vehicle (3.4mM ethanol), B and E) high Ca-P medium with either the vehicle, 1nM, 10nM or 100nM calcitriol or C and F) high Ca-P medium with either the vehicle, 10nM calcitriol or 10nM paricalcitol. RNA was extracted and ALP expression determined by qPCR analysis and normalised to expression of 18S. A, C, E) ALP expression in control and dialysis VSMCs combined. B, D, F) Comparison of ALP expression in control and dialysis VSMCs. Data shown as mean $\pm$ SE and statistical significance was determined by an unpaired two-tailed t-test.

5.5.3 Calcitriol increased ALP activity in VSMCs explanted from control and dialysis children.

The previous section showed that only the highest dose of 100nM calcitriol increased ALP mRNA expression in VSMCs, and that this upregulation was significant in control but not dialysis VSMCs. To consider the functional importance of this, next I consider the level of ALP activity in control and dialysis VSMCs.

VSMCs were treated as before and their ALP activity determined by a colorimetric assay. Data is shown as fold change in enzyme units relative to ALP activity in VSMCs from the same patient cultured in normal Ca-P medium.

High Ca-P did not affect ALP activity in VSMCs, in addition, the vehicle, 1nM and 10nM calcitriol as well as 10nM paricalcitol had no effect on ALP activity in VSMCs, whereas 100nM calcitriol significantly increased the fold change in ALP activity from 1.39$\pm$0.23 fold in high Ca-P with the vehicle to 3.22$\pm$0.58 fold in high Ca-P with 100nM calcitriol ($p=0.0077$), as shown in Figure 5-9B.

The trend for 100nM calcitriol to increase ALP activity was evident in both control and dialysis VSMCs (NS) as shown in Figure 5-9 E. There was a trend for 10nM paricalcitol to increase ALP activity in control but not in dialysis VSMCs (NS), consequently, in VSMCs treated with 10nM paricalcitol, the fold change in ALP activity was significantly higher in controls (2.36 0.26 fold) than in dialysis VSMCs (1.42$\pm$0.25 fold) ($p=0.025$).

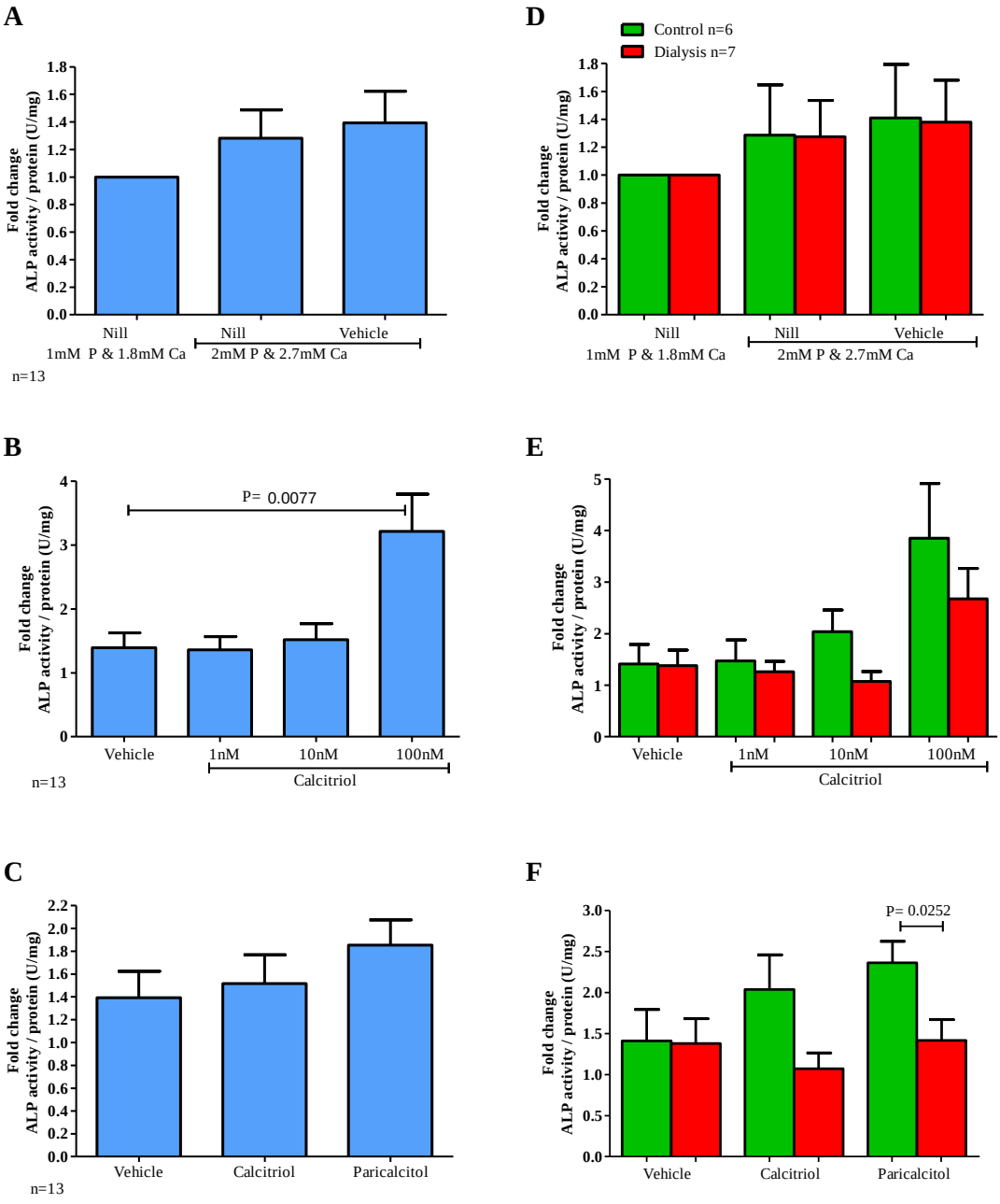


Figure 5-9: Effect of VDRAs on ALP activity in VSMCs.

VSMCs were cultured in A and D) normal Ca-P medium, high Ca-P or high Ca-P and the vehicle (3.4mM ethanol), B and E) high Ca-P medium with either the vehicle, 1nM, 10nM or 100nM calcitriol or C and F) high Ca-P medium with either the vehicle, 10nM calcitriol or 10nM paricalcitol. ALP activity was determined by colourimetric assay and shown as fold change in ALP activity after culture in treatment conditions relative to normal Ca-P medium. A, B, C) All VSMCs. D, E, F) Comparison of control and dialysis VSMCs. Data shown as mean $\pm$ SE and statistical significance was determined by an unpaired two-tailed t-test.

5.5.4 VDRAs had no effect on expression of osteoblast enzyme Runx2 in VSMCs from control and dialysis children.

As shown above, the higher dose of 100nM calcitriol upregulated both ALP expression and activity. ALP is associated with an osteoblast like phenotype. Next I consider whether the osteoblast related transcription factor Runx2 was also upregulated by VDRAs.

QPCR analysis showed that expression of osteoblast transcription factor Runx2 was not affected by the culture of VSMCs in any of the treatment conditions tested (high Ca-P medium only or with the vehicle, 1nM, 10nM or 100nM calcitriol and 10nM paricalcitol). This was true for VSMCs from both control and dialysis patients, as shown in Figure 5-10.

Interestingly there was a trend for Runx2 expression to be higher in dialysis than control VSMCs in each of the treatment conditions. In VSMCs treated with 1nM calcitriol Runx2 expression was significantly higher in dialysis VSMCs (0.41$\pm$0.03 copies/100 000 18S) than control VSMCs (0.13$\pm$0.08 copies/100 000 18S) ($p=0.037$). In VSMCs cultured in high Ca-P medium with 10nM paricalcitol Runx2 expression was also significantly higher in dialysis (0.50$\pm$0.12 copies/100 000 18S) than control (0.13$\pm$0.06 copies/100 000 18S) ($p=0.047$).

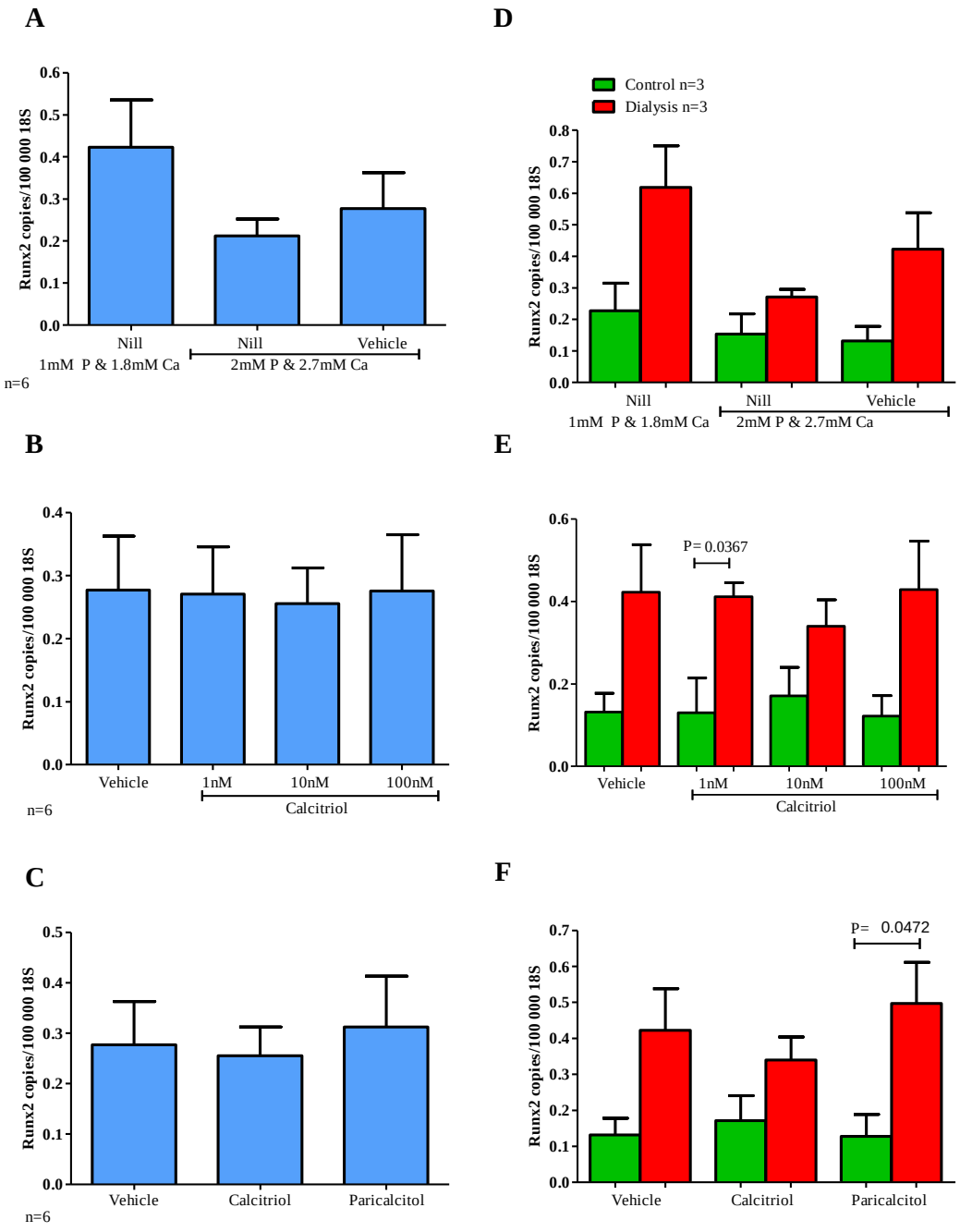


Figure 5-10: Runx2 mRNA expression in VSMCs.
VSMCs were cultured in A and D) normal Ca-P medium, high Ca-P or high Ca-P and the vehicle (3.4mM ethanol), B and E) high Ca-P medium with either the vehicle, 1nM, 10nM or 100nM calcitriol or C and F) high Ca-P medium with either the vehicle, 10nM calcitriol or 10nM paricalcitol. RNA was extracted and Runx2 expression determined by qPCR analysis and normalised to expression of 18S. A, C, E) Runx2 expression in VSMCs. B, D, F) Comparison of Runx2 expression in control and dialysis VSMCs. Data shown as mean ± SE and statistical significance was determined by an unpaired two-tailed t-test.

5.6 VDRAs had no effect on expression of the calcification inhibitor MGP in VSMCs from control and dialysis children.

Earlier in this chapter I showed that both 10nM calcitriol and 10nM paricalcitol had a protective effect on Ca load in VSMCs. Physiological doses of calcitriol have been shown to upregulate the MGP promoter in rat VSMCs (Farzaneh-Far et al., 2001). MGP is an endogenously expressed inhibitor of calcification (as discussed in the introduction). Here I consider the effect of calcitriol and paricalcitol on MGP expression in VSMCs to determine if these VDRAs could have a protective effect on Ca load by regulating MGP expression.

QPCR analysis showed that the culture of VSMCs in any of the treatment conditions tested (high Ca-P medium only, high Ca-P with 1nM, 10nM or 100nM calcitriol and 10nM paricalcitol) had no effect on their MGP mRNA expression VSMCs.

In control VSMCs, MGP expression was reduced from 1.13$\pm$0.08 copies/1000 18S in normal Ca-P medium to 0.47$\pm$0.13 copies/1000 18S in high Ca-P medium ($p=0.012$). High Ca-P medium did not have a significant effect on MGP expression in dialysis VSMCs. There was a consistent trend in each of the treatment conditions for MGP expression to be higher in dialysis than control VSMCs (NS). All of the VDRA treatment conditions tested did not affect MGP expression in either the control or dialysis VSMCs.

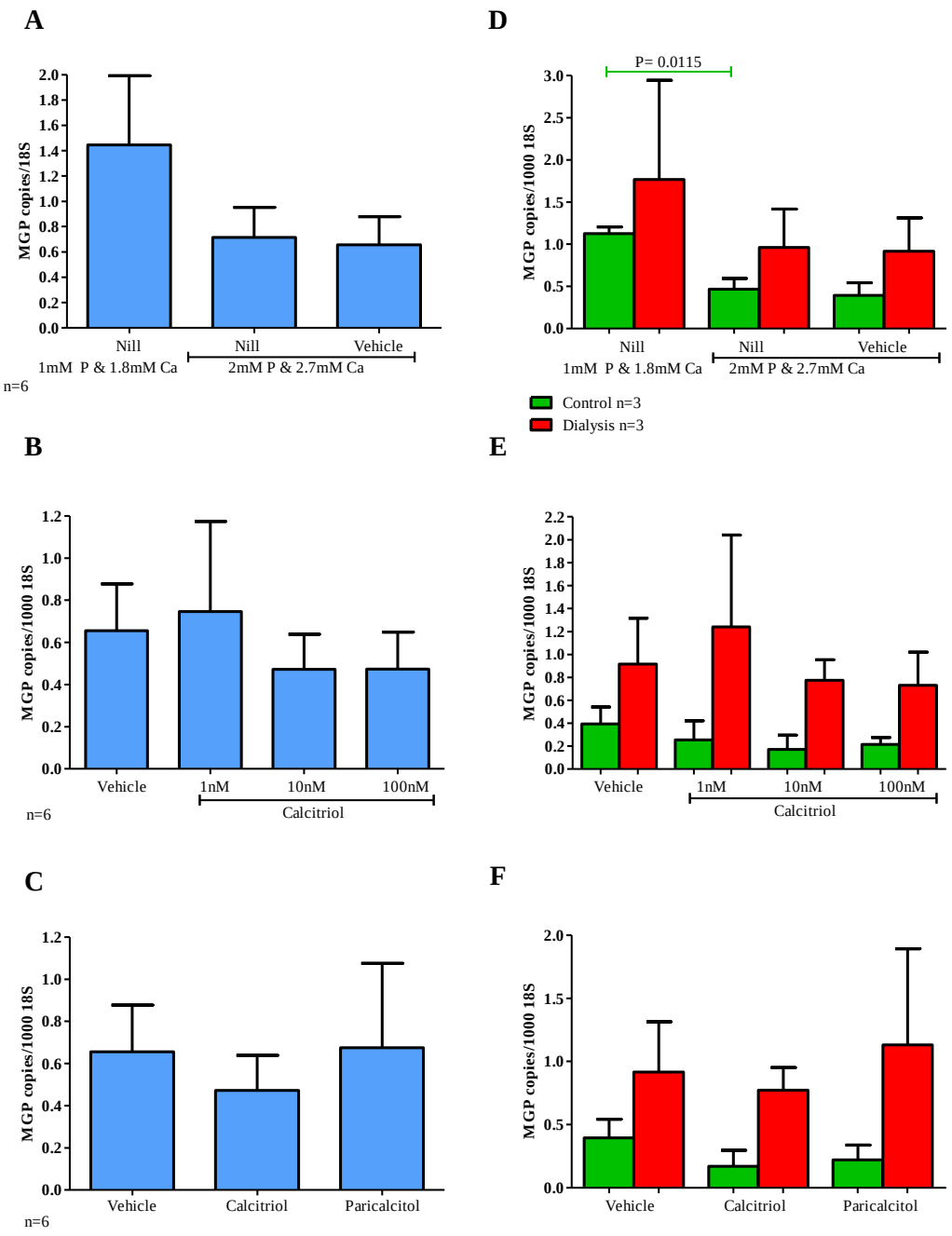


Figure 5-11: MGP mRNA expression in VSMCs.
VSMCs were cultured in A and D) normal Ca-P medium, high Ca-P or high Ca-P and the vehicle (3.4mM ethanol), B and E) high Ca-P medium with either the vehicle, 1nM, 10nM or 100nM calcitriol or C and F) high Ca-P medium with either the vehicle, 10nM calcitriol or 10nM paricalcitol. RNA was extracted and MGP expression determined by qPCR analysis and normalised to expression of 18S. A, C, E) MGP expression in VSMCs. B, D, F) Comparison of MGP expression in control and dialysis VSMCs. Data shown as mean ± SE and statistical significance was determined by an unpaired two-tailed t-test.

5.7 Discussion

5.7.1 Summary

Data in this chapter showed that the effect of calcitriol on calcification in VMSCs was dose dependent and the dose-response curve formed a U-shape. Both calcitriol and paricalcitol had a protective effect on calcification in explanted VSMCs, this effect was observed equally in VSMCs explanted from control and dialysis patients.

The key findings were:

1. At baseline, control VSMCs expressed more SMA than dialysis VSMCs. In addition, some of the dialysis VSMCs had higher expression levels of the osteogenic markers Runx2 and BSP.

2. The physiological doses of both 10nM calcitriol and 10nM paricalcitol were protective of calcification; they reduced calcification by the same extent in both control and dialysis VSMCs. Each of the extreme doses of 1nM and 100nM calcitriol were protective of calcification in only 2 of the individual VSMC explants.

3. MRNA expression of CYP24A1 increased in a concentration dependent manner in response to 1nM, 10nM and 100nM calcitriol in both control and dialysis VSMCs. CYP24A1 expression in VSMCs treated with 10nM paricalcitol was similar to that with 10nM calcitriol.

4. The highest dose of 100nM calcitriol increased ALP mRNA expression as well as fold change in ALP activity in VSMCs. The increase in ALP mRNA expression was significant in control but not dialysis VSMCs.

5. Neither mRNA expression of MGP nor Runx2 were affected by any of the VDRA treatments in either control or dialysis VSMCs. There was a trend for dialysis VSMCs to express both more MGP and Runx2 mRNA than control VSMCs.

5.7.2 Baseline characteristics of control and dialysis VSMCs

The VSMC explants utilised in this chapter all expressed SMA, SMMHC and SM22α as shown in Figure 5-2; expression of these multiple markers identifies these cells of VSMC origin. SMMHC expression is particularly important as it is considered to be explicitly expressed in the VSMC lineage (Madsen et al., 1998). Control and dialysis VSMCs in this study expressed similar levels of SMMHC confirming their VSMC lineage.

The phenotype of VSMCs change throughout development. They do not terminally differentiate and are able to respond to environmental cues leading to a spectrum of VSMC phenotype. The extremes of this spectrum are considered to be 'contractile' and 'synthetic', with the respective primary functions of contraction and synthesis of extracellular matrix proteins which are required in growth and repair (Metz et al., 2012). Many signalling pathways are involved in the complex regulation of VSMC differentiation including growth factors/inhibitors, mechanical influences, cell-cell and cell-matrix interactions and inflammatory mediators (Owens et al., 2004). In culture VSMCs are known to switch from a contractile to a synthetic phenotype with increasing passage number before eventually reaching senescence (Thyberg, 1996). In this study, VSMCs of similar passage numbers, between passage 6 and 10, were compared in order to minimise the variation that changes in VSMC phenotype through extensive passaging may introduce.

Many vascular calcification studies have been performed on human VSMCs, as discussed in the introduction, here I am able to compare the phenotype of human VSMCs from aged matched healthy control and CKD patients on dialysis. Dialysis VSMCs expressed less SMA than control VSMCs. Expression of SMA is specifically associated with a

contractile phenotype (Weissberg et al., 1995) therefore indicating VSMCs from dialysis patients had a reduction in contractile function. Despite this dialysis VSMCs were not predisposed to calcification relative to control VSMCs.

As discussed in the introduction, vascular calcification is a highly regulated process that resembles bone formation, including osteogenic differentiation induced by Runx2 and characterised by the expression of bone related molecules (Kurabayashi, 2015). Control VSMCs and the majority of dialysis VSMCs showed low level expression of Runx2 and BSP, however dialysis VSMCs 05.1.5M.04.OM, 06.8F.36.IE and 15.12F.126.IE had noticeably higher expression of Runx2 and/or BSP as shown in Figure 5-2, which is suggestive of osteogenic differentiation in these dialysis VSMCs at baseline.

For this and other qPCR analysis all expression levels were normalised to the housekeeping gene 18s. 18s is a ribosomal RNA that is stable and highly conserved between species, with only a 0.1% divergence between mice and humans (Gonzalez and Schmickel, 1986). It is constitutively expressed in all cells therefore widely accepted as an appropriate housekeeping gene to represent the relative total mRNA and normalise expression levels to (Goidin et al., 2001, Bas et al., 2004).

5.7.3 The U-shaped curve of calcitriol concentration versus VSMC Ca load.

The effect of different concentrations of calcitriol on Ca load in VSMCs was determined. The concentration range of calcitriol included the sub-physiological dose of 1nM, physiological dose of 10nM and supra-physiological dose of 100nM. The mean Ca load of VSMCs treated with this concentration range of calcitriol formed a U-shaped curve; the highest and lowest dose had no effect on Ca load whereas the middle dose of 10nM calcitriol reduced Ca load.

A U-shaped association between calcitriol and calcification has been observed previously in multiple scenarios, clinically in dialysis children (Shroff et al., 2008a) as well as *in-*

vitro in human VSMCs where 1nM calcitriol had the greatest protective effect by upregulation of CaSR expression (Mary et al., 2015).

Potential explanations of the U-shaped concentration to Ca load response include:

- Theory 1: Calcitriol has a protective effect on Ca load, however at high doses it is rapidly converted to inactive metabolites.

- Theory 2: Calcitriol activates counteracting protective and causative effects on calcification in VSMCs, the contribution of each is concentration dependent.

- Theory 3: Calcitriol has a protective effect on calcification in VSMCs at a narrow concentration window only.

5.7.3.1 Theory 1: Calcitriol has a protective effect on Ca load, however at high doses it is rapidly converted to inactive metabolites.

CYP24A1 encodes the 24 hydroxylase enzyme which hydroxylates calcitriol to form metabolites for degradation. Transcription of CYP24A1 is upregulated by calcitriol to form a negative feedback mechanism. The transcriptional regulation of the CYP24A1 gene is highly responsive to VDRAs. 2 VDREs were identified in its promoter region by cloning of the 24 hydroxylase promoter; either of the VDREs were able to direct a reporter gene independently however both VDREs were required to induce optimal 24 hydroxylase expression (Chen and DeLuca, 1995). The region up stream of the VDREs, between nucleotides -548 and -294 was found to have a synergistic function in expression of the CYP24A1 gene in human fibroblasts (Tashiro et al., 2007). Furthermore, a cluster of enhancers located downstream of the CYP24A1 gene were identified by Chip-sequencing and shown to contribute to the activation of the gene in response to VDRAs (Meyer et al., 2010). CYP24A1 is highly inducible with 100 to 1000 fold increases in expression, therefore is often used as a positive control to demonstrate VDRA activity. In this study a

typical dose response is observed with incremental doses of calcitriol (1nM, 10nM and 100nM) eliciting greater transcriptional upregulation of CYP24A1. Consequently, the 24 hydroxylase expression was upregulated and the production of metabolites for the degradation pathway such as $1\alpha,24,25(OH)_3D_3$, and eventually calcitroic acid, were increased. The 24 hydroxylated metabolites may themselves affect calcification. In osteoblast cells, $1\alpha,24,25(OH)_3D_3$ as well as $24R,25(OH)_2D_3$ increased ALP activity, increased osteocalcin production and enhanced mineralisation (van Driel et al., 2006). $24R,25(OH)_2D_3$ was also shown to increase ALP activity and mineralisation in human mesenchymal stem cells (Curtis et al., 2014).

In future work it would be interesting to compare the significance of this greatly increased CYP24A1 expression on the levels of active vitamin D remaining in the system as well as the concentration and effect of 24 hydroxylated metabolites. Throughout these experiments media was replaced every 3 days, therefore prior to replenishment the VSMCs may have experienced peak levels of 24 hydroxylated metabolites and troths in levels of original VDRA concentration.

5.7.3.2 Theory 2: Calcitriol activates counteracting protective and causative effects on calcification in VSMCs; the extent of each is concentration dependent.

The lowest dose of 1nM calcitriol had no effect on Ca load in explanted human VSMCs, however the physiological dose of 10nM calcitriol had a protective effect; it reduced Ca load. Vitamin D has previously been shown to upregulate transcription of the calcification inhibitor MGP (Farzaneh-Far et al., 2001). QPCR analysis of MGP expression showed that, in this study, MGP mRNA expression was not affected by 1nM, 10nM or 100nM calcitriol. In other studies VDRAs have been shown to down-regulate MGP leading to an increased Ca load, for example in both sham operated and 5/6 nephrectomy Wistar rats, calcitriol increased Runx2 protein and reduced MGP protein expression, corresponding to an increase in calcification (Koleganova et al., 2009). In our model of children's VSMCs

an alternative, as yet undefined, protective mechanism must be contributing to the reduced Ca load observed with 10nM calcitriol treatment.

The supra-physiological dose of 100nM calcitriol had no effect on Ca load, however it did upregulate ALP mRNA expression and activity. As discussed previously ALP is an osteoblast marker. It also dephosphorylates and inactivates the calcification inhibitor pyrophosphate therefore ALP upregulation is associated with calcification. 10nM calcitriol did not affect ALP expression or activity but it reduced Ca load in VSMCs, this differed from 100nM calcitriol which upregulated ALP expression and activity, however it had no overall effect on Ca load. In VSMCs treated with 100nM calcitriol the causative effect of ALP on increasing calcification may counteract the calcification protective mechanisms observed with 10nM calcitriol.

As the VDR is a transcriptional regulator it was of interest to compare the mRNA expression of ALP in control and dialysis VSMCs treated with VDRAs, however ALP is not known to be a target gene of VDRAs. Post-translational modifications or activation of ALP may affect its functional activity, therefore ALP mRNA expression and ALP activity may not be directly linked and both were studied.

In this model of human VSMCs from both control and dialysis children, the causative and protective mechanisms of calcitriol are balanced at both 1nM and 100nM; at these concentrations there was no overall effect on Ca load. It would be of interest in future studies to identify the protective mechanism of calcitriol that reduced Ca load at the 10nM calcitriol dose and counteracted the pro-calcification effect of upregulated ALP at the 100nM calcitriol dose that results in an overall null effect on Ca load.

5.7.3.3 Theory 3: Calcitriol activates a protective effect on calcification in VSMCs at a narrow concentration window only.

In the explanted VSMCs in this study, calcitriol was shown to have a protective effect on Ca load when used at 10nM concentration only, the lower or higher dose of 1nM or 100nM respectively had no effect on Ca load. This suggests that there is a narrow concentration window at which calcitriol is effective at reducing Ca load. This narrow effective concentration window was also observed in a study by the Mentaverri group. In their study, 1nM calcitriol had a greater protective effect on calcification than either 0.1nM or 10nM calcitriol (Mary et al., 2015). This supports the theory that calcitriol is protective of calcification at a narrow concentration window only, creating a U-shaped dose response curve, however there is a shift in the most effective concentration in different experimental set ups.

The Mentaverri group showed that 1nM calcitriol effectively increased the expression of CaSR hence increased Ca sensitivity of VSMCs and protected against calcification in human VSMCs (Mary et al., 2015). This study used an excessively high dose of 5mM Ca to induce calcification; 5mM Ca but not 2mM Ca reduced CaSR expression in arterial SMCs from adults by approximately 4 fold (Molostvov et al., 2015). This study induced calcification with a more modest 2.7mM Ca and 2mM P therefore any reduction in CaSR expression is likely to be much smaller. CaSR expression is reduced in CKD, which could contribute to their higher risk of vascular calcification observed *in-vivo* (Massy et al., 2014). In future it would be of interest to compare the concentration of CaSR in these control and dialysis VSMCs and to consider the effect of calcitriol concentration on CaSR in my experimental setup.

5.7.4 Effect of paricalcitol versus calcitriol on calcification

The mean effect of VDRAs on Ca load was for the physiological doses of both 10nM paricalcitol and 10nM calcitriol to protect against Ca load by a similar extent. Individual

patients' VSMCs responded to different extents to each of these VDRAs. In both of the control VSMCs the Ca load was significantly less in response to 10nM calcitriol than 10nM paricalcitol. Whereas in one of the dialysis VSMCs 10nM paricalcitol, but not 10nM calcitriol, significantly reduced Ca load, there was no significant difference between Ca load after treatment with 10nM calcitriol or 10nM paricalcitol in the remaining 3 dialysis VSMCs. This demonstrates variation in the amplitude of response to VDRA treatment between VSMCs from different individuals.

5.7.5 Differences between control and dialysis VSMCs

Similar mean Ca loads were observed between control and dialysis VSMCs in each of the VDRA treatment conditions tested as summarised in Table 5-2. Despite this, differences between control and dialysis VSMCs in response to VDRAs were observed in regards to expression of CYP24A1 as well as expression and activity of ALP.

There was a consistent trend for CYP24A1 expression to be higher in dialysis VSMCs than controls both prior to VDRA treatment and in response to each of the VDRA treatment conditions. With 100nM calcitriol, where the greatest increase in CYP24A1 expression was observed, it was significantly greater in dialysis than control VSMCs.

The differential expression levels both prior to and in response to VDRA treatment in VSMCs explanted from control and dialysis VSMCs may be attributed to the difference in prescribed vitamin D in these patients. CKD patients, particularly children, are routinely prescribed VDRAs; in this cohort each of the patients that dialysis VSMCs were explanted from were on alfacalcidol supplements. VSMCs explanted from these patients have already been exposed to high levels of vitamin D. This may have increased their responsiveness to calcitriol as demonstrated by the significantly greater increase in CYP24A1 expression when treated with 100nM calcitriol.

In contrast to CYP24A1 upregulation, VDRAs upregulated ALP expression to a greater extent in control than dialysis VSMCs. ALP is not known to be transcriptionally regulated by the VDR and the mechanism for its upregulation in this system is currently not known. A circulating factor in uremic serum was thought to upregulate ALP activity as aortas from uremic rats, as well as control aortas exposed to serum from uremic rats, had upregulated ALP activity (Lomashvili et al., 2008). In my study, neither control nor dialysis VSMCs were exposed to uremic serum however there was a trend for higher ALP mRNA expression in dialysis VSMCs than control VSMCs in most conditions.

Calcitriols upregulation of CYP24A1 expression was greater in dialysis VSMCs whereas its upregulation of ALP mRNA expression and activity was greater in control VSMCs. The mechanism by which VDRAs regulate ALP is not known whereas CYP24A1 is known to be regulated by the ligand bound VDR binding to VDRE in its promoter region. Difference in VDR expression between control and dialysis VSMCs will be explored in Chapter 4.

Table 5-2: Summary of effects of VDRAs and the type of VSMC most affected.

Effect of VDRAs:	Cell type most affected:
↓ Calcification	Control = Dialysis
↑ CYP24A1 mRNA	Dialysis > Control
↑ ALP mRNA	Control > Dialysis
↑ ALP activity	Control > Dialysis

5.7.6 Patient variation

There was variation in this chapter between individual patients' Ca load in their response to VDRAs with the U-shaped response shifting to either the left or the right, this suggests

that different concentrations of calcitriol are required to evoke the same response in VSMCs from different patients. Analysis of Ca load in VSMCs from individual patients showed that, in both of the control but none of the 4 dialysis VSMCs, 1nM calcitriol as well as 10nM calcitriol significantly reduced Ca load. This suggests that control VSMCs may be more sensitive to calcitriol treatment than dialysis VSMCs. Children on dialysis are routinely prescribed VDRAs, therefore the dialysis VSMCs have had prior exposure *in-vivo* to VDRAs. The effect of this is unknown but dialysis VSMCs may be desensitised to calcitriol treatment, therefore require a higher dose to evoke a similar protective effect on Ca load.

Further individual patient variation was observed with 100nM calcitriol significantly reducing Ca load in one of the control and one of the dialysis VSMCs but having no effect in the remaining four VSMCs. Individual patient variation in the calcitriol concentration that elicits a protective effect on Ca load suggests that there may be differences in the VDR expression between VSMCs from individual patients' VSMCs. This will be explored in Chapter 6.

5.7.7 Limitations.

In this chapter control and dialysis VSMCs were compared, ideally these VSMCs would be explanted from vessels of the same vascular bed and from patients of the same age and gender. As discussed in section 3.8.6, the control vessels were obtained from patients undergoing routine abdominal surgery and a different vascular bed was accessed, this was usually the mesenteric or omental arteries rather than the inferior epigastric artery obtained from CKD patients.

Chapter 6 Expression of the VDR in vessel rings and VSMCs

6.1 Introduction

Previous chapters have shown large variations in the response of both vessel rings and isolated VSMCs to VDRAs. Chapter 3 showed that there was no significant difference between pre-dialysis and dialysis vessel rings in their extent of calcification in response to VDRAs. However, there was variability within each group, particularly in response to 10nM calcitriol with some vessel rings having increased calcification and others showing no response. Contrary, in the VSMCs cultured in high Ca-P 10nM calcitriol had a protective effect and had a lower Ca load compared to VSMCs without calcitriol. Interestingly the extent to which the lower and higher doses of calcitriol protected against calcification was hugely varied between VSMCs from different individuals. For example, 1nM calcitriol reduced the Ca load in control but not in dialysis VSMCs. Control and dialysis VSMCs also differed in the extent to which VDRAs upregulated expression of CYP24A1 and ALP.

It is well documented that VDRAs regulate the transcription of multiple genes via the VDR, ligand bound VDR forms a heterodimer with the RXR and interacts with the VDRE in the promoter region of target genes (as described in the introduction). Here I will focus on the VDRA calcitriol. This chapter investigates the expression of the VDR in vessel rings and VSMCs from different individuals to address whether the patient variation observed in response to calcitriol treatment could be attributed to a difference in the level of VDR expression.

6.1.1 Aim

1. To determine whether VDR expression in vessels varies between individuals giving rise to the different responses to calcitriol treatment in both arterial vessel rings and explanted VSMCs.

This will be addressed with the following key questions;

1. Is there a difference in VDR expression at baseline and after calcitriol treatment in vessel rings that calcified more in response to calcitriol compared to those that did not respond to calcitriol?

2. Is there a difference in VDR mRNA and protein expression between control and dialysis VSMCs, either at baseline and in response to calcitriol?

3. Is there a difference in VDR isoform expression between control and dialysis VSMCs either at baseline or in response to calcitriol?

4. Is there a difference between VDRE activation between control and dialysis VSMCs? Are any differences in VDRE activation determined by the VDR localisation in the cell or the ratio of VDR isoforms expressed at baseline or in response to calcitriol?

6.2 Method

Inferior epigastric arteries were harvested from children with CKD at the time of renal transplant, and cultured as whole vessel rings as described in Chapter 3. Vessel rings were fixed, embedded and sectioned for immunostaining for the VDR. Pictures were taken at 40x magnification. The number of VDR positive nuclei were counted in 3 frames per vessel and the mean count compared between vessel rings whose Ca load either increased in response to calcitriol (Responders: 07.16F.46.IE, 07.6M.44.IE, 13.5M.110.IE) or did not respond to calcitriol (Non-responders: 14.8M.114.IE, 14.11M.118.IE, 14.13M.119.IE). All patient information for each vessel ID is shown in the Methods.

In VSMCs the expression of the VDR was analysed at baseline and after culture in sub physiological (1nM), physiological (10nM) or supra physiological (100nM) doses of calcitriol for 5 days, both in control and dialysis VSMCs. This included qPCR analysis of total VDR mRNA expression and western blot analysis of VDR-A and VDR-B protein expression, which was quantified by optical density analysis. The NCBI reference sequence database has identified 3 transcript variants of the human VDR gene (Pruitt et

al., 2014). The exons in each transcript are shown in Figure 6-1, together with the binding sites for the forward and reverse primers as well as the location of all ATG start codons to initiate translation. The primers detected all 3 transcripts therefore qPCR analysis represented total VDR expression.

Control and dialysis VSMCs were transfected with a reporter construct containing the firefly luciferase gene under a VDRE promoter. Transfected VSMCs were cultured with 100nM calcitriol for 24 hours and their transcriptional activation capacities compared. Associations between VDRE mediated luciferase activity and both VDR subcellular location and VDR isoform expression were analysed.

The VSMCs used in each experiment are shown in Table 6-1 and their patient information is shown in Chapter 2.

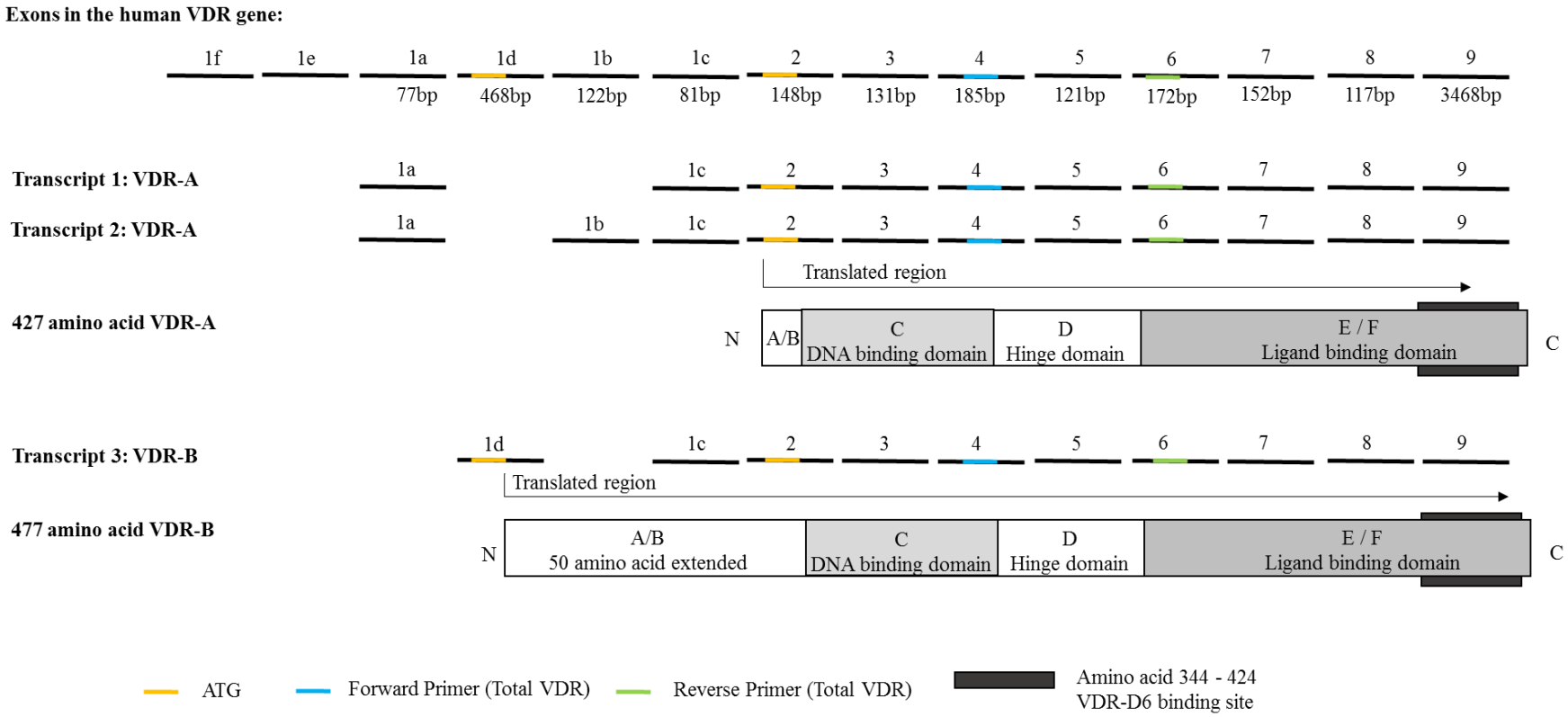


Figure 6-1: VDR transcripts and isoforms.

There are two VDR mRNA transcripts (transcript 1 and 2) that encode the same VDR-A isoform, both of these transcripts are translated from the ATG start codon in exon 2 to give the 427 amino acid VDR-A isoform. Transcript 3 encodes the VDR-B isoform which is translated from the ATG start codon in exon 1d to give the 477 amino acid VDR-B isoform, which has a 50 amino acid N terminal extension.

Table 6-1: Summary of VSMCs used in each experiment.

VSMC ID: Year. Age and gender. Unique number. Vessel type.	**Baseline VDR mRNA**	**Baseline VDR protein**	**VDR mRNA (treated)**	**VDR protein (treated)**	**Luciferase assays**	**Luciferase activated isoform ratio**
Controls VSMC ID						
13.10F.108.OM	✓	✓	✓	✓#	✓	✓
13.0.1F.106.OM	✓	✓	✓	✓		
13.1F.28.OM*	✓	✓				
06.8M.20.IE**	✓					
06.1M.39.RA**	✓					
13.3M.31.OM*		✓			✓	✓
13.5F.32.OM*		✓			✓	✓
13.5M.33.OM*		✓			✓	
14.2M.123.ME		✓				
15.2F.127.ME		✓	✓#	✓#	✓	
35F (adult aorta)					✓	✓
Dialysis VSMCs						
13.16M.101.IE	✓	✓	✓	✓	✓	✓
12.13M.16.IE*	✓	✓	✓		✓	✓
14.13M.119.IE	✓	✓			✓	✓
13.15F.112.IE	✓	✓		✓#	✓	✓
13.15F.24.IE*	✓	✓	✓			
06.8F.36.IE**	✓					
06.9M.34.IE**	✓					
05.1.5M.04.OM**	✓					
14.14M.124.IE		✓		✓#	✓	✓
14.8F.122.IE		✓				
14.17M.125.IE			✓#	✓#	✓	

*VSMCs explanted by Alex Todd. ** VSMCs explanted by Rukshana Shroff. All other VSMCs (except 35F) were explanted by myself, and experiments on all VSMCs were performed by myself, Joanne Laycock unless indicated by #.
Experimental repeat on this VSMC isolate performed under my supervision by Annelies DeMare, a master's student that I, Joanne Laycock trained, planned experiments for and assisted in the lab. All data interpretation and analysis was performed by myself.

6.3 There was no difference in VDR expression between vessel rings that responded and did not respond to calcitriol with an increased Ca load.

In Chapter 3 I showed that vessel rings could be sorted into 2 distinct groups, responders, whose Ca load increased in response to calcitriol and non-responders whose Ca load did not change. This difference was independent of dialysis status, renal diagnosis, gender, and age as well as vitamin D supplements at the time of transplant.

The response to calcitriol might be affected by differences between the patients may at a molecular level. Therefore, immunostaining was used to determine the VDR density in the medial layer of patients whose Ca load responded or did not respond to calcitriol. As shown in Figure 6-2, VDR positive nuclei were identified in both non-responder and responder vessel rings at baseline and after culture in high Ca-P medium with calcitriol.

For each condition the number of VDR positive nuclei per $10\mu m^2$ was counted, a mean VDR count from 3 frames per vessel was taken for 3 non-responder and 3 responder vessel rings. As shown in Figure 6-3, there was no difference in the number of VDR positive nuclei either at baseline or after culture in high Ca-P medium with calcitriol between non-responder and responder vessel rings.

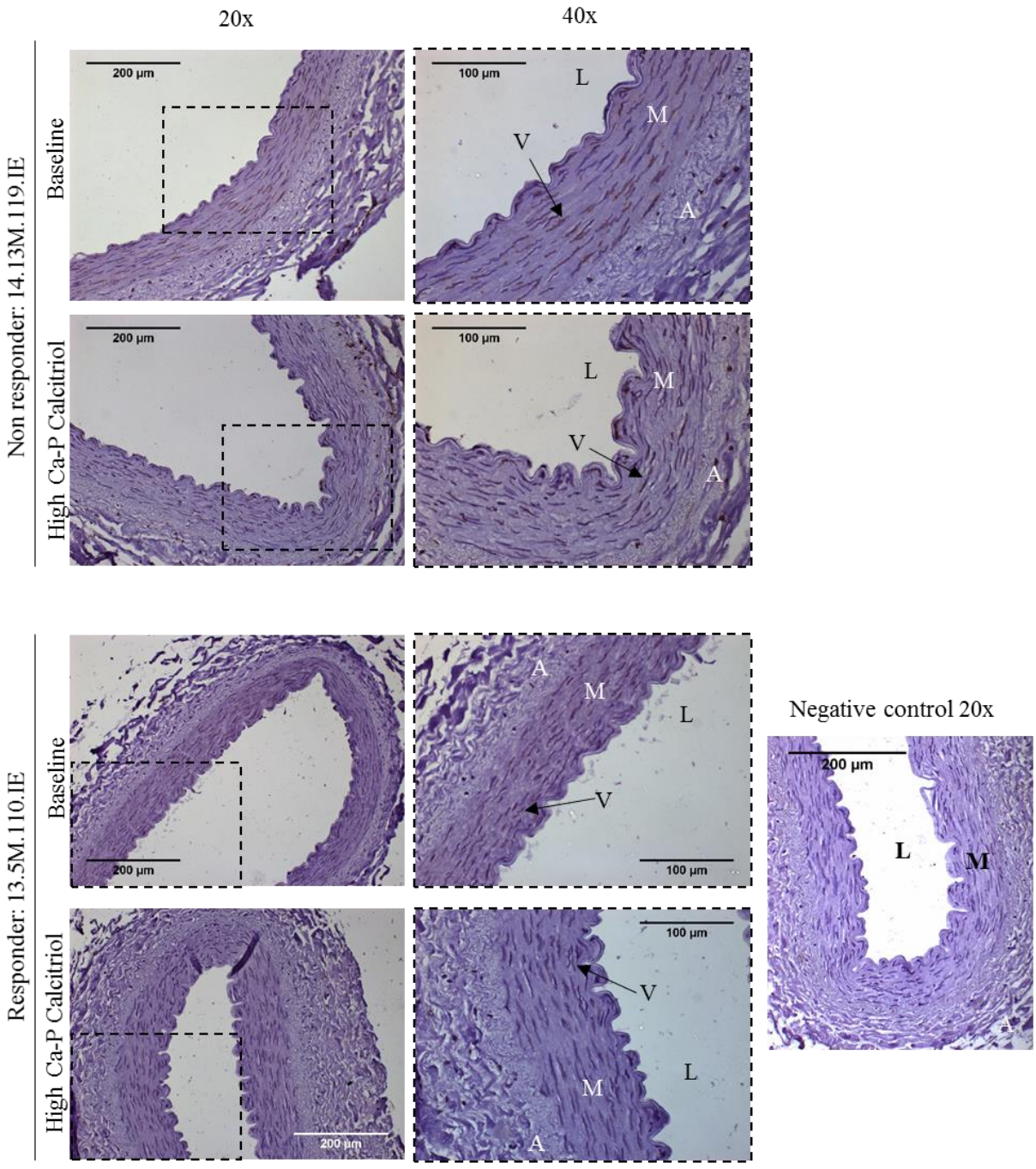


Figure 6-2: VDR expression in responder and non-responder vessel rings.

Vessel rings that were harvested from children with CKD were isolated at baseline or cultured for 14 days in high Ca-P medium (2mM P and 2.7mM Ca) with 10nM calcitriol. Vessel ring sections were stained for VDR (Brown) and counter stained with haematoxylin. Negative control with no primary antibody. Images taken at 20x or 40x magnification. **A-** Adventitia. **M** – Tunica Media. **L** – Lumen. **V** – VDR positive nuclei.

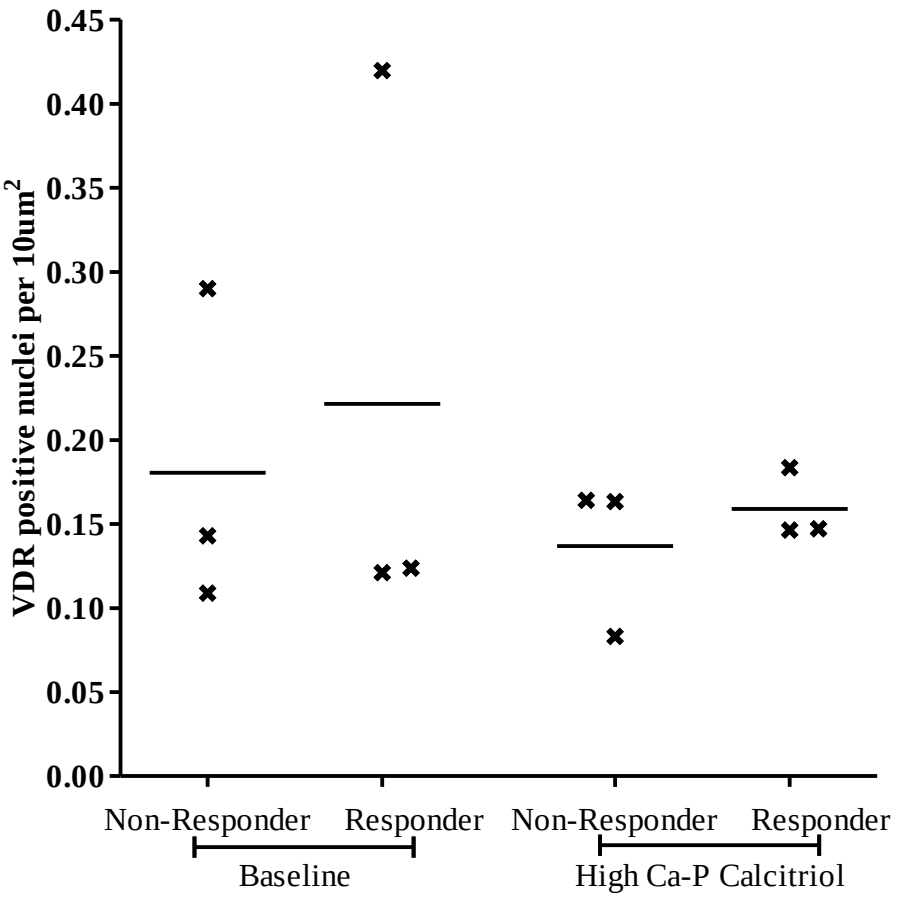


Figure 6-3: Quantification of VDR positive nuclei in responder and non-responder vessel rings.

Vessel rings harvested from children with CKD were stained for VDR. For each vessel ring the number of VDR positive nuclei was counted in 3 frames and a mean is shown. Nuclei were counted in 3 different vessel rings per condition to compare VDR expression in non-responders and responders at baseline and after culture in high Ca-P medium (2mM P and 2.7mM Ca) with 10nM calcitriol for 14 days. Mean is shown and statistical significance was determined by unpaired two-tailed t-test.

6.4 VDR expression in control and dialysis VSMCs

6.4.1 Control and dialysis VSMCs expressed different VDR isoforms at baseline.

The previous section showed that there was no difference in VDR protein expression between vessel rings whose Ca load responded and did not respond to calcitriol. This section will compare the VDR expression in explanted VSMCs from control and dialysis VSMCs.

It was shown in Chapter 5 that there were significant differences between control and dialysis VSMCs in the extent of their responses to VDRAs as summarised in Table 6-2. For example, ALP mRNA expression was upregulated by 100nM calcitriol in all VSMCs but it was significantly increased in control and not dialysis VSMCs. In addition, the fold change in ALP activity was greater in control than dialysis VSMCs treated with VDRAs. CYP24A1 expression was also increased in both control and dialysis VSMCs, however a greater upregulation was seen in dialysis than control VSMCs.

Table 6-2: Summary of effects of VDRAs on VSMCs.

Effect of VDRAs:	Cell type most affected:	Effect statistically significant with:
↓ Calcification	Control = dialysis	10nM calcitriol
↑ ALP mRNA	Control	100nM calcitriol
↑ ALP activity	Control	10nM calcitriol 10nM paricalcitol
↑ CYP24A1 mRNA	Dialysis	100nM calcitriol

Here it was considered whether there was any difference in expression of the VDR mRNA or protein between control and dialysis VSMCs at baseline that could contribute to these differences in response to VDRAs between the two groups.

To determine the VDR expression of VSMCs at baseline, RNA and protein were harvested from control and dialysis VSMCs cultured in normal Ca-P medium. QPCR and western blot analysis showed that here was no difference in total VDR expression between control and dialysis VSMCs at either the mRNA or the protein level as shown in Figure 6-4 A to C.

Interestingly western blot analysis showed that some VSMC isolates expressed the larger VDR-B isoform as well as the standard VDR-A isoform. As shown in the example in Figure 6-4D controls tended to express more of the large 54kDa VDR-B, whereas dialysis VSMCs expressed a higher proportion of the 48 kDa VDR-A isoform. The fractional distribution of each isoform was quantified. The fraction of VDR-A in control VSMCs was 0.30 ± 0.065, this was over doubled in dialysis VSMCs where the fraction of VDR-A was 0.65 ± 0.12, as shown in Figure 6-4 E and F.

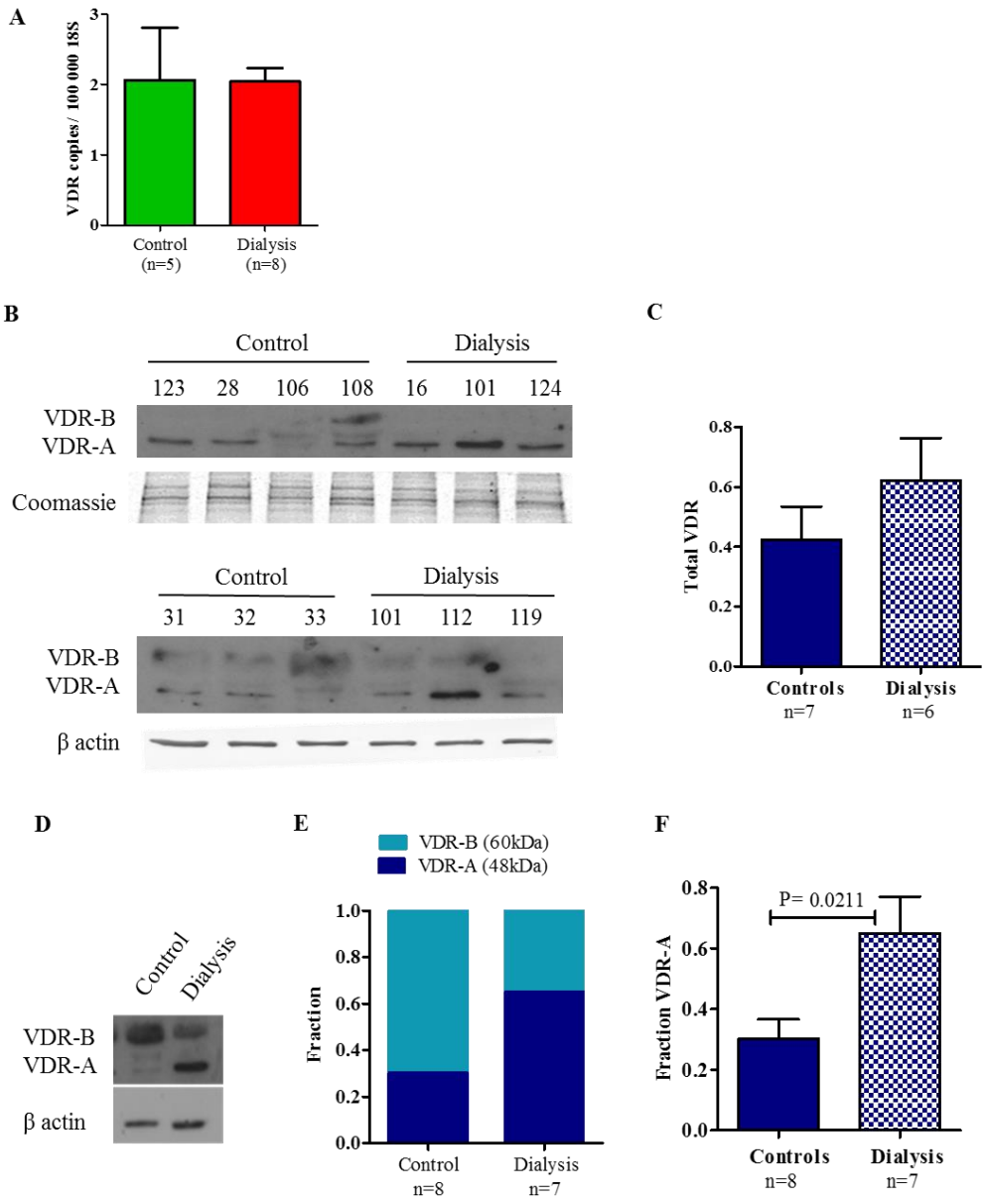


Figure 6-4: Expression of the VDR in control and dialysis VSMCs.

MRNA and protein were harvested at baseline from control and dialysis VSMCs isolated from different patients. A) Total VDR mRNA normalised to 18S RNA expression. B) Western blot analysis of VDR-A and VDR-B isoforms, C) Relative expression of Total VDR quantified from the sum of VDR-B and VDR-A densities after being normalised to loading control (β-actin). D) Example western blot of VDR-A and VDR-B isoform expression in control and dialysis cell line. The fraction of VDR-A and VDR-B isoforms were quantified from the optical density of western blots in different control (n=8) and dialysis (n=7) VSMCs, shown as E) mean fraction of VDR-A and VDR-B and F) fraction of VDR-A. C and F data shown as mean $\pm$ SE and statistical significance determined by unpaired two-tailed t-test**.**

6.4.2 Calcitriol increased expression of the total VDR mRNA in VSMCs.

The previous section showed that there was no difference in total VDR mRNA or protein expression between control and dialysis VSMCs, however the ratio of VDR isoforms between the 2 groups differed. Next the effect of VDRA treatment for 5 days on expression of the VDR was considered.

As shown in Figure 6-5A, high Ca-P medium with or without the vehicle did not affect expression of total VDR mRNA, nor did treatment of VSMCs with either 1nM calcitriol, 10nM calcitriol or 10nM paricalcitol (Figure 6-5 B and C). The higher concentration of 100nM calcitriol significantly increased expression of total VDR (copies/1000 18S RNA) from 2.6$\pm$0.5 with the vehicle in high Ca-P medium to 6.3$\pm$1.4 with 100nM calcitriol in high Ca-P medium ($p=0.028$).

It was important to consider whether VDRAs up regulate transcription of the total VDR to the same extent in control and dialysis VSMCs. As shown previously in Figure 6-4A, there was no difference in the baseline expression of total VDR mRNA between control and dialysis VSMCs. Here I show that despite a trend for higher total VDR mRNA expression in dialysis than control VSMCs in each of the treatment conditions, as shown in Figure 6-5D-F there was no significant differences between total VDR expression in control and dialysis VSMCs.

Next it was considered whether the increase in total VDR mRNA expression with calcitriol led to an increased VDR protein expression of both VDR-A and VDR-B isoforms.

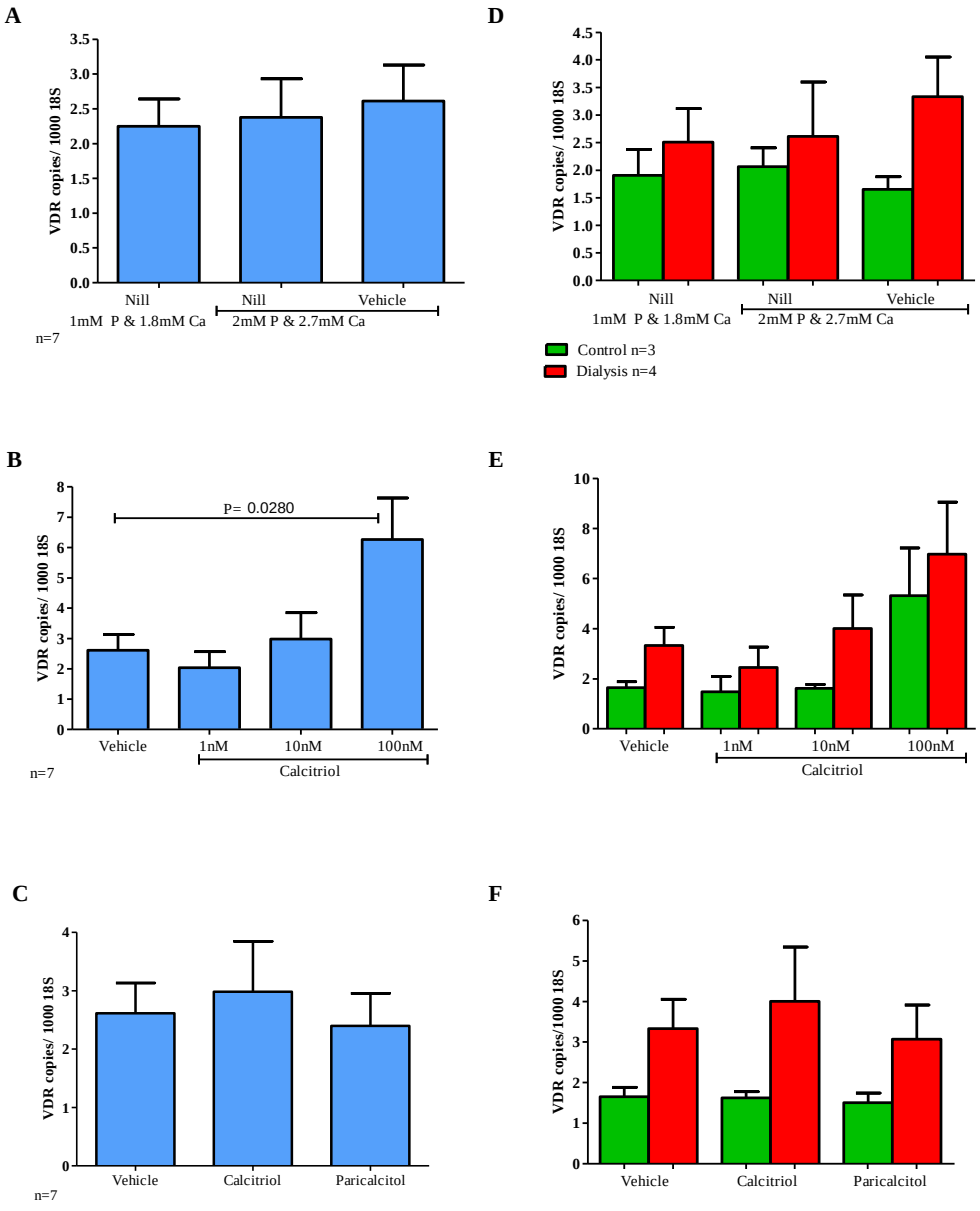


Figure 6-5: Total VDR mRNA expression in VSMCs treated with VDRAs.

VSMCs were cultured for 5 days in normal Ca-P medium, high Ca-P medium or high Ca-P medium with vehicle (3.4mM ethanol) (A and D), high Ca-P medium with either vehicle, 1nM, 10nM or 100nM calcitriol (B and E) or high Ca-P medium with either vehicle, 10nM calcitriol or 10nM paricalcitol (C and F). RNA was extracted and VDR expression determined by qPCR analysis and normalised to expression of 18S RNA. A, C and E show VDR expression in control and dialysis VSMCs combined. B, D and F show the comparison of VDR expression in control and dialysis VSMCs. Data shown as mean $\pm$ SE and statistical significance determined by unpaired two-tailed t-test. Data shown as mean $\pm$ SE and statistical significance determined by unpaired two-tailed t-test.

6.5 Transcriptional activation of the VDRE.

6.5.1 There was no difference between control and dialysis VSMCs transcriptional activation of the VDRE.

Previous sections in this chapter have shown that dialysis VSMCs express a higher proportion of the VDR-A isoform than control VSMCs. Furthermore, 100nM calcitriol increased expression of the VDR-A isoform and reduced expression of the VDR-B isoform in both control and dialysis VSMCs. This suggests that the ratio of VDR isoforms expressed is functionally significant however the mechanism of this is not understood. As discussed in the introduction, the relative transcriptional activation capacity of the VDR-A and VDR-B isoforms have not been consolidated in the literature.

Here I investigate whether dialysis VSMCs, which predominantly express the VDR-A isoform at baseline, have a greater transactivation capacity of the VDRE than control VSMCs which predominantly express the VDR-B isoform at baseline.

To compare the transactivation capacity of the VDRE in control and dialysis VSMCs, cells were transfected with a firefly luciferase construct with either a vitamin D-inducible promoter containing multiple repeats of the VDRE, a constitutively active CMV promoter as a positive control, or negative control without any promoter sequence. In each case a construct constitutively expressing Renilla luciferase was included as a transfection control for normalisation. Transfected VSMCs were cultured with and without 100nM calcitriol for 24 hours.

In the VSMCs transfected with the vitamin D inducible construct, 100nM calcitriol was able to significantly increase luciferase activity in only 4 out of the 12 VSMC isolates tested. This was independent of the VSMC type as it included 2 out of 6 control VSMCs as shown in Figure 6-6 and 2 out of the 6 dialysis VSMCs as shown in Figure 6-7.

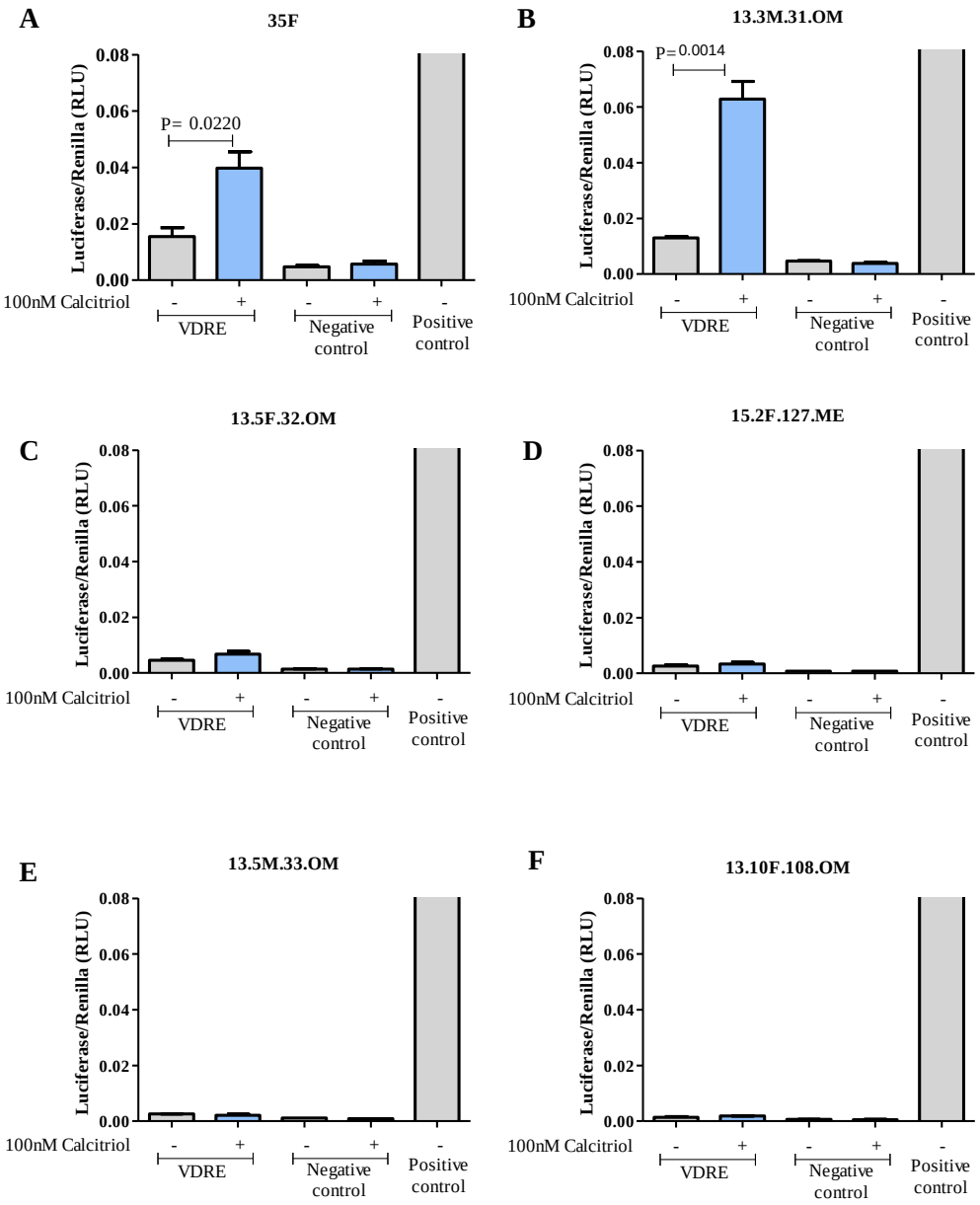


Figure 6-6: Activation of the VDRE-luciferase in control VSMCs.

Control VSMCs were transfected in triplicate wells with a firefly luciferase construct either under a vitamin D-inducible promoter containing VDREs, or with the negative control non-inducible promoter or the constitutively active positive control promoter. All VSMCs were transfected with constitutively active Renilla luciferase as a transfection control. Post transfection VSMCs were treated with or without 100nM calcitriol for 24 hours to induce the VDRE-firefly luciferase. A) Adult 35F VSMC line. B-F) Children's VSMC explants from control patients**.** Data shown as mean $\pm$ SE and statistical significance determined by unpaired two-tailed t-test.

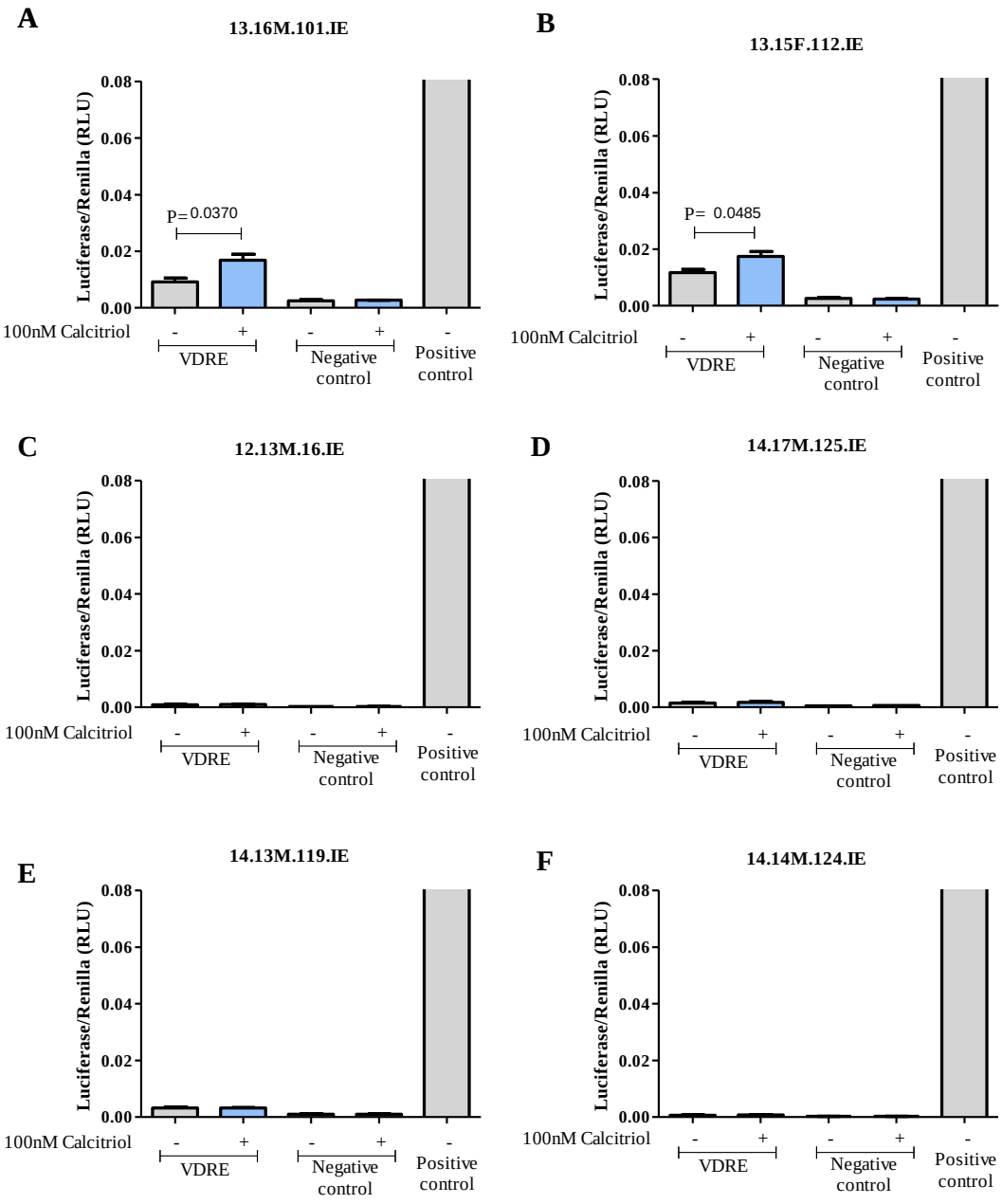


Figure 6-7: Activation of the VDRE-luciferase in dialysis VSMCs.

VSMCs were transfected in triplicate wells with the Firefly luciferase either under a vitamin D-inducible promoter containing VDREs, or with the negative control non-inducible promoter or the constitutively active positive control promoter. All VSMCs were transfected with constitutively active Renilla luciferase as a transfection control. Post transfection VSMCs were treated with or without 100nM calcitriol for 24 hours to induce the VDRE-firefly luciferase. Data shown as mean $\pm$ SE and statistical significance determined by unpaired two-tailed t-test.

Interestingly the 4 VSMC isolates that had VDRE-luciferase that was inducible by 100nM calcitriol also had a higher level of background luciferase activity in the VSMCs transfected with the VDRE-luciferase construct and treated with the vehicle only. It was necessary to confirm that the transfection efficiency was consistent between all experiments by comparing activity of the Renilla luciferase. A Renilla luciferase construct under a constitutively active CMV promoter was transfected alongside each of the firefly luciferase constructs to act as a transfection control. The firefly luciferase activity in each treatment condition was normalised to Renilla activity. Comparison of Renilla activity in each cell type, showed that transfection was successful in each of the VSMC isolates and there was no significant difference in transfection efficiency between experiments, data shown in the Appendix Figure 10-3 and Figure 10-4.

As shown above, only 4 out of 12 VSMCs transfected with the VDRE luciferase had increased luciferase activity in response to 100nM calcitriol for 24 hours. An experimental control was required to confirm that the 100nM calcitriol used was active in each experiment. Non-transfected VSMCs were treated in parallel for 24 hours with 100nM calcitriol or vehicle, RNA was isolated and the expression of vitamin D responsive CYP24A1 gene was analysed by qPCR. Each VSMC isolate upregulated CYP24A1 expression as shown in Figure 6-8A, therefore it is evident that the 100nM calcitriol used in each experiment was active. CYP24A1 expression was significantly upregulated by 100nM calcitriol in VSMCs in which it also activated VDRE-luciferase as well as those that it did not. Furthermore, there was no significant difference in CYP24A1 expression after calcitriol treatment in VSMCs in which the VDRE-luciferase was activated or not activated (Figure 6-8B).

Expression of the VDR in transfected VSMCs would also be essential for calcitriol to activate the VDRE-luciferase construct, therefor VDR mRNA expression was analysed in these VSMCs. There was a tendency for cell lines that activated the VDRE-luciferase to express higher levels of total VDR mRNA as shown in Figure 6-8 C and D, however this was not significantly different. In addition, 100nM calcitriol treatment for 24 hours did not upregulate the expression of total VDR in either VSMCs that activated or did not activate luciferase expression. This differs from 100nM calcitriol treatment for 5 days

which did increase expression of total VDR mRNA in VSMCs as shown previously in Figure 6-5.

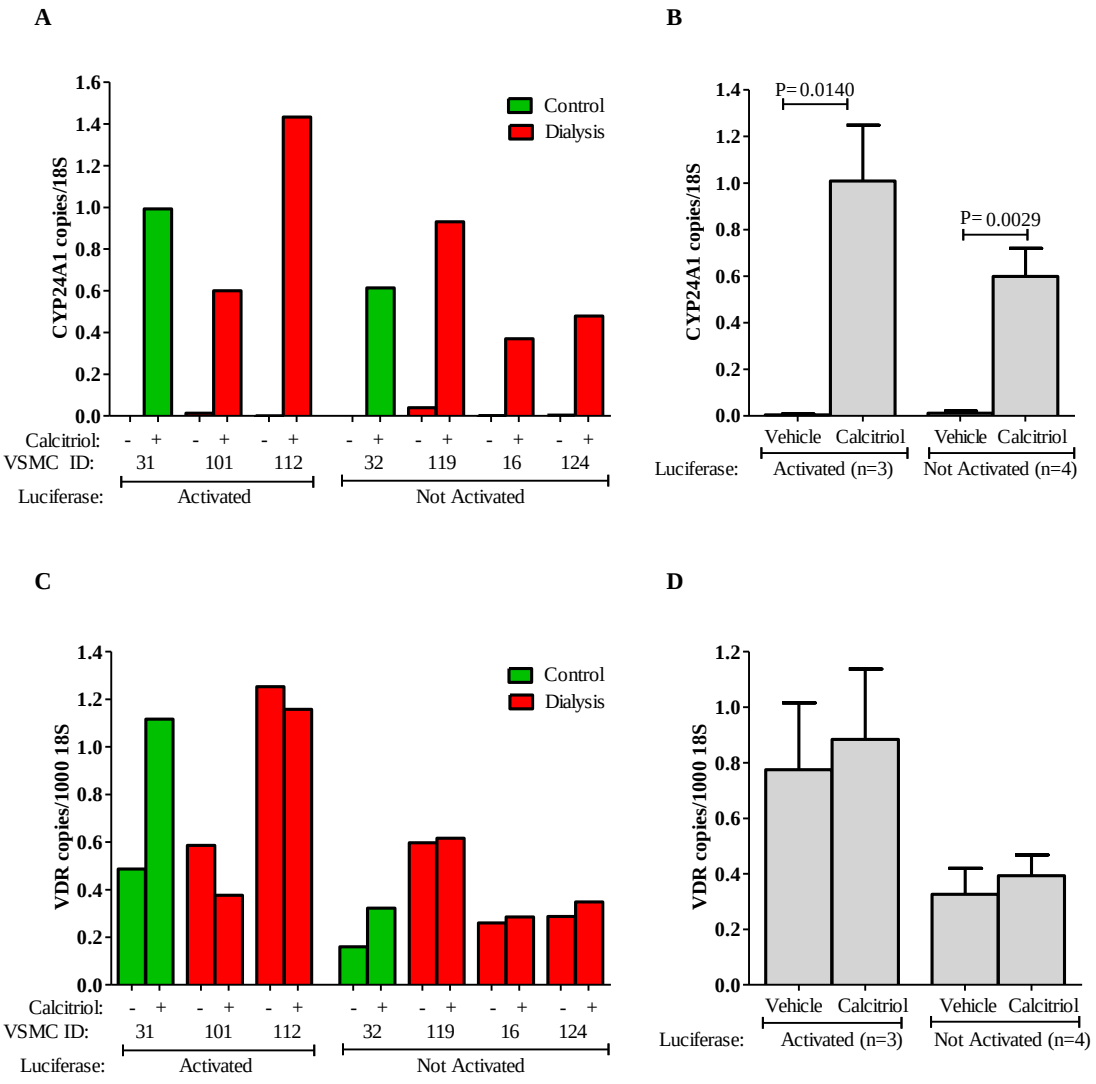


Figure 6-8: CYP24A1 and VDR mRNA expression in VSMCs.
Control and dialysis children's VSMCs were cultured and treated with or without 100nM calcitriol in parallel to those transfected with luciferase constructs. MRNA was harvested and CYP24A1 (A and B) and VDR (C and D) expression quantified by qPCR. Expression in each VSMC type is shown in A and C. B and D compare expression levels in VSMCs that had VDRE-luciferase expression activated by 100nM calcitriol and those in which expression was not activated. Data shown as mean ± SE and statistical significance determined by unpaired two-tailed t-test.

6.5.2 VDRE-dependent luciferase expression coincides with a higher fraction of VDR-A expression.

As shown in the previous section, there was no difference in total VDR mRNA expression between VSMCs in which 100nM calcitriol treatment for 24 hours activated VDRE luciferase and VSMCs in which it was not activated.

In the following section the ratio of VDR isoforms expressed was compared between VSMCs in which 100nM calcitriol activated the VDRE-luciferase and those VSMCs in which it did not. An example of the VDR protein isoforms expressed with and without 100nM calcitriol was determined by western blot analysis as shown in Figure 6-9 A. Optical density of VDR-A and VDR-B was calculated and the expression of each as a fraction of total VDR is shown in Figure 6-9 B. Each of the 4 VSMCs that activated the VDRE-luciferase had a higher fractional expression of VDR-A than those that did not activate VDRE-luciferase. This was evident in Figure 6-9 C which showed the mean fraction of the VDR isoforms. In addition, Figure 6-9 D shows that the fraction of VDR-A expressed was significantly higher in VSMCs that activated the expression of VDRE-luciferase (0.70 ± 0.17) than those that did not (0.07 ± 0.02).

Earlier in this chapter I demonstrated that treatment with 100nM calcitriol for 5 days caused a switch from expression of VDR-B to the VDR-A isoform in both control and dialysis VSMCs. Here I showed that treatment of VSMCs with 100nM calcitriol for 24 hours did not alter the ratio of VDR isoforms expressed in either VSMCs that activated luciferase or those that did not activate luciferase, as shown in Figure 6-9 D.

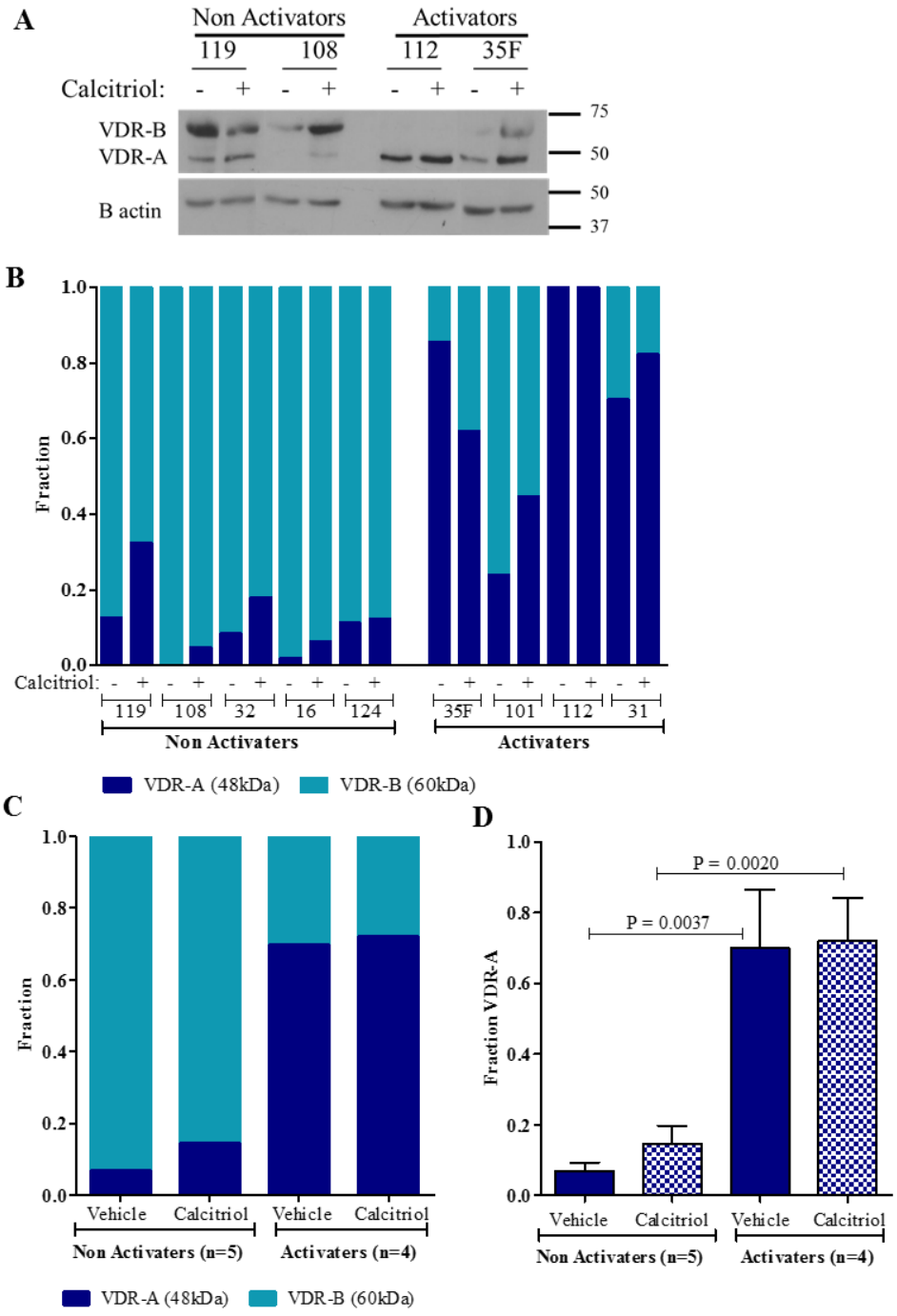


Figure 6-9: VSMCs that activated the VDRE-luciferase expression, expressed a higher fraction of the VDR-A isoform.

Control and dialysis children's VSMCs (n=8), as well as adult VSMC line 35F were treated with or without 100nM calcitriol for 24 hours in parallel to those transfected with luciferase constructs. Protein was harvested and VDR isoform expression visualised by western blotting. The optical density of each isoform was quantified and calculated as a fraction of total VDR expression. D) Data shown as mean ± SE and statistical significance determined by unpaired two-tailed t-test.

6.5.3 Calcitriol increased expression of the VDR-A isoform in VSMCs.

The previous section showed that the highest tested concentration of calcitriol (100nM) increased expression of total VDR mRNA in VSMCs after culture for 5 days. Protein was also harvested from VSMCs cultured in the same treatment conditions and VDR expression analysed by western blot.

It was shown in Figure 6-4 D-F that at baseline, dialysis VSMCs expressed a higher fraction of VDR-A, and control VSMCs expressed a higher fraction of the VDR-B isoform. The effect of high Ca-P and calcitriol treatment on VDR isoform expression was explored in both control and dialysis VSMCs. As shown in Figure 6-10, there appears to be no change in total VDR protein expression after treatment with 1, 10 or 100nM calcitriol however there was a clear shift in the VDR isoform expressed with increasing calcitriol dose.

The control VSMCs predominantly expressed the VDR-B isoform at baseline and in high Ca-P medium only, with the vehicle and with 1nM calcitriol. Expression of VDR-A was increased by 10nM calcitriol, at this dose similar levels of VDR-A and VDR-B isoforms were expressed in the control VSMCs as shown in Figure 6-10. 100nM calcitriol further increased expression of the VDR-A isoform and the expression of VDR-B was drastically reduced to undetectable levels.

Dialysis VSMCs expressed a higher fraction of VDR-A isoform than VDR-B at baseline. VDR-B was detectable in dialysis VSMCs in: normal Ca-P medium, high Ca-P medium, with the vehicle, 1nM or 10nM calcitriol. As with the control VSMCs, 100nM calcitriol reduced VDR-B expression to undetectable levels.

In both control and dialysis VSMCs, high doses of calcitriol resulted in a shift in the expression of VDR isoforms from VDR-B to VDR-A. Calcitriol favoured expression of the VDR-A isoform.

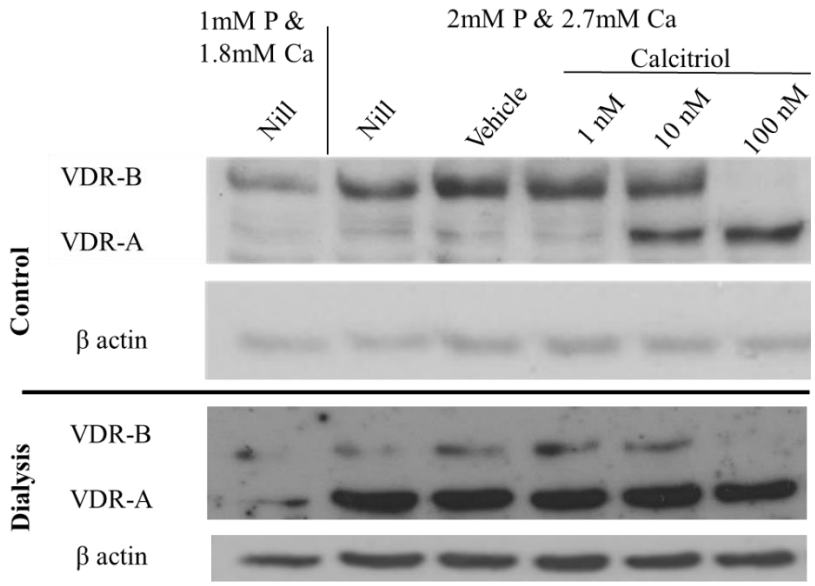


Figure 6-10: Calcitriol favoured expression of the VDR-A isoform.

VSMCs were cultured for 5 days in normal Ca-P medium, or high Ca-P medium with; no additives (nil), vehicle (3.4mM ethanol), 1nM, 10nM or 100nM calcitriol. Protein was extracted and VDR expression determined by western blot analysis, representative western blots from a control (13.10F.108.OM) and dialysis (13.16M.101.IE) VSMC are shown.

6.5.4 There was no difference in the subcellular localisation of the VDR in VSMCs that activate VDRE-luciferase expression and not.

As discussed in the introduction, inactive VDR can be found in the cytoplasm and in order to regulate transcription the VDR has to translocate to the nucleus. Therefore, as well as the expression level, the cellular localisation of the VDR is important for its function and activity.

Immunofluorescence staining of the total VDR protein was used to compare the cellular localisation of VDR in the control VSMC isolate 13.3M.31.OM with high VDRE-luciferase activation and the dialysis VSMC isolate 14.14M.124.IE that did not activate VDRE-luciferase. Both isolates were treated with or without 100nM calcitriol. As shown in Figure 6-11, in each VSMC isolate and treatment condition the VDR was specifically located in the nucleus and there was no difference in the cellular localisation of the VDR.

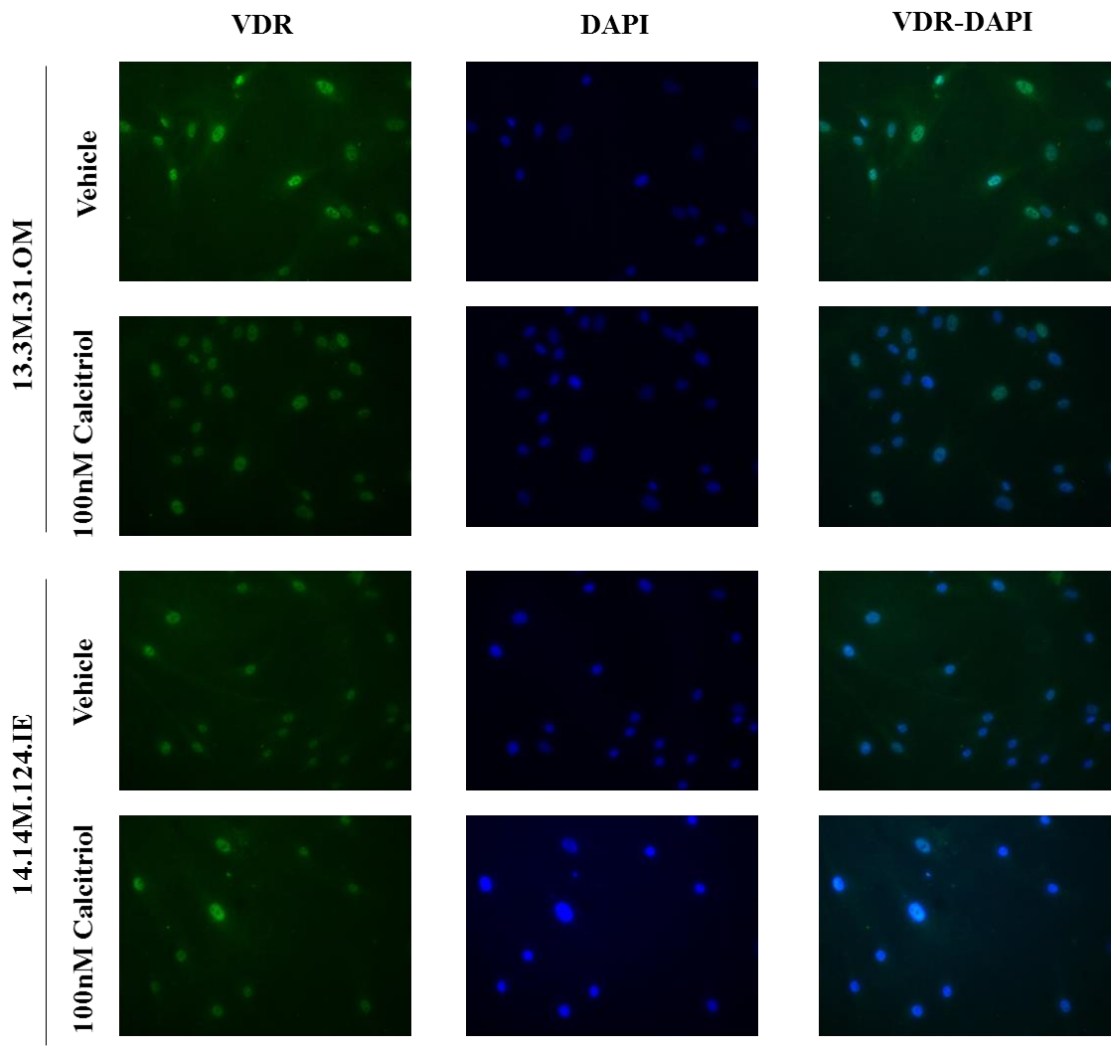


Figure 6-11: VDR localised to the nuclei of VSMCs.

VSMC isolates 13.3M.31.OM and 14.14M.124.IE were selected as extreme examples of high expression activation and no activation, respectively, of the VDRE-luciferase construct. VSMCs were treated with or without 100nM calcitriol, formalin-fixed and stained by immunofluorescence for the VDR and the nuclei counterstained with DAPI.

6.6 Discussion:

6.6.1 Summary

There was no variation in total VDR expression between individual's vessel rings and VSMCs. However, differences in the ratio of VDR isoforms expressed may contribute to the patient variation in response to calcitriol observed in previous chapters.

The key findings of this chapter were:

1. Immunostaining and quantification of VDR positive nuclei showed that there was no difference in total VDR expression between vessel rings that either showed no response in their Ca load to calcitriol or responded with an increased Ca load.

2. There was no difference in total VDR mRNA or protein expression between control and dialysis VSMCs either at baseline or after culture with 1nM, 10nM or 100nM calcitriol or with 10nM paricalcitol.

3. Control and dialysis VSMCs expressed different ratios of the VDR isoforms. Control VSMCs predominantly expressed the 54KDa VDR-B whereas dialysis VSMCs predominantly expressed the 48KDa VDR-A. In both control and dialysis VSMCs high doses of calcitriol decreased VDR-B and increased VDR-A expression.

4. In a selection of control and dialysis VSMCs 100nM calcitriol activated the VDRE-dependent luciferase expression. There was no difference in VDR localisation between activators and non-activators however they differed in their VDR isoform expression.

6.6.2 Control VSMCs predominantly express VDR-B whereas dialysis VSMCs predominantly express VDR-A.

This study for the first time compares the VDR expression in VSMCs from control and dialysis patients. Despite there being no difference in total VDR at either the RNA or protein level, there was a significant difference in the ratio of VDR isoforms expressed. Dialysis VSMCs predominantly expressed VDR-A whereas control VSMCs predominantly expressed the N-terminally extended VDR-B.

The VDR was previously shown by western blot analysis to be expressed in human VSMCs, however in these VSMCs only 1 band was identified at approx. 48KDa corresponding to the VDR-A isoform (Mary et al., 2015). Using the same mouse anti-VDR antibody (mAb VDR (D-6), sc-13133) I identified two VDR isoforms in the children's VSMCs. There are multiple VDR antibodies available, Wang et al. tested these by extensive immunoassay analysis and the D-6 antibody was preferred for its high specificity and extreme sensitivity (Wang et al., 2010). The VDR D-6 antibody is raised against VDR-A amino acids 344 – 424, VDR-B has the same amino acid sequence as VDR-A with the addition of a 50 amino acid extension at the N-terminus, therefore both isoforms are detectable by this antibody as shown in Figure 6-1.

6.6.3 Calcitriol increased VDR mRNA expression and shifted the ratio of VDR isoforms to increase VDR-A expression.

It has been documented that VDRAs increase the expression of the VDR itself, for example intramuscular injections of the VDRA cholecalciferol in New Zealand white female rabbits increased their expression of aortic VDR (Rajasree et al., 2002). In line with this study my results show that, in the control and dialysis VSMCs 100nM calcitriol significantly increased expression of total VDR mRNA. It was not possible to accurately quantify the total VDR protein expression after treatment with 100nM calcitriol due to the shift in VDR isoform expression observed. There is a limited number of studies dedicated

to the expression of the different VDR isoforms; here for the first time the effects of different doses of calcitriol on VDR isoform expression were observed. Both in control and dialysis VSMCs, treatment with 100nM calcitriol reduced expression of the VDR-B protein and increased expression of the VDR-A protein.

It is of interest to note that the dialysis VSMCs but not the control VSMCs were explanted from patients who were receiving vitamin D supplements in the form of alfacalcidol at the time of vessel collection. The effect of alfacalcidol treatment *in-vivo* on VDR isoform expression in VSMCs is not known. As with calcitriol treatment *in-vitro*, the prescribed alfacalcidol *in-vivo* may also favour the expression of the VDR-A isoform. Hence, alfacalcidol supplements given to dialysis patients prior to vessel collection may account for the higher fraction of VDR-A isoform expressed in dialysis VSMCs relative to control VSMCs. To test this theory, it would be necessary to investigate the effect of vitamin D supplementation *in-vivo* on VDR isoform expression, this could be examined in either control mice or 5/6 nephrectomised mice as a model for CKD.

In future studies it is important to understand the functional significance of this change in VDR isoform composition induced by calcitriol for processes regulating calcification. As discussed previously, the dose-dependent effect of calcitriol on calcification forms a U shaped curve. Therefore, it could be speculated that the change in expression of VDR isoforms at different doses of calcitriol alters the pattern of downstream gene activation, hence the effect of calcitriol on Ca load. In particular, moderate doses of calcitriol may protect against calcification via activation of VDR-B, whereas higher doses of calcitriol, which favour the expression of VDR-A, may have causative effects on calcification. This hypothesis could to be tested by overexpressing VDR-A or VDR-B in cells with low endogenous expression of the VDR and then inducing calcification.

6.6.4 Activation of VDRE luciferase in some VSMCs only.

The functional significance of the different ratios of VDR isoforms expressed in control and dialysis VSMCs was explored by luciferase assay. Control and dialysis VSMCs from children were transfected with a construct containing the firefly luciferase gene under a VDRE promoter and the effect of calcitriol on transcriptional activation of the luciferase was compared.

100nM calcitriol was able to activate the VDRE luciferase at detectable levels in only 3 of the 11 children's VSMCs and one adult VSMC line. Transfection efficiency measured by transfection with a Renilla luciferase under a constitutively active promoter was comparable in cells that activated expression from VDRE and did not. In addition, the 100nM calcitriol was evidently active as it upregulated CYP24A1 expression.

VSMCs that activated VDRE-dependent luciferase expression included both control and dialysis VSMCs. Despite this, activators of luciferase expression had different fractional distribution of VDR isoform expression to non-activators. The VDR-A isoform was predominantly expressed by VSMCs that activated the VDRE luciferase promoter, suggesting that this isoform may be required for luciferase activation where as either VDR isoform is able to activate the CYP24A1 promoter in the presence of calcitriol.

In neither activators nor non-activators did 100nM calcitriol induce a shift in isoform expression after 24 hours of treatment. This differs from previous data in this chapter where 5 days of treatment with 100nM calcitriol caused a shift in VDR expression to favour the VDR-A isoform. Hence, duration of calcitriol treatment is evidently important for its biological function. It would be interesting to determine if with a prolonged 5-day treatment with calcitriol these VSMCs switched to express a higher fraction of VDR-A, and were then able to activate the VDRE-luciferase. Unfortunately, this was not tested in the present study as the harsh nature of the transfection procedures limited VSMC survival duration post transfection.

Once activated, the VDR is translocated to the nucleus where it functions as a transcriptional regulator. The nuclear localisation of the VDR is important for its function as a transcriptional regulator. Immunofluorescence showed that the VDR was diffusely expressed in the nucleus both in VSMC isolate 13.3M.31.OM that had high expression of the VDR-A and activated VDRE-luciferase expression as well as in the VSMC isolate 14.14M.124.IE that had high expression of VDR-B and did not activate VDRE-luciferase expression. This differed from findings by E M Gardiner's group, which showed that the cellular distribution differed between VDR isoforms when transfected in COS-1 cells. VDR-A was diffusely expressed in the nuclei, whereas VDR-B was expressed in nuclear foci, that were dispersed by 10nM calcitriol, giving a diffused nuclear distribution similar to VDR-A (Sunn et al., 2001). It was thought that VDR-B nuclear foci represented their physical association with cellular cofactors in the absence of ligand, enabling a more rapid response to ligand activation (Flanagan et al., 2002).

6.6.5 What is the functional significance of the VDR-A and VDR-B isoform?

In chapter 5 it was shown that dialysis VSMCs were more responsive to calcitriol than control VSMCs in their upregulation of CYP24A1 and VDR mRNA. This chapter has shown that dialysis VSMCs express a higher proportion of VDR-A than control VSMCs. It is possible to speculate the existence of an association between higher transcriptional activity and higher VDR-A expression, in this scenario VDR-A has a greater transcriptional activation capacity than VDR-B. Original data from J A Eisman's lab supports this theory, they found that VDR-B had only 60% the transcriptional activation capacity of VDR-A. This was determined by co transfection of the 24-hydroxylase promoter reporter construct with each of the VDR isoforms in Cos-1 and P19 cells that had low endogenous expression of the VDR (Sunn et al., 2001). However, subsequent data collected by the same group showed the opposite, that VDR-B had a greater transcriptional activation than VDR-A. Further investigation revealed that VDR-B was more susceptible to interference from CMV and SG5 viral promoters, which compete for transcriptional co-factors, and reduced the transactivation capacity of the VDR-B isoform

(Gardiner et al., 2004). Therefore, the relative transcriptional activation capacities of the two VDR isoforms may be dependent on the availability of cellular co-factors and transcriptional regulators in different cell types. A comparison of 7 different cell types confirmed that the relative VDR-A and VDR-B activity was cell type-dependent and even varied between different cell lines from the same tissue bed (Esteban et al., 2005). The N-terminal extension of VDRB-1 may interact with specific cell co-factors. Pull down assays in COS-1 cells showed that the C terminal ligand binding domain of VDR-B had a stronger interaction with calcitriol than VDR-A did (Flanagan et al., 2002). As well as being dependent on cellular cofactors the relative activity of the two VDR isoforms varied between different promoters (CYP24A1 and CYP3A4) as well as different activating ligands (1nM calcitriol and lithocholic acid) (Esteban et al., 2005).

In the literature many factors are reported to affect the relative activation properties of the two VDR isoforms. Experimental manipulation of VDR isoform expression by the viral promoters utilised in the experiments discussed above subsequently affected the function of the VDR isoforms being investigated, rendering the experiment biased. Here, with the control and dialysis VSMCs naturally expressing different ratios of the VDR-A and VDR-B isoforms it is possible to compare the transcriptional regulation of naturally occurring downstream genes such as CYP24A1, without the need for interference from experimental promoters. However, this would be made challenging by the natural variation in the fraction of VDR isoforms expressed in VSMC explants from different patients, and the shift in VDR isoform expression upon calcitriol treatment.

6.6.6 Limitations

The VDR-A and VDR-B isoforms are translated from 3 different mRNA transcripts, as shown in Figure 6-1. The primers used in this chapter do not differentiate between the transcripts and the PCR product represents the quantification of total VDR mRNA. In future work, it would be of great interest to use isoform-specific primers in order to determine if the differences in VDR isoform expression arise at the transcriptional or post-

transcriptional level. Furthermore, a VDR-B isoform-specific antibody as previously produced by the Gardiner group (Sunn et al., 2001), that interacted with the N-terminal extended region of the VDR-B isoform would enable the comparison of isoform expression in vessel rings by immunohistochemistry and identification of the subcellular localisation of each isoform in VSMCs.

Chapter 7 The effects of VDRAs on vascular calcification in arterial rings isolated from adults with CKD.

7.1 Introduction

This thesis so far has focused on the effect of vitamin D on calcification in paediatric patients with CKD, this is of particular interest and clinical concern as children are not generally exposed to traditional cardiovascular risk factors and young CKD patients still suffer from medial calcification or Monckeberg sclerosis as discussed in the introduction. However, it is also important to consider the effects of VDRAs on calcification in the adult CKD population to determine if this mirrors the effects observed in children. In the adult cohort it is likely that patients have been exposed to traditional cardiovascular risk factors such as diabetes, smoking, dyslipidaemia and hypertension in addition to uraemia-related cardiovascular risks. The vessels may have underlying atherosclerosis which may complicate the pattern of calcification observed at baseline and after treatment. Moreover, adult renal patients may have a longer duration of CKD, longer time on dialysis, previous failed transplants and also different causes of their CKD compared to children. It was of particular importance to consider the general health and Ca load of the vasculature at baseline, as well as the patients' past medical history with regards to cardiovascular health.

To compensate for the patient variation, numerous rings were obtained from each patient's vessel and the effects of different treatment conditions were compared against the baseline vessel characteristics, such that each vessel was its own internal control.

7.1.1 Aim

1. To elucidate whether VDRAs accelerate the progression of vascular calcification in arterial rings from adults with CKD.

This will be addressed using the following key questions;

1. What effect do physiological doses of the VDRAs, calcitriol and paricalcitol, have on vascular calcification in vessels from adults with CKD?

2. Are there any differences in susceptibility to vascular calcification between vessel rings from pre-dialysis and dialysis patients?

3. Do any patient related factors affect the response of adult vessel rings to VDRA induced calcification, including gender, age, primary renal diagnosis, previous cardiovascular health and current vitamin D supplements?

4. What effect do physiological doses of the VDRAs, calcitriol and paricalcitol, have on activity of the osteoblast enzyme ALP in vessels from adults with CKD?

7.2 Method

For this study the inferior epigastric artery was collected from patients listed in Table **7-1** who were undergoing renal transplantation at the Royal Free Hospital. Vessel rings were cultured in either normal Ca-P medium (M199 tissue culture medium which contains 1mM P and 1.8mM Ca) or high Ca-P medium (M199 with added P & Ca to a final concentration of 2mM P and 2.7mM Ca) for 2 weeks, with the medium changed every 3 days. Vessels were exposed to physiological doses of either 10nM calcitriol or 10nM paricalcitol, or 3.4mM ethanol as the vehicle control for both VDRAs. Up to 3 vessel rings were cultured per condition, they were either; homogenised and their Ca content quantified by a colorimetric σ-cresolphthalein assay, formalin fixed and their Ca load visualised by von Kossa staining or homogenised and their ALP activity determined by colorimetric assay. Each experimental design is described in detail in the Chapter 2. All data shown as mean $\pm$ SE.

Table 7-1: Patient information for adult vessels studied.
Vessel ID format: Year. Age and gender. Unique number. Vessel type.
IE – Inferior Epigastric artery; CAKUT – Congenital Anomalies of the Kidneys and Urinary Tract. RVD – Renovascular Disease.

Vessel ID	Age Years	Gender	Primary renal Diagnosis	Type of & time on dialysis (months)	Vitamin D supplements		HTN	IHD / PVD / Stroke	DM
					Alfacalcidol	Cholecalciferol			
13.46M.1.IE	46	M	Glomerulopathy	1 HD	NO	NO	YES	NO	NO
13.45M.2.IE	45	M	Glomerulopathy	Pre-dialysis Failed Tx	YES	NO	YES	NO	NO
13.57F.3.IE	57	F	Unknown (Other)	48 PD	YES	YES	YES	NO	NO
13.19F.4.IE	19	F	Glomerulopathy	12 PD Failed Tx	YES	YES	NO	NO	NO
13.31M.6.IE	31	M	Vasculitis (Other)	12 HD	NO	NO	NO	NO	NO
13.67F.7.IE	67	F	RVD (Other)	6 PD	YES	NO	YES	NO	NO
14.61M.11.IE	61	M	Glomerulopathy	Pre-dialysis	NO	YES	YES	YES	NO
14.45F.12.IE	45	F	Glomerulopathy	6 PD	NO	YES	YES	NO	NO
14.35M.13.IE	35	M	CAKUT (Other)	Pre-dialysis	YES	YES	NO	NO	NO
14.27M.14.IE	27	M	Fabrys (Other)	Pre-dialysis	YES	YES	NO	NO	NO
14.71M.15.IE	71	M	Glomerulopathy	12 HD	NO	NO	YES	YES	NO

HD – Haemodialysis; PD - Peritoneal-Dialysis. Failed Tx - Prior renal transplant failed. HTN – Hypertension. IHD – Ischaemic Heart Disease. PVD - Peripheral Vascular Disease.

7.3 Adult vessel rings at baseline

7.3.1 Vessels were in general good health at baseline.

Adult vessels were collected from patients aged 19 to 71 years. These patients are likely to have been exposed to traditional cardiovascular risk factors to different extents. As shown in Table **7-1**, 64% of patients suffered from hypertension and 18% had a previous cardiovascular complication. Unusually, none of the patients in this cohort were diabetic. Unfortunately, I do not have data on their history of smoking. In the adult cohort it was particularly important to observe baseline histology for general vascular health and atherosclerotic regions which lead to intimal calcification. The Ca load at baseline by σ-cresolphthalein assay and observed by von Kossa were also considered. Despite the advancing age, vessels from each patient were generally in good health and no atherosclerosis was observed, examples of the vasculature at baseline are shown in Figure 7-1. Next I looked for evidence of arteriosclerosis at baseline in the adult vessel rings.

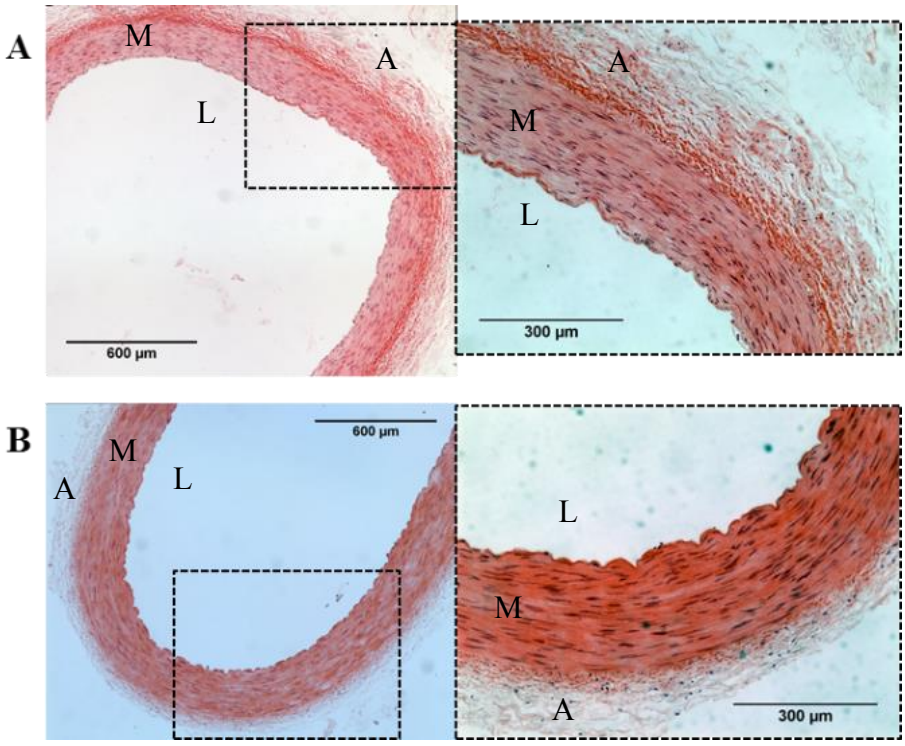


Figure 7-1: Hematoxylin and Eosin at baseline:

Inferior epigastric arteries were harvested from adults with CKD, vessel rings at baseline were formalin fixed, sectioned and stained with haematoxylin and eosin. A) 13.57F.3.IE B) 13.31M.6.IE at 4x and 10x magnification. A: Adventitia. L: Lumen. M: Tunica Media.

7.3.2 Advancing age correlated with an increased Ca load at baseline.

In this adult CKD population patient ages ranged from 19 to 71 years. Older patients have had more time to be exposed to traditional cardiovascular risk factors and may have lived for longer with CKD. In addition, age itself is considered a risk for cardiovascular disease. Despite vessels being free from atherosclerosis, it was important to look for any baseline calcification in the adult vessel rings that may represent arteriosclerosis.

Von Kossa staining detected Ca deposition in the tunica media of 3 baseline vessels, indicative of arteriosclerosis; an example is shown in Figure 7-2A. The remaining 8 adult vessels had no von Kossa positive areas as per the example in Figure 7-2B.

The baseline Ca load as determined from the σ-cresolphthalein assay ranged from 1.8 to 130 µg / µg/µl. Interestingly the Ca load at baseline as determined by the σ-cresolphthalein assay was found to have significant positive correlation with patient age in years as shown in Figure 7-3.

No other patient variables shown in Table **7-1** had an association with baseline Ca load in the adult vessel rings.

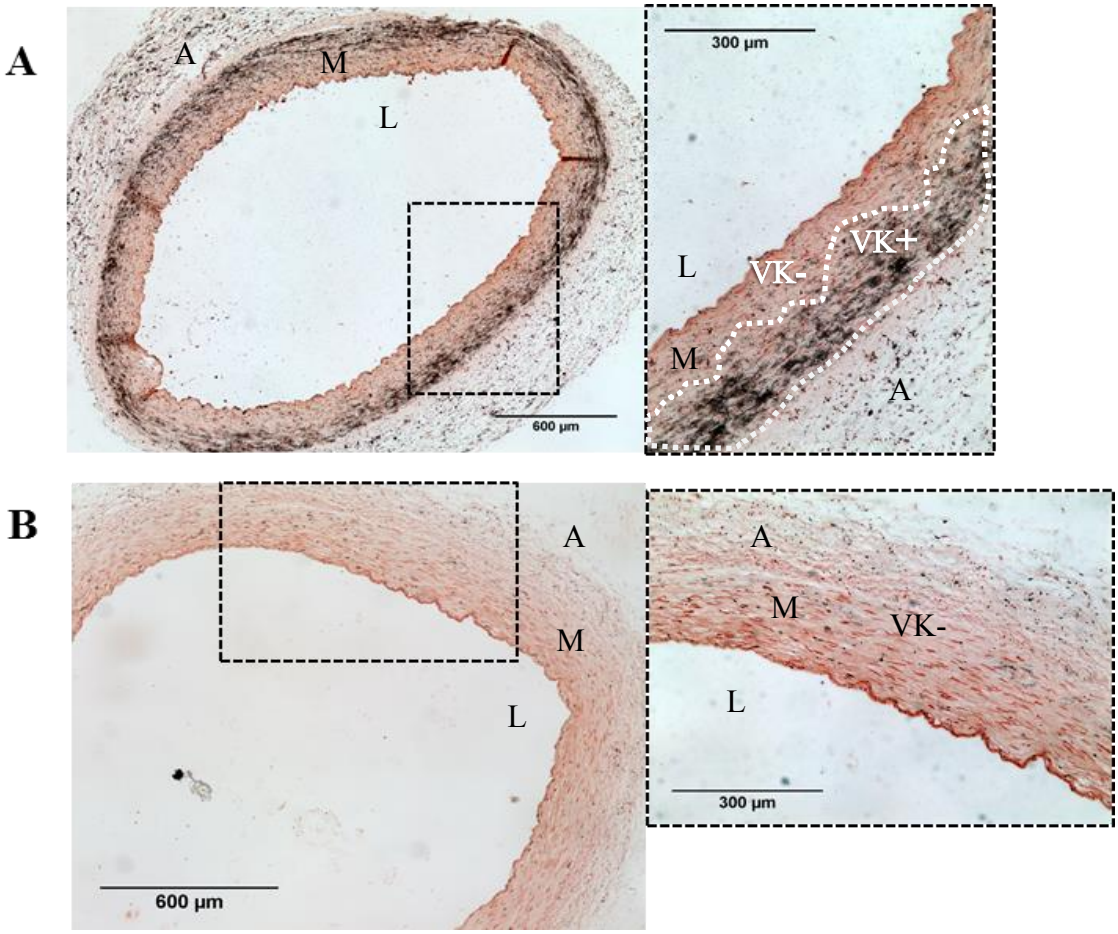


Figure 7-2: Ca deposition in baseline vessel rings:

Inferior epigastric arteries were harvested from adults with CKD, A&B) vessel rings at baseline were formalin fixed, sectioned and stained with von kossa for calcium deposition (black) and counterstained with neutral red. A) 13.45M.2.IE B) 13.57F.3.IE B) at 4x and 10x magnification. A: Adventitia. L: Lumen. M: Tunica Media. VK+: Von Kossa positive area. VK-: Von Kossa negative area.

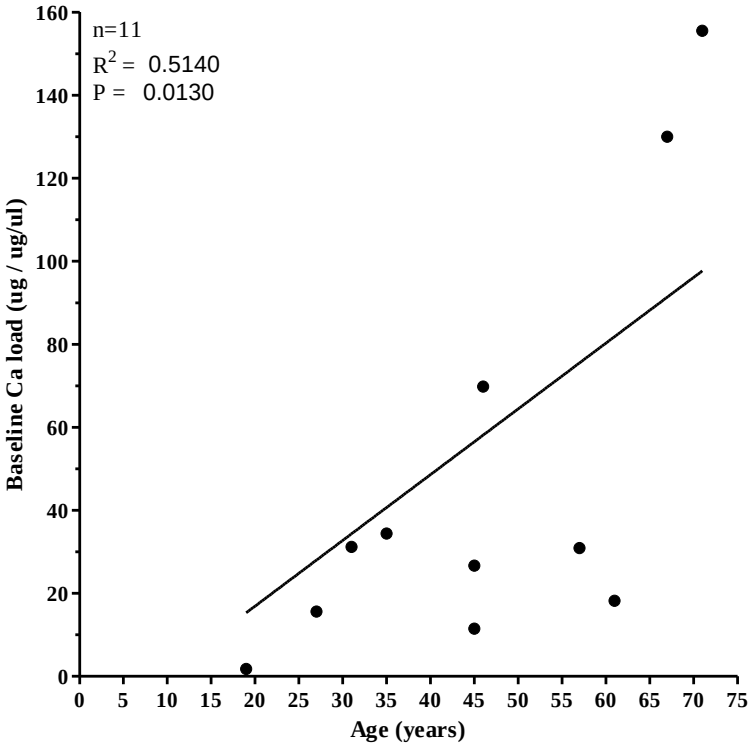


Figure 7-3: Advancing age correlates with increased baseline Ca load.

Inferior epigastric arteries were harvested from adults with CKD, a baseline vessel ring was homogenized and the Ca load analysed by σ-cresolphthalein assay and plotted against the patients' age at time of vessel collection. The Pearson R correlation coefficient was calculated along with its two-tailed P value to determine the statistical significance.

7.4 Adult vessel rings in culture.

7.4.1 Adult vessel rings can survive in culture *ex-vivo.*

As this was the first time that vessel rings from adult patients had been cultured *ex-vivo*, it was important to consider if they could remain viable in culture. H&E staining was comparable at baseline and after culture in M199 for 14 days as shown in Figure 7-4, there was no change in VSMC nuclei density or any structural changes to the vessel rings that would indicate cell death.

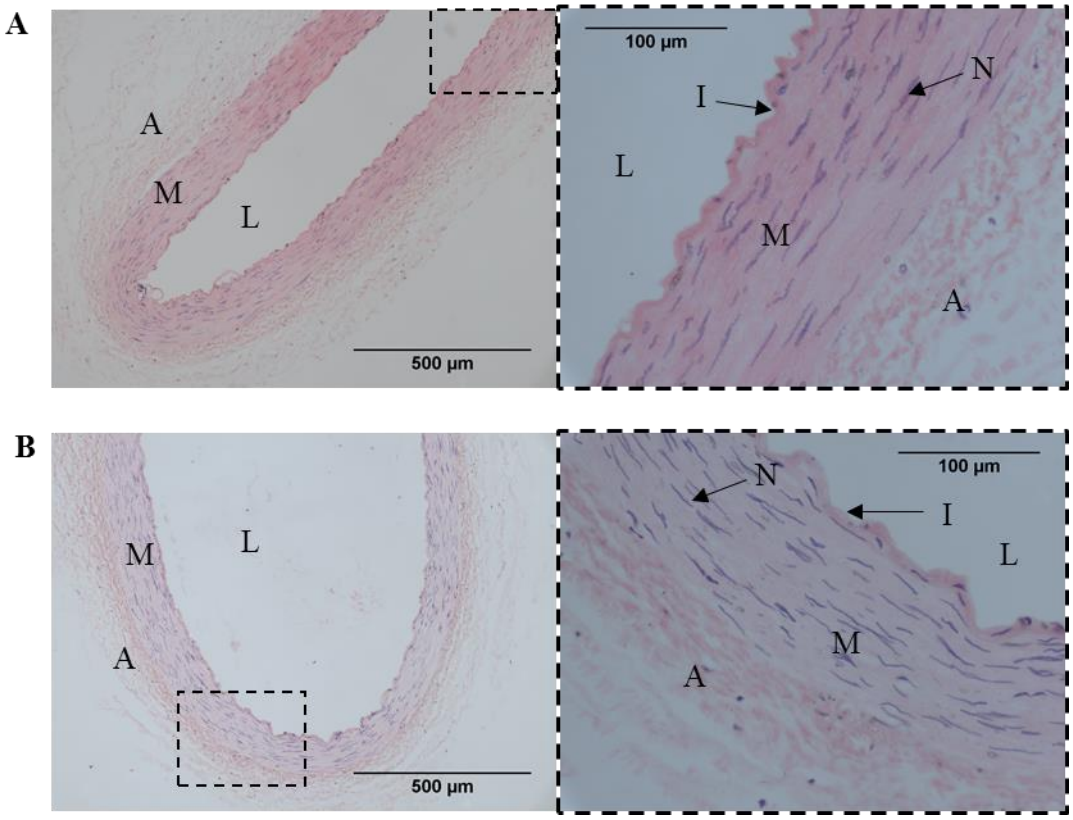


Figure 7-4: Adult vessel rings before and after culture for 14 days.

Vessel rings from inferior epigastric artery (13.67F.7.IE) were formalin fixed, sectioned and stained with H&E. A) At baseline and B) after cultured in M199 for 14 days. (10x and 40x) **A**: Adventitia. **L**: Lumen. **M**: Tunica Media. **I:** Internal elastic lamella. **N:** VSMC Nuclei.

7.4.2 High Ca-P medium induced calcification in vessel rings isolated from adults.

The previous section has shown that adult vessel rings were in good general health and able to survive in culture for 14 days, therefore it was possible to study the effect of VDRAs on vascular calcification in *ex-vivo* adult vessel rings. As with the children's vessel rings, the Ca load was studied both in normal Ca-P medium and in high Ca-P medium to mimic the mineral disturbances observed in CKD.

Culture of adult vessel rings in normal Ca-P medium did not affect Ca load in the vessel wall relative to baseline. As shown in Figure 7-5. The Ca load was significantly increased by the raised mineral content from 57.8±14.2 µg/ µg/µl in normal Ca-P medium to 1077±390.8 µg/ µg/µl in high Ca-P medium ($P<0.05$).

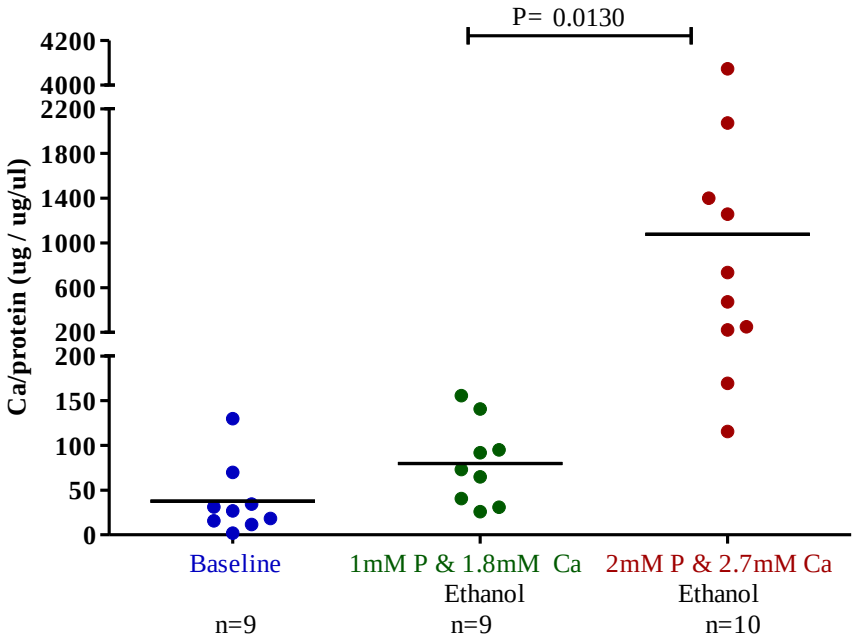


Figure 7-5: High Ca-P medium increased Ca load in vessel rings from adults with CKD.
Vessel rings harvested from adults with CKD were isolated at baseline or cultured for 14 days in either normal Ca-P medium (1mM P and 1.8mM Ca M199) or high Ca-P medium (2mM P and 2.7mM Ca) to induce calcification. Both were in the presence of 3.4mM ethanol, which will be used as the vehicle in this project. Ca load in the vessel wall analysed by σ-cresolphthalein assay. D'Agostino & Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined unpaired two-tailed t-test.

7.4.3 VDRAs had no effect on vascular calcification in vessel rings isolated from adults with CKD.

High Ca-P increased the Ca load in adult vessel rings. Next the effect of 10nM calcitriol and 10nM paricalcitol on Ca load were assessed using this *ex-vivo* adult vessel ring model in both normal Ca-P and high Ca-P medium.

In the presence of each of the VDRAs, high Ca-P medium significantly increased the Ca load in the vessel wall as shown in Figure 7-6. However, neither calcitriol nor paricalcitol had an effect on Ca load in the vessel wall in normal Ca-P or high Ca-P medium.

As with the baseline vessel rings the localisation of the Ca deposition was determined by von Kossa staining. In each vessel ring the Ca deposition was specifically in the tunica media. The von Kossa staining was semi-quantitatively scored (0, +, ++, +++, ++++) and assigned a colour dependent on the estimated percentage of tunica media positive for von Kossa, and therefore Ca deposition. This gives rise to the key in Figure 7-7. The Ca load determined by σ-cresolphthalein assay, and its corresponding colour for von Kossa positive areas were represented graphically in Figure 7-7 and summarised in Table 7-2.

After culture in normal Ca-P medium, 4 of the 11 vessel rings had small areas of von Kossa positivity. Culture in high Ca-P medium increased this to 8/10 vessels showing some von Kossa positive area with 4 of those having very dense von Kossa staining in the tunica media (Figure 7-7). The rings with a higher Ca load quantified by σ-cresolphthalein assay tended to be grouped as +++ or ++++ for on Kossa representing the highest density of von Kossa.

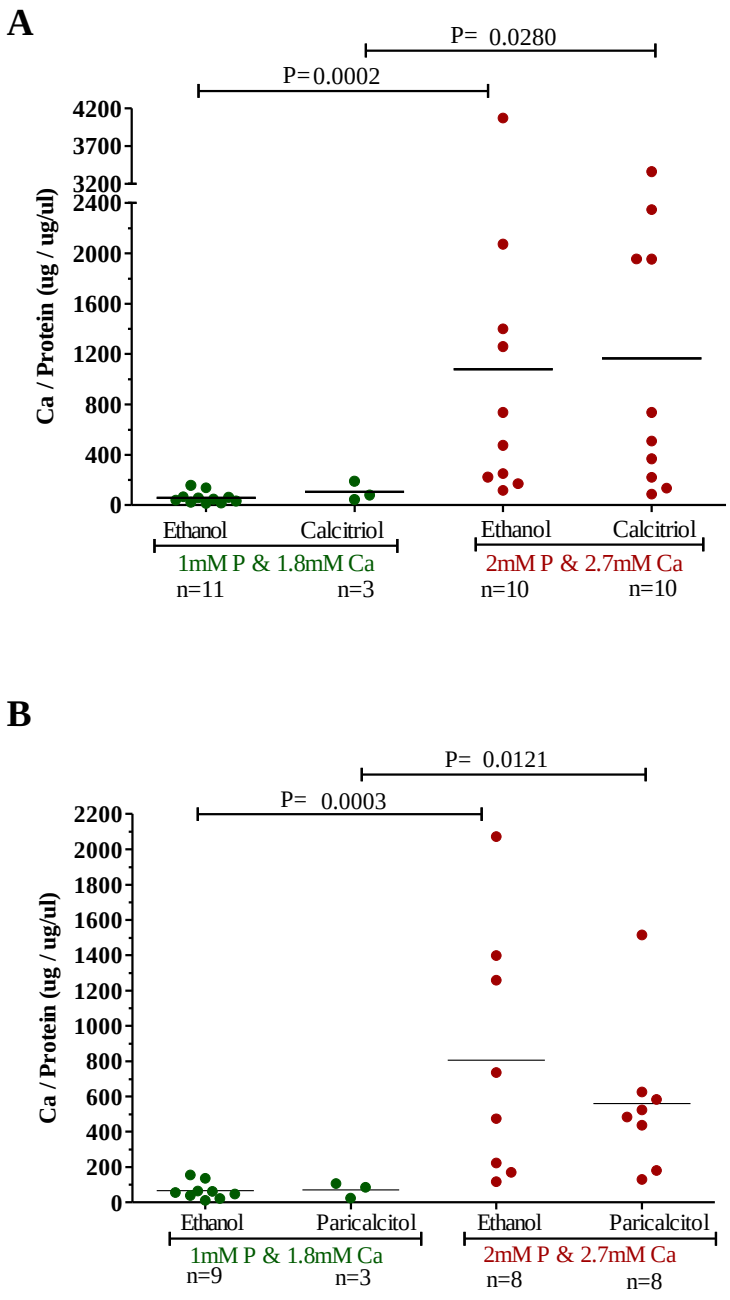


Figure 7-6: Ca load in vessel rings from CKD adults cultured with VDRAs.

Vessel rings isolated from adults with CKD were cultured in both normal Ca-P medium (1mM P and 1.8mM Ca) or high Ca-P medium (2mM P and 2.7mM Ca), as well as either the vehicle of 3.4mM ethanol or A) 10nM calcitriol, and B) 10nM paricalcitol. The different y-axis each graph accommodates the different calcification potentials of the two VDRAs. D'Agostino & Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by A) Two-tailed Mann Whitney test and B unpaired two-tailed t-test.

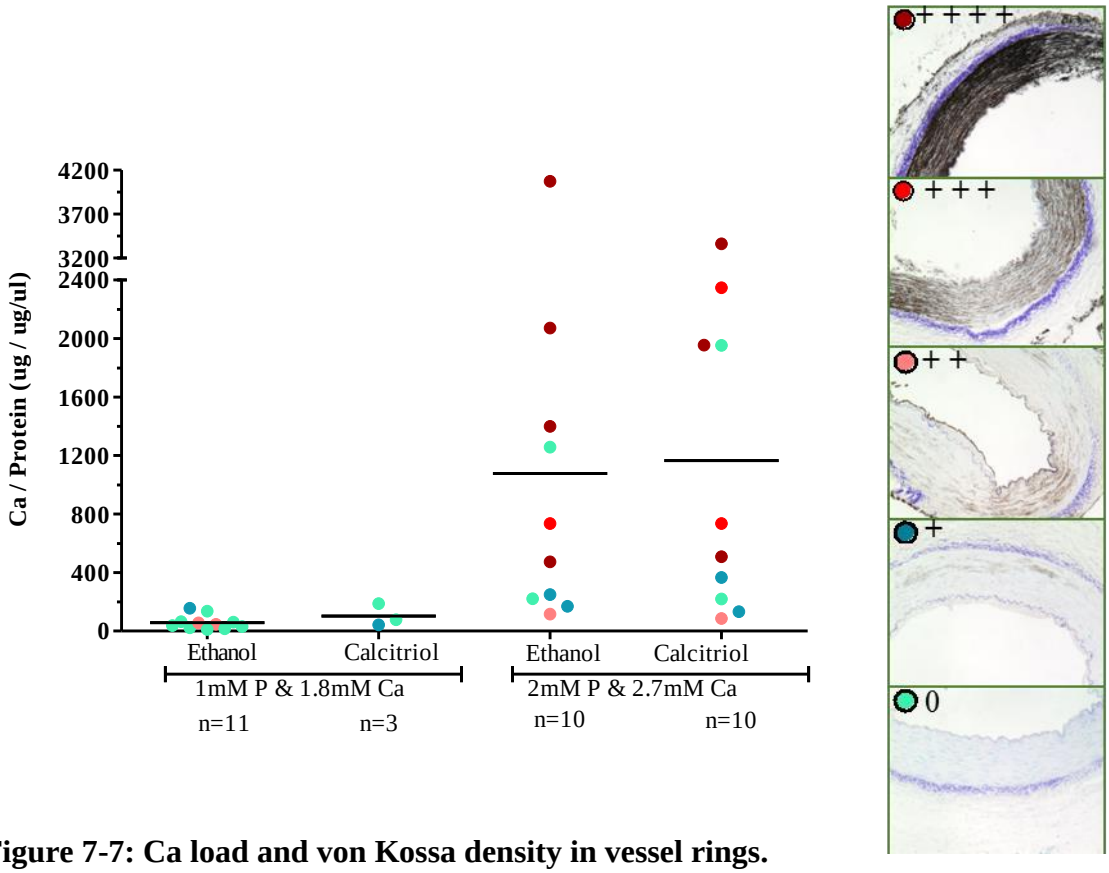


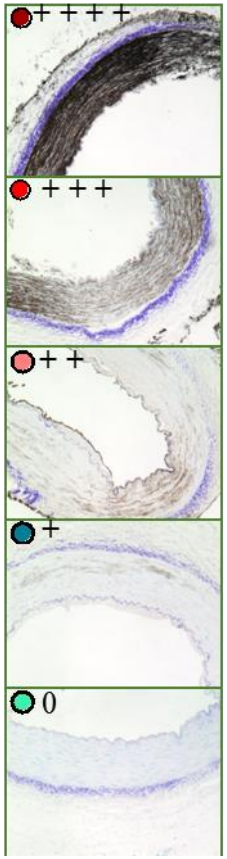
Figure 7-7: Ca load and von Kossa density in vessel rings.

Vessel rings isolated from adults with CKD were cultured for 14 days with 3.4mM ethanol or 10nM calcitriol, rings were harvested and their Ca load analysed by σ-cresolphthalein assay and Ca deposition visualised by von Kossa stain. Rings were colour coded dependent on their von Kossa positive area (black), a representative key is shown above. D'Agostino & Pearson omnibus normality test was performed as a guide to analyse the distribution of Ca load scores and statistical significance was determined by two-tailed Mann Whitney test (NS).

Table 7-2: Summary of pre-dialysis and dialysis adult's vessels Ca load.

Vessel IDs and their corresponding von Kossa score and Ca load determined by the σ-cresolphthalein assay. Data for each vessel shown at baseline and after treatment for 14 days in each condition.

	Vessel ID	Method		1mM P and 1.8mM Ca			2mM P and 2.7mM Ca		
			Baseline	Ethanol	Calcitriol	Paricalcitol	Ethanol	Calcitriol	Paricalcitol
Pre - Dialysis	13.45M.2.IE	Von Kossa	++	++			++++	+++	+++
		Ca load	26.7	64.9			115.6	85.9	130.0
	14.61M.11.IE	Von Kossa	+	0	+	+	+++	+++	++
		Ca load	18.2	25.9	42.3	24.3	735.9	2346.6	624.6
	14.35M.13.IE	Von Kossa	0	0	0	0			
		Ca load	34.4	95.1	78.3	105.1			
	14.27M.14.IE	Von Kossa	0	0		0	++++	++++	++++
		Ca load	15.6			46	4073.4	3361.4	
Dialysis	13.46M.1.IE	Von Kossa	++	++			++++	+++	+++
		Ca load	69.8	40.7			1399.2	735.4	483.0
	13.57F.3.IE	Von kossa	0	+			+	+	0
		Ca load		30.9			249.5	365.9	
	13.19F.4.IE	Von Kossa	0	0			0	0	0
		Ca load	1.8	73.1			1257.8	1953.5	5067.5
	13.31M.6.IE	Von Kossa	0	0			+	+	+
		Ca load	31.2	91.9			169.5	132.5	582.4
	13.67F.7.IE	Von Kossa	0	0	0		0	0	0
		Ca load	130.0	140.7	187.3		221.8	218.7	180.1
	14.45F.12.IE	Von Kossa	0				++++	++++	++++
		Ca load	11.5				5980.0	1954.9	523.8
	14.71M.15.IE	Von Kossa		+	+	+	++++	++++	++++
		Ca load		155.5		84.7	473.4	508.6	437.3



7.4.4 VDRAs had no effect on ALP activity in adult vessel rings.

This chapter so far has shown that neither calcitriol nor paricalcitol had any significant effect on Ca load in adult vessel rings. Next I considered whether the VDRAs had an effect on ALP activity, indicative of osteogenic transformation in the vessel rings. The osteogenic enzyme ALP is normally expressed in bone and is a marker of increased osteoblast activity.

As shown in Figure 7-8 culture of vessel rings in high Ca-P medium did not increase ALP activity relative to that observed in normal Ca-P medium. Furthermore, neither calcitriol nor paricalcitol had an effect on ALP activity in adult vessel rings.

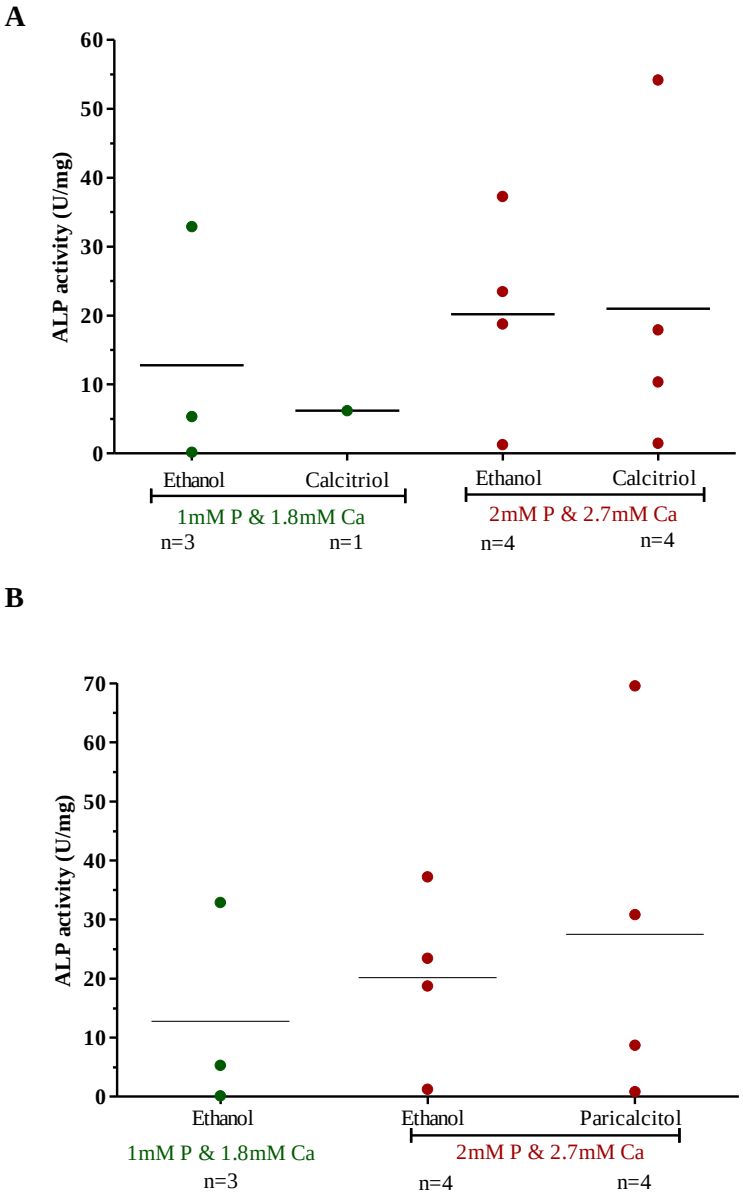


Figure 7-8: ALP activity in adult vessel rings.
Vessel rings were isolated from adults with CKD. Rings were cultured for 14 days in either normal Ca-P medium with 1mM P and 1.8mM Ca or high Ca-P medium with 2mM P and 2.7mM Ca to induce calcification. Rings were cultured with A) calcitriol or B) paricalcitol. Vessel rings were homogenised and the ALP activity in the vessel wall was determined by a colorimetric assay. Statistical significance was analysed by unpaired two-tailed t-test (NS).

7.5 The effect of patient related variables on Ca load in adult vessels.

The previous section showed that neither 10nM calcitriol or 10nM paricalcitol had an effect on mean Ca load in adult vessel rings. As discussed previously there are many patient variables to be considered when analysing this data. The following section will take into consideration whether patients are pre-dialysis or dialysis, their prescribed vitamin D supplements, age, gender, primary renal diagnosis and history of hypertension or other cardiovascular events.

7.5.1 There was no difference in Ca load in adult vessel rings from pre-dialysis and dialysis patients.

The Ca load as determined by σ-cresolphthalein assay was compared between pre-dialysis (n=4) and dialysis (n=7) vessel rings. No difference in Ca load was found either at baseline, in normal Ca-P medium or in high Ca-P medium. The effect of VDRAs on Ca load in pre-dialysis and dialysis vessels was also considered and no difference was found between them after either calcitriol or paricalcitol treatment.

The duration which dialysis patients had been receiving their renal replacement therapy was between 1 and 48 months, this was taken into consideration and no correlation was found between time on dialysis and Ca load at baseline or after any treatment condition. Further variation arises in the mode of dialysis given, patients were either receiving haemodialysis (n=3) or peritoneal dialysis (n=4). No significant difference in Ca load was found between the two modalities at baseline or after treatment of vessel rings.

7.5.2 Prior cholecalciferol supplements increased Ca load in response to calcitriol.

CKD patients are often deficient in vitamin D and are prescribed supplements to compensate for this. In this study it was important to consider if vitamin D supplements *in-vivo* altered the response of *ex-vivo* vessel rings to VDRAs in culture. Two different vitamin D supplements were prescribed in this cohort, cholecalciferol and alfacalcidol as shown in Table **7-1**.

Cholecalciferol supplements had no effect on baseline Ca load in the adult vessels or on Ca load after culture in normal Ca-P medium. There was a trend (NS) for patients on cholecalciferol supplements to have a higher Ca load in their vessel rings cultured in high Ca-P medium alone or with paricalcitol. As shown in Figure 7-9B cholecalciferol supplements prescribed *in-vivo* did significantly increase the Ca load in CKD vessel rings cultured in high Ca-P medium with calcitriol ($p=0.0103$). Vessel rings from patients not given cholecalciferol (n=5) had a Ca load of 336.2 $\pm$ 123.9 µg / µg/µl after culture in high Ca-P with calcitriol, while those treated with cholecalciferol (n=6) had vessel Ca loads of 1996 $\pm$ 482.1 µg / µg/µl *ex-vivo.*

Some patients were prescribed alfacalcidol either instead of or as well as 25OHD. The Ca load in patients that were prescribed alfacalcidol (n=6) or not (n=5) was compared. Alfacalcidol prescribed *in-vivo* had no effect on Ca load either at baseline or after culture in normal Ca-P or high Ca-P medium with or without calcitriol or paricalcitol (data not shown).

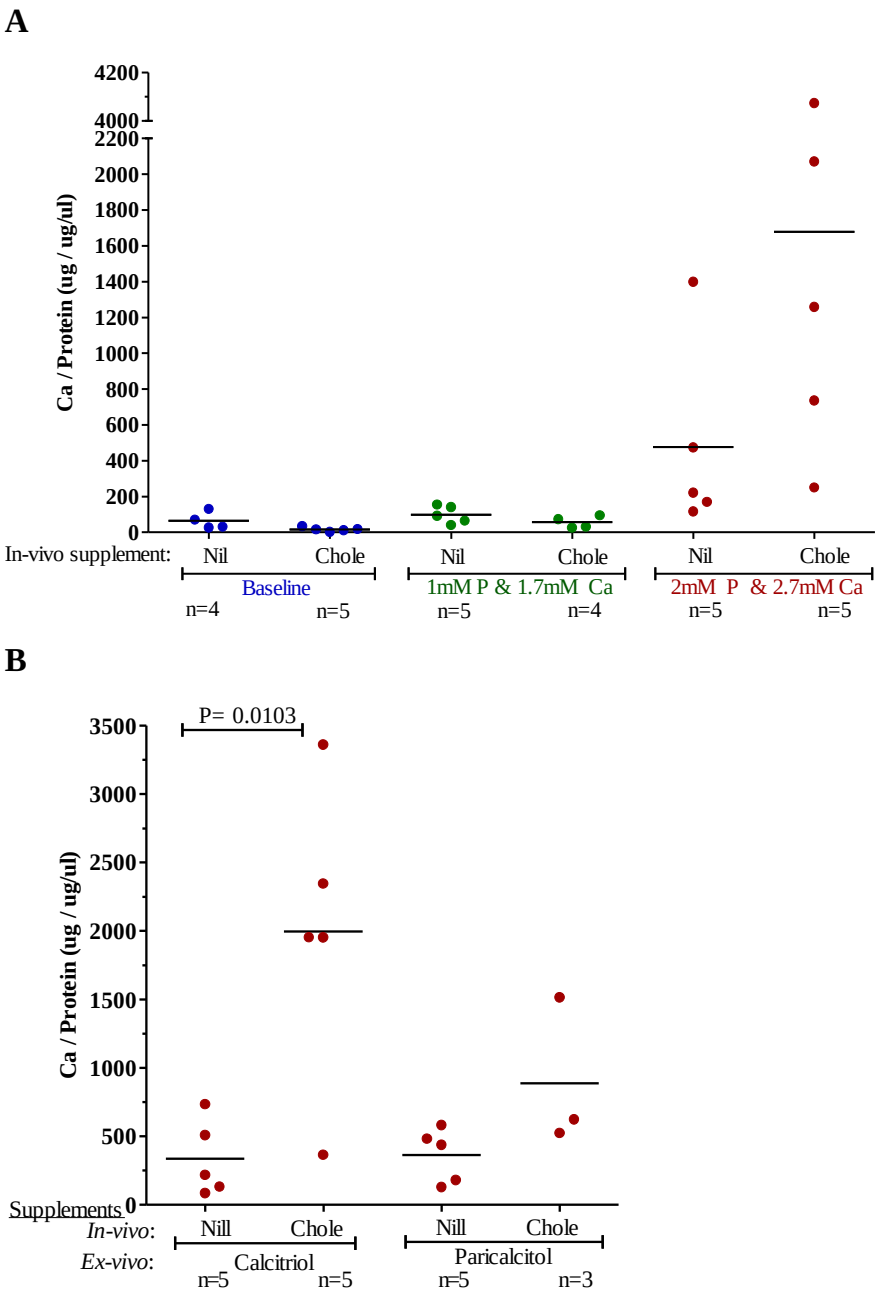


Figure 7-9: Comparison of Ca load in vessel rings from patients receiving 25OHD supplements or not.

Vessel rings were isolated from adults with CKD, some who were receiving cholecalciferol supplements as part of their routine treatment and others who were not. Ca load in the vessel walls were analysed by σ-cresolphthalein assay. **A)** At baseline and after culture for 14 days in either normal Ca-P medium (M199 with 1mM P and 1.8mM Ca) or high Ca-P medium (M199 with 2mM P and 2.7mM Ca) to induce calcification. **B**) Rings cultured in high Ca-P medium with calcitriol or paricalcitol. Statistical significance was determined by unpaired two-tailed t-test. Chole – cholecalciferol supplements.

7.5.3 No other patient variables had an effect on Ca load in adult vessels.

Other patient variables that were considered and found to have no significant effect on Ca load either after culture in normal Ca-P or high Ca-P medium only or with calcitriol or paricalcitol included:

Age: Despite the positive correlation between age and Ca load at baseline, there was no relationship between age and Ca load after culture in any of the treatment conditions.

Primary renal diagnosis: There was no difference in Ca load between patients that were diagnosed with glomerulopathy (n=6) compared to patients with other diagnosis (n=5).

Hypertension: There was no difference in Ca load between patients who had a history of hypertension (n=7) compared to those that had not (n=4)

Other cardiovascular events: No statistical analysis was performed as only 2 patients had suffered a cardiovascular event such as stroke, ischaemic heart disease or peripheral vascular disease.

Gender: There was no difference in Ca load between males (n=7) and females (n=4).

7.5.4 Low VDR expression in adult vessel rings.

Earlier in this chapter it was shown that neither calcitriol nor paricalcitol had an effect on the mean Ca load in adult VSMCs. This differs from results in Chapter 3 where a subgroup of children's vessel rings responded to calcitriol in high Ca-P medium and an increase in Ca load was observed. As discussed in the introduction and in chapter 6, VDRAs such as calcitriol act via the nuclear VDR to elicit a response. In the following section, expression of the VDR was analysed in adult vessel rings.

Immunohistochemistry staining for the VDR in adult vessel rings was not able to detect any VDR positive nuclei in any vessel rings either at baseline or after culture in any of the treatment conditions. Therefore, expression of the VDR in adult vessel rings was below detectable levels by immunostaining, an example is shown in Figure 7-10A. A positive control of VDR immunostaining in children's vessel rings is shown in Figure 7-10B. A further positive control in Figure 7-10D shows that adult vessel rings were successfully immuno-stained with other antibodies, here the tunica media but not the adventitia is positive for SMA.

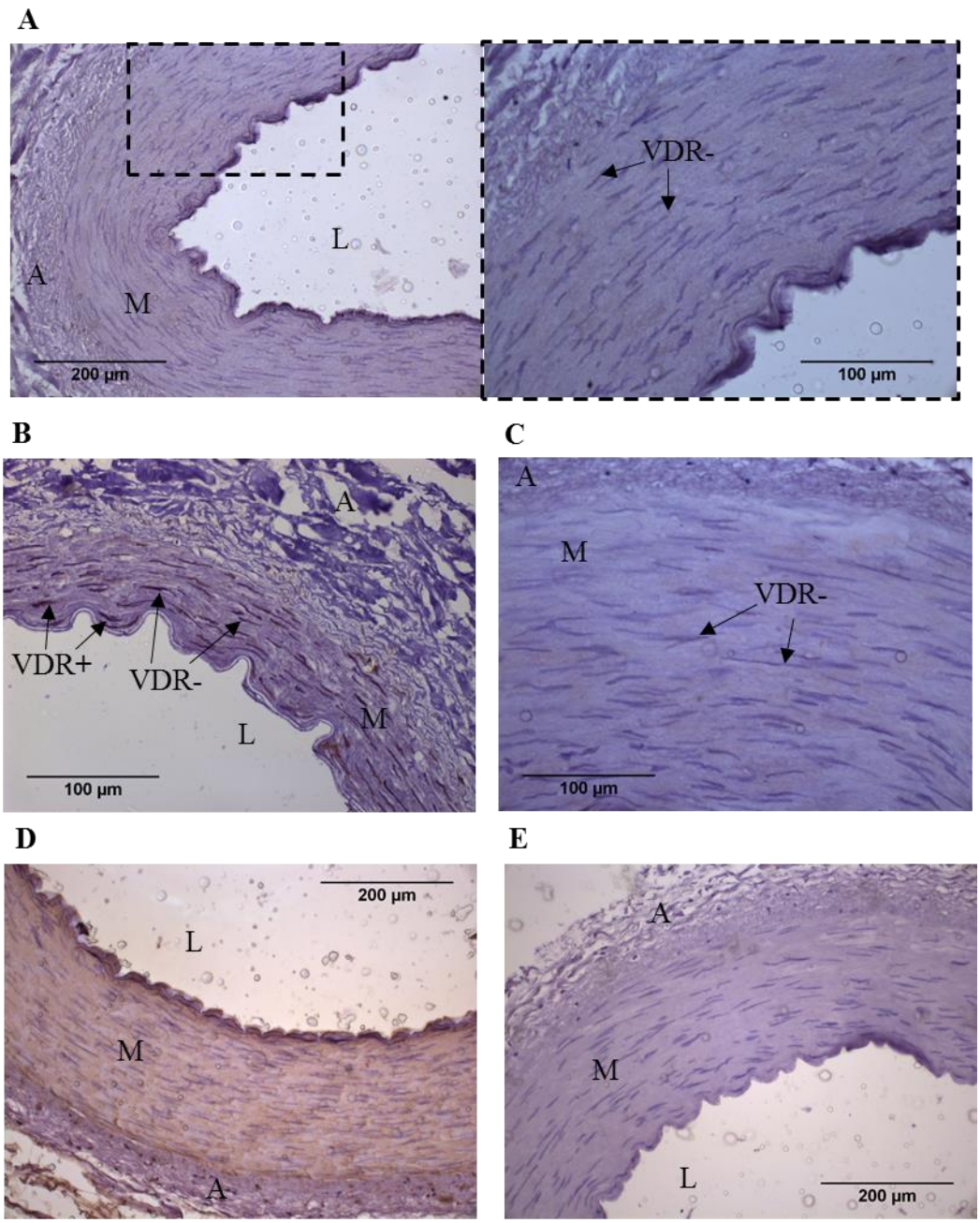


Figure 7-10: VDR not expressed at detectable levels in adult vessel rings.

Vessel rings from inferior epigastric arteries were formalin fixed, sectioned and immune-stained with A,B,C) VDR. A) Adult vessel 14.45F.12.IE at baseline (20x and 40x). B) Positive control: Children's vessel 07.6M.44.IE at baseline (40x). C) Negative control, no primary antibody: Adult vessel 14.45F.12.IE at baseline (40x). D) SMA immune-stain of 14.45F.12.IE (20x) and E) negative control for SMA (no primary antibody) 14.45.12.IE (20x) A: Adventitia. L: Lumen. M: Tunica Media. VDR+: VDR positive nuclei. VDR-: VDR negative nuclei.

7.6 Discussion

7.6.1 Summary

The VDRAs calcitriol and paricalcitol had no overall effect on Ca load in the adult vessel rings, they did not accelerate the progression of vascular calcification in these vessels.

The key findings were:

1. Physiological doses of calcitriol and paricalcitol had no effect on vascular calcification in vessels from adults with CKD.

2. Vessel rings from pre-dialysis and dialysis patients were equally susceptible to vascular calcification.

3. Variability in patient gender, age, primary renal diagnosis and previous cardiovascular health had no effect on calcification in response to VDRA treatment. Vessel rings from patients receiving cholecalciferol supplements had a higher Ca load after treatment in high Ca-P with calcitriol than patients not prescribed cholecalciferol.

4. Calcitriol and paricalcitol had no effect on activity of the osteoblast enzyme ALP in vessels from adults with CKD.

7.6.2 Baseline vessel characteristics and survival in culture.

This was the first time that vessel rings from adult patients have been cultured *ex-vivo*. Histological analysis of the vessel rings at baseline showed that each patient's vasculature was in general good health and no atherosclerotic regions were observed in the 11 vessels studied. This was unexpected as adults are at increased risk of atherosclerosis due to their prolonged exposure to traditional cardiovascular risk factors. Age itself also predisposes patients to atherosclerosis, which is supported by

CT studies on ancient human mummies. These mummies while never exposed to modern cardiovascular risk factors, and presumably without CKD, their odds of increased severity of atherosclerosis still increased by 70% every decade lived (Thompson et al., 2013). The incidence of atherosclerosis observed in this study of *ex-vivo* vessel rings from a section of inferior epigastric artery was 0% (n=11) compared to (Thompson et al., 2013) who found 34% of mummies had atherosclerosis. They did not analyse atherosclerosis in the inferior epigastric artery, instead they examined 5 different vascular beds per mummy which may account for the higher incidence of atherosclerosis recorded. The inferior epigastric artery may be less prone to atherosclerosis than other vascular beds.

Despite the lack of calcified atherosclerotic regions in patient's vessels, there was varying levels of Ca deposition in the tunica media at baseline identified by von Kossa positive regions and semi quantitatively analysed as ranging from 0 to ++. Medial calcification is characteristic of Monckeberg sclerosis and is associated with CKD.

A modern, cross sectional study of 150 patients that used radiography to measure intimal and medial calcification (Gelev et al., 2008) found that males and patients over 55 years had a higher frequency of arterial calcification. Although I found no difference between genders, a positive correlation between age and Ca load at baseline was observed in the adult vessel rings.

After 2 weeks in culture in normal Ca-P medium, adult vessel rings showed no apoptotic or necrotic regions, and continued to express SMA demonstrating for the first time that it is possible to study vessel rings from adult patients of all ages in culture for 2 weeks.

7.6.3 Physiological doses of the VDRAs, calcitriol and paricalcitol, have no effect on vascular calcification in vessels from adults with CKD.

In adult vessel rings neither paricalcitol nor calcitriol had a significant effect on calcification, there was no change in either mean Ca load or ALP activity. For paricalcitol this reflects the effect observed in children's vessel rings however the effect of calcitriol in adults differs from children's vessel rings. In adult vessel rings,

calcitriol had no effect on the mean Ca load whereas it increased Ca load in children's vessel rings. Calcitriol had no effect or a minor protective effect in some adult vessels and slightly increased calcification in others, leading to either low or high Ca loads in the calcitriol treated vessels. There may be a set of vessel ring responders and non-responders to calcitriol as observed in the children's vessel rings, however in this case it is less defined.

Interestingly the VDR expression in all adult vessels was either undetectable or sparse as shown by immunohistochemistry, this differed from the children's vessel rings where the VDR was detected by the same method. It has been shown previously that the VDR expression was reduced with advancing age in human skeletal muscle biopsies (Bischoff-Ferrari et al., 2004). A reduced expression of VDR in adult VSMCs could prevent the VDRAs having an effect on calcification.

7.6.4 Patient related factors and their effect on calcification of cultured adult vessel rings.

At baseline and after each treatment condition there was a wide range of Ca loads observed, this may be explained by the high degree of patient variation. The effect of patient variation was minimised by comparing vessel rings from the same patients in each treatment condition, therefore each vessel acts as its own control. In addition, the implication of patient related factors on response to treatment was taken into consideration.

Adult patients varied in the vitamin D supplements prescribed, some were given cholecalciferol either alone or in combination with alfacalcidol. Cholecalciferol is easily absorbed from the small intestine lowering the risk of hypercalcaemia (Brandi and Minisola, 2013). At baseline there was no difference in Ca load between vessels from patients prescribed cholecalciferol and those that were not. This agrees with findings from a randomised trial in Germany on 105 adults that found no difference in cardiovascular risk including markers of vascular calcification (Runx 2, osteocalcin and osteopontin) between groups given either 20µg cholecalciferol per day or a

placebo (Seibert et al., 2015). In addition, (Gelev et al., 2008) found no association between vitamin D supplementation and presence of calcification in their cross sectional study of 150 patients. Interestingly after culture in high Ca-P medium with calcitriol the Ca load was significantly higher in patients that were prescribed 25OHD relative to those who were not. This suggests that although 25OHD does not affect baseline Ca load it may prime the adult vessels to be more susceptible to calcitriol induced calcification.

As discussed in Chapter 3 it has previously been documented that patients on dialysis are more prone to vascular calcification than pre-dialysis CKD patients, in this study of adult vessel rings no difference was found between pre-dialysis and dialysis vessels in any treatment conditions.

The correlation between age and Ca load did not persist after culture in either normal Ca-P or high Ca-P medium, this suggests that any effect of culture must strongly outweigh the effects of aging on Ca load.

7.6.5 Limitations

This chapter focuses on vascular calcification in whole vessel rings from adult patients with CKD. As with the children's vessels and any human tissue, it is a scarce resource but incredibly valuable in providing a clinically relevant model for translational research. This creates its own challenges and data in this chapter are collected from very few vessels (11 in total) with high patient variability. Vessels were collected from both male and female patients with a wide age range of 19-71 years, some patients had a past medical history of hypertension or other cardiovascular events. Patients had different primary renal diagnosis and were either pre-dialysis or had a dialysis vintage of between 1 and 48 months. In addition, patients were on different combinations of vitamin D supplements at the time of vessel collection. Unfortunately, I was unable to collect data on patients' smoking history therefore could not take into consideration its impact on vascular health.

In this adult cohort no patients had co-morbidity with diabetes, this is atypical of the general adult CKD population, as diabetes is the leading cause of CKD in adults and 40% of people with diabetes also have CKD (Van Huffel et al., 2014).

The length of inferior epigastric artery that could be dissected during renal transplant determined how many 1mm vessel rings were obtained for *ex-vivo* culture, this was the limiting factor in determining the number of *ex-vivo* treatment conditions. Unfortunately, there was insufficient tissue to test the effects of the 3 different VDRAs used in the treatment of children's vessels. Calcitriol treatment was prioritised as this is the natural active form of vitamin D therefore an important reference point for comparing other VDRAs. For clinical use it is important to identify a VDRA that does not cause calcification in vessels. Alfacalcidol increased Ca load in the children's vessels where as paricalcitol did not, based on this data paricalcitol would be favoured for clinical use therefore testing its effect on calcification in adult vessels was prioritised over alfacalcidol.

It can be assumed that calcitriol and paricalcitol were biologically active as they provoked the responses observed in the previous chapters. It would be of interest to confirm that the VDRAs were able to evoke a response in adult vessel rings, CYP24A1 is well documented as being upregulated in response to vitamin D therefore an upregulation of CYP24A1 protein shown by immunohistochemistry would confirm their activity in the adult vessel rings.

Here for the first time adult vessel rings were cultured *ex-vivo*, histological analysis suggests that they remained viable for 2 weeks in culture. In future it would be of interest to quantify VSMC viability in the vessel rings by β-glucuronidase assay.

Unfortunately, I was unable to obtain vessels from non-renal controls. As with the children's vessels, VSMCs were explanted from the vessel rings however only 2 of the vessels successfully established VSMC cultures. Therefore, further molecular analysis on VSMCs explanted from adults with CKD was not performed.

Chapter 8 Final Discussion

The effects of VDRAs on vascular calcification in CKD has emerged as a controversial topic in the literature, with different models and experimental designs showing opposing effects of VDRAs on calcification. This thesis focussed on a clinically relevant, *ex-vivo* model of intact vessel rings obtained from children or adults with CKD, as well as the *in-vitro* study of VSMCs explanted from the children's vessels. Calcification was induced with high Ca-P medium to mimic the dysregulated mineral metabolism observed in CKD and vessel rings were treated with the VDRAs, calcitriol, alfacalcidol or paricalcitol which are routinely used in clinical practice.

In the children's vessel rings, alfacalcidol and to a greater extent calcitriol increased Ca load whereas paricalcitol had no effect on Ca load. 25-hydroxylase, required for the activation of alfacalcidol was expressed in VSMCs, therefore it was assumed that alfacalcidol was functioning in its active, di-hydroxylated form. Despite calcitriol naturally being in its active di-hydroxylated form, there was no correlation between the Ca load in vessel rings cultured in high Ca-P medium with calcitriol and alfacalcidol suggesting that they were acting via different mechanisms. This was also evident as calcitriol but not alfacalcidol promoted vascular calcification in the vessel rings through increased ALP activity and reduced cell viability.

The VDR is a transcriptional regulator, therefore to further understand the mechanisms of action of VDRAs on vascular calcification I examined the expression of VDR downstream genes in the explanted VSMCs. Physiological doses of both calcitriol and paricalcitol had a protective effect on Ca load in the VSMCs, however neither of them had an effect on expression of the calcification inhibitor MGP. Nor did physiological doses of calcitriol or paricalcitol affect expression of osteoblast markers Runx2 or ALP. Both VDRAs increased expression of CYP24A1 mRNA, required for their own inactivation. Further work is required to understand the mechanism by which 10nM calcitriol and paricalcitol had a protective effect on Ca load in this model of explanted VSMCs. A potential mechanism is that VDRAs upregulate expression of the CaSR and increased sensitivity to Ca therefore protecting against calcification, this was recently observed in human VSMCs at a narrow concentration range (Mary et al., 2015).

My data showed that the effects of VDRAs on calcification was not comparable between the different experimental models of *ex-vivo* children's vessel rings and explanted VSMCs, despite the fact that they were both harvested from the same cohort of paediatric patients. This is most notable with 10nM calcitriol which reduced Ca load in explanted VSMCs but increased Ca load in vessel rings. Furthermore, 10nM calcitriol increased ALP activity in the vessel rings but had no effect on ALP in VSMCs in which 100nM calcitriol was required to increase ALP activity. I also observed that the effect of paricalcitol was different between the 2 experimental models, it had no effect on Ca load in the vessel rings, whereas it was protective of calcification and reduced Ca load in VSMCs. The different characteristics of the 2 models may give rise to the different responses observed in response to VDRAs. Explanted VSMCs lose their contractility in culture and develop a synthetic phenotype similar to that observed in dialysis VSMCs. In contrast, VSMCs in the vessel rings maintain their contractile phenotype in culture and have their adventitia and internal lamella intact therefore closely resemble *in-vivo* vessels.

In both the children's vessel ring and the VSMC model variation was observed between individual patients in their response to VDRAs. Despite there being no difference between vessel rings from pre-dialysis and dialysis patients in their Ca load in response to calcitriol, paricalcitol or alfacalcidol, there was patient variation within the whole CKD cohort. Children's vessel rings naturally segregated into those that responded to 10nM calcitriol treatment with an increased Ca load and those that did not respond showing no change in Ca load. There was no difference in SMA, ALP or total VDR expression between responders and non-responders however they did differ in their expression profile of 24 hydroxylase which is highly regulated by the VDRE.

The dose dependent effect of calcitriol was studied in control and dialysis VSMCs, the physiological dose of 10nM calcitriol was protective of calcification. In addition 1nM and/or 100nM calcitriol also reduced Ca load in VSMCs from some individuals. These doses were less effective than 10nM calcitriol at reducing Ca load and the Ca load to calcitriol dose response curve produced a U-shaped. Individual patient variation was observed within both control and dialysis VSMCs and the U-shaped dose response curve shifted to either the left or the right in some patients.

Control and dialysis VSMCs differed in their baseline mRNA expression profiles with dialysis VSMCs having a higher expression of osteoblast markers ALP, BSP and Runx2 than control VSMCs, along with a lower expression of SMA and other smooth muscle cell markers. Despite this indication of osteogenic conversion in the dialysis VSMCs, there was no difference between control and dialysis VSMCs' Ca load in response to high Ca-P, calcitriol or paricalcitol. There was no difference between control and dialysis VSMCs in the expression of total VDR mRNA or total VDR protein. However, 2 isoforms of the VDR protein were expressed in the VSMCs. Control and dialysis VSMCs differed in the ratio of the VDR-A and the N terminally extended VDR-B isoforms expressed at baseline, with dialysis VSMCs expressing a higher proportion of the VDR-A isoform. Calcitriol dose dependently increased expression of the total VDR mRNA and caused a shift in the ratio of VDR protein isoforms expressed. More specifically, it reduced the expression of the VDR-B isoform and increased expression of the VDR-A isoform in both control and dialysis VSMCs. I speculate that the administration of VDRAs *in-vivo* may cause a similar shift in the ratio of the VDR isoforms expressed, this would account for the higher ratio of VDR-A expressed in dialysis VSMCs relative to control VSMCs as CKD patients on dialysis were all prescribed the VDRA alfacalcidol.

There is still much to learn in order to understand the functional significance of the VDR-A and VDR-B isoforms, current literature suggests that the relative transcriptional activation capacity of the VDR-A and the VDR-B isoform is dependent on the cell type and cellular cofactors available (Esteban et al., 2005, Gardiner et al., 2004). Control and dialysis VSMCs differed in the extent of their upregulation of downstream genes in response to calcitriol: there was a greater upregulation of both ALP mRNA expression and activity in control VSMCs, whereas there was a greater upregulation of CYP24A1 mRNA expression in dialysis VSMCs. CYP24A1 is well documented as being highly regulated by VDRAs, it encodes the 24-hydroxylase enzyme for the inactivation of VDRAs. The increased responsiveness of dialysis VSMCs to upregulate CYP24A1 could suggest that they are primed to respond to VDRA treatment, including the shift in expression of VDR isoforms and being more efficient at inactivating VDRAs. This leads to the relatively limited response in the upregulation of ALP in comparison to control VSMCs. Despite these differences in control and dialysis VSMCs, there is much overlap between the 2 groups in terms of

the ratio of their VDR isoform expression as well as their responses to calcitriol. The VDRE luciferase assay showed that an equal number of control and dialysis VSMCs were able to activate the VDRE in this system. Those that activated the VDRE luciferase expressed a higher fraction of the VDR-A isoform, supporting the theory that the ratio of VDR isoforms expressed is functionally significant and this hypothesis should be explored further.

The patient variation in the ratio of VDR isoforms expressed, suggests that there is not a single solution for the optimal vitamin D treatment regime for all patients. Individualised treatments based on the patients VDR expression profile may be required, this would be complex and require continuous monitoring as once VDRAs are prescribed, a shift to favour expression of the VDR-A isoform may be observed. For now, the differential expression of VDR isoforms has only been observed *in-vitro*, therefore is far from being translated directly into patients.

Due to the limited tissue availability it was outside the scope of this thesis to explore the ratio of VDR isoforms expressed in the CKD vessel rings. In future work it would be of great interest to compare the VDR isoform expression profile in children's vessel rings that either responded or did not respond to calcitriol. Sufficient tissue would be required to extract protein for western blot analysis. As the VDR-B isoform is a 50 amino acid N-terminal extension of the shorter VDR-A isoform, it would also be of interest to produce a VDR-B specific antibody for both western blot analysis and immunohistochemistry comparison of responder and non-responder vessel rings. Furthermore, if sufficient tissue could be acquired for RNA extraction, the mRNA expression profile of the different VDR isoforms could be investigated using primers specific to their unique first exons. This would be of great interest in both the VSMCs and the vessel rings to determine if the different VDR isoform expression profiles observed, were a consequence of a transcriptional or a post transcriptional modification.

A third model of vessel rings from adult CKD patients was studied in this thesis. Despite the adult vessel rings surviving in culture and having an increased Ca load when exposed to high Ca-P medium, their Ca load did not respond to calcitriol treatment. This differs from analysis of Ca load in the children's vessel rings where a

group of non-responders and responders emerged, where responders to calcitriol had an increased Ca load. Immunohistochemistry analysis of total VDR expression showed that the VDR was not expressed at detectable levels in the adult vessel rings, whereas the VDR was detected in the majority of VSMC nuclei in the children's vessel rings. Advancing age and a decline in VDR expression may play a key role in the lack of response to calcitriol observed in the adult vessel rings. We must bear in mind that this is a small cohort of only 11 adult vessels, this may represent a group of non-responders. With a larger cohort a group of responders may also emerge, as was observed in the children's vessel ring model. The paediatric patients with CKD were all prescribed alfacalcidol whereas the adults were prescribed either alfacalcidol, cholecalciferol or a combination of both. Vessel rings from those patients given cholecalciferol alone or in combination had a higher Ca load in high Ca-P with calcitriol than patients not receiving cholecalciferol, this is further evidence that prior *in-vivo* administration of VDRAs may alter the response of these vessels to subsequent treatment. CKD is a chronic disorder and patients are currently prescribed VDRAs on a long term basis to help maintain homeostatic levels of PTH and mineral metabolism. It is therefore important that future work considers not only the short term but also the long term effects of VDRAs on the susceptibility of vessels to calcification.

This thesis addressed the hypothesis that VDRAs play a key role in driving vascular calcification in vessels from CKD patients and the differential expression pattern of the VDR can determine the extent of mineralisation in an *in vitro* and *ex vivo* model. The 2 main conclusions from this thesis are;

1. VDRAs can have either a protective, causative or null effect on vascular calcification in CKD, this response is highly complex and is dependent on the model studied (children's vessel rings, adult vessel rings or explanted VSMCs), as well as the type of VDRA (calcitriol, paricalcitol or alfacalcidol), and the VDRA concentration (as demonstrated with 1nM, 10nM and 100nM calcitriol).

2. VSMCs were shown to express 2 different isoforms of the VDR, VDR-A and VDR-B. The ratio of these expressed isoforms differed between control and

dialysis VSMCs, was altered by exposure to calcitriol and was functionally important for the activation of the VDRE in the luciferase promoter.

There is a vast collection of previous literature that use different models and show contradictory results in the study of the effects of VDRAs on vascular calcification in CKD. This thesis focused on the clinically relevant model of intact vessel rings from CKD patients as well as VSMCs explanted from them. Within each of these models a high degree of patient variation was observed that could not be accounted for by any of the patient characteristics analysed. This patient variation in the effect of VDRAs on calcification may arise from differences in the ratio of VDR isoforms expressed in each patient. I have demonstrated that the ratio of VDR isoform expression is functionally important for activation of the VDRE luciferase. It would be of great interest in future studies to compare the effects of VDR-A and VDR-B activation on vascular calcification. This could determine if the ratio of these isoforms expressed in a patients' vessels is a predictor of whether VDRAs will have a protective or causative effect on calcification, and patients' individual treatment courses could be adapted appropriately.

Chapter 9 References

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Chapter 10 Appendix

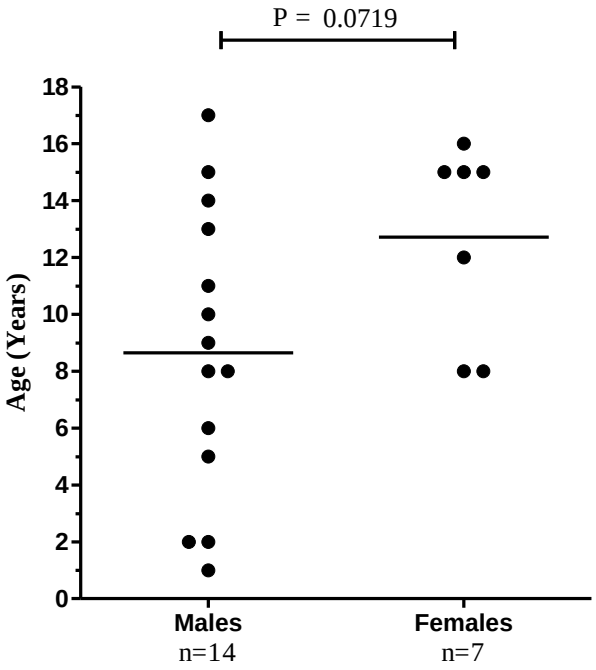

Figure-10-1: Is there an age difference between the CKD children's male and female cohorts?

Vessel rings were isolated from both male and female children with CKD, the age distribution for each gender was compared. Statistical significance was determined by two-tailed unpaired t-test.

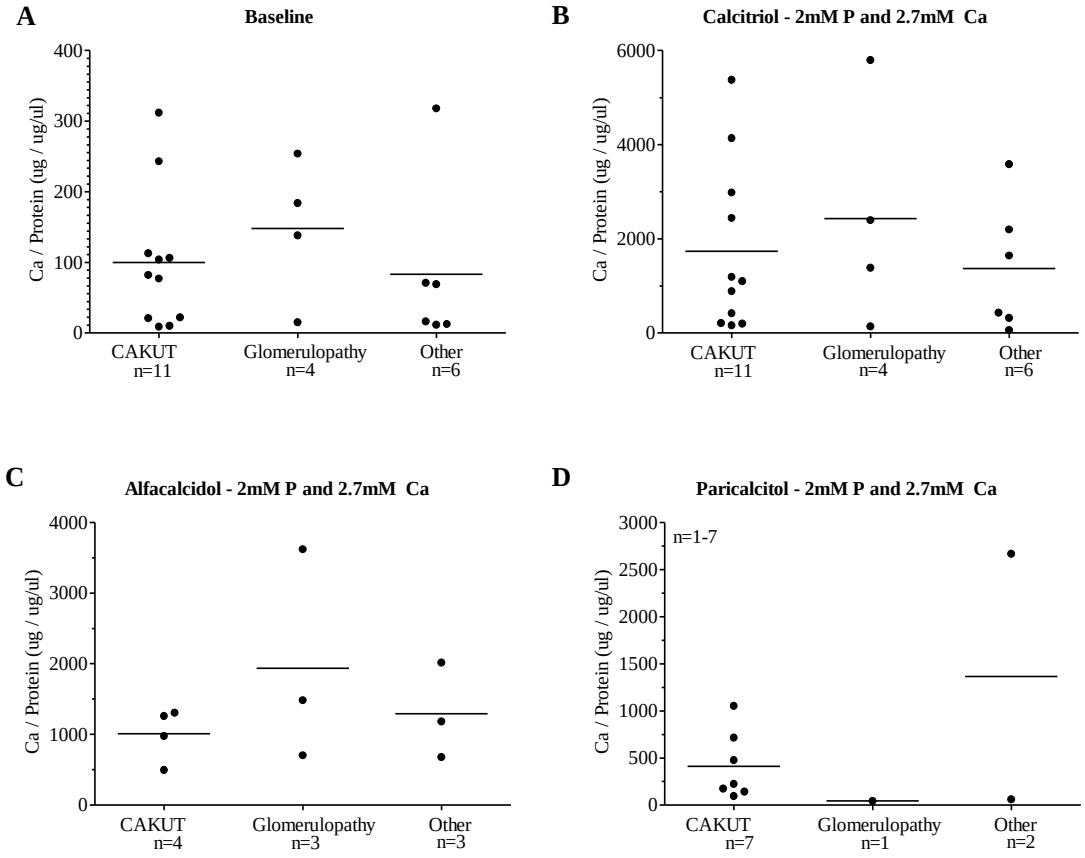

Figure -10-2: Effect of primary renal diagnosis on Ca load.

Vessel rings were isolated from CKD children with different primary renal diagnosis, grouped as CAKUT (congenital anomalies of the kidneys and urinary tract), glomerulopathy or other. A ring was taken at A) baseline or cultured for 14 days in high Ca-P medium (M199 with 2mM P and 2.7mM Ca) to induce calcification and cultured with B) calcitriol, C) alfacalcidol or D) paricalcitol. Ca load in the vessel wall analysed by cresolphthalein assay. D'Agostino & Pearson omnibus normality test was performed as a guide to analyse the distribution and statistical significance was determined by A, B, C) Kruskal Wallis test with Dunn's multiple comparison post hoc test. No statistical significance. D) n numbers too small for statistical analysis.

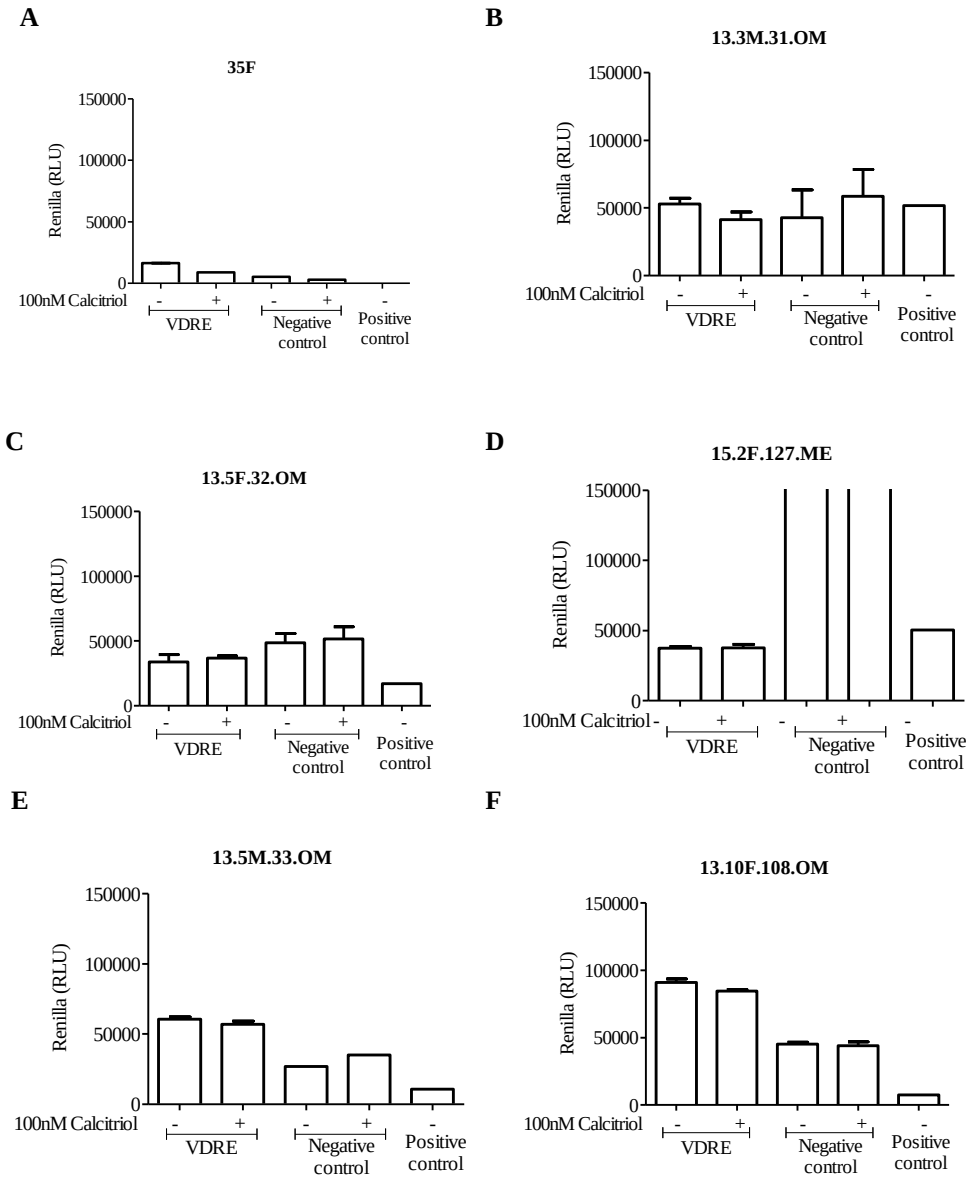

Figure 10-3: Transfection control: Renilla activity in control VSMCs

VSMCs were transfected in triplicate wells with the constitutively active Renilla luciferase under a cmv promoter as a transfection control for Firefly luciferase (either VDRE, negative control or positive control constructs). Post transfection VSMCs were treated with or without 100nM calcitriol to induce the VDRE- Firefly luciferase.

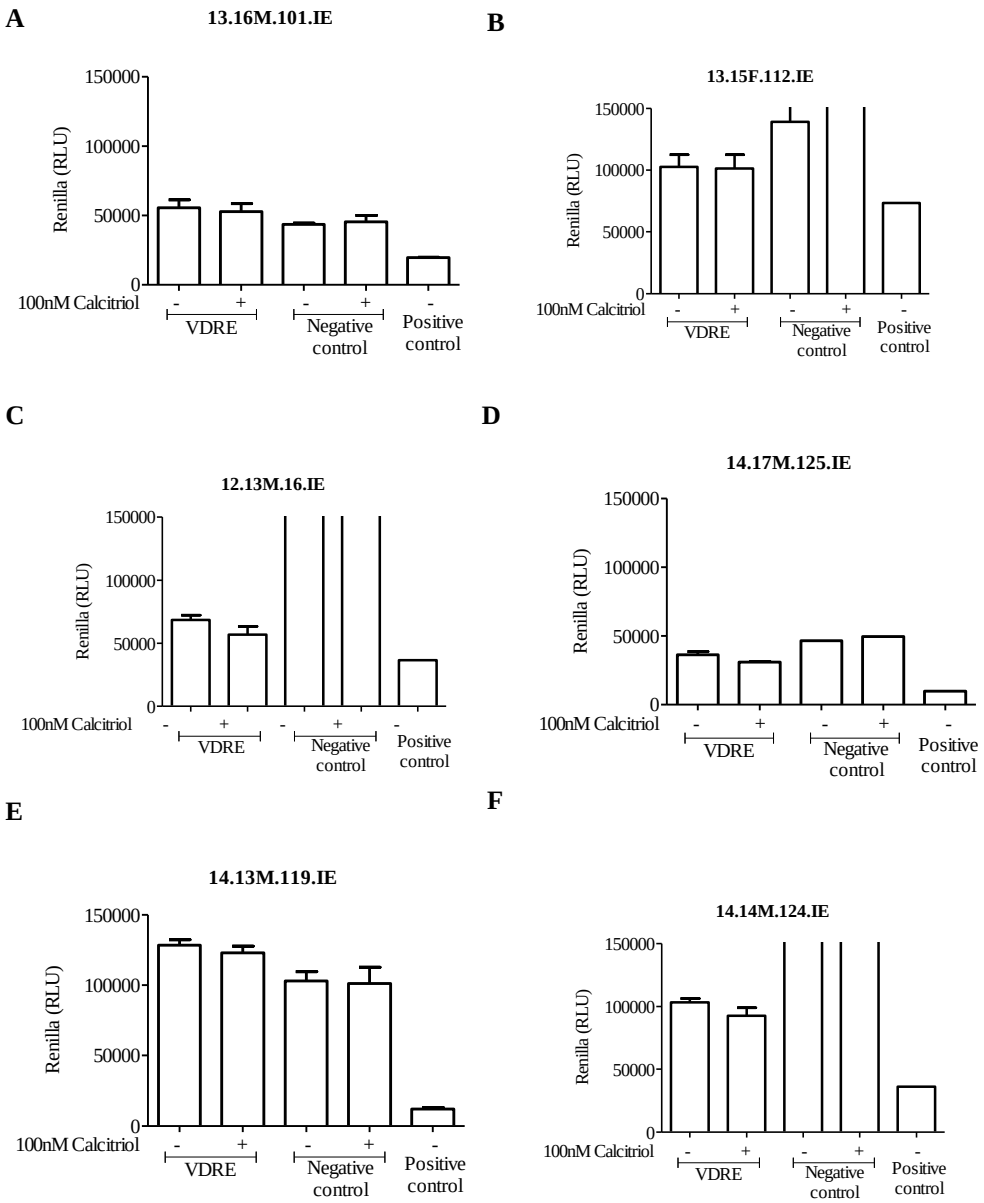

Figure 10-4: Transfection control: Renilla activity in dialysis VSMCs.

VSMCs were transfected in triplicate wells with the constitutively active Renilla luciferase under a cmv promoter as a transfection control for Firefly luciferase (either VDRE, negative control or positive control constructs). Post transfection VSMCs were treated with or without 100nM calcitriol to induce the VDRE- Firefly luciferase.