Uraemic Vascular Damage and Calcification in Children with Chronic Kidney Disease

Presented for the degree of Doctor of Philosophy to

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Declaration

The work reported in this dissertation was undertaken at the Great Ormond Street Hospital and Institute of Child Health, London and the Division of Cardiovascular Medicine, University of Cambridge.

This dissertation describes the outcome of my own work, none of which has been submitted for a degree, diploma or other qualification here, or at any other university. Any work done in collaboration and all help that I have received in the course of this thesis is specified in the text and acknowledgements.

Signed .....

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SUMMARY OF THESIS

Cardiovascular disease is the most common cause of death in patients with chronic kidney disease. Structural and functional vascular abnormalities and arterial calcification begins early in the course of renal decline and can be found even in children, contributing to their high mortality risk. Through clinical and laboratory studies, this thesis sought to investigate the causes of uraemic vascular damage and calcification in children with chronic kidney disease and on dialysis.

Dysregulated mineral metabolism, manifested by hyperparathyroidism and high phosphate, in association with low vitamin D levels, is key to the pathophysiology of ectopic vascular and soft tissue calcification. In addition, a number of treatment-related factors can potentially lead to a high calcium load, contributing to an increased risk of calcification. Importantly, these are modifiable risk factors and have been associated with an increased mortality risk in adult dialysis patients.

Using established surrogate measures of vascular damage, carotid artery intima media thickness, pulse wave velocity and multi-slice CT scan, I have studied a cohort of children on chronic dialysis, and shown that those with mean parathyroid hormone levels above twice the upper limit of normal had increased vascular thickness, stiffer vessels and a higher prevalence of coronary artery calcification, whereas those with lower levels had vascular measures that were similar to age-matched controls. Also, a higher vitamin D dosage was associated with thicker vessels and coronary calcification. To explore this association, in a further study I have measured the levels of 25-hydroxy and 1,25-dihydroxy vitamin D and shown that both low and high levels of 1,25-dihydroxy vitamin D are associated with thicker vessels and calcification.

Also, 1,25-dihydroxy vitamin D showed a strong inverse association with high sensitivity CRP, and we speculate that vitamin D's influence on calcium-phosphate homeostasis and inflammation may be lead to this bimodal effect. Levels of the circulating calcification inhibitors, fetuin-A, osteoprotegerin and Matrix Gla-protein, may influence an individual patients' susceptibility to calcify, and but have not been described in children. I found that these levels influenced vascular stiffness and calcification, and that there may be a protective upregulation of fetuin-A in the early stages of exposure to a pro-calcific and pro-inflammatory uraemic environment.

In a subsequent translational study I have sought to find direct evidence of vascular damage and calcification in the vessels. Using intact human arteries removed at the time of routine surgery, I have shown that calcium accumulation begins pre-dialysis, but dialysis induced vascular smooth muscle cell apoptosis coupled with osteo/chondrocytic transformation and a loss of the normal calcification inhibitors leads to overt calcification. Our currently available clinical measures are not sensitive enough to detect the earliest stages of calcification. On *in vitro* culture in calcifying media, dialysis but not control vessels showed accelerated time-dependent calcification, suggesting that these vessels had lost their smooth muscle cell defence mechanisms and were primed to undergo rapid calcification. Apoptotic cell death was a key event that triggerred calcification, and this was a vesicle mediated process, possibly involving oxidative DNA damage.

This thesis investigates the role of modifiable risk factors in uraemic vascular damage and calcification in children with CKD and explores the earliest changes in the pathophysiology of uraemic medial calcification in intact human vessels.

ABREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AIx	augmentation index
Ca	calcium
CaSR	calcium sensing receptor
cIMT	carotid intima-media thickness
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease – mineral and bone disorder
eGFR	estimated Glomerular Filtration Rate
FGF-23	Fibroblast Growth Factor 23
HD	haemodialysis
hs-CRP	High sensitivity C reactive protein
KDIGO	Kidney Disease Improving Global Outcomes
NKF-K/DOQI	National Kidney Foundation - Kidney Disease Outcome Quality Initiative
MGP	Matrix γ-carboxy glutamic acid protein
OPG	Osteoprotegerin
PD	peritoneal dialysis
PO_4	phosphate
PTH	parathyroid hormone
iPTH	intact parathyroid hormone
PWA	pulse wave analysis
PWV	pulse wave velocity
RANK	receptor activator of nuclear factor-κB
RANK-L	receptor activator of nuclear factor κβ ligand
RRT	renal replacement therapy
SDS	standard deviation scores
VSMC	vascular smooth muscle cells

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Chapter 1

General Introduction

Introduction

Since the inception of paediatric dialysis programmes approximately 40 years ago, there have been vast improvements in both technology and expertise in the care of these patients. Nevertheless, children on dialysis continue to have an unacceptably high mortality, and cardiovascular disease is the most common cause of death in this population. Calcification of blood vessels is a significant contributor to the cardiovascular risk: it begins early in the course of renal decline and is present even in children and young adults. Epidemiological data and observational studies have consistently shown that dysregulated mineral metabolism is central to the ectopic calcification process, and most importantly, is a modifiable risk factor. Furthermore, we now know that vascular calcification is not mearly a passive process in dead or dying cells, but a highly regulated cell-mediated process that involves a complex interplay between promoters and inhibitors of calcification. Identifying potentially modifiable damage-inducing agents in the uraemic milieu and understanding their role in the pathophysiology of vascular calcification may allow us to inhibit progression or even induce regression of existing vascular injury.

In this chapter I have discussed the epidemiology, risk factors, clinical studies and *in vitro* and animal studies investigating the pathophysiology of ectopic vascular calcification that have contributed to our current knowledge and generated the research questions addressed in this thesis.

Section I – Definitions and risk factors for vascular calcification in chronic kidney disease

1.1 Definitions: Chronic kidney disease – mineral and bone disorder (CKD-MBD)

With the growing awareness that mineral dysregulation in CKD is closely linked to abnormal bone pathology, and that these in turn lead to extra-skeletal calcification, the KDIGO (Kidney Disease Improving Global Outcomes) have proposed a broad and encompassing term chronic kidney disease - mineral and bone disorder (CKD-MBD) to describe this clinical entity *(Moe at al, 2006)*.

CKD-MBD is defined as a systemic disorder of mineral and bone metabolism that is manifested by either one or a combination of the following:

- Abnormalities of calcium, phosphorus, PTH, or vitamin D metabolism
- Abnormalities in bone turnover, mineralization, linear growth, or strength
- Vascular or other soft tissue calcification

A proposed framework for classifying CKD-MBD divides patients into four types based on the presence or absence of abnormalities in the three primary components used in the definition of the disorder: laboratory abnormalities, bone disease, and calcification of extraskeletal tissue (Figure 1.1).

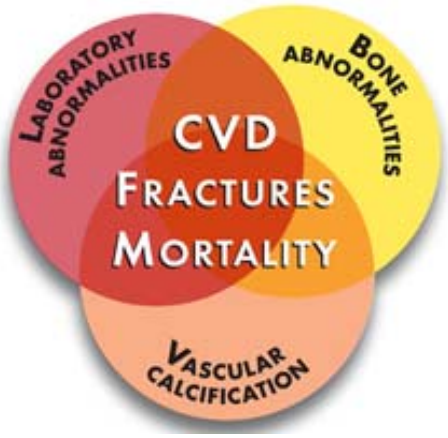


Figure 1.1 – The spectrum of abnormalities described in the KDIGO definition of CKD-MBD.

Adapted from the KDIGO website http://www.kdigo.org/clinical-practice-guidelines.

Also, KDIGO have recommended that the term renal osteodystrophy, that has been used traditionally to describe the abnormalities in bone morphology in renal disease, should be used exclusively to define the bone pathology associated with CKD. Thus, they have defined renal osteodystrophy as an alteration of bone morphology in patients with CKD. It is quantifiable by histomorphometry of bone biopsy, and it is suggested that the results should be reported based on a classification system that includes parameters of turnover, mineralization, and volume *(Moe et al, 2006)*.

1.2 Epidemiology of cardiovascular disease in CKD patients

A seminal paper by Foley et al drew the attention of the medical community to the very high rate of cardiovascular deaths in patients on dialysis *(Foley et al, 1998)*. This epidemiological study compared the mortality of maintenance dialysis patients with that of age, gender and race matched healthy controls. The standardized mortality rate was described in deciles for age, and the authors showed that the mortality of young adults (25 – 34 years old) on dialysis was approximately 700-fold higher than age

related mortality and equivalent to that of an 80 year old (Figure 1.2). Other reports have confirmed these findings: the mortality from cardiovascular disease is 1,000 times more common in children with CKD stage V than in the general paediatric population, in which accidents are responsible for most deaths *(Parekh et al, 2002)*. Comparable figures for adults show a considerably higher mortality: 54% of adult dialysis patients die within 5 years of starting dialysis *(USRDS 2002)*, and adult patients are more likely to die of a cardiovascular event before there is even a need for renal replacement therapy *(Sarnak et al, 2003)*.

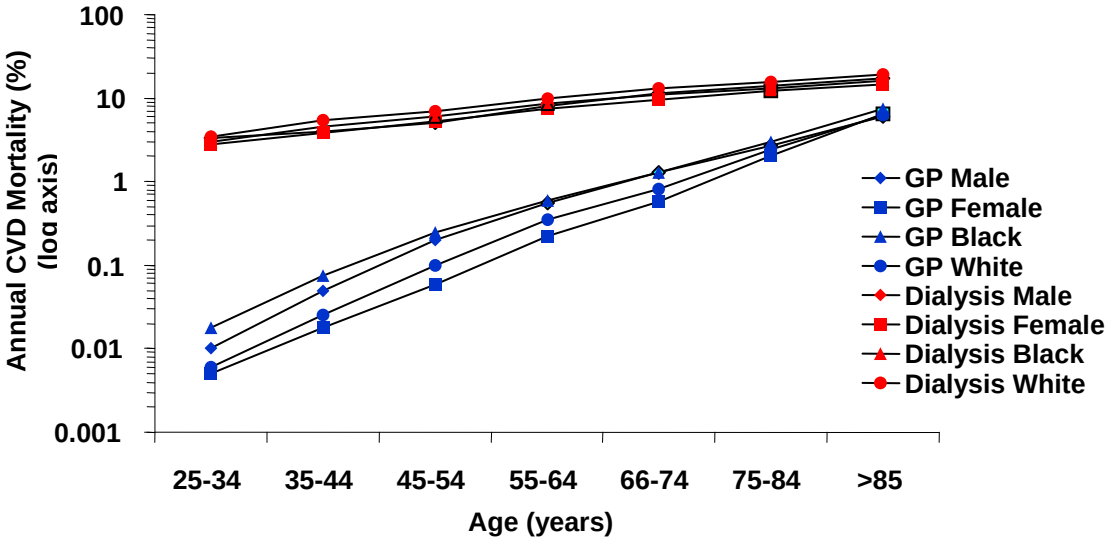


Figure 1.2 – Cardiovascular mortality in patients on dialysis is significantly higher than in the age, gender and race matched general population.
GP – general population

Adapted from Foley RN et al, Am J Kidney Dis, 1998.

Subsequently, several large national registries have published similar findings for paediatric dialysis recipients. The United States Renal Data Systems (USRDS) analyzed 1380 deaths over a 5 year period from 1990 – 1996 among patients who had started renal replacement therapy (RRT) as children and died before 30 years of age

(Parekh at al, 2002). 23% of all deaths were from cardiovascular causes, and deaths on haemodialysis (HD) were approximately twice as common as on peritoneal dialysis (PD) [49% vs 22% respectively]. The percentage of cardiac deaths varied by age: the 0 – 4 years, 5 to 9 years, 10 – 14 years, 15 – 19 years and 20 – 30 years age groups at the time of death accounted for 10.2%, 6.2%, 8.8%, 21% and 54% respectively. Also, this study showed that the mortality in dialysis patients was approximately 78% higher than that in transplant recipients.

The Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry has reported on mortality rates and causes of death in all children who received dialysis between 1963 and 2002, with a median follow-up of 9.7 years *(McDonald et al, 2004)*. Children on dialysis had a 4-fold higher mortality than renal transplant recipients. 45% of all deaths were due to cardiovascular disease, with 57% of deaths on HD and 43% on PD from cardiovascular causes. 25% were attributed to cardiac arrest (uncertain etiology), 16% to cerebrovascular accident, 14% to myocardial ischaemia, 12% to pulmonary oedema, 11% to hyperkalaemia and 22% to other cardiac causes.

The Dutch cohort study reported very similar findings: amongst children who received RRT between the ages of 0 to 14 years, after a median follow up of 8 years the standardized mortality rate was 31.0, and cardiovascular or cerebrovasular causes accounted for 24% of all deaths *(Groothoff et al, 2002)*. Interestingly, this study has also looked at the mortality rate of long-standing hypertension in children, and shown that children on long-term dialysis have almost double the mortality rate of this group, suggesting that factors other than hypertension and the ensuing left ventricular

hypertrophy contribute to a high cardiovascular mortality in dialysis patients. In a subsequent review, this group have suggested that left ventricular hypertrophy, aortic valve calcification and increased arterial stiffness, but not increased arterial intima media thickness are the most frequently observed alterations in young adult survivors of childhood onset CKD *(Groothoff et al, 2005)*. Importantly, this group did not include adolescents, the age-group with the highest proportion of cardiovascular deaths as reported by both the USRDS and ANZDATA, and may thus have lower reported mortality rates than other paediatric studies.

A large single-centre long term follow-up study from the Heidelberg group have shown that of the 283 children who received end-stage renal failure treatment between 0 – 14 years of age, there were 42 deaths, and approximately half of these were from a cardiovascular or cerebrovascular cause *(Oh et al, 2002,* Figure 1.3). Unfortunately, a large number of patients in this study were lost to follow-up, and the causes of death were not further evaluated.

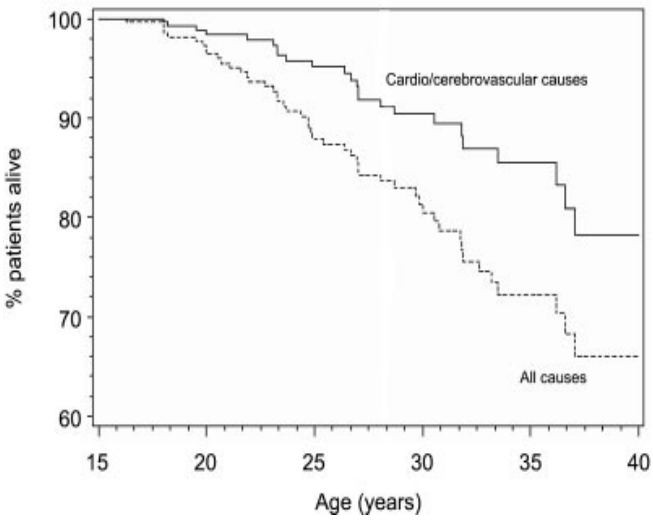


Figure 1.3 – The cardiovascular mortality in young adults who received end-stage renal disease treatment in childhood.
50% of deaths were from cardiovascular or cerebrovascular causes.

Broken line - survival rate considering all causes of death
Solid line, survival rate considering cardiovascular / cerebrovascular causes of death only.
Adapted from Oh et al, Circulation, 2002.

Cardiovascular morbidity in paediatric dialysis recipients

Chavers et al have used the large USRDS database to examine the incidence and extent of cardiovascular disease in incident paediatric (0 – 19 years) dialysis patients from 1991 to 1996 *(Chavers et al, 2002)*. 31% of the 1454 children developed a cardiac-related event. Arrhythmia was the most common (20%), followed by valvular heart disease (12%), cardiomyopathy (9%) and cardiac arrest (3%). 38% of the deaths during the study period were cardiac deaths. The incidence of valvular heart disease and arrhythmias was highest in the teenagers.

Mortality risk of peritoneal vs hemodialysis

The dialysis modality (peritoneal vs hemodialysis) has been shown to influence mortality. Foley et al compared the outcomes of 433 incident dialysis patients using intention to treat analysis, and found that there was no difference in the adjusted mortality rates for the first 2 years, but mortality amongst PD patients was significantly higher thereafter (PD/HD adjusted hazards ratio = 1.57 (95%CI 0.97 to 2.53). Interestingly, clinically symptomatic or echocardiographically proven cardiac disease was not responsible for this late mortality, but hypoalbuminaemia in PD patients accounted for a large proportion of the increase in mortality *(Foley et al, 1998).*

1.3 Cardiovascular disease begins early in the course of CKD

While it was previously believed that cardiovascular disease occurs only in the late stages of CKD and on dialysis, recent studies have shown disturbing evidence of its development even in very early CKD. In 1998 the National Kidney Foundation convened a task force on cardiovascular disease in CKD. It concluded that in terms of

risk stratification, individuals with CKD *per se* should be considered to be at very high risk from cardiovascular disease *(Levey et al, 1998)*. Go et al followed up over one million adults in a large community-based study for up to 4 years and showed that both the risk of death and the risk of cardiovascular events increased as the estimated GFR dropped below 60ml/min/1.73m^2 (*Go et al, 2004*; Figure 1.4). For eGFR levels of 45 – 60, the adjusted hazards ratio for death was 1.2, and increased to 1.8, 3.2 and 5.9 for eGFR levels of 30 – 45, 15 – 30 and < 15 ml/min/1.73m^2 respectively. Similarly, the adjusted hazards ratio for cardiovascular events also increased inversely with the eGFR: 1.4, 2.0, 2.8 and 3.4 for the above eGFR categories respectively. This independent and graded association between renal function and cardiovascular disease and death highlights the importance of recognising and controlling modifiable risk factors from the earliest stages of CKD.

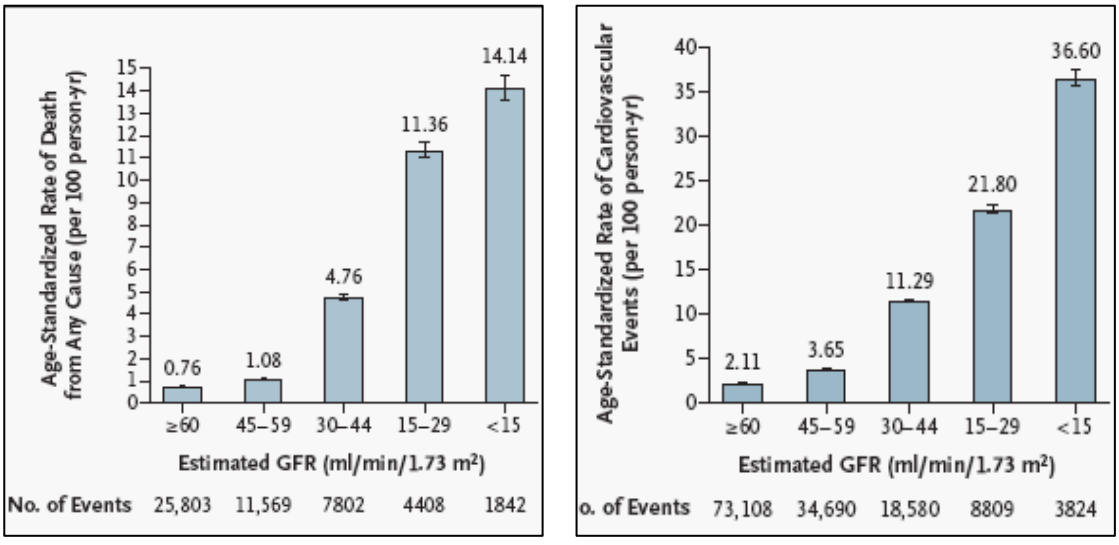


Figure 1.4 Age standardized rates of death (left) and cardiovascular disease (right) according to estimated GFR in a large population-based longitudinal study.
Adapted from Go et al, New England Journal of Medicine, 2004.

Other groups have shown similar associations between declining renal function and cardiovascular events in the original Framingham Heart Study cohort *(Culleton et al, 1999).* Similarly, Levin et al have shown that hyperparathyroidism and vitamin D deficiency begin early in the course of CKD *(Levin et al, KI, 2006)* and our group has shown that endothelial dysfunction, a surrogate marker of early cardiovascular disease, is present as early as the first decade of life in children with pre-dialysis CKD *(Kari et al, 1997)*. These studies have been discussed at length in later sections.

1.4 Risk factors for the development of cardiovascular disease

CKD patients have a higher prevalence of both the 'traditional' Framingham risk factors as well as non-traditional risk factors that increase their cardiovascular risk (Table 1.1). 'Traditional' risk factors have been defined and validated in the general population through prospective cohort studies, notably the Framingham cohort.

TABLE 1.1 Cardiovascular risk factors in chronic kidney disease

Traditional risk factors	CKD-specific risk factors
Old age	Abnormal Ca and PO_4 levels
Male gender	Abnormal PTH levels
Hypertension	Vitamin D deficiency
Diabetes	Anaemia
Higher total cholesterol	Extracellular fluid overload
Higher LDL cholesterol	Inflammation
Lower HDL cholesterol	Oxidative stress
Family history of cardiovascular disease	Perturbation in the circulating calcification inhibitors
Lipoprotein (a)	Albuminuria
Smoking	Hyperhomocysteinemia
Physical inactivity	Abnormal Fibroblast Growth Factor 23 (FGF-23)
	Malnutrition and hypoalbuminemia
	Altered nitric oxide / endothelin balance

'Traditional' risk factors

Not only are the 'taditional' cardiac risk factors, such as older age, dyslipidemia, hypertension, diabetes, and physical inactivity more prevalent in adults with CKD, but they are more likely to be clustered in these subjects. The Framingham coronary risk score has consistently underestimated the risk of cardiovascular events risk in both pre-dialysis *(Parfey et al, 1996; Sarnak et al, 2002)* and dialysis patients *(Longenecker et al, 2002)*, and it has been suggested that the 'traditional' risk factors may have a qualitatively and quantitatively different risk relationship with cardiovascular disease in CKD compared to the general population. Also, a

phenomenon of reverse epidemiology or risk factor reversal has been reported between body mass index *(Kalantar-Zadeh et al, 2003; Kopple et al, 1999)*, systolic and diastolic blood pressure *(Salem et al, 1999)*, serum total and LDL cholesterol levels *(Kalantar-Zadeh et al, 2004)* and the hazard ratio for morbidity or mortality in CKD patients. The relationship between these factors and the hazard ratio for mortality and morbidity vary from a change in the normal incremental linear relationship (e.g. blood pressure and mortality) to a 'J' shaped relationship, wherein low levels are also a mortality risk, to a complete mirror image reversal (e.g. that for body mass index and mortality risk). One of the major causes for this risk factor reversal may be the confounding effects of protein energy malnutrition and inflammatory disorders that are prevalent in maintenance dialysis patients *(Kalantar-Zadeh et al, 2003)*.

Unlike this plethora of risk factors in adults, children have considerably fewer cardiovascular risk factors. However, hypertension remains the single most prevalent and significant 'traditional' risk factor for left ventricular hypertrophy *(Mitsnefes et al, 2003; Mitsnefes et al, 2005)* as well as for vascular damage and re-modelling as measured by carotid artery ultrasound imaging *(Litwin et al, 2008)*. The UK Renal Registry report a high prevalence of hypertension and anaemia (~25% and 33% in prevalent dialysis patients respectively), but a very low prevalence of obesity, diabetes, and smoking in the UK paediatric dialysis population *(UK Renal Registry Report, 2006)*.

Uraemia-related risk factors

The non-traditional risk factors can be further divided into two groups: those that are recognized as cardiovascular risk factors in the general population but have a higher prevalence in CKD (e.g. hypertension) and those factors that are primarily present in CKD patients, such as anemia or elevated Ca x PO_4 product. Furthermore, there are a number of potential iatrogenic or treatment-related risk factors such as exposure to a high Ca load from dialysate, calcium-based phosphate binders and vitamin D therapy, advanced glycation end-products, metabolic acidosis and warfarin therapy that can all contribute to the pro-calcific uraemic milieu. Dysregulations in the Ca - PO_4 - PTH axis are central to the vascular damage and calcification in CKD patients.

1.5 Mineral dysregulation and the Ca - PO_4 - PTH axis in CKD

Normal regulation of the Ca - PO_4 - PTH axis

Serum free (ionized) Ca is required for many vital processes, such as myocardial and smooth muscle cell contractility and enzymatic reactions, and is tightly regulated by the calciotropic hormones PTH and vitamin D. Parathyroid hormone is synthesized in the parathyroid cells and stored in secretory granules, providing a reservoir of hormone that is available for immediate release into the blood. A decrease in the availability of either 1,25$(OH)_2$D or Ca promotes pre-pro-PTH gene transcription, whereas PTH synthesis decreases when 1,25$(OH)_2$D and /or Ca are abundant *(Rostrand et al, 1999)*. PTH release itself is regulated through a negative feedback mechanism via the Ca sensing receptor (CaSR) that is abundantly present on the

parathyroid gland and can detect even minute changes in the free serum Ca level *(Brown et al, 1991)*. The CsSR is a 1078-amino acid protein composed of seven membrane spanning segments and a long extracellular domain that contains clusters of amino acids that serve as binding sites for Ca. The receptor is coupled to G-proteins and is abundantly expressed in the plasma membrane of parathyroid cells. Increases in blood ionized Ca concentrations activate the CaSR, triggering a rise in cytosolic Ca concentrations through the release of Ca from the endoplasmic reticulum. This rapid rise in intracellular Ca transiently diminishes PTH release. Similarly, a fall in the plasma free Ca inactivates the CaSR and enhances PTH release *(Brown et al, 1995)*.

There is an inverse sigmoidal relationship between blood ionized Ca concentrations and the serum PTH level in subjects with normal renal and parathyroid gland function (*Ramirez et al, 1993*; Figure 1.5). Although minute to minute variations in PTH secretion are regulated by the CaSR, a component of PTH release by the parathyroid glands cannot be regulated by the CaSR, and as shown in Figure 1.5, basal amounts of PTH are released into the circulation even in the presence of high Ca levels.

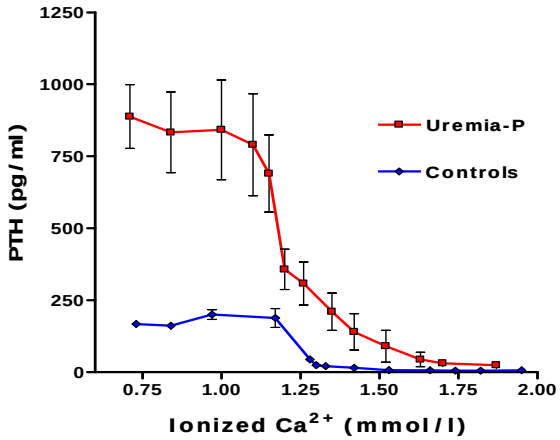


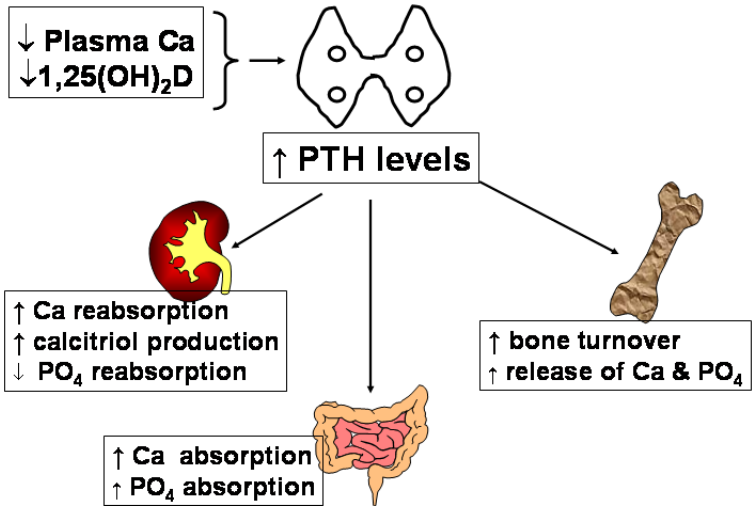
Figure 1.5 The relationship between blood ionized Ca levels and PTH in healthy subjects (blue line) and uraemic patients (red line).

In uraemia there is a shift to the right so that higher Ca levels are required to suppress PTH release.

Adapted from Lewin et al, KI,1997

The PTH receptors are present in the bone, gastrointestinal tract, kidneys and vasculature amongst other organ systems, and regulate the absorption of Ca from the gut, its reabsorption from the renal tubules and its mobilization from bone (Figure 1.6). Thus, the main stimulus for PTH release is low ionized Ca levels, and PTH release attempts to correct this by increasing the serum Ca. The half-life of circulating PTH is only approximately 10 minutes, as it is rapidly degraded by the liver and its target organs.

Figure 1.6 The effects of PTH on Ca homeostasis



Abnormal Ca - PO_4 – PTH regulation in uraemia

Several factors contribute to the secondary hyperparathyroidism of CKD, and the earliest amongst these are PO_4 retention and and reduced production of $1,25(OH)_2D$ by the failing kidneys. In a recent population-based study Levin et al showed that hyperparathyroidism and 25(OH)D and $1,25(OH)_2D$ deficiency begins at eGFR levels of approximately 40 – 50 ml/min/1.73m^2 *(Levin et al, 2006)*. In addition, with

advancing renal failure, both the number and the function of the CaSR are perturbed. Immunohistochemistry of hyperparathyroid tissue from CKD patients has shown that expression of the CaSR is reduced by 30 – 70% *(Kifor et al, 1996)*. *In vivo* studies of parathyroid function in patients with CKD stage V have shown that Ca regulated PTH release is altered, with higher Ca levels required to suppress PTH release *(Lewin et al, 1997;* Figure 1.5).

Skeletal resistance to the calcaemic actions of PTH further compromise the ability to maintain normal serum Ca levels in renal disease; higher serum PTH levels are required to elicit equivalent biological responses in patients with advanced CKD *(Massry et al, 1973)*. Abnormalities in vitamin D metabolism may account for some of these changes, but alterations in the vitamin D receptor expression may also contribute *(Cohen-Solal et al, 1991)*. Reductions in vitamin D receptor expression are well documented in secondary hyperparathyroidism *(Korkor et al, 1987)*, and this disrupts the normal feedback inhibition of pre-pro-PTH gene transcription by $1,25(OH)_2D$. Finally, expression of the PTH/PTH-related protein receptor is downregulated in renal failure, and this may contribute to the tissue resistance of PTH.

Long-standing secondary hyperparathyroidism leads to parathyroid hyperplasia. $1,25(OH)_2D$ is a potent inhibitor of cell proliferation, and reduced $1,25(OH)_2D$ production by the failing kidney as well as downregulation of vitamin D receptor expression within the parathyroid tissue may be an important determinant of the degree of parathyroid hyperplasia *(Szabo et al, 1989)*. Once established, parathyroid

enlargement is difficult to reverse because the rate of apoptosis in parathyroid glands is very low; the half – life of a parathyroid cell is estimated to be 30 years *(Parfitt et al, 1997)*. In addition to its effects on Ca and PO_4 homeostasis, PTH has a direct action on the vascular smooth muscle cells and the cardiac fibroblast, causing arteriolar thickening and myocardial fibrosis respectively *(Rostrand et al, 1999).*

Although a number of studies have shown the deleterious effects of high PO_4 levels, few have discussed the associations of PTH and mortality or surrogate cardiovascular end-points. Patients with higher PTH levels are also likely to have more advanced renal failure, have higher serum PO_4 levels and need larger doses of PO_4 binders, making it impossible to discern the true effect of any single parameter. While previous studies using time-dependent models only showed an association between low PTH levels and mortality *(Block et al, 2004; Avram et al, 2001; Panuccio et al, 2002)*, using time-dependent Cox-models with repeated measures, Kalantar-Zadeh et al have shown that there was a strong association between incrementally higher PTH levels and an increased risk of death, that was masked almost entirely by the case-mix characteristics of the population *(Kalantar-Zadeh et al, 2006)*. If this association is causal, it may explain why vitamin D analogues that lower PTH are associated with better survival outcomes *(Teng et al, 2003, Tentori et al, 2005)*. Also, it must be kept in mind that a proportion of the 'low PTH' values recorded in all of these studies are in patients who have undergone parathyroidectomies for tertiary hyperparathyroidism, and in whom cardiovascular damage from exposure to a prolonged period of high PTH and the ensuing mineral ion dysregulation has already occurred. The concept of 'optimal' PTH levels and the controversies surrounding this are discussed in section 1.8 in this chapter.

1.6 Phosphate is a uraemic toxin

Phosphorous retention and hyperphosphataemia have been recognised for many years as important factors in the pathogenesis of secondary hyperparathyroidism and central to the pathophysiology of ectopic calcification.

Phosphorus is an intracellular anion and its serum levels are a poor reflection of total body stores: approximately 85% of PO_4 is present in bone, 14% intracellularly and 1% extracellularly. The average diet contains 900 – 1400 mg/day of phosphorus, and the main route of excretion is via the kidneys. In the dialysis patient, PO_4 balance depends on its intake (minus PO_4 binding) and its removal by dialysis (although residual renal function may contribute). The fractional PO_4 intestinal absorption is ~65 – 80%, depending on serum PO_4 and calcitriol levels. The K/DOQI guidelines recommend restricting dietary PO_4 intake to 800 – 1000mg/day in CKD stage V *(K/DOQI, Bone and Mineral metabolism guidelines, 2003)*, but limiting PO_4 intake can also result in restricting protein intake.

The major hormones regulating PO_4 metabolism are PTH, vitamin D and FGF-23. As described above, PTH inhibits renal PO_4 reabsorption in the renal tubules, but indirectly increases PO_4 absorption by stimulating the synthesis of $1,25(OH)_2D$. In turn, PO_4 retention and hyperphosphataemia promote PTH secretion through several mechanisms:

- excess amounts of inorganic phosphorus form complexes with free Ca ions and thus lower the blood ionized Ca levels

- Large amounts of transepithelial PO_4 transport in the proximal tubule can impair renal 1-alphahydroxylase activity thus reducing active $1,25(OH)_2D$ production *(Parfitt et al, 1997)*
- PO_4 can directly enhance PTH synthesis by the parathyroid cells through a post-transcriptional mechanism *(Denda et al, 1996)*

The regulation of PO_4 homeostasis in the kidney occurs primarily in the proximal tubule, with approximately 85% of the filtered PO_4 reabsorbed via the Na-P cotransporter IIa located in the proximal brush border membranes. Also, as renal failure progresses, levels of the phosphaturic hormone Fibroblast Growth Factor 23 (FGF-23) increase in an attempt to increase PO_4 excretion *(Schiavi et al, 2004)*.

Since the kidneys are the main route of PO_4 excretion, hyperphosphataemia in the anuric dialysis patient is extremely common and difficult to manage. PO_4 removal during dialysis is limited largely to the intracellular location of most inorganic phosphorus, and depends on PO_4 transfer from different body compartments, particularly bone (~350mg/day). The amounts removed by conventional thrice-weekly HD, ~800mg/treatment or 2400mg/week, or by daily PD, 300 – 400 mg/treatment or 2100 – 2800mg/week, are far less than ingested by most patients, 800 – 1200mg/day or 5600 – 9600mg/week. PO_4 removal during dialysis is time-dependent and only long daily or nocturnal HD can achieve normal PO_4 levels. Thus, limiting dietary PO_4 intake and use of PO_4 binders are the first steps in a PO_4 control strategy.

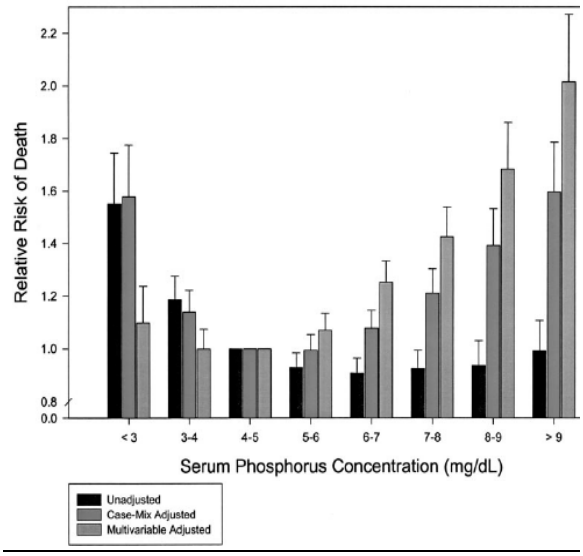
Block et al first reported that elevated PO_4 levels are an independent risk factor for increased mortality in adult dialysis patients (Figure 1.7; *Block et al, 1998)*: in a

cohort of >6400 prevalent HD patients, as serum PO_4 levels increased above 5.6mg/dL (= 1.8 mmol/L) the hazards ratio for mortality increased by 6% for every 1mg/dL increase in serum PO_4. Two studies have shown that PO_4 is an independent risk factor for death in the pre-dialysis population, and stressed the importance of maintaining normal levels even in pre-dialysis patients. Kestenbaum et al have measured serial serum creatinine levels in over 7000 adults and shown serum PO_4 levels >3.5 mg/dl [> 1.5mmol/L] were associated with an increased risk of death, with the mortality risk increasing linearly with each subsequent 0.5 mg/dl increase in PO_4 *(Kestenbaum et al, 2005)*. In a smaller study of pre-dialysis CKD II – IV patients with a mean eGFR of 13 (± 5) ml/min/1.73m^2 who were followed up for a minimum of 2 years, for each 1mg/dl higher PO_4 concentration, the decline in eGFR increased by 0.154 ml/min/month, and the adjusted mortality rate was 1.62 fold higher (95% CI 1.02 to 2.59) *(Voormolen et al, 2007)*.

Although no clinical trial has examined the impact of lowering serum PO_4 on mortality, several large observational studies have shown that hyperphosphataemia and secondary hyperparthyroidism are associated with increased mortality *(Block et al, 2004).* While most previous studies have examined associations between baseline values and survival without accounting for variations in clinical and laboratory measures over time, Kalantar-Zadeh et al have examined associations between survival and quarterly lab values using both time-dependent and fixed-covariate Cox models *(Kalantar-Zadeh et al, 2006)* in over 58,000 maintenance HD patients. They have shown that higher serum Ca and PO_4 were consistently associated with a higher mortality risk, however, the threshold Ca level that predicted mortality was higher, and some of the previous observations between low PO_4 and mortality may in fact be

due to the confounding effect of the malnutrition-inflammation-atherosclerosis syndrome *(Kalantar-Zadeh et al, 2006).* A number of paediatric studies have also linked serum PO_4 levels with adverse vascular changes and coronary calcification, and are discussed in section II in this chapter.

Figure 1.7 Increased mortality risk with increasing phosphate levels



Unadjusted, case mix–adjusted, and multivariable-adjusted relative risks (RR) of death and 95% CI for eight categories of serum PO_4.

Referent range, 4.0 to 5.0 mg/dl; to convert to mmol/l multiply by 0.32.

Case mix adjustment refers to adjustment for age, gender, race or ethnicity, diabetes, and vintage. Multivariable adjustment refers to case mix plus body weight, URR*, serum albumin, creatinine, predialysis BUN*, bicarbonate*, cholesterol, hemoglobin, ferritin*, and aluminum.

Adapted from Block et al, J Am Soc Nephrol, 2004.

Several *in vitro* studies using vascular smooth muscle cell explant cultures have shown the direct causal role of PO_4 in inducing and promoting vascular calcification *(Giachelli et al; Reynolds et al, 2004)*, and are discussed in section V.

1.7 Calcium homeostasis in CKD

Disorders in Ca homeostasis are common in CKD patients, and closely linked with PO_4 and PTH dysregulation and ectopic calcification as described above.

A healthy adult has ~25,000mmol (~1 kg) of Ca, of which >99% is in the bone, and <1% (~ 20mmol) is in the extracellular fluid. Approximately 50% of the circulating Ca is bound to albumin, a further 10% to other anions and ~40% is free or ionized Ca that is available for enzymatic reactions *(Houillier et al, 2006)*. The Ca homeostatic system aims at regulating the extracellular fluid Ca levels and maintaining this within a very tight range, never deviating >2% from its set-point in healthy individuals *(Nordin et al, 1976)*. Thus, it is important to remember that serum Ca levels are a poor, and sometimes misleading, marker of total body Ca.

The intestine, bone and kidneys are involved in maintaining Ca homeostasis, under the regulation of the calciotropic hormones, PTH and vitamin D. Ca balance studies have shown that the minimal dietary Ca requirement is ~600mg (15 mmols) per day, and large obligatory losses in faeces (~300 mg) and urine (~100mg) occur *(Kurokawa et al, 1994)*. In the presence of calcitriol, intestinal Ca absorption increases from approximately 40% to 80%, through the synthesis of calbindin proteins in the duodenum and jejunum *(Walters et al, 1989)*. Ca absorption essentially replenishes the bone Ca mass and plays only a very small role in regulating serum Ca levels *(Nordin et al, 1976)*. A defect in intestinal Ca absorption, particularly if sustained,

will result in a reduction in bone mineral content, without any change in the serum Ca levels *(Nordin et al, 1997)*.

The negative feedback control of serum Ca levels by PTH provides an efficient means of maintaining serum Ca levels within a narrow range *(Parfitt et al, 1976)*. The Ca equilibrium level is maintained by an inflow of Ca from the bone pool into the extracellular compartment and a net outflow from the extracellular compartment into the urine. Bone Ca release, orchestrated by PTH, is rapid and of marked amplitude, but of limited capacity, as only the superficial layers of bone are involved. In healthy individuals who have completed their growth, the urinary Ca excretion is equal to the net amount absorbed by the intestine.

CKD patients are thought to be in a net positive Ca balance as a result of iatrogenic Ca loading from Ca-based phosphate binders, vitamin D therapy and dialysate Ca, and reduced or absent Ca removal via the kidneys. Current K/DOQI guidelines recommend an absolute maximum elemental calcium load of 2,000 mg/d, including calcium-containing medication (maximum 1500 mg/d) and a maximum dialysate calcium concentration of 1.25 mmol/L (to avoid intradialytic Ca loading) *(K/DOQI Bone and mineral metabolism guidelines 2003)*. Sigrist et al have performed careful Ca balance studies during hemodialysis and shown that the majority of HD patients are continually experiencing Ca overload. Also, the amount of Ca removed during dialysis was independent of exogenous Ca load from diet or binders *(Sigrist et al, 2006)*.

Studies in maintenance HD patients by Block and Kalantar-Zadeh *(Block et al, 2006; Kalantar-Zadeh et al, 2006)* have both shown that higher serum Ca levels are associated with an increased mortality risk, although the threshold serum Ca associated with mortality was higher (10.5 vs 8.5 mg/dl) in the non-time dependent models used by Block et al. The associations between low serum Ca and mortality that were shown in previous studies were likely due to the confounding effects of malnutrition *(Kalantar-Zadeh et al, 2006).*

Concerns with Ca loading has led to the development of new non-calcium based PO_4 binders like sevelamer and lanthanum carbonate. Studies have shown that sevelamer has an equivalent PO_4 binding capacity to calcium acetate, but is associated with less ectopic calcification *(Chertow et al, KI 2002; Block et al, 2005; Block et al, 2007; Spiegel et al, 2007; Raggi et al, 2005)*. Nevertheless, despite the reduction in vascular calcification and also the lipid-loweing benefits of sevelamer, it does not have a survival advantage when compared to conventional therapy *(Suki et al, 2007)*.

Importantly, the serum Ca level does not reflect the total body Ca load. In the 'Treat-to-Goal' study the Ca treated group had virtually identical serum Ca levels compared to the sevelamer treated group despite ingesting ~500 grams more elemental Ca during the year, and with intestinal absorption being facilitated by concomitant vitamin D therapy *(Chertow et al, 2002)*. Also, the growing skeleton of children 'mops up' a large amount of Ca, and most adult studies are performed in older, often post-menopausal, patients in whom the skeleton is no longer able to cope with large Ca loads. Finally, in the presence of high serum PO_4 levels, transient increases in

serum Ca, such as seen during dialysis therapy and after ingestion of vitamin D analogues or Ca containing PO_4 binders, may influence ectopic calcification. Reynolds et al have shown that Ca and PO_4 act synergistically to increase calcification: when vascular smooth muscle cells are incubated in high PO_4 media, even a small increase in the Ca concentration will significantly increase calcification *(Giachelli et al, 2004)*. These transient increases that inevitably occur in clinical practise may go unrecorded, but can impact on ectopic calcification, particularly in the setting of high PO_4 conditions. London et al have reported that the extent of arterial calcification was directly related to the number of episodes of hypercalcaemia during the preceeding 6 months *(London et al, 2003),* and in the 'Treat-to-Goal' study the Ca treated group had significantly more hypercalcaemic episodes than the sevelamer group *(Chertow et al, 2002).*

The Ca x PO_4 product

Although the Ca x PO_4 product is frequently quoted as an outcome parameter in many clinical studies and the K/DOQI have produced an evidence based guideline on its regulation, it is simply an 'artificial' number that does not reflect the chemical properties of the two minerals. The precipitation of Ca and PO_4 from physiologic saline solutions is a second order reaction consistent with the formation of $CaHPO_4$ and chemical analyses of hydroxyapatite crystals $[(CaMg)_{10}(PO_4CO_3)_6(OH)_2]$ from ectopic calcification has shown that it is not simply a precipitation of Ca and PO_4, but a complex reaction that depends on the solubility product of Ca^{2+} and HPO_4^{2-} *(Shear et al, 1928)*. Precipitation of $CaHPO_4$ does not occur in plasma until the Ca x PO_4 is at least three times the K/DOQI threshold of 55 mg^2/dl^2 *(O'Neill et al, 2007)*. Thus, the

Ca x PO_4 product, although convenient, is an oversimplified and incorrect approach to describing the complex interplay between these minerals.

1.8 Recommended Ca – PO_4 – PTH levels in CKD

While most physicians now accept that high PO_4 levels have deleterious cardiovascular effects, there is much controversy over what 'optimal' PTH levels should be. 'Optimal' PTH levels may be defined as levels that maintain normal bone turnover without increasing the risk of ectopic calcification. As bone biopsies are infrequently performed in clinical practise, circulating PTH levels have been used as a surrogate marker of bone turnover, however, the specificity of PTH as an indicator of bone turnover has been questioned *(Rees L, 2008)*.

A correlation of bone histomorphometry with circulating PTH levels has shown that high PTH levels are associated with high bone turnover, although the range of PTH values was very wide, from just above the upper limit of normal to 16-fold normal *(Rees L, 2008; Mathias et al, 1993; Salusky et al, 1994; Goodman et al, 1994; Ziolkowska et al, 2000; Yalcinkaya et al, 2000, Waller et al, 2008)*. However, the ability of PTH levels to differentiate between normal and low bone turnover is less clear *(Mathias et al, 1993; Salusky et al, 1994; Ziolkowska et al, 2000)*: small patient numbers, a diverse patient population with very few young children with active skeletal growth, prior use of aluminium, parathyroidectomies, and a wide scatter of results makes it very difficult to interpret these results. Moreover, the role of PTH as a

marker of bone turnover is questioned: its short half-life means that fluctuations are very likely, and the presence of PTH fragments in advanced CKD and hyperparathyroidism may give spurious results *(Waller et al, 2006)*. Also, given that long-standing secondary hyperparathyroidism leads to a skeletal resistance to the calcaemic actions of PTH, such that increasingly higher PTH levels are necessary to maintain normal bone turnover, the 'optimal' PTH level may differ between patients. Most importantly, there are only a few small observational studies that have examined the role of PTH on the cardiovascular system and these report deleterious effects of high PTH *(Oh et al, 2002; Linhartova et al, 2008)* but there is conflicting data on its association with cardiovascular mortality *(Kalantar-Zadeh et al, 2006; Block et al, 2005; Avram et al, 2001; Panuccio et al, 2002)*.

Guidelines on the optimal levels of Ca, PO_4 and PTH levels and all aspects of their control have been proposed by the K/DOQI and the European Paediatric Dialysis Working Group *(K/DOQI, Bone and Mineral Metabolism guidelines, 2003; Klaus et al, 2006)*. The European recommendations are more conservative and advice keeping PTH levels in the normal range until CKD stage V, when 2 – 3 times the upper limit of normal is recommended. The K/DOQI have set higher levels of up to twice the upper limit of normal in CKD stage IV and 3 – 5 times the upper limit of normal on dialysis. It must be remembered that in the absence of randomised controlled studies many of these guidelines are based on expert opinion.

Section II – Clinical studies

1.9 Surrogate measures of cardiovascular risk in CKD patients

Unlike studies in adult CKD patients where 'hard' end-points like death or cardiovascular events are used, paediatric studies have to rely on surrogate measures of vascular damage. These include vascular measures of structure and function and biomarkers from blood and urine.

Measures of vascular damage include the carotid artery intima media thickness (cIMT) and direct evidence of coronary artery calcification (CAC) on CT scan. Functional changes in the vasculature can be determined by the pulse wave velocity (PWV), aortic augmentation index (AIx) and carotid distensibility. The IMT, PWV and CAC have been extensively used in many studies of vascular outcome and in the work in this thesis. I have described each method and relevant clinical studies in depth under the 'General Methods' in Chapter 2.

Numerous biomarkers of vascular damage and future cardiovascular events have been described and some validated against 'hard end-points'. In our current state of knowledge, these can best serve as corroborative evidence of vascular injury or predictors of future cardiovascular events, but cannot replace the better established vascular measures. In the work in this thesis, I have used vitamin D levels (25-hydroxyvitamin D and 1,25-dihydroxyvitamin D) and levels of the circulating calcification inhibitors (fetuin-A, Matrix Gla-protein and osteoprotegerin) as

biomarkers, and described their associations with vascular damage and calcification. These are described in detail in sections III and IV in this chapter.

1.10 Studies in paediatric dialysis patients

A number of cross-sectional observational studies in paediatric dialysis patients or young adult survivors of paediatric dialysis programmes have described surrogate measures of cardiovascular damage and sought to find associations with these. Children provide an ideal opportunity to study uraemic influences on the vasculature as they seldom have confounding pro-atherosclerotic risk factors such as diabetes and dyslipidaemia that are major confounders in similar adult studies.

A summary of these studies, the vascular measures described and the key findings are presented in Table 1.2, and their salient findings discussed below.

Table 1.2 Vascular measures and their correlations in paediatric and young adult dialysis patients (in chronological order of publication date)

No.	Author, Journal, Year	No. of patients	Mean age (yrs)	Duration of dialysis (yrs)	Vascular measures	Clinical and biochemical correlations	Key message
1.	Goodman et al, NEJM, 2000	39	19 ± 7 (range 7–30)	7 ± 6 (range 0.3–21)	CAC	Presence of CAC correlated with - Age - dialysis duration - mean serum PO_4 and Ca x PO_4 - Ca intake from binders	No CAC in any patients <20 yrs age, but 14/16 patients >20 yrs had CAC. CAC doubled on follow-up scan at 20 months.
2.	Eifinger et al, NDT, 2000	16	26.5 (range 14 – 39)	RRT for 2.5 to 21 years.	CAC	None found.	CAC in 6/16 (37%) patients. All children asymptomatic despite high CAC burden.
3.	Oh et al, Circulation, 2002	39	27.3 (range 19 – 39) (Young adults with childhood onset ESRD)	5.0 (range 0 – 22)	CAC + cIMT	CAC and cIMT correlated with - ESRD duration - dialysis duration - mean serum Ca x PO_4 CAC correlated with - PTH levels - hs-CRP - homocysteine levels	50% of deaths are due to cardiovascular or cerebrovascular causes. High prevalence of arteriopathy in young adult survivors of CKD. Vascular damage correlates with Ca – PO_4 load, hyperparathyroidism and microinflammation, but not 'traditional' risk factors.
4.	Groothoff et al, JASN, 2002	130 29 dialysis	29 (range 20.7 to 40.6)	RRT – 18 yrs Dialysis – 4.5 yrs	cIMT, stiffness measures	Hypertension main determinant of abnormal arterial wall properties. No biochemical data available.	No increase in cIMT compared with controls, but reduced distensibility and increased vascular stiffness parameter in all CKD groups.

			(Young adults with childhood onset ESRD)	Tx (n = 101) – 13.5 yrs			No difference in cIMT or arterial wall stiffness between dialysis and transplant groups.
5.	Litwin et al, JASN, 2005	55– CKD 2-4 37– dialysis 34– Transplant	range 10 – 20 yrs	Pre-dialysis CKD – 7.1 ± 5.1 yrs Dialysis – 2.2 ± 2.9 yrs Transplant- 2.8 ± 3.2 yrs	Carotid & femoral IMT, Wall & lumen cross-sectional areas	cIMT correlated with - dialysis duration - mean serum Ca x PO_4 - Ca intake from binders - Mean calcitriol dose	Increased cIMT in all CKD groups - significantly greater in dialysis compared with transplant patients. Suggest partial reversibility post-Tx. Carotid lumen increased post-Tx – possibly as a result of higher BP post-Tx.
6.	Mitsnefes et al, JASN, 2005	44– CKD 2-4 16– dialysis		Pre-dialysis CKD – ? Dialysis – 1.2 ± 1.3 yrs (range 0.3 – 3.7 yrs)	IMT, distensibility and stiffness of carotid artery and ECHO	cIMT correlated with - dialysis duration - mean serum Ca x PO_4 - Ca intake from binders - Mean calcitriol dose Stiffness correlated with - mean serum Ca x PO_4 - mean PTH levels	Increased cIMT in dialysis compared with pre-dialysis patients. No change in vessel stiffness pre-dialysis, but increased carotid artery stiffness noted in the dialysis group, suggesting that structural changes preceed functional abnormalities.
7.	Covic et al, NDT, 2006	14	14.1 ± 2.6 yrs	1 month to 6 yrs (all HD)	cIMT, PWV and aortic augmentation index	PWV correlated with - mean PO_4 levels - mean serum Ca x PO_4 Age was the only significant predictor of aortic augmentation index.	PWV and aortic augmentation index significantly higher in patients than controls, and comparable with adult values. No reversibility after a dialysis session, suggesting that structural changes underly the loss of function.

8.	Briese et al, NDT, 2006	40	23.6 yrs (Young adults who developed ESRD at ~11yrs age)	9-dialysis – 2.9 ± 3.5 yrs 31 – transplant 9.2 ± 4.3 yrs	cIMT, ECHO and CAC	Patients with calcification were - older - longer dialysis duration - increased cIMT - higher mean serum Ca x PO_4 - increased Ca intake from binders - increased mean calcitriol dose	No difference in cIMT between dialysis patients, transplant recipients and controls. 10% had moderate to severe CAC, and 9% had mild CAC. cIMT was higher in patints with calcification
9.	Civilibal et al, Ped Nephrol, 2006	53	15.7 yrs (range 6.9 – 22.7 yrs)	39-dialysis – 4.9 ± 2.7 yrs 14 – transplant 3.4 ± 2.7 yrs	CAC	Presence of CAC correlated with - longer dialysis duration - higher mean serum PO_4 and Ca x PO_4 - higher mean PTH levels - higher Ca intake from binders - higher mean calcitriol dose	CAC was present in 8 of 53 (15%) – 6 currently on dialysis and 2 transplanted.
10.	Civilibal et al, Ped Nephrol, 2007	39	14.8 ± 3.8 yrs	4.8 ± 2.6 yrs	cIMT, endothelium dependent dilatation and ECHO	cIMT correlated with - diastolic BP - higher mean serum Ca x PO_4 - higher total & LDL cholesterol - higher homocysteine levels - higher mean calcitriol dose	Increased cIMT, hs-CRP and homocysteine levels in patients compared with controls, but no difference in endothelium dependent dilatation between the groups. Endothelium dependent dilatation correlated with cIMT.
11.	Poyrazoglu et al, Ped Nephrol, 2007.	34	18.0 ± 4.3 yrs	4.6 ± 2.9 yrs	cIMT and ECHO	cIMT correlated with - mean BP - left ventricular mass index - inversely with PTH (negative correlation) (No data available for phosphate binder or calcitriol dosage)	Increased cIMT, left ventricular hypertrophy and higher left ventricular mass index in the dialysis as compared to control groups. Significant negative correlation between cIMT and PTH.

Key findings from the paediatric studies

A. Carotid intima media thickness

Most studies have shown a significant increase in cIMT signifying structural changes in the vessel wall. These changes have been shown to begin even at a young age and have been reported in adolescents on dialysis *(Litwin et al, 2005; Mitsnefes et al, 2005; Civilibal et al, 2006)*. Also, an increase in cIMT above control levels has been shown in pre-dialysis CKD stages II – IV patients as well, suggesting that vascular damage begins very early in the course of GFR decline *(Litwin et al, 2005; Mitsnefes et al, 2005)*. Importantly, Mitsnefes et al have shown that although structural vascular change in the form of an increased cIMT is found in pre-dialysis patients, the vessel retains its normal compliance and distensibility properties as compared to controls *(Mitsnefes et al, 2005)*. However, with progressive duration and severity of uraemic damage as found in dialysis patients, a further deterioration in cIMT coupled with increased vascular stiffness occurs. Interestingly, an increase in the vessel wall thickness or cIMT is coupled with a re-modelling of the vessel so that an increase in the carotid artery lumen occurs, possibly to counter the stiffness or loss of compliance of the vessel *(Litwin et al, 2005).* It may be this compensatory re-modelling in the early stages of CKD and the more plastic vessels of children that protect them against the deleterious consequences of vascular damage.

None of the studies in children and young adults have reported the presence of intimal plaques, and although ultrasound is not an accurate means of assessing intimal vs medial changes in the vessel wall, it appears that uraemic vasculopathy, at least in young adults, is a predominantly medial process. Also, although hypertension is a

significant determinant of carotid artery properties, especially the diameter of the vessel *(Litwin et al, 2005)*, associations with other 'traditional' risk factors for cardiovascular disease were seldom found.

Although described in a cross-sectional design, it appears that removal, or at least a reduction, of uraemic 'toxins' after transplantation, can lead to lower cIMTs in the transplanted population as compared to dialysis patients *(Litwin et al, 2005)*, but results are conflicting *(Briese et al, 2006)*. In a longitudinal study of pre-dialysis, dialysis and transplanted children, Litwin et al have described an improvement in cIMT after transplantation *(Litwin et al, 2008);* this study is described in detail in the next section.

Three studies have reported on cIMT levels in young adult survivors of paediatric dialysis or end stage renal disease programmes, and interestingly shown some conflicting results. While Oh et al found that cIMT was significantly increased as compared to controls *(Oh et al, 2002)*, similar studies by Groothoff and Briese have not shown any increase in cIMT above control levels *(Groothoff et al, 2002; Briese et al, 2006)*. Furthermore, Groothoff et al have shown that despite a normal cIMT, patients had increased vascular stiffness. The patients in the two studies were similar in age at study and the total duration of ESRD, but the patients in Oh's study had spent nearly twice as long on dialysis. They speculate that functional abnormalities preceed structural damage to the vessel, and a longer dialysis vintage resulted in more severe damage and an increase in cIMT in Oh's study *(Groothoff et al, 2002)*. Briese et al, who also report normal cIMTs in their patients, had a shorter duration of pre-

dialysis CKD and dialysis vintage in their patients, as well as a markedly lower dosage of calcium containing PO_4 binders and calcitriol (8-fold and 30-fold lower) as compared to the study by Oh et al, that may have accounted for a normal cIMT as well as a significantly lower CAC score in their patients *(Briese et al, 2006).*

A number of associations with cIMT have been shown, but most studies consistently report worsening cIMT with older age, longer dialysis vintage, and higher mean serum PO_4 and Ca x PO_4 levels as well as higher doses of Ca intake from PO_4 binders and calcitriol (Table 1.3). It must be remembered that cIMT is an age-dependent measure and increases by ~ 0.01 to 0.02 mm/year *(Johnson et al, 2007),* hence must be compared with an age-matched population or described as standard deviation scores for age *(Jourdan et al, 2005).* In all studies cIMT has consistently and significantly correlated with Ca, PO_4 and PTH levels, as well as medication dosages of Ca based PO_4 binders and vitamin D compounds, suggesting that dysregulated mineral metabolism is central to the vasculopathy of CKD, and that these modifiable risk factors require careful monitoring and strict control from the earliest stages of CKD.

Finally, all of these studies are cross-sectional observational studies in small and diverse CKD populations, and the associations drawn must be interpreted with caution. These studies can best serve as hypothesis – generating studies, and prospective longitudinal follow-up data in a large cohort of CKD patients is required before any definitive recommendations on management decisions can be made.

B. Pulse wave velocity (PWV)

Only one paediatric study has described PWV and augmentation index (AIx) in children on dialysis and shown that both were consistently higher in their haemodialysis cohort as compared to controls *(Covic et al, 2006)*. Importantly, there was no real improvement in PWV or the AIx after a dialysis session, suggesting that structural and not simply functional alterations determine the increased arterial stiffness. In adult dialysis patients, aortic PWV and AIx have been shown to be the strongest predictors of cardiovascular mortality: for each 1 m/sec increase in PWV the all-cause mortality adjusted odds ratio was 1.39 (95%CI 1.19 to 1.62), and similarly for each 10% increase in AIx the risk ratio was 1.51 (95%CI 1.23 to 1.86) *(Blacher et al, 1999; London et al, 2001).* Although paediatric studies have no data to support the poor prognostic effects of increased vascular stiffness, an association with increased cIMT and greater left ventricular mass index were shown *(Covic et al, 2006)*.

Functional changes in the large arteries can also be studied by measuring the carotid artery distensibility, stiffness and elastic modulus *(Groothoff et al, 2002; Mitsnefes et al, 2005)* as well as re-modelling of the vessel *(Litwin et al, 2005; Mitsnefes et al, 2005)*. These studies have shown increased vascular stiffness as well as increased diameter of the carotid artery in dialysis patients, and importantly, these changes show a greater correlation with the systolic and diastolic blood pressure than with biochemical measures *(Litwin et al, 2005; Mitsnefes et al, 2005).*

C. Coronary artery calcification score (CAC) on CT scan

Direct evidence of Ca deposition in the coronary arteries of young adults was first described by Goodman et al *(Goodman et al, 2000)*. They showed that there was no evidence of calcification on CT scan in the coronary vessels in any of the 25 patients younger than 20 years of age, but 14 of 16 patients above 20 years had CAC. A careful analysis of their data reveals that not only were the patients with CAC older, but that they also had a significantly longer median dialysis vintage (13 vs 2 years), higher mean serum Ca x PO_4 levels (5.2 vs 4.5 $mMol^2/L^2$) and almost double the intake of Ca from binders (6456 vs 3325 mg/day) as compared to the group without calcification. Interestingly, both groups had very high PTH levels (36.1 and 44.5 pmol/L), although there was no statistically significant difference between them, and in fact 9 patients had undergone parathyroidectomy. The serum PO_4, Ca x PO_4 and PTH levels are very high in both groups and the Ca intake from PO_4 binders significantly above the K/DOQI recommended limit of 1500mg/day, suggesting that these patients were at a substantial risk of ectopic calcification. Traditional risk factors such as diabetes, hypertension, inflammation and male sex were not associated with calcification. Despite these risk factors and the presence of CAC, none of the patients had overt cardiovascular disease, although 5 had ischaemic changes on ECG, and one had a first degree heart block. What is perhaps the most crucial finding in this study is that the arteriopathy in dialysis patients is rapidly progressive: when a repeat CT scan was performed after a mean interval of 20 months, the calcification score almost doubled in the 10 patients who had evidence of initial calcification.

Evidence of CAC in children was first shown by Eifinger et al. In their small and uncontrolled study of 16 children and young adults on RRT (CKD, dialysis and post-transplant), they showed that 37% had calcification, and this was present even in teenagers. Unfortunately, their small and very diverse cohort and the lack of any correlations with clinical or biochemical data does not allow us to draw further conclusions from their work *(Eifinger et al, 2000)*. In a subsequent study, Civilibal et al have described CAC in a larger cohort of paediatric chronic dialysis patients, and found that 15% of their patients had CAC *(Civilibal et al, 2006)*. Interestingly the presence of CAC correlated with all of the parameters originally described by Goodman et al in their cohort of older dialysis patients: dialysis vintage, dysregulated mineral metabolism and higher Ca intake from binders as well as with the dose of activated vitamin D compounds.

In the study by Oh et al, CAC was present in 92% *(Oh et al, 2002)* while Briese et al have reported a considerably lower prevalence of 19% in a similar cohort young adult survivors of paediatric dialysis programmes *(Briese et al, 2006):* the 30-fold higher dosage of vitamin D in Oh's study may account for the greatly increased CAC. In the study by Oh et al the presence of CAC correlated more closely with the PTH levels than with the serum PO_4 or the Ca x PO_4 product. They postulate that PTH has a direct effect on vascular smooth muscle cells, not only increasing Ca entry into these cells, but also inducing arteriolar thickening and fibrosis *(Rostrand et al, 1999)*, and this may explain the preferential medial calcification in uraemia. The very high prevalence of CAC in this study supports the finding by Goodman et al that in the pro-calcific and pro-inflammatory uraemic milieu 'calcium begets calcium'.

1.11 Progression of vascular calcification through different stages of CKD

Some cross-sectional studies have compared groups of pre-dialysis CKD stages IV and V patients with dialysis and transplant cohorts and shown that the carotid IMT and coronary calcification scores are higher in dialysis patients compared to pre-dialysis CKD or transplant patients. Despite a plethora of observational cross-sectional studies, there are very few longitudinal studies that have followed patients through pre-dialysis – dialysis - transplantation phases and described changes in surrogate markers at different stages of uraemia.

Russo et al have shown that coronary artery calcification is present in 40% of adult patients in CKD stages II – IV and that the calcification scores doubled in 8 of 10 patients when followed-up after a mean of 8 months while still in pre-dialysis CKD *(Russo et al, 2004)*. The annualized progression rate of coronary artery calcification, after adjusting for all confounders, was 28%. Calcification in pre-dialysis patients has been reported in 27 to 64% of patients in other studies *(Kramer et al, 2005; Tomiyama et al, 2006);* it is influenced by age, diabetes and triglyceride levels.

Calcification progresses rapidly in patients on dialysis as first shown by Goodman et al *(Goodman et al, 2000)*. In a subsequent study on stable peritoneal dialysis patients, the coronary calcification scores quadrupled in one year and were influenced by Ca – PO_4 abnormalities *(Stompor et al, 2004)*. Importantly, approximately one third of the patients did not have any baseline calcification, and at one-year follow-up remained free of calcification. Three studies in haemodialysis patients, all comparing the effects of sevelamer with calcium-based phosphate binders, have shown that coronary

artery calcification progresses in patients on calcium based phosphate binder treatment but can be arrested with sevelamer *(Chertow et al, 2002; Block et al, 2005; Spiegel et al, 2007).* Fascinatingly, in all these studies patients who did not have baseline calcification continued to remain free of calcification despite exposure to similar uraemic conditions.

By ameliorating the uraemic milieu, renal transplantation could intuitively be thought to reverse some of the cardiovascular damage from dialysis. In a small observational study of 22 renal transplant recipients, de Lima et al showed that although left ventricular wall morphology showed significant improvement within 12 months of successful transplantation, carotid IMT normalized only after 40 months but carotid distensibility and ventricular wall thickness and dysfunction continued to remain abnormal *(de Lima et al, 2002).* An extended period on dialysis and high blood pressure adversely affected the rate of post-transplantation improvement.

In the only paediatric study offering long-term follow-up, Litwin et al have shown that carotid IMT thickening and remodelling of the vessel wall begin early in CKD and progress rapidly on dialysis, correlating with the blood pressure and mean serum phosphate levels. Successful transplantation can improve the cIMT towards pre-dialysis values, but cannot normalise it *(Litwin et al, 2008).* Changes in cIMT were observed within 1 year post-transplantation in this study, whereas in the above mentioned study by de Lima et al, significant regression of the cIMT was not noted until the third year post-transplantation, suggesting that a shorter exposure to uraemia, and possibly better repair mechanisms in children, favour rapid improvement.

Section III – The role of vitamin D in cardiovascular health

Vitamin D deficiency is common in the general population as well as in CKD patients *(Holick M, 2007; Levin et al, 2007)*. The discovery that most tissues and cells in the body have a vitamin D receptor and also have the enzymatic machinery to convert the primary circulating form of vitamin D, 25-hydroxyvitamin D [25(OH)D], to the active form, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], has renewed interest in the functions of this vitamin, particularly its role in inflammatory and immune-mediated disorders and cardiovascular health *(Holick M, 2007; Zittermann A et al, 2008)*.

In this section I will discuss the sources, metabolism and pluripotent actions of vitamin D, with particular reference to its actions on the cardiovascular system and the relevant literature in CKD patients.

1.12 Sources of vitamin D

Approximately 80 – 90% of an individual's vitamin D requirement is obtained through sunlight. Solar ultraviolet B (UV-B) radiation (wavelength 290 to 315 nm) converts 7-dehydrocholesterol in the epidermis to pre-vitamin D_3, which is immediately converted to vitamin D_3 in a heat-dependent process *(Holick et al, 2006)*. Skin pigment, sunscreen use, clothing, time of day, season, latitude and altitude dramatically affect pre-vitamin D_3 synthesis. In the United Kingdom (latitude 51.5 – 54° North), the UV-B radiation in sunlight is minimal from October to April, resulting in a well documented seasonal variation in the circulating levels of 25(OH)D.

Few foods naturally contain or are fortified with vitamin D. Vitamin D_2 is manufactured through the ultraviolet irradiation of ergosterol from yeast, and vitamin D_3 through the ultraviolet irradiation of 7-dehydrocholesterol from lanolin. Oily fish are the richest natural source of vitamin D, but unless consumed regularly, will not provide adequate vitamin D levels.

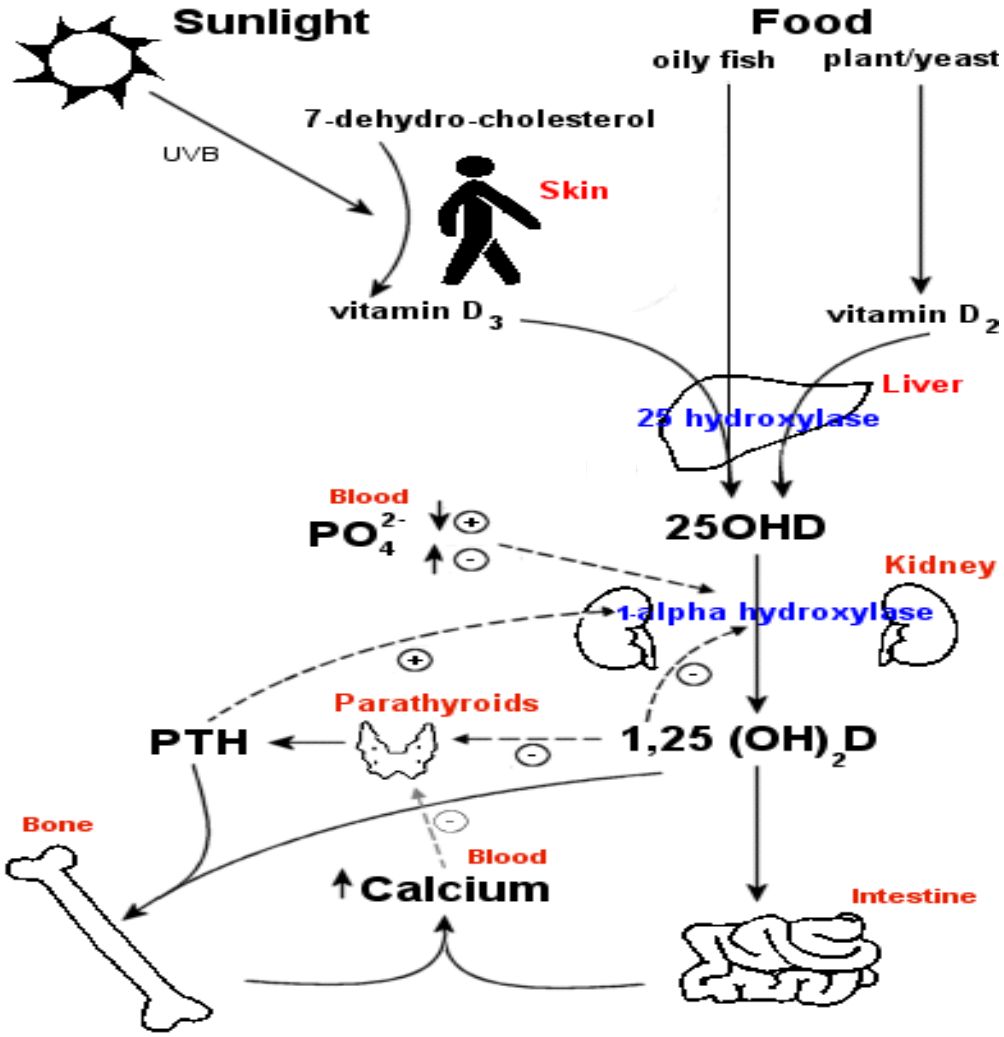
1.13 Vitamin D metabolism

25 hydroxyvitamin D (hereafter 'D' represents D_2 or D_3) made in the skin or ingested in the diet can be stored in fat cells, and as required, incorporated into chylomicrons and transported by the lymphatic system into the venous circulation. Vitamin D requires two successive hydroxylations, first in the liver (CYP27A1 and CYP2R1) on carbon 25 to form 25(OH)D, and then in the kidney, by the enzyme 25-hydroxyvitamin D-1α-hydroxylase [CYP27B1], for a hydroxylation on carbon 1 to form the biologically active form $1,25(OH)_2D$. Importantly, in renal patients the 1α-hydroxylase enzyme is substrate dependent.

Unlike 25-hydroxylation in the liver that is an unregulated step (no negative feedback), the renal production of 1,25-dihydroxyvitamin D is tightly regulated by PTH, Ca and PO_4 levels *(Bouillon et al, 2001; DeLuca et al, 2004).* $1,25(OH)_2D$ decreases its own synthesis through negative feedback: it can increase the expression of 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) to catabolize $1,25(OH)_2D$ to the water-soluble, biologically inactive calcitroic acid, which is excreted in the bile. Also, $1,25(OH)_2D$ decreases the secretion of PTH via the vitamin D receptor on the parathyroid gland. Fibroblast growth factor 23 (FGF-23), secreted by osteoblasts,

causes the sodium–phosphate cotransporter to be internalized by the cells of the kidney and small intestine and also suppresses 1,25-dihydroxyvitamin D synthesis *(Hruska K, 2006*). Figure 1.8 shows the metabolic pathway for vitamin D synthesis and its regulation.

Figure 1.8 Vitamin D synthesis and its role in Ca homeostasis



1.14 Role of vitamin D in Ca – PO_4 – PTH regulation

Vitamin D analogues regulate PTH secretion via both the vitamin D receptor and also the calcium sensing receptor on the parathyroid glands. Low $1,25(OH)_2D$ levels would be expected therefore to result in low plasma Ca and hyperparathyroidism, whereas high $1,25(OH)_2D$ levels cause hypercalcaemia, hyperphosphataemia and over

suppression of PTH *(Rostrand et al, 1999; Dusso et al, 2005; Feldman et al, 2005)*. In turn, high bone turnover (due to hyperparathyroidism) results in an efflux of Ca and PO_4 from the bones into the soft-tissues, whereas low bone turnover (due to over-suppression of PTH) results in an inability of the bone to buffer fluxes in serum Ca and PO_4.

$1,25(OH)_2D$ enhances intestinal Ca absorption in the small intestine by interacting with the vitamin D receptor–retinoic acid x-receptor complex (VDR-RXR) to enhance the expression of the epithelial Ca channel (transient receptor potential cation channel, subfamily V, member 6 [TRPV6]) and calbindin 9K, a calcium-binding protein. Without vitamin D, only 10 to 15% of dietary Ca and about 60% of PO_4 is absorbed. The interaction of 1,25-dihydroxyvitamin D with the vitamin D receptor increases the efficiency of intestinal Ca absorption to 30 to 40% and PO_4 absorption to approximately 80% *(Hruska K, 2006; Holick M, 2007).*

In bones, $1,25(OH)_2D$ is recognized by its receptor in osteoblasts, causing an increase in the expression of the receptor activator of nuclear factor-κB ligand (RANKL) *(Holick M, 2007; Dusso et al, 2005).* RANK, the receptor for RANKL on preosteoclasts, binds RANKL, which induces pre-osteoclasts to become mature osteoclasts. Mature osteoclasts remove Ca and PO_4 from the bone so as to maintain their respective levels in the blood.

1.15 Autocrine / paracrine effects of vitamin D

The vitamin D receptor as well as the 1-hydroxylase enzyme system is ubiquitous and highly conserved through the species *(Haussler et al, 1998).* Directly or indirectly,

1,25-dihydroxyvitamin D controls more than 200 genes, including genes responsible for the regulation of cellular proliferation, differentiation, apoptosis, and angiogenesis *(Holick M, 2007).* Both 25(OH)D and $1,25(OH)_2D$ can act in an autocrine / paracrine manner on virtually all tissues *(Holick M, 2007; Andress et al, 2006)*. However, some tissues such as macrophages and muscles *(Birge et al, 1975)* may be able to utilise only 25(OH)D *(Andress et al, 2006)*, making it important to ensure adequate levels of both, especially in the CKD patient where activated vitamin D compounds are used.

On a molar basis, $1,25(OH)_2D$ is the most potent vitamin D metabolite. Dose-response studies indicate a molar potency of $1,25(OH)_2D$ relative to 25(OH)D ranging from 125:1 to 400:1 in increasing Ca absorption from the gut *(Barger-Lux et al, 1995)*. However, the serum levels of 25(OH)D are approximately 1:500 to 1:1000 fold higher than those of $1,25(OH)_2D$ [nanomolar vs picomolar concentrations] *(Zittermann et al, 2003).* Thus, 25(OH)D also serves as a substrate for the 1-α hydroxylase of various tissues, and it has been suggested that tissues that are not responsible for regulating extracellular Ca levels probably utilize circulating 25(OH)D to synthesise their own calcitriol *(Holick M, 2002).* However, extrarenal 1-α hydroxylase activity is not sufficient to maintain adequate circulatory levels of $1,25(OH)_2D$ as seen in anephric individuals.

1.16 Measurement of vitamin D levels and their significance

Serum 25(OH)D is the barometer for vitamin D status – it has a plasma half-life of 12 – 19 days *(Zittermann et al, 2003)*, and reflects the person's vitamin D status. As the metabolism of ergocalciferol and cholecalciferol by the liver is not regulated (no

negative feedback), serum 25(OH)D lvels are an accurate assessment of cutaneous synthesis and dietary intake. Serum $1,25(OH)_2D$ has a very short half-life (~15 hours), hence it provides no information about vitamin D status and is often normal or even increased when secondary hyperparathyroidism associated with vitamin D deficiency.

Although there is no consensus on optimal serum levels of 25(OH)D, vitamin D deficiency is defined by most experts as a 25(OH) D level of less than 20 ng /ml (= 50 nmol/L) *(Holick et al, 2007; Thomas et al, 1998)*. In healthy subjects, 25(OH) D levels are inversely associated with PTH until the former reach 30 to 40 ng/ml, at which point PTH levels begin to level off at their nadir *(Thomas et al, 1998; Holick et al, 2005).* Furthermore, intestinal calcium transport increases by 45 to 65% when 25(OH)D levels are increased from ~ 20 to 32 ng/ml *(Heaney et al, 2003)*, suggesting that 25(OH)D levels between 20 – 30 ng/ml can be considered to indicate a relative insufficiency of vitamin D. Vitamin D intoxication is observed when serum levels of 25-hydroxyvitamin D are greater than 150 ng/ml *(Holick et al, 2007)*. With the use of such definitions, it has been estimated that 1 billion people worldwide have vitamin D deficiency or insufficiency *(Holick et al, 2007)*! Table 1.3 shows the currently used terminology to describe the vitamin D status of patients based on 25(OH)D levels.

Table 1.3 Definitions of vitamin D status based on 25(OH)D levels

Stages of vitamin D status	25(OH)D concentrations (nmol/l)	Biochemical/clinical symptoms
Deficiency	0–25	Severe hyperparathyroidism, calcium malabsorption, rickets, osteomalacia, myopathy
Insufficiency	>25–50.0	Elevated PTH levels, low intestinal calcium absorption rates, reduced bone mineral density, subclinical myopathy
Hypovitaminosis D	>50–70 to100	Low body stores of vitamin D, slightly elevated PTH levels
Adequacy	70–100 to 250	No disturbances of vitamin D-dependent functions
Toxicity	>250	Intestinal calcium hyperabsorption, hypercalcemia

To convert values for 25-hydroxyvitamin D to ng/ml, divide by 2.50.

Adapted from Zittermann et al, British Journal of Nutrition, 2003.

The vitamin D assays performed in the course of my study are discussed in chapter 2, section II. Radioimmunoassays measure total 25-hydroxyvitamin D, which includes levels of both $25(OH)D_2$ and $25(OH)D_3$. Some commercial laboratories measure $25(OH)D_2$ and $25(OH)D_3$ with liquid chromatography and tandem mass spectroscopy and report the values separately. As long as the combined total is 30 ng per milliliter or more, the patient has sufficient vitamin D. The $1,25(OH)_2D$ assay should normally not be used for detecting vitamin D deficiency, but if a patient is supplemented with activated vitamin D analogues, this level becomes relevant.

1.17 Vitamin D deficiency in CKD patients

Virtually all studies in dialysis patients have reported 25(OH)D and $1,25(OH)_2D$ deficiency to the order of 50 - 90% *(Goldsmith D, 1997; Address, D, 2005; Holick M, 2007).* In a recent population based study of >1800 adults, Levin et al showed that 20% of subjects with eGFRs between 60–70ml/min/1.73m^2 and 60% with eGFRs between 30–40ml/min/1.73m^2 had raised PTH levels *(Levin et al, 2007).* Hyperparathyroidism was associated with low levels of $1,25(OH)_2D$, which was found to be extremely common even at higher GFRs than previously reported: thus 13% of patients with eGFRs >80ml/min/1.73m^2 had $1,25(OH)_2D$ deficiency and 60% of patients with eGFRs <30ml/min/1.73m^2 were $1,25(OH)_2D$ deficient. Significant differences in PTH and $1,25(OH)_2D$ levels were seen across the deciles of eGFRs, and importantly, these were not associated with any change in serum Ca, PO_4 or 25(OH)D levels. Thus, this study highlights the role of vitamin D deficiency in the development of secondary hyperparathyroidism in CKD patients, and the importance of its early detection and supplementation.

Figure 1.9A The prevalence 25(OH)D and 1,25(OH)$_2$D deficiency and of secondary hyperparathyroidism by GFR intervals

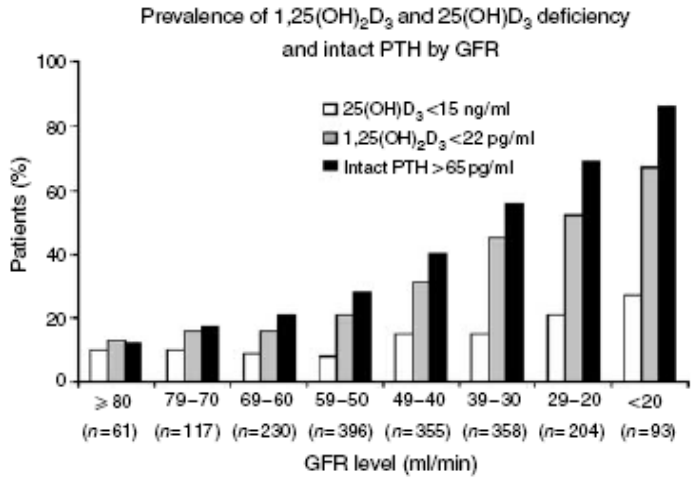


Figure 1.9B Median values of 25(OH)D, 1,25(OH)$_2$D and iPTH by GFR levels.

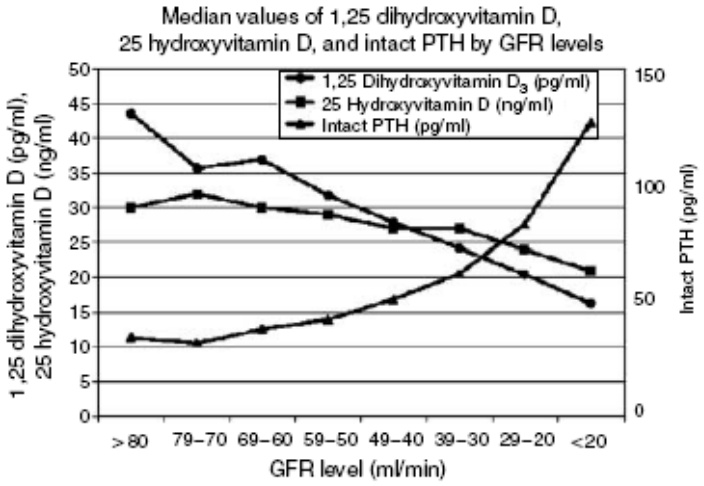


Figure 1.9A and B are adapted from Levin et al, KI, 2007.

CKD patients can have low 25(OH)D levels for many reasons *(Andress D, 2008):-*

(i) they may be less active and have less sunlight exposure.

(ii) the endogenous synthesis of vitamin D in the skin is reduced in CKD

(iii) ingestion of foods that are natural sources of vitamin D may be diminished

(iv) proteinuria may be accompanied by high urinary losses of 25(OH)D, D-binding protein, and megalin *(Sato et al, 1982; Levin et al, 2007)*

(v) 25(OH)D and D-binding protein may be lost in peritoneal dialysis fluid.

In addition, when the GFR falls to <50ml/min/1.73m^2, the kidney cannot convert 'nutritional' 25(OH)D to 1,25$(OH)_2$D *(Rostrand et al, 1999)*; 1,25$(OH)_2$D deficiency is widely prevalent in CKD patients *(Levin et al, 2007; London et al, 2007; Zittermann A, 2006)*. Importantly, in CKD patients, unlike healthy subjects, the 1-α hydroxylase enzyme is substrate dependent, so 25(OH)D levels are crucially important.

In the absence of robust evidence, an opinion based guideline from the National Kidney Foundation's Kidney Disease Outcome Quality Initiative (K/DOQI) recommends that if PTH levels are elevated and serum 25(OH)D levels low, ergocalciferol supplements should be prescribed *(K/DOQI, Clinical Practise Guidelines, 2003)*. Randomised studies have shown that ergocalciferol provides adequate substrate for the synthesis of 25(OH)D and can reduce PTH levels without any risk of hypercalcaemia in CKD 2-3 *(Al-Aly et al, 2007; Saab et al, 2007)* but not in advanced CKD, by which point the 1-α hydroxylase activity of the kidney is incapable of sustaining 1,25$(OH)_2$D production.

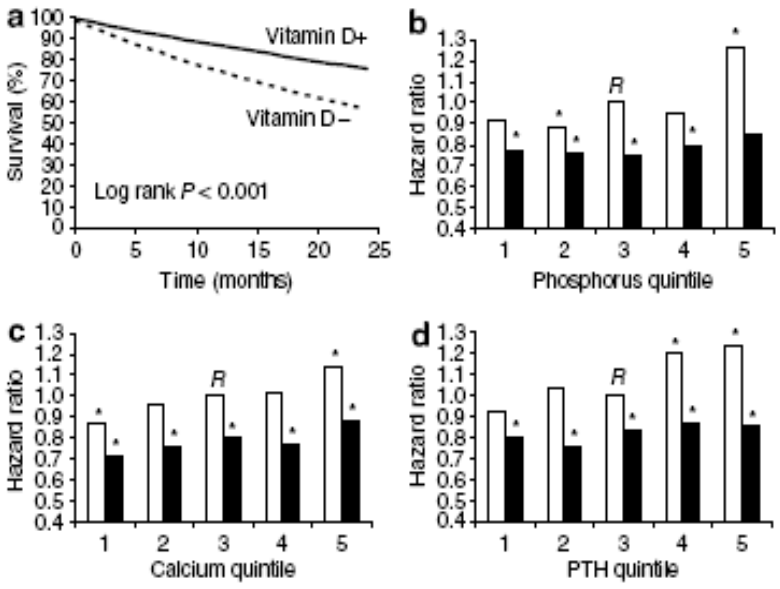
1.18 Vitamin D supplementation and survival in dialysis patients

The effects of vitamin D treatment on all-cause and cardiovascular mortality have been reported from several large epidemiological studies on HD patients. All the studies have consistently shown that HD patients receiving any activated vitamin D

treatment have a significant survival advantage to the order of 20 – 25% as compared to untreated patients *(Teng et al, 2003; Teng et al, 2005; Tentori et al, 2006; Shoji et al, 2004; Wolf et al, 2007).* Although there are conflicting reports on this, there is unlikely to be a significant difference in survival between different vitamin D analogues (doxercalciferol vs paricalcitriol) *(Teng et al, 2005; Tentori et al, 2006).* Most importantly, vitamin D treatment could mitigate the effects of high Ca, PO_4 and PTH on cardiovascular mortality: the survival advantage of vitamin D was present across all quintiles of Ca, PO_4 and PTH levels *(Teng et al, 2005),* suggesting that vitamin D has important effects beyond its role in mineral metabolism. In a prospective study on incident dialysis patients, severe 25(OH)D deficiency was associated with an increased all-cause, but not cardiovascular, mortality *(Wolf et al, 2007).* Importantly, all of these studies are non-randomised, and physician bias may have confounded the results.

Figure 1.10 a. Survival curves in dialysis patients treated with injectable vitamin D compared with untreated patients. *Adapted from Teng et al, JASN, 2005.*

Figure 1.10 b, c and d Mortality hazard ratios across all quintiles of phosphate (b), calcium (c) and PTH (d). The first quintile represents the lowest levels and the fifth the highest levels. Solid bars represent treated patients.



1.19 Effects of vitamin D on the cardiovascular system

Vitamin D deficiency is associated with increased cardiovascular morbidity and mortality both in the general population *(Zittermann et al, 2003; Zittermann et al, 2006)* and in CKD patients as discussed above *(Teng et al, 2003; Teng et al, 2005; Tentori et al, 2006; Shoiji et al, 2004; Wolf et al, 2007).* The vitamin D receptor is present in the vascular smooth muscle cells *(Carthy et al, 1989)*, cardiomyocytes *(Zitterman A, 2006; Xiang et al, 2005)* and cells of the monocyte/macrophage lineage *(Mathieu et al, 2002),* where it has important anti-inflammatory and immunomodulatory effects *(Towler D, 2007; Tabata et al, 1988; Tokuda et al, 2000).* Vitamin D is a negative endocrine regulator of the renin-angiotensin system *(Li et al, 2003)*, inhibits atrial natriuretic peptide *(Bodyak et al, 2007),* increases myocardial contractility *(Zitterman A, 2003)* and reduces cardiomyocyte hypertrophy *(Li et al, 2003).* Also, a reno-protective effect of Vitamin D, exerted via the TGF-ß pathway, leads to reduced proteinuria and fibrosis *(Mizobuchi et al, 2007).*

1.20 Effects of vitamin D on the vasculature

Although several clinical studies have discussed the effects of vitamin D therapy on vascular measures *(Milliner et al, 1990; Litwin et al, 2005; Shroff et al, 2007)* and calcification, only one study has correlated these with vitamin D levels *(London et al, 2007).* London et al showed that in a cohort of 52 prevalent HD patients who were naïve to vitamin D therapy, low 25(OH)D and 1,25$(OH)_2$D levels were associated with increased arterial stiffness and endothelial dysfunction but not with vascular calcification as seen on plain x-rays *(London et al, 2007).*

The effects of vitamin D analogues on the vascular smooth muscle cells have been described in animal models. Calcitriol (1,α-25 dihydroxyvitamin D_3) upregulates the VDR and increases cellular Ca uptake in a dose dependent manner *(Wu-Wong et al, 2006)*, decreases VSMC proliferation that is mediated through an increase in vascular endothelial growth factor expression *(Cardus et al, 2006)* and induces VSMC migration *(Carthy et al, 1989)*. Also, calcitriol can upregulate the expression of osteochondrocytic genes, including core-binding factor 1 (cbfa-1) and osteopontin, that mediate osteoblastic conversion of VSMCs *(Giachelli et al, 2004; Shalhoub et al, 2006; Wu-Wong et al, 2006)*. However, some of these studies used pharmacological doses of calcitriol that are clearly toxic and result in severe hypercalcaemia and hyperphosphataemia.

1.21 Newer vitamin D analogues

Newer vitamin D analogues such as 19-nor-1α25$(OH)_2D_2$ (paricalcitol) and 1α-hydroxyvitamin-D_2 (doxercalciferol) effectively suppress PTH, but are reportedly less calcaemic than calcitriol, and are now commonly used in clinical practice. Their effects have been tested in animal models of smooth muscle cell explant cultures as well as intact vessels, but conflicting results are reported: in a model of human VSMC explant cultures calcitriol induced a significant increase in Ca uptake whereas paricalcitriol had no appreciable effect on calcification *(Wu-Wong et al, 2006,)* but in bovine artery smooth muscle cells calcitriol and paricalcitol had similar pro-calcific effects *(Shalhoub et al, 2006)*. A study in uraemic rats has shown that doxercalciferol induces aortic calcification even at low doses that do not raise the Ca x P product, whereas high doses of paricalcitol did not cause calcification *(Mizobuchi et al, 2007)*.

This suggests that vitamin D analogues mediate vascular calcification via different mechanisms, with possible species differences, and this needs further evaluation in humans.

The distinct survival advantages of the vitamin D analogues and their multiple effects on the cardiovascular system need further investigations to determine their optimal therapeutic levels in dialysis patients and to study the mechanisms of their action on the VSMCs.

1.22 Fibroblast Growth Factor 23 (FGF-23)

FGF-23, the most potent amongst of the phosphatonins, plays a key role in the Ca-PO4-PTH–vitamin D axis. Like PTH, it increases urinary PO_4 excretion, and by suppressing renal 1-α hydroxylase activity it reduces $1,25(OH)_2D$ production *(Hsu C et al, 2008)*. FGF-23 levels are raised even in the early stages of CKD, and persists in dialysis patients, and has been linked with increased mortality *(Gutierrez et al, 2008)*. Moreover, elevated FGF-23 levels are seen in non-CKD patients with high PO4 levels. Given its phosphaturic effect and effect on down-regulating $1,25(OH)_2D$ production, FGF-23 decreases bone mineralisation, but this is mainly due to hypophosphataemia and not a direct effect on bone *(Urena-Torres et al, KI 2008).* FGF-23 has not been studied in children with CKD to date.

Section IV – The role of calcification inhibitors in CKD

1.22 The discovery of calcification inhibitors: animal knock-out models

All extracellular fluid contains calcium and phosphate in concentrations exceeding their solubility product for spontaneous precipitation, suggesting that under normal conditions protein inhibitors of ectopic soft tissue calcification prevent the development or progression of vascular calcification *(Ketteler et al, 2003; Moe et al, 2003)*. There is a body of evidence showing that calcification is a highly regulated cell mediated process, involving a complex interplay of promoters and inhibitors of calcification. *In vitro* studies have shown that, whereas serum from normal subjects is a potent inhibitor of VSMC calcification, serum from CKD patients lacks this protective effect *(Moe et al, 2005; Reynolds et al, 2005)*. Although this might be due to the presence of toxins in uremic serum, an alternate explanation may be that serum from dialysis patients lacks inhibitors.

Animal knock-out models and human single gene defects have confirmed the role of a number of proteins in regulating vascular calcification. In addition, they have focused attention on proteins involved in the regulation of both vascular calcification and bone mineralization and suggested that these processes may in some way be mechanistically linked. Table 1.4 summarises the principal calcification inhibitors, the outcome of gene disruption studies in mice, and single gene defects or genetic polymorphisms in humans.

Table 1.4 Calcification inhibitors - outcome of gene disruption studies

Inhibitors	Gene disruption studies in mice	Human single gene studies and genetic polymorphisms
Fetuin-A	Ectopic calcification of small blood vessels, most organs (e.g., myocardium, lung, kidney, skin	Polymorphisms may predispose patients to vascular calcification
Matrix-Gla protein	Medial calcification of arteries, aortic valves (not arterioles, capillaries, or veins), cartilaginous metaplasia within the vessel wall	Keutel syndrome-extensive vascular calcification and abnormal calcification of cartilage. MGP gene polymorphisms may be prognostic for vascular calcification
Osteoprotegerin	Medial and subintimal calcification of the aorta and renal arteries, presence of multinuclear osteoclast-like cells within the vascular wall	Juvenile Paget's disease-an autosomal recessive osteopathy, but no clear association with vascular disease. Polymorphisms in the promoter region of OPG are associated with atherosclerosis.
Klotho	All calibers of arteries affected, intimal thickening of medium-sized arteries	Polymorphism may be a genetic risk factor for coronary artery disease.
Nucleotide pyrophosphatase / phosphodiesterase 1	Aortic medial calcification, intraaortic cartilaginous differentiation of VSMC	Infantile idiopathic arterial calcification — calcification of the internal elastic laminae of large vessels, often with death in the first year of life.

In this section I have described the physiological calcification inhibitors, in particular Fetuin-A, Osteoprotegerin (OPG) and matrix γ-carboxyglutamic acid protein (MGP), focusing on their role in calcification inhibition, the association of their circulating levels with underlying disease processes, and their prognostic relevance, if any.

1.23 Fetuin-A

Fetuin-A (a2-Heremans-Schmid glycoprotein) is a 62kD protein that belongs to the cystatin superfamily of cysteine protease inhibitors. It is a circulating glycoprotein that is produced by the liver and contributes to almost 50% of the calcification inhibitory capacity of human plasma *(Ketteler et al, 2006)*.

Animal Knock-out studies

Fetuin-A-/- mice develop mild ectopic calcification, but when crossed onto a DBA/2 calcification-susceptible mouse strain or when these mice are fed on a mineral- and vitamin D-rich diet or on high-fat diet, they develop widespread soft-tissue and intra-arterial calcification, including calcification of parenchymal organs like the heart, lungs and kidneys *(Schafer et al, 2003)*. Calcification was more likely to involve the smaller vessels whereas larger vessels such as the aorta were spared, possibly as a result of the protective effects of other calcification inhibitors like MGP.

Mechanisms of action

Fetuin-A acts systemically by binding excess mineral and inhibiting basic Ca-PO_4 precipitation in serum and extracellular fluids *(Heiss et al, 2003)*. Price et al showed that a complex of fetuin (80%), MGP (2%) and Ca-PO_4 (18%) efficiently prevented the growth, aggregation and precipitation of hydroxyapatite in etidronate treated rats *(Price et al, 2003)*. This suggests that the calcification inhibitory properties of fetuin and MGP may be related to their ability to form stable complexes with nascent crystals. In addition, Reynolds et al have shown that fetuin-A is a multifunctional protein that can also modulate the calcification processes locally. At sites of vascular damage fetuin-A is taken up by VSMCs, incorporated into intracellular vesicles, and then released within matrix vesicles where it potently inhibits mineral nucleation *(Reynolds et al, 2005)*. In addition, fetuin-A inhibits VSMC apoptosis and aids in phagocytosis of extracellular vesicles, thus further limiting mineralization *(Reynolds et al, 2005)*. It is hypothesized that while fetuin-A blocks crystal growth during transcellular transport of Ca x PO_4 rich vesicles, it will eventually be co-precipitated

with hydroxyapatite mineral outside the cell, where this material can be safely deposited without triggering apoptosis. This may explain how fetuin-A, one of the most potent calcification inhibitors in solution, regularly co-localises with pathologically calcified lesions, and indeed with all mineralized tissue including bone *(Triffit et al, 1976; Schinke et al, 1996)*.

In addition to these calcification inhibitory effects, fetuin-A ia a negative acute phase reactant; various interleukins, particularly interleukin-1 β, decrease its synthesis. Also, fetuin-A acts as a soluble TGF-β antagonist, as a result of structural similarities to TGF-β II receptor via a β-glycan like domain *(Demetriou et al, 1996)*. This property allows for cytokine-dependent osteogenesis, but may potentially also antagonize other TGF-β effects such as fibrogenesis and inhibition of cell proliferation. Finally, fetuin-A can impair insulin receptor tyrosine kinase signaling, thus reducing insulin sensitivity *(Mathews et al, 2006)*.

Influence on the vasculature

Several small observational studies have examined the associations between the circulating fetuin-A level and vascular measures and shown that low circulating fetuin-A levels are associated with greater vascular stiffness and calcification *(Heremans et al, 2006; Mori et al, 2007; Ix et al, 2007)*. However, cross-sectional studies are not able to determine a cause-effect relationship, and there are no longitudinal studies to date. Moreover, a single session of haemodialysis can significantly lower the fetuin-A level *(Cozzolino et al, 2007; Ciaccio et al, 2008)*, and levels also need to be interpreted keeping in mind the inflammatory status of the patient *(Stenvinkel et al, 2005)*.

Fetuin-A levels in CKD and associations with outcome

Several cross-sectional observational studies have reported low fetuin-A levels in dialysis patients, possibly as a combined result of reduced fetuin-A production in the face of a pro-inflammatory uraemic milieu or increased fetuin consumption in a pro-calcif environment. Fetuin-A levels are also particularly low in patients with calcific uraemic arteriolopathy *(Schafer et al, 2003)*. Fetuin-A levels are also low in renal transplant recipients as compared to age matched healthy controls, but did not correlate with any vascular measure *(van Summeren et al, 2008)*.

Circulating fetuin-A levels are significantly lower in dialysis patients than in healthy controls, and this has been linked with cardiovascular mortality, presumably as a result of accelerated vascular calcification *(Ketteler et al, 2003; Stenvinkel et al, 2005; Wang et al, 2005)*. In a cohort of ~1000 incident dialysis patients, Hermans et al showed that an increase in serum fetuin-A by 0.1g/L was associated with a 9% lower adjusted risk for death after a median follow-up of 2.8 years, and this effect was independent of serum CRP levels *(Hermans et al, 2007)*. Moreover, in this study Hermans also found an association of low fetuin-A levels with non-cardiovascular mortality. While there were too few patients in each subgroup to meaningfully dissect the association of low fetuin-A with non-cardiovascular causes of death, infectious causes constitute the most common cause of non-cardiovascular deaths, and fetuin-A levels are depressed in the presence of inflammation. In early CKD (stages 3 and 4), fetuin-A levels did not correlate with all cause or cardiovascular mortality *(Ix et al, 2007)*.

However, it may also be that patients with calcification have genetically lower levels or perturbations in the fetuin-A functional activity that predisposes them to calcify. Polymorphisms in the fetuin-A gene may determine the magnitude of decrease in fetuin-A production in the face of inflammation and an individual's susceptibility to calcify. Fetuin-A levels correlate with PO_4 levels even in the general population *(Osawa et al, 2005)*, but the only two studies in this field have shown conflicting results *(Cozzolino et al, 2007; Osawa et al, 2005)*

Importantly, all of the above studies are cross-sectional in nature, and measures at a single time-point cannot reflect the complex relationship between fetuin-A and vascular disease at different stages of CKD. In the Heart and Soul study, which included patients mostly with intact renal function, high fetuin-A levels were associated with hyperlipidaemia and features of the metabolic syndrome, but not with outcome parameters, and no correlation between fetuin-A and declining renal function could be detected *(Ix et al, 2006)*. Fetuin-A levels are not low in early CKD, and normal fetuin-A levels have been reported in a cohort of well-controlled dialysis patients with low inflammatory activity and a low Ca x PO_4 burden *(Hermans et al, 2005)*. Importantly, all of the above studies include a large proportion of diabetic patients, and fetuin-A levels are in fact higher in patients with diabetic nephropathy *(Mehrotra et al, 2006)*. Thus, further longitudinal studies are needed to fully appreciate the complex interplay between fetuin-A and the calcification process at different stages of CKD.

1.24 Osteoprotegerin

Osteoprotegerin (OPG) is a member of the tumour necrosis factor receptor superfamily and acts as a decoy receptor for receptor activator of nuclear factor-κB ligand (RANKL), which stimulates all aspects of osteoclast function, including differentiation, activation, fusion and survival, that together mediate bone resorption *(Simonet et al, 1997; Lacey et al, 1998)*. By blocking RANKL, OPG inhibits osteoclastic bone resorption, but OPG is produced by a number of tissues, particularly in the media of arteries *(Simonet et al, 1997)*.

Animal Knock-out studies

Targeted deletion of OPG in mice leads to early onset osteoporosis from unrestrained osteoclast function and also medial calcification of great arteries *(Bucay et al, 1998)*. Calcification in these mice could be rescued by introduction of an opg transgene from mid-gestation but not by parenteral application of OPG after mineralized lesions were established. This is in contrast to osteoporosis, which could be efficiently treated by a parenteral OPG regimen *(Collin-Osdoby et al, 2004)*.

Mechanisms of action

The precise role of OPG in the vascular wall and its possible interaction with VSMCs has yet to be determined. Evidence from animal models suggests that OPG may be protective against vascular calcification: OPG-deficient mice develop vascular calcification *(Bucay et al, 1998)* and OPG inhibits warfarin-induced vascular calcification in rats *(Price et al, 2001)*. Interestingly, although OPG is deposited at sites of calcification and globally downregulated in the diseased vasculature, circulating OPG levels are increased in patients on hemodialysis, and have been

linked to the presence and extent of coronary artery disease *(Collin-Osdoby et al, 2004)*. Given that OPG is produced by a variety of cell types, the source of elevated OPG remains elusive and it is unclear whether increased systemic OPG levels reflect the cause or consequence of vascular calcification or are an epiphenomenon of processes harboured in calcifying tissue.

Role in CKD and influence on the vasculature

OPG levels have been associated with increased aortic stiffness as shown in haemodialysis patients *(Othmane et al, 2007)* and in non-CKD diabetics *(Kim et al, 2005)*. A number of studies have shown that OPG is elevated in vascular disease and that OPG seems to be a biomarker for increased vascular mortality and an increased risk for cardiovascular disease, especially in adult populations of renal failure patients *(Browner et al, 2001; Kiechl et al, 2004; Hjelmesaeth et al, 2006)*. In the last study, serum OPG taken from renal transplant recipients shortly after transplant was a significant independent predictor of cardiovascular death in these adult patients *(Hjelmesaeth et al, 2006)*. HD patients have higher OPG, and this may be raised as a compensatory response to PTH driven bone resorption *(Albalate et al, 2006)*.

Associations with cardiovascular outcome

Studies in adults with normal renal function have shown that OPG is elevated in stable coronary artery disease *(Schoppet et al, 2003)*, is associated with the progression of carotid atherosclerosis *(Kiechl et al, 2004)* and coronary calcification *(Abedin et al, 2007)* and increased cardiovascular mortality *(Kiechl et al, 2004)*. Two prospective studies in the non-CKD population have shown that elevated OPG levels are independently associated with an increased risk of cardiovascular events *(Ueland*

et al, 2004; Kiechl et al, 2004). Finally, atherosclerosis risk factors have been associated with OPG independent of the measured atherosclerosis burden, suggesting a pathogenic rather than a compensatory role for OPG in the vasculature *(Abedin et al, 2007).* It is interesting to speculate that circulating OPG may in itself be a damaging agent for VSMCs in a manner analogous to glucose in diabetes and PTH in CKD. As genetically engineered OPG is now being given to older patients with osteoporosis in pilot trials *(Bekker et al, 2001)*, further studies to determine the effects of elevated OPG on the vasculature are urgently required.

1.25 Matrix Gla-protein

Matrix Gla [γ-carboxyglutamic acid] protein (MGP) is an extracellular matrix protein, that is synthesised by chondrocytes and vascular smooth muscle cells, that belongs to a family of proteins that contain γ-carboxyglutamate residues *(Shanahan et al, 1998)*. These proteins require a vitamin K dependent γ-carboxylation to convert their inactive undercarboxylated form (uc-MGP or Glu-MGP) into the active γ-carboxylated form (Gla- MGP). γ-carboxyglutamic acid has unique metal binding properties, and confers these properties to the proteins into which it is incorporated *(Burnier et al, 1981)*.

Animal knock-out studies

Homozygous mice deficient in MGP develop calcification and cartilaginous metaplasia of the aorta and its branches and typically develop aortic rupture as a direct consequence of vascular calcification *(Luo et al, 1997)*. Mutations of MGP in humans

leading to absent or non-functional MGP manifests as Keutel syndrome *(Munroe et al, 1999)*, which is characterized by abnormal calcification of cartilage in the ears, nose, larynx, trachea, and ribs. These patients on post-mortem show extensive medial vascular calcification at an early age.

Mechanisms of action

MGP is highly expressed in the tunica media of healthy arteries and MGP mRNA expression is upregulated in intimal and medial vessel calcification *(Shanahan et al, 1994; Shanahan et al, 1998),* and also with VSMC mineralization *in vitro (Proudfoot et al, 1998)*. MGP is found in matrix vesicles, wherein it acts like fetuin-A to limit mineral nucleation *(Reynolds et al, 2004)*. Along with fetuin-A, it forms part of the 'fetuin-mineral complex' (fetuin (80%), MGP (2%) and Ca-PO_4 (18%)) that was shown to prevent the growth, aggregation and precipitation of hydroxyapatite in etidronate treated rats *(Price et al, 2003)*.

Importantly, MGP is also a regulatory protein for BMP-2 *(Zebboudj et al, 2002):* higher levels of MGP will inhibit BMP-2 activity. BMP-2 is a potent morphogen of the TGF-β superfamily that can induce ectopic bone and cartilage when implanted in soft tissues due to its capacity to regulate mesenchymal progenitor cell differentiation. It is possible that in MGP-deficient mice, the unopposed action of BMP-2 results in mesenchymal progenitor cells or local VSMC to develop instead into chondrocytes. Thus, MGP acts as a regulator of both calcification and cell differentiation in the vasculature.

Transgenic studies have suggested that impaired γ-carboxylation of MGP and not MGP levels *per se* are associated with vascular calcification. In rats, treatment with the vitamin K antagonist, warfarin, at doses that inhibit the vitamin K dependent γ-carboxylation of MGP, there was a rapid calcification of elastic lamellae of the arterial media with increased MGP mRNA expression *(Price et al, 1998)*. In humans, small observational studies have suggested that the use of oral anticoagulants is associated with increasing coronary artery and valvular calcification *(Koos et al, 2005; Schurgers et al, 2004)*.

Influence on the vasculature

As with OPG, the role of circulating MGP, if any, remains largely unknown and the influence of circulating MGP levels on vascular calcification is unclear. Increased serum levels of MGP without a concomitant increase in MGP expression in the arterial walls, does not inhibit the ectopic mineralization observed in mice lacking MGP; rescue of calcification only occured if MGP was expressed in VSMCs, not when it was present systemically *(Murshed et al, 2004).*

There are conflicting reports on the associations between circulating MGP levels and atherosclerosis in humans, *(Braam et al, 2000; Jono et al, 2004)*, with one study reporting an association between serum MGP levels and coronary risk factors even in patients with no pre-existing cardiovascular disease *(O'Donnell et al, 2006)*. In patients with known coronary artery disease, MGP levels have shown an inverse correlation with the coronary artery calcification score on CT scan in one study *(Jono*

et al, 2004), but after adjustment for cardiovascular risk factors, no consistent association was found in a second study *(O'Donnell et al, 2006)*.

One reason for the conflicting results from the above studies may be that we are measuring the 'wrong' type of MGP in the circulation. Using many of the currently available assays, no distinction can be made between the carboxylated active (Gla-MGP) and the undercarboxylated inactive (Glu-MGP) forms of MGP. Using immunohistochemistry, Schurgers et al found a strong association between vascular calcification and local deposition of Glu-MGP in arteries with Monckeberg's sclerosis, whereas healthy uncalcified arteries had abundant amounts of Gla-MGP *(Schurgers et al, 2005)*. In the only published study measuring serum uc-MGP levels, it was found that lower circulating levels of uc-MGP were inversely associated with phosphate levels and the aortic augmentation index but not the aortic pulse wave velocity *(Hermans et al, 2007)*.

Levels and outcome in CKD patients

In the face of different measures of MGP and conflicting reports on associations of low serum levels with calcification, there is no outcome data available. Also, the cross-sectional nature of this study would make it difficult to draw any conclusions about a cause-effect relationship. However, MGP polymorphisms have been associated with increased mortality in dialysis patients *(Brancaccio et al, 2005)*. As MGP levels can be potentially modulated with dietary supplementation of vitamin K *(Schurgers et al, 2001)*, the role of circulating MGP, if any, needs to be further explored.

Section V - The vascular biology of calcification

Over a century ago Virchow described vascular calcification as an ossification, not a mere deposition of calcium: calcium deposits in both the tunica intima and tunica media of sclerotic arteries were accompanied by the presence of osteoblast-like cells, lamellar structures, and hematopoietic cells *(Virchow, 1863)*. Since then, calcification has been described as a complication of many vascular diseases and clinical conditions, but is most spectacularly evident in uraemic patients, where a 'perfect storm' of mineral dysregulation, inflammatory insults and often co-existing atherosclerotic diseases are present.

In this section I have described the *in vitro* and animal studies that have contributed to our current knowledge of the vascular biology of calcification, with particular relevance to uraemic vascular disease.

1.26 The normal VSMC phenotype

VSMCs within the normal tunica media are responsible for maintaining vascular tone and this depends on the interaction between actin and myosin myofibrils. Consequently, they express a number of unique contractile proteins, agonist receptors and signal transduction molecules *(Chamley-Campbell et al, 1979)*. α– SM actin is the first marker of differentiated VSMCs expressed during vasculogenesis, and is the most abundant actin isoform in mature VSMCs *(de Groot et al, 1999)*.

On the other hand, intimal VSMCs resemble immature, dedifferentiated VSMCs and express low levels of VSMC contractile proteins, contain fewer myofilaments, and exhibit a gene expression profile that differs from that of medial VSMCs *(Shanahan et al, 1993; Shanahan et al, 1998)*. Also, the intimal and medial phenotypes of VSMCs are not confined to distinct areas of the vessel wall, but rather a mixture of phenotypes is seen in all areas of the vessel *(de Groot et al, 1999)*.

1.27 The phenotypic plasticity of VSMC

Unlike other skeletal and cardiac muscle cells, VSMCs do not terminally differentiate. They have an extraordinary capacity to undergo phenotypic change during development, *in vitro*, and in response to injury *(Shanahan et al, 1998; Iyemere et al, 2006)*. Under normal conditions, blood vessels have low rates of cell proliferation and turnover. Hence, the phenotype of a medial VSMC is used as a reference point, and the relative levels of expression of contractile proteins (α-SM actin, smooth muscle myosin heavy chain, SM-22, calponin and caldesmon) are used as markers to describe the adult medial VSMC phenotype *(Eddinger et al, 1991; Shanahan et al, 1993)*. More recently, studies on VSMC heterogeneity have given rise to the concept of a continuum or spectrum of VSMC phenotypes from development to maturity: early fibroblast-like proliferating cells to neonatal matrix-producing VSMCs to the mature contractile phenotype *(Shanahan et al, 1999; Chamley-Campbell et al, 1981)*. Studies have shown that, at least in some vertebrates, VSMCs in different loci in the arterial tree may have different embryonic origins: VSMCs in the upper thoracic aorta are derived from a neuro-ectodermal source, those in the abdominal aorta from a

mesenchymal source and those in the coronary vessels from an intracardiac mesemchyme *(Ross et al, 1999; Topouzis et al, 1996).*

Recent studies have shown that the phenotypic changes in VSMCs *in vitro* or in response to injury are associated with their ability to acquire the characteristics of a diverse range of mesenchymal lineages, such as osteoblastic, chondrocytic or adipocytic cell types, that all originate from the same mesenchymal cell lineages and precursors *(Shanahan et al, 1999; Tyson et al, 2003; Davies et al, 2005)*. These de-differentiated phenotypes may lead to calcification, altered matrix production and lipid accumulation, and importantly, the end-point for some of these processes is VSMC death or senescence *(Shanahan et al, 1999).*

In response to injury, medial VSMCs can migrate into the intima, and in doing so lose their 'contractile' phenotype and dedifferentiate into a 'synthetic' phenotype that is essential for repair *(Chamley-Campbell et al, 1981; Davies et al, 1990)*. This de-differentiation allows VSMCs to migrate, proliferate and produce an extracellular matrix. In atherosclerotic disease, this process of vascular repair leads to the formation of a VSMC-rich fibrous cap that acts as a barrier to shield the lipid-rich pro-thrombogenic core from blood flow (*Weissberg et al, 1996)*. Factors that regulate VSMC phenotypic heterogeneity and initiate phenotypic change remain unanswered.

In *in vitro* culture VSMCs demonstrate a similar process of de-differentiation as they do in response to injury *(Bjorkerud et al, 1987).* VSMCs in culture can rapidly de-differentiate, losing their myofilaments, increasing their proportion of biosynthetic organelles and losing their ability to contract *(Chamley-Campbell et al, 1979)*. In *in*

vitro culture human VSMCs spontaneously mineralise and co-express chondrocytic and bone markers alongside VSMC contractile proteins *(Shanahan et al, 1999)*.

1.28 Intimal vs medial calcification

Calcification can occur at two sites in the vessel wall: the tunica intima and the tunica media *(Shanahan et al, 1999)*. The anatomical site of calcification determines the nature and extent of its clinical manifestations *(London et al, 2005)*.

- **Intimal calcification** is seen with dyslipidemia, hypertension and smoking and takes the form of atherosclerotic vascular disease. It is a patchy and discontinuous process that involves macrophages and VSMCs in lipid-rich regions. Atherosclerotic plaques often contain apatite crystals, and may display a histological appearance similar to that of lamellar bone (Bostrom et al, 1995).
- **Medial calcification**, also known as Monckeberg's sclerosis, is focal in distribution, organized along the elastic lamellae and is almost exclusively associated with VSMCs *(Proudfoot et al, 2001; London et al, 2005)*. It is seen with advancing age and in diabetes and uraemia.

Although a combination of intimal and medial calcification has been observed in patients with CKD, either process may occur independently of the other and, at least in adolescents and young adults with CKD, the involvement is almost exclusively medial *(London et al, 2005)*. The differences between intimal and medial calcification imply different etiologies, or at least different mechanisms of initiation

of VSMC damage, however, a common feature of both forms of calcification is the phenotypic modulation of VSMCs in response to injury *(Shanahan et al, 1999)*.

1.29 Vascular calcification is an active cell-mediated process

Mineralisation of the extracellular matrix in skeletal and dental tissues is a cell-mediated process that is required for the normal development of bones and teeth *(Schinke et al, 1999)*. Yet, the precipitation of Ca and PO_4 ions as hydroxyapatite mineral in bones or at ectopic sites is in disequilibrium with the ionic Ca and PO_4 concentrations in the surrounding environment *(Ng et al, 1976)*. Thus a variety of factors are required to create a unique environment that allows mineral deposition both physiologically and pathologically. For many years ectopic vascular calification was thought to be a passive degenerative process in dead or dying cells, but converging evidence from numerous *in vitro* studies and animal knock-out models has shown that calcification is a highly regulated, cell-mediated process with similarities to bone mineralisation *(Ikeda et al, 1993; Shanahan et al, 1994)*.

I - Histopathology and gene expression in human calcified vascular lesions

- X-ray crystallography and electron microprobe analysis have shown that the mineral deposited in the vessel wall is basic Ca - PO_4 in apatitic form *(Schmid et al, 1980; Reynolds et al, 2004)*, some of which is hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], the same crystal found in bone.
- VSMCs *in vivo* have been observed to bud matrix vesicles from their plasma membrane *(Proudfoot et al, 2000; Reynolds et al, 2004)*. These are small

membrane bound particles, first described in chondrocytes and osteoblasts during developmental osteogenesis, that form a microenvironment capable of concentrating Ca and PO_4, thus allowing crystal nucleation to occur.

- In advanced atherosclerotic lesions, and more commonly in the peripheral arteries of diabetic patients, osteoid has been observed which mineralises to form mature bone tissue complete with vascular canals and marrow spaces within the vessel wall *(Shanahan et al, 1999)*. The cellular origin of this ossification, as opposed to calcification, is unknown. It is possible that stem cells from the circulation that are trapped in the plaque, or present in the vessel wall, are exposed to signals within the calcified lesions that lead to the initiation of a developmental osteogenic differentiation program *(Tintut et al, 2003)*.
- Expression of osteogenic markers by VSMCs in association with dystrophic calcification provides further evidence of its active nature. Matrix γ-carboxyglutamic acid protein [MGP] *(Bostrom et al, 2001; Shanahan et al, 1998)*, osteopontin [OPN] *(O'Brien et al, 1994)* and bone morphogenetic protein-2 [BMP-2] *(Zebboudj et al, 2002)* were the first proteins shown to be associated with vascular calcification *in vivo*. Studies using RT-PCR and immunohistochemical analysis of gene expression in normal and calcified arteries, including arteries from uraemic patients, have shown that VSMCs express Cbfa1/Runx2 and Sox 9 *(Proudfoot et al, 2000; Moe et al, 2002)*, obligate transcription factors for osteoblastic and chondrocytic differentiation as well as their target genes, alkaline phosphatase, bone sialoprotein, osteocalcin and collagen II, at sites of calcification *in vivo*. Analysis of the temporal pattern of expression of these proteins in relation to the onset of calcification demonstrated that in the normal vessel wall VSMCs expressed constitutive inhibitors of

calcification such as MGP *(Tyson et al, 2003)*, but these inhibitors were downregulated in calcified vessels and VSMCs upregulated expression of mineralization regulators normally expressed in bone *(Shanahan et al, 1999)*.

II - Mouse gene knockouts models and human single gene defects

Animal knockout models have confirmed the role of a number of proteins in regulating vascular calcification. In addition, they have focussed attention on proteins involved in the regulation of both vascular calcification and bone mineralization and suggested that these processes may in some way be mechanistically linked.

- The knockout mouse for MGP was the first amongst these models *(Luo et al, 1997),* and has been discussed extensively in section 1.12 of chapter 1. Homozygous mice deficient in MGP develop calcification and cartilaginous metaplasia of the aorta and its branches and typically develop aortic rupture as a direct consequence of vascular calcification *(Luo et al, 1997)*. Importantly, MGP is also a regulatory protein for Bone Morphogenetic Protein – 2 [BMP-2] (*O'Brien et al, 1994; Zebboudj et al, 2002)*: higher levels of MGP will inhibit BMP-2 activity. BMP-2 is a potent morphogen of the TGFβ-superfamily that can induce ectopic bone and cartilage when implanted in soft tissues due to its capacity to regulate mesenchymal progenitor cell differentiation. It is possible that in MGP deficient mice the unopposed action of BMP-2 results in mesenchymal progenitor cells, or local VSMC to develop instead into chondrocytes. Thus, MGP acts as a regulator of both calcification and cell differentiation in the vasculature. Mutations of MGP in humans leading to absent or non-functional MGP manifests as Keutel syndrome *(Munroe et al, 1999)*

characterised by abnormal calcification of cartilage in the ears, nose, larynx, trachea and ribs. These patients on post-mortem show extensive medial vascular calcification at an early age.

- Fetuin-A (α-2-Heremens-Schmid glycoprotein) is an important circulating factor and has been described at length in section IV in this chapter. It is a very potent inhibitor of apatite crystal formation *(Price et al, 2003)* and also regulates apoptosis, vesicle calcification and phagocytosis *(Reynolds et al, 2005)*. The Fetuin-A knockout mouse develops extensive, fatal vascular and parenchymal calcification, predominantly of the kidneys, heart and lungs *(Schafer et al, 2003)*.

- Osteoprotegerin (OPG), a soluble TNFα receptor mimic, can inhibit osteoclastogenesis and has paradoxical effects on bone mineralization and vascular calcification. OPG binds to and inhibits RANKL [receptor activator of nuclear factor kappa B (NFKB) ligand] *(Collin-Osdoby et al, 2004)*, which is expressed on the surface of osteoblast-like cells. RANKL activation of its receptor RANK is essential for the maturation of osteoclast progenitors. Mice deficient in OPG develop calcification and osteoporosis as a result of unopposed stimulation of RANKL receptors leading to increased osteoclastic activity *(Bucay et al, 1998)*. The role of OPG in calcification and clinical associations with its levels are described in section IV.

- Mice defective in fibrillin, an important microfibril associated with elastin, develop medial aortic calcification *(Ramirez et al, 1999)*. Variations in the fibrillin-1 genotype in humans cause Marfan's syndrome which is associated with increased aortic stiffness and an increased risk of cardiovascular disease (Fietta et al, 2002).

- The role of pH in promoting or inhibiting calcification is also important: crystal growth is favoured in an alkaline medium. The carbonic anhydrase isoenzyme II (CA II) knockout mouse develops an age-dependent medial calcification of small arteries in a number of organs, with the male genital tract developing the most extensive arterial calcinosis *(Spicer et al, 1989)*. CA II deficiency in humans causes a rare autosomal recessive disorder characterised by osteopetrosis, renal tubular acidosis, and cerebral calcification *(Cotter et al, 2005)*.

- Perturbation of the pathway involved in the generation of pyrophosphate by inactivation of the enzyme ecto-nucleotide pyrophosphatase/ phosphodiesterase 1 (ENPP1) leads to ossification of the aorta. The mouse knockout for ENPP1 *(Johnson et al, 2005)* develops upregulated alkaline phosphatase, decreased expression of osteopontin, increased calcification of aortic smooth muscle cells and chondrogenesis in mesenchymal precursors. In humans mutations in ENPP1 causes infantile idiopathic arterial calcification *(Rutch et al, 2003)*, a condition in which the internal elastic lamina of muscular arteries calcifies resulting in death usually within the first year of life.

- Klotho, a membrane protein which is thought to regulate human aging, plays a critical role in the regulation of Ca – PO_4 homeostasis by negatively regulating active Vitamin D synthesis *(Ikushima et al, 2006)*. Knockout mice for the Klotho gene (β-glucosidase) *(Nabeshima et al, 2002)* develop hyperphosphataemia, vascular calcification and impairment of both osteoblast and osteoclast differentiation leading to low-turnover osteopenia.

Table 1.4 (page 71) describes the human single-gene defects in calcification inhibitors and their clinical phenotypes.

1.30 Initiation of vascular smooth muscle cell calcification – insights from *in vitro* studies

Mechanistic insights into the process of vascular calcification have come from *in vitro* studies. When human VSMCs are cultured *in vitro* they spontaneously convert to an osteo/chondrocytic phenotype *(Shanahan et al, 1999)*, mimicking the phenotypic changes observed in calcified arteries. They form multicellular nodules that spontaneously calcify and upregulate expression of markers of bone and cartilage differentiation including Cbfa1 and Sox9 *(Tyson et al, 2003)*. Using these *in vitro* models it has been shown that apoptosis and vesicle release by phenotypically modified VSMCs produce the initial nidus for mineral nucleation *(Proudfoot et al, 2000)* and that this calcification process can be actively inhibited by a variety of multi-functional regulatory proteins *(Shanahan et al, 1999; Tyson et al, 2003)*.

I - The formation of matrix vesicles

One of the earliest events associated with calcification is VSMC death and apoptotic body release as well as matrix vesicle release from living cells *(Proudfoot et al, 2000)*. Using human aortic VSMC explants, Proudfoot et al have demonstrate that apoptosis occurs before the onset of calcification in VSMC nodules, and this was confirmed by nuclear morphology, the TUNEL technique and external display of phosphatidyl serine. In these nodular VSMC cultures apoptosis was apparent by day 7 and increased coincident with the onset of calcification *(Proudfoot et al, 2000; Reynolds et al, 2004)*. Inhibition of apoptosis experimentally with the caspase inhibitor ZVAD.fmk reduced calcification in nodules by ~40%, and stimulation of apoptosis with anti-Fas IgM, caused a10-fold increase in calcification. Also, this study

showed that apoptotic bodies derived from VSMCs can act as nucleating structures for calcium crystal formation: when VSMC-derived apoptotic bodies were incubated with ^{45}Ca, they were able to concentrate calcium *(Proudfoot et al, 2000)*. Reynolds et al have shown that the addition of increased levels of extracellular Ca and /or PO_4 to cultures induces VSMC apoptosis and that the inhibition of apoptosis in this model ameliorates calcification *(Reynolds et al, 2004)*. The apoptotic bodies accumulated calcium in a manner comparable to chondrocyte matrix vesicles, suggesting their ability to initiate calcification in a similar manner *(Reynolds et al, 2004)*. In a subsequent study it was shown that Fetuin-A inhibited *in vitro* VSMC calcification in part through inhibition of apoptosis and associated caspase cleavage *(Reynolds et al, 2005).*

These *in vitro* studies have lead to the hypothesis that factors that cause VSMC death or damage are likely to induce or accelerate the calcification process. In addition, factors that inhibit the normal phagocytosis of apoptotic bodies are also likely to increase the probability that these bodies will calcify within the vascular matrix *(Massy et al, 2008)*. Studies *in vitro* have shown that modified lipids are a key factor in inhibiting phagocytosis *(Proudfoot et al, 2004)* and lipids also contribute to osteogenic differentiation of VSMCs, explaining the presence of lipid and calcification in atherosclerotic plaques *(Demer et al, 2002)*. Finally, in a very recent publication using a mouse model of inducible VSMC-specific apoptosis, Clarke et al have shown that low-level VSMC apoptosis induced in apolipoprotein (Apo)E(-/-) mice fed a high fat diet induced the development of calcified plaques in younger

animals and promoted calcification within established plaques and also diffuse calcification of medial VSMCs *(Clarke et al, 2008)*.

II - Expression of osteo/chodrocytic markers by modified VSMCs

The expression of bone-associated proteins is not confined to bone, and the discovery of skeletal matrix proteins in atherosclerotic arteries and calcified heart valves has led to a search for the mechanisms and cell types responsible for their ectopic expression. Macrophages were initially considered to be the source of bone associated proteins, but there is now substantial evidence to suggest that VSMCs themselves can synthesise these proteins in vivo *(O'Brien et al, 1994; Shanahan et al, 1999)*, even in the course of medial calcification where there are no macrophages *(Shanahan et al, 1999)*. Some bone matrix proteins such as MGP and osteopontin are constitutively expressed by VSMCs in normal arteries and downregulated in calcified vessels *(Tyson et al, 2003)*. In association with this, some bone matrix proteins that are not normally expressed in the vessel wall, such as alkaline phosphatise (ALK), bone sialoprotein (BSP) and bone Gla-protein (BGP) are induced *(Tyson et al, 2003)*.

In vitro studies have characterised the VSMC expression of bone matrix proteins and provided some support for osteoblast-like VSMCs in regulating vascular calcification. Evidence suggests that the osteo/chondrocytic conversion of VSMCs, characterized by the expression of Cbfa1/Runx2, may be a phenotypic change induced in response to injury. Core binding factor α-1 (Cbfa-1, now known as Runx2), a transcription factor from the runt homology domain, is essential for osteoblast differentiation *(Otto*

et al, 1997), and regulates the expression of multiple genes expressed in osteoblasts such as BGP, BSP, OPN and collagen type II. Cbfa-1 / Runx2 is expressed at very low levels in normal vessels and significantly upregulated in calcified vessels *(Steitz et al, 2001)*. Expression of this plethora of mineralization-regulating proteins by VSMCs may represent an attempt by the cells to further regulate the calcification process or a direct transdifferentiation of the cell in response to pathological stimuli. However, the exact role of these multi-functional proteins and their expression at different stages of uraemic vascular injury remains unknown.

Although it is unclear exactly what regulates expression of Runx2 in VSMCs, it is upregulated *in vitro* by phosphate and lipids, i.e. damage inducing agents. Mineralisation of bovine VSMCs *in vitro* is shown to be encouraged by the addition of β–glycerophosphate, a PO_4 donor in vitro. These mineralising cells express cbfa-1, providing evidence of their osteogenic phenotype *(Steitz et al, 2001)*. Proudfoot et al have shown that human VSMCs in vitro spontaneously mineralise, and as they do so express bone associated proteins such as ALK, BSP, BGP, MGP and OPN, while simultaneously maintaining the expression of smooth muscle contractile markers *(Proudfoot et al, 1998; Severson et al, 1995; Shanahan et al, 1999)*. This model of VSMC calcification bears similarities to in vitro models of osteoblast differentiation, and the expression of cbfa-1 by human VSMCs suggests that the transcriptional pathways that regulate osteogenic differentiation may be present in human VSMCs *(Giachelli et al, 2001; Jono et al, 2004)*.

III - Mineral imbalance – the role of calcium and phosphate

More recently *in vitro* models have been developed to determine the factors specific to CKD that might induce VSMC calcification. Exposure of VSMCs to media containing elevated levels of Ca and /or PO_4 rapidly induced calcification with synergistic effects if both ions were elevated *(Reynolds et al, 2004)*. Apoptosis accounted for part of the accelerated calcification observed in this model. However, in response to extracellular Ca viable VSMCs were induced to release vesicles in a manner analogous to growth plate chondrocytes and these vesicles contained preformed Ca – PO_4 apatite accounting for their increased calcification capacity *in vitro (Reynolds et al, 2004; Proudfoot et al, 2000)*.

Giachelli et al have shown that in the presence of increased intracellular phosphate the sodium-dependent phosphate co-transporter, Pit-1, signals through Cbfa-1 to induce osteoblastic differentiation of vascular cells *(Giachelli et al, 2001; Jono et al, 2000)*. Thus, PO_4 can act as a signalling molecule and induce phenotypic changes in the VSMCs as well as directly contribute to the mineralisation process. Likewise, elevated Ca levels in the culture media enhance mineralisation and phenotypic transformation of VSMCs, also via the sodium-dependent phosphate co-transporter *(Yang et al, 2004)*. Elevated Ca levels were not able to increase PO_4 uptake acutely, but prolonged exposure of smooth muscle cell cultures to elevated Ca induced Pit-1 mRNA levels, suggesting that elevated Ca regulates PO_4 sensitivity of VSMCs (*Giachelli et al, 2001)*. These findings were extended by Reynolds et al, who showed that elevated Ca stimulated apoptosis as well as the release of mineralisation competent matrix vesicles from human VSMCs, further suggesting that elevated Ca has pro-mineralising effects beyond simply raising the Ca x PO_4 product, and

regulates multiple systems in smooth muscle cells that promote susceptibility to matrix mineralisation *(Giachelli et al, 2001; Reynolds et al, 2004)*. The disturbances in mineral metabolism in CKD possibly have similar effects *in vivo*: the heightened state of supersaturation that exists in plasma favours the deposition of mineral in soft-tissues by promoting vesicle release and VSMC osteogenic differentiation.

IV - Inhibitors of calcification – the role of regulating proteins

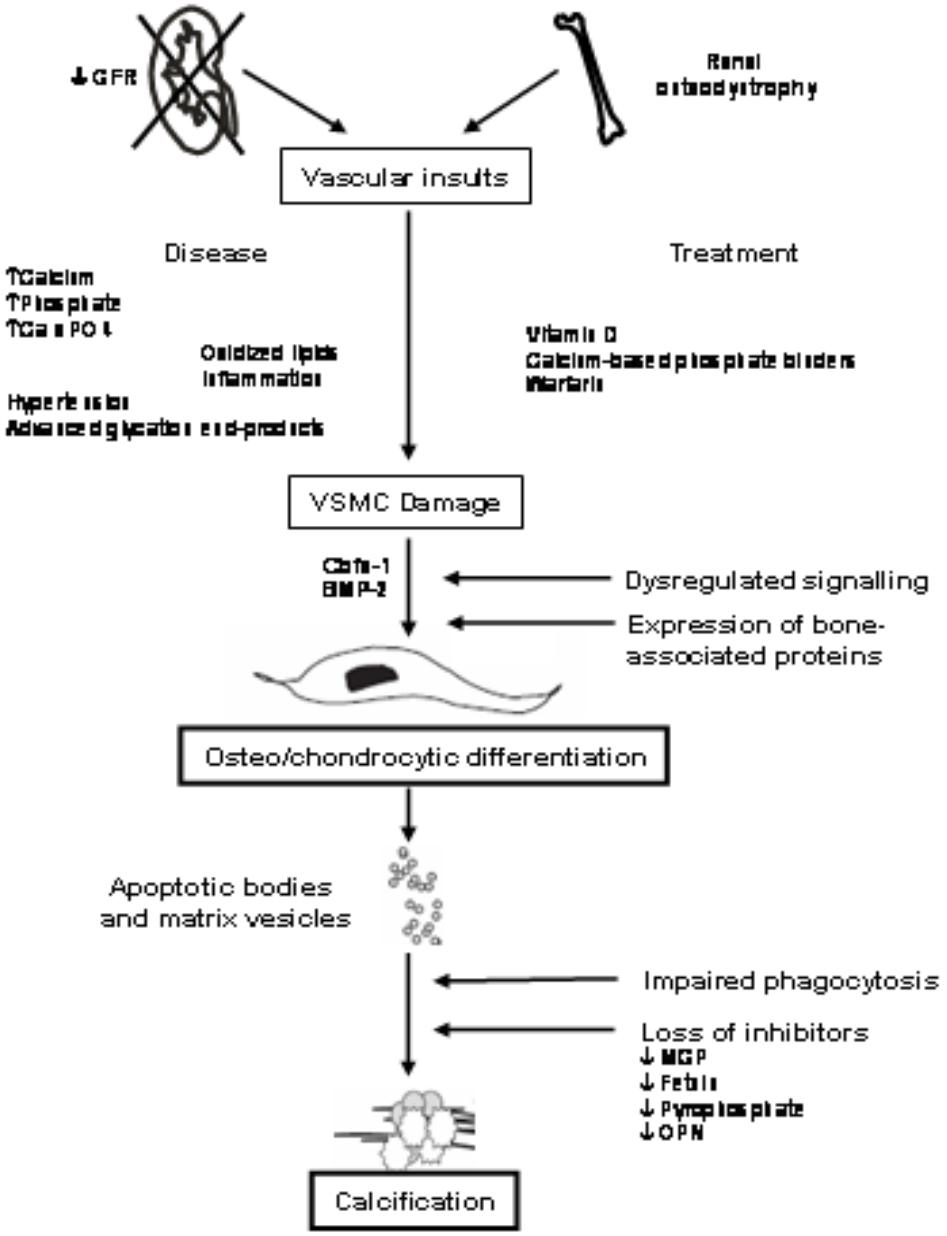
All extracellular fluids, even under normal circumstances, are saturated with respect to calcium and phosphate suggesting that potent inhibitors of vascular calcification are normally circulating to prevent ectopic soft tissue calcification *(Ketteler et al, 2002; Schafer et al, 2003)*. The addition of normal human serum to VSMC *in vitro* in the presence of elevated calcium and phosphate significantly inhibits the time-course and extent of calcification when compared to VSMCs treated in the absence of serum *(Reynolds et al, 2005)*. Serum acts by inhibiting apoptosis and by reducing the calcification potential of shed membrane vesicles, suggesting that under normal conditions vesicles may contain inhibitors of calcification derived from serum *(Reynolds et al, 2005)*. Subsequent analyses identified the serum protein fetuin-A as a key component of VSMC derived vesicles *(Ketteler et al, 2003; Reynolds et al, 2005)*. This protein is taken up by VSMCs and stored in cytoplasmic vesicles before being incorporated into released extracellular vesicles. Reynolds et al showed that Fetuin-A can inhibit apoptosis, enhance phagocytosis and its incorporation into vesicles completely abrogates their ability to calcify *(Reynolds et al, 2005)*. Potentially other as yet unidentified serum components may also act to inhibit soft tissue calcification.

Figure 1.11 is a schematic representation of the processes involved in vascular calcification in CKD.

1.31 Currently available models to study VSMC calcification

Although explants of VSMCs have been very useful in vitro models to study vascular damage and calcification, these cells lack the matrix and architecture of a normal vessel. The collagen matrix of the tunica media and the elastic lamina, the initial site of medial artery calcification *in vivo,* are lacking. To address this, Lomashvilli et al have developed an *in vitro* model of rat aortic rings *(Lomashvilli et al, 2005).* When the aortic rings were incubated in a medium with elevated PO_4 no calcification occurred, but mechanical injury resulted in extensive medial calcification. Pyrophosphate was identified as a potent endogenous inhibitor of calcification on the basis that normal aortas produce inhibitory levels of pyrophosphate and the addition of pyrophosphate could inhibit calcification in injured aortas. Pyrophosphate was found to be inactivated by alkaline phosphatase, a protein expressed by osteo/chondrocytic VSMCs *in vitro* in response to injury and at sites of calcification *in vivo* further suggesting that the induction of an osteogenic programme of gene expression in VSMCs may be a response to injury and reducing vascular damage may be a key target for reducing calcification.

Figure 1.11 A schematic representation of vascular calcification in CKD



Section VI – Project design

The work in this thesis broadly involves clinical studies to investigate the role of modifiable risk factors in cardiovascular disease in children on dialysis, translational research that directly correlates the *ex vivo* changes in the vessels with clinical parameters, and an *in vitro* study that uses a model of intact human arteries to determine the role of Ca and PO_4 in vascular injury and calcification.

The research questions examined in the course of this work are:

1. What is the cardiovascular mortality of chronic dialysis patients in our centre?
2. Can keeping the PTH levels at less than 2-fold the upper limit of normal throughout the course of CKD reduce the prevalence of vascular damage and calcification in children on dialysis?
3. Are abnormal vitamin D levels associated with vascular damage and calcification in children on dialysis?
4. Are the circulating calcification inhibitors, Fetuin-A, Osteoprotegerin and matrix Gla-protein, associated with vascular damage and calcification in children on dialysis?
5. Is there direct evidence for calcification in the blood vessels of pre-dialysis and dialysis children? Does the calcium load in the blood vessels correlate with clinical and biochemical parameters and clinical measures of vascular damage?
6. Is there a difference in the calcification potentials of pre-dialysis and dialysis vessels in an *in vitro* model of intact human vessels? What is the role of Ca and PO_4 in uraemic vascular injury and calcification?

Chapter 2

General Methods

As vascular calcification is increasingly recognized as an important risk factor for cardiovascular morbidity and mortality in CKD patients, it has led to an increased interest in vascular imaging methods to allow early detection, accurate risk stratification and monitoring of the disease process. Structural changes to the vasculature have been studied by plain radiography, CT scan and ultrasound, and have been correlated with functional changes in the vessels *(Raggi et al, 2002; Blacher et al, 2001; Haydar et al, 2004)*, vessel anatomy on angiography *(Haydar et al, 2004)* and also cardiovascular morbidity and mortality *(London et al, 2002; Goldsmith et al, 1997)*. In parallel with improved imaging technology, a number of biomarkers of cardiovascular disease events and morbidity have been identified. These include vitamin D levels, the circulating calcification inhibitor proteins, fetuin-A, osteoprotegerin and matrix gla-protein, high sensitivity C reactive protein and other markers of inflammation, and cardiac troponin levels.

In this chapter I have discussed vascular imaging techniques, assays that have been performed in the course of this thesis and the process of collecting, processing and performing the vessel studies, including *in vitro* studies, histology and immunohistochemistry. I have independently performed and analysed all of the imaging techniques and performed the vessel analysis myself, but received help from lab technicians in biochemical assays and preparation of slides for histology and immunohistochemistry, for which I am extremely grateful. All the work that is not independently performed by myself has been acknowledged in the appropriate sections.

Section I - Assessment of vascular structure, function and calcification

2.1 Carotid Artery Intima Media Thickness (cIMT)

Arterial vessel wall changes occur during a long subclinical lag phase and are characterized by gradual thickening of the intima-media and functional disturbances, possibly associated with remodelling of the vessel wall. Over the past decade the measurement of cIMT using high resolution B mode ultrasonography has emerged as one of the methods of choice for determining the anatomic extent of atherosclerotic and arteriosclerotic disease and its progression and for assessing cardiovascular risk. cIMT is now a well established measure of the presence and extent of coronary atherosclerosis (*Aminbakhsh et al, 1999)*. cIMT has been used in several studies of adult and paediatric CKD patients, and is considered a well established surrogate marker of cardiovascular disease events.

Measurement of cIMT is non invasive, free of ionizing radiation and well accepted by patients. Measurements can be performed serially and cIMT has the advantage of visualizing the full thickness of the arterial wall, in contrast with angiographic techniques which provide only an outline of the arterial lumen. Several observational studies have demonstrated that there is a relationship between ultrasonic and histological determination of cIMT although some studies suggest that ultrasound estimation gives a slighter higher IMT reading *(Pignoli et al, 1986)*, possibly as a result of contraction during histological fixation or post-mortem shrinkage of the tissue.

Carotid IMT increases significantly with age and is greater in men compared with women. Rates of progression in control groups have ranged from 0.006mm/year in asymptomatic adults to 0.06mm/year in subjects with coronary artery disease *(Salonen et al, 1991 and Crouse et al, 1994)*. The association with cardiovascular risk factors is evident even at a young age, as demonstrated in the Bogalusa Heart Study *(Johnson et al, 2007)*. Racial differences in the carotid wall thickness have been described: Afro-Caribbeans have significantly greater carotid IMT than Caucasian adults *(Wagenknecht et al, 1998)*. Well established cardiovascular risk factors such as diabetes mellitus *(Taniwaki et al, 1999)*, hypercholesterolaemia *(Pauciullo et al, 2004; Fisicaro et al, 1994)* and hypertension *(Bots et al, 1993 and Suurkula et al, 1994)* have also been associated with increased cIMT. Of all these 'traditional' risk factors, hypertension seems to have the greatest impact on cIMT, probably via medial hypertrophy *(Suurkula et al, 1994)*.

All of the above 'traditional' Framingham risk factors are present in the adult CKD population, but factors specific to the uraemic milieu also have a direct causal effect on vascular changes and increased cIMT. Studies in paediatric dialysis patients that have utilized cIMT are discussed at length in chapter 1 *(Oh et al, 2002; Groothoff et al, 2002; Mitsnefes et al, 2005; Litwin et al, 2005, Briese et al, 2006).* As children have few, if any, of the traditional risk factors, they provide an ideal opportunity to study the effects of uraemic changes *per se* on the vasculature.

Importantly, cIMT has been shown to be responsive to interventions. Therapeutic interventions with blood pressure lowering agents, lipid lowering agents and multifactorial interventions in diabetics can slow the progression or even reduce

carotid IMT *(de Groot et al, 1998 and Smilde et al, 2001)*. Apart from pharmacological interventions, lifestyle modifications such as weight loss and smoking cessation have been associated with 0.13mm/year reduction in progression of carotid IMT *(Markus et al, 1997)*. Longitudinal studies in CKD patients have shown improvement, but without complete regression to normal, of the cIMT but not functional properties of the vessel *(Litwin et al, 2008; de Lima et al, 2002)* after renal transplantation.

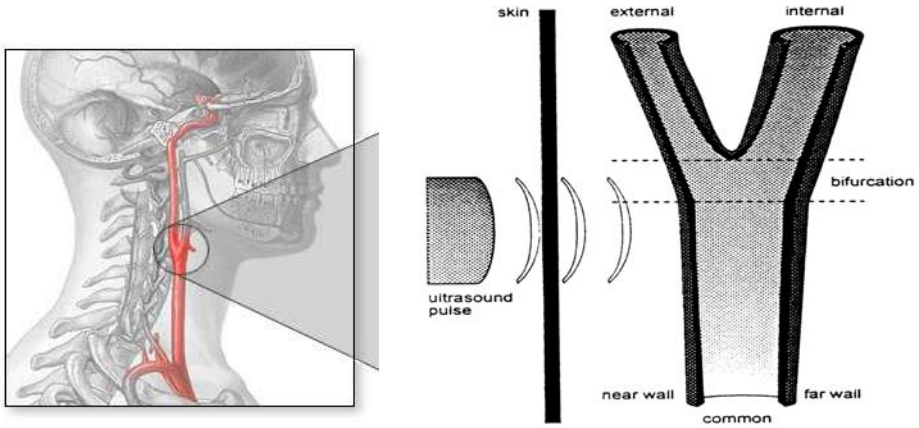
Image acquisition

Different ultrasound techniques have been used to measure IMT in the carotid artery. Although most groups use the B-mode to calculate the average IMT of an arterial segment *(Bots et al, 1997)*, some others used M-mode techniques to assess IMT at a discrete arterial position *(Saba et al, 1999)*. More recently, echo-tracking devices have also been used that calculate IMT at a fixed time-point and also the average IMT during one cardiac cycle *(van Bortel et al, 2005)*.

Most studies have measured IMT in the carotid artery but the most appropriate site of carotid IMT measurement is still a matter of debate (Figure 2.1). The common carotid artery (CCA), 1-2 cm proximal to the carotid bulb, was examined in most studies whereas the internal carotid artery [ICA] and carotid bulb have been studied less often. The CCA is easier to image as it is relatively close and parallel to the skin surface. In contrast IMT measurements in the ICA can be quite challenging. Although atherosclerotic lesions appear later in the CCA compared to ICA or bifurcation *(Solberg et al 1971)*, changes in all sites seem to be equally strongly associated with

risk of subsequent cardiovascular events *(Solberg et al 1971)*. Other arterial sites such as the common femoral, brachial or radial arteries have also been proposed for IMT measurements. There is conflicting information in the literature as to whether IMT thickening in the muscular arteries has the same meaning as changes in the elastic common carotid artery *(Kanters et al, 1997)*.

Figure 2.1 Schematic representation of the CCA, the bifurcation, the ICA, and the external carotid artery



Discrepancies exist also as to which arterial wall should be measured. Most studies have measured IMT on the far wall, while others averaged these measurements with those of near wall *(Baldassarre et al, 1994)*. Reliable IMT measurements can be obtained from the far wall whereas near wall measurements are not equally reliable *(Salonen et al, 1993)*. This is due to the different order in which the interfaces of the intima-lumen and media-adventitia are exposed to the incoming ultrasound beam, generating different B-mode images of the near and far wall. In general, a good image of the near wall depends on the gain settings, and is more difficult to standardize. Measurements from the far wall are more reproducible than those of the near wall.

Experimental protocol

i. The study procedure was explained to the child, and demonstrated on a doll in younger children.

ii. Children were scanned in the supine position in a temperature controlled room.

iii. A 3-lead ECG was attached and continuously recorded.

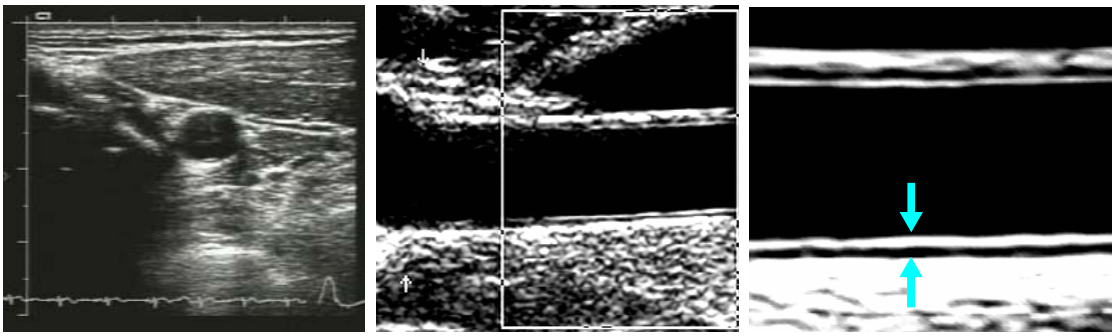
iv. Both the right and left carotid arteries were scanned using a 5-10 MHz linear array transducer (*Vivid 7; GE Medical, Horton, Norway*) in order to detect vessel wall changes and to measure common carotid IMT.

v. The proximal part of the carotid bulb was identified, and a segment of the common carotid artery 1 cm proximal to the bulb was scanned (Figure 2.2). The transducer was manipulated such that the near wall of the carotid artery was parallel to the transducer footprint and the lumen maximised in the longitudinal plane. The image was focused on the posterior (far) wall and the resolution box function was used to magnify the arterial far wall.

vi. Since it has been shown that IMT of the carotid artery changes during the cardiac cycle (decreases during systole due to the larger vessel diameter and increases during diastole), the optimal longitudinal image was acquired on the R

wave of the ECG and videotaped for 5 seconds to minimize variation, and also for the subsequent calculation of carotid distensibility.

Figure 2.2 Image acquisition and analysis of the IMT measurement



A. The length of the common carotid artery is scanned and the carotid bulb identified.
B. A segment of the common carotid artery 1 cm proximal to the bulb is scanned in the longitudinal plane and the posterior (far) wall of the artery focussed on.
C. IMT is measured between the first bright line and the leading edge of the second bright line (shown by the blue arrows) in the far wall of the common carotid artery.

Analysis of IMT measurements

IMT can be measured using electronic callipers or by an automated computerized edge tracking method *(de Groot et al, 2004)*. Measurements can be performed on a video image or on a digitally frozen image. Off-line analysis has the advantage of separating data acquisition from data interpretation, making patient examination more efficient, and also allowing for the analysis to be performed in a blinded fashion.

In studies described in this thesis measurements of the far wall of the common carotid artery were made from stored images using electronic calipers. IMT was calculated as the distance between the first bright line (lumen-intima interface) and the leading edge of the second bright line (media-adventitia interface) (Figure 2). Six measurements,

the three maximum measurements of the right common carotid artery in three different frames and the three maximum measurements of the left common carotid artery in three different frames, were averaged.

Reproducibility

Phantom studies have shown that distances similar to the intima-media thickness of the carotid arterial wall can be measured with B-mode ultrasound system with an axial resolution of 0.2 to 0.4mm at a precision of about 0.03 to 0.05mm *(Salonen et al, 1993)*. In general reproducibility of IMT measurement was better in studies limited to the common carotid artery far wall than in studies including multiple measurements at different carotid sites *(Salonen et al, 1993)*. Intraobserver and interobserver reproducibility of measurements for the studies in this thesis were determined by measurement of cIMT twice, 2 weeks apart in 10 randomly selected children, and were was 1.9 and 2.2 micrometres (= 1.8 and 2.1%) respectively.

2.2 Pulse Wave Velocity (PWV) and Pulse wave analysis (PWA)

Physiology of the pulse pressure and pulse wave velocity

With each heart-beat a pressure waveform is generated which travels forward in the arterial tree. The ventricular ejection (force / time) and cushioning effects of the aorta determine the waveform characteristics. The aorta functions not simply as a conduit vessel, but also as a capacitance device for translating the 'on-off' blood flow characteristics generated by the left ventricle into a smooth non-pulsatile blood flow pattern at the capillary level: in a healthy young person, approximately 40% of a stroke volume is forwarded and the remainder stored in the elastic aorta, to be released during diastole so as to ensure continuous flow. Tempering the rise in aortic systolic blood pressure, and maintaining aortic diastolic blood pressure not only protects the distal circulation against barotrauma, but also allows for adequate coronary perfusion. This capacitance function of the aorta depends on its distensibility, and this principally determines the degree of energy absorbed from the forward travelling pulse waveform in systole, and the elastic recoil of the aorta in diastole *(Goldsmith et al, 2002)*.

'Arterial stiffness' is a measure of the relationship between pressure response and change in volume. The stiffer the artery the greater is the difference between systolic and diastolic blood pressure, as there is a higher pressure wave generated into the stiff vessel during systole and decreased elastic recoil during diastole, resulting in a lower diastolic blood pressure. The resultant high systolic and low diastolic blood pressures give rise to a wide pulse pressure *(O'Rourke et al, 1980)*. The pulse pressure is a

stronger predictor of future cardiovascular mortality than either systolic or diastolic blood pressures *(Benetos et al, 1997; Franklin et al, 1999).*

The pulse waveform characteristics are also determined by 'backward' travelling waves that are reflected at various points in the arterial tree, and that collide with the direct forward travelling wave. The pressure pulse at any point of the arterial tree is hence the summation of the direct wave and reflected waves from the periphery *(McVeigh et al, 2002)*. Reflected waves have their origin at points where the flow and pressure waves are not perfectly matched. Common reflection sites are branch points, constrictions or areas of turbulence *(Izzo et al, 2004)*. At some point along the aorta the incident and reflected wave forms summate. When (in relation to the cardiac cycle) and at what point along the aorta this happens depends on the speed of energy transfer along the aorta (or the pulse wave velocity [PWV]), the degree of arterial luminal diameter mismatch (branch points and areas of stenoses) and the aortic length. In young healthy subjects the pulse waveform summation takes place low in the abdominal aorta in early diastole, allowing for adequate coronary perfusion. A stiffer aorta will allow for a more rapid PWV, and when this is coupled with constricted aortic branches and a short subject height, wave summation occurs at a more proximal point in the aorta, closer to the aortic valve and coronary sinus, and also occurs earlier in the cardiac cycle, thereby compromising coronary perfusion *(London et al, 1996)*.

Although pulse pressure is simple to measure, it is influenced by both the cardiac function and also by the aortic wall properties, and a more accurate assessment of the vessel properties can be determined from pressure wave contour analysis. The PWV is a real-time measure of the time taken for aortic mural energy waves (from cardiac

contraction, aortic dilatation then recoil) to propagate down the aorta. Increased PWV is thus mechanistically linked with systolic hypertension, an increase in systolic and a decrease in diastolic blood pressures and left ventricular hypertrophy. A higher demand for coronary blood flow as a result of left ventricular hypertrophy coupled with a decrease in diastolic BP leads to an increased susceptibility to myocardial ischaemia *(Wilkinson et al, 2000)*.

An assessment of vessel compliance can also be obtained from an ultrasound image by measuring the distensibility of the vessel is measure in a specific area of artery, essentially by determining the difference between its systolic and diastolic diameters. The Moens - Koertweg equation, PWV = 1 / √ Distensibility, gives the relationship between the two.

Augmentation index (AIx)

Indices to characterise the pressure pulse can be derived from the contour of the pulse waveform. The feature of the pulse contour, which has received the most interest, is the peak (or shoulder) that occurs in systole due to reflected wave. AIx is a measure of the increase in BP caused by the return of the reflected wave during systole. AIx will depend on the characteristics of the reflected wave such as its timing (large artery stiffness), amplitude (related to peripheral vascular tone) and the reflectance point.

Augmentation = ΔP

Augmentation Index = (Δ P / PP)

Figure 2.3 Schematic representation of an aortic waveform in a young (compliant) and old (stiff) vessel

Associations with increased PWV

A key outcome study by Blacher et al showed that aortic PWV and AIx were independent predictors of morbidity and mortality in patients on haemodialysis. For each 10% increase in AIx the risk ratio for all cause mortality was 1.51 and for cardiovascular mortality 1.48, and for each 1 m/s increase in PWV the all cause mortality-adjusted odds ration was 1.39 *(Blacher et al, 1999)*. In a recent study Taal et al have shown that increased arterial stiffness is in itself a risk factor for progression to end stage renal disease in pre-dialysis patients, presumably through impairing renal perfusion *(Taal et al, 2007)*. PWV is strongly correlated to the degree of EBCT-derived coronary artery calcium score *(Haydar et al, 2004; Raggi et al, 2007; Sigrist et al, 2007)* as well as to the number of arterial sites with calcification as determined by plain x-ray *(Guerin et al, 2000)*. Tillin et al have recently shown that the site of PWV measurement influences results: the carotid – femoral PWV is a better indicator of atherosclerosis and correlates closely with the coronary artery calcium load than the carotid – radial or femoral – posterior tibial PWV *(Tillin et al, 2007)*. Finally, reflecting the associations between calcification and vessel stiffening as well as those

between the bone - vascular axis in CKD, a low spinal bone mineral density has been associated with an increased PWV in CKD stage V patients *(Raggi et al, 2007).*

Carotid and radial pulse pressure waveform

The contour of the pulse pressure waveform in different arteries differs considerably: the propagation, timing and reflection of pressure waves cause the peripheral pressure pulse to differ from the central pressure pulse *(Latham et al, 1985 and O'Rourke et al, 1980)*. There is however little difference between the carotid pulse and the aortic pulse because the distance between the two arteries is relatively small. The carotid pulse has a short sharp incisura denoting aortic valve closure and the end of ventricular systole as does the ascending aortic pulse (Figure 2.4).

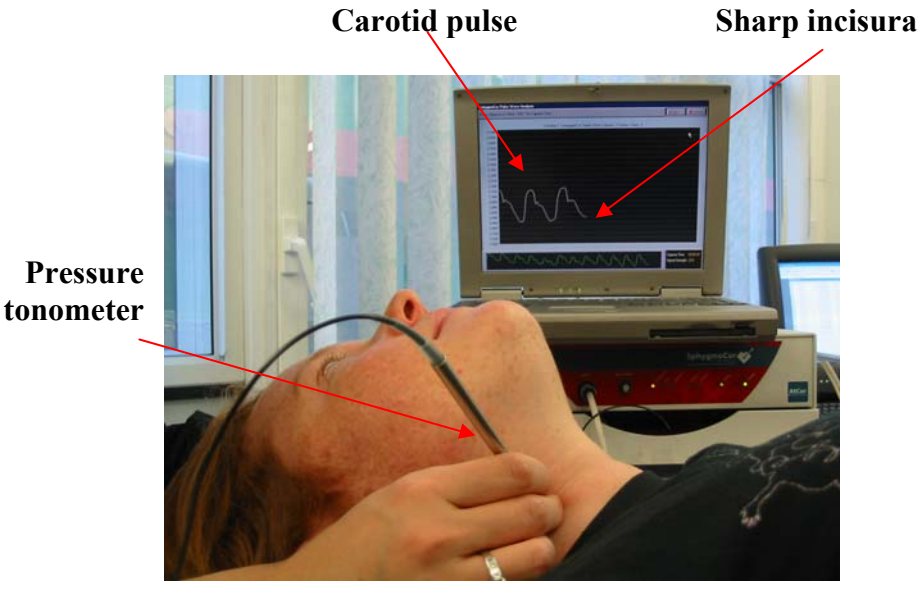


Figure 2.4 Carotid pulse pressure waveform

Although the carotid pulse is easily accessible, it can be a challenge to obtain accurate recordings. The carotid artery is sited deeply in the neck and is poorly supported laterally. Application of the probe to the carotid artery can lead to cardiac slowing due to baroreceptor stimulation or to gagging and coughing due to pressure on the neck near the throat, and can be quite unpleasant, especially for children. Thus it is difficult to obtain consistent records from the carotid artery.

By contrast, the radial and femoral pulse is easily accessible and the arteries can be applanated against the radial bone or the inguinal ligament respectively without difficulty or discomfort to the subject. The shape of the radial pulse is very different from the aortic pulse. The propagation, timing and reflection of pressure waves in the vasculature cause the peripheral pressure pulse to differ from the central pressure pulse *(O'Rourke et al, 1980)*. Mechanical and elastic properties of the arterial tree distort the direct and reflected waves during their propagation and, especially in young subjects, the peripheral pulse pressure is increased compared to central pulse pressure *(Mahmud et al, 2003 and Cockroft et al, 2003)*. In the radial waveform, the reflected wave usually causes a shoulder on the down-slope of the systolic part of the radial pressure pulse (Figure 2.5).

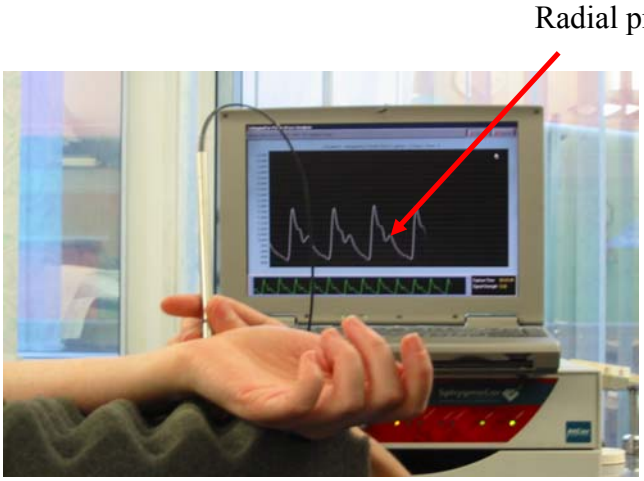


Figure 2.5 The radial pulse pressure waveform

Applanation tonometry and the SphygmoCor apparatus

Applanation tonometry is a non-invasive technique for measuring arterial pressure. A piezo-resistive transducer is applied over an artery. When the piezoelectric crystal is deformed by arterial pressure transmitted through the overlying superficial tissue and skin, its resistance changes. This change in resistance is directly related to the pressure applied on the crystal. There are different ways of accommodating the piezoelectric transducer: it can be mounted on the tip of pen-type probe (Millar micromanometer, Texas US) or an array of transducers can be strapped around the wrist (Colin wrist probe, Japan). Applanation tonometry requires the artery to be flattened against a hard surface such as the head of the radial bone (radial artery), the inguinal ligament (femoral artery) or the vertebral column and ligaments in the neck (carotid artery). Applanation tonometry has been validated and the radial artery pressure waveform obtained is very close to that obtained using invasive intraarterial measurements of the radial artery pressure waveform.

For the work in this thesis, I have recorded the radial, femoral and carotid artery pressure waveforms and amplitude non-invasively using the Millar probe. Applanation tonometry by this technique has been validated in children and the radial artery pressure waveform obtained is very close to that obtained using invasive intra-arterial measurements of the radial artery pressure waveform.

Experimental technique

Pulse wave velocity measurements were performed between the carotid and femoral artery sites to measure the aortic stiffness and between the carotid and radial artery sites to measure the brachio-radial pulse wave velocity.

- All subjects were studied after 10 minutes of resting in the supine position.
- The blood pressure was measured non-invasively using Omron automated sphygmomanometer recordings on the left arm (or non-fistula arm as appropriate).
- Three ECG leads were attached and the heart beat continuously recorded.
- The radial artery pulse was palpated and the probe positioned directly above the point where the strongest pulse was felt.
- Care was taken to ensure that the operator was comfortably positioned so that their elbow or the wrist was resting against a solid surface to minimise movements.
- ***Radial measures:*** The tonometer was placed on the wrist directly above the right radial artery (or the left radial artery if there was an arterio-venous fistula on the right arm), and the waveform recorded directly onto a laptop computer running proprietary waveform analysis software (SphygmoCor Px Version 6.0, ATCOR, Sydney Australia). The data collection screen allows the operator to see the peripheral signal and display of the last 10 seconds of data, and recordings are taken when a reproducible signal with high amplitude excursion is obtained.
- ***Carotid artery measure:*** The subjects were asked to extend their neck so that the carotid artery was flattened against the muscles of the neck. To improve the recording, subjects were asked to hold their breath in expiration, if possible, to minimise the often marked respiratory variation seen in young subjects.
- ***Femoral artery measures:*** With minimal exposure in the right groin area, the femoral artery was palpated against the inguinal ligament, the tonometer

placed directly perpendicular to the point of maximal pulsation, and recordings obtained as described above.

- ***Pulse wave analysis:*** The radial arterial pressure waveform was recorded directly onto the SphygmoCor laptop. Once a satisfactory signal was been obtained (pressure waves consistent from beat to beat, amplitude optimised and the pulse waveform of the same character as one would expect in the artery) from at least ten consecutive pulses, a recording was taken to determine AIx. The carotid artery AIx was recorded in a similar fashion.

Analysis of PWV measurement

The SphygmoCor device uses successive measurements of pressure waveforms and a simultaneous ECG. For each measurement site, the delay from the R-wave of the ECG to the foot of the pressure pulse is determined. Subtraction of the R-wave to pulse foot time at the proximal and distal sites provides the transit time (Figure 2.6). PWV is then calculated from the arterial length and transit time using the intersecting tangent algorithm *(Millasseau et al, 2003)*. This algorithm uses the point formed by the intersection of a line tangent to the initial systolic upstroke of the pressure waveform and a horizontal line through the minimum point. The distance travelled by the pulse wave was measured over the body surface with a tape measure. PWV was calculated as the distance: transit time ratio and is expressed as meters per second.

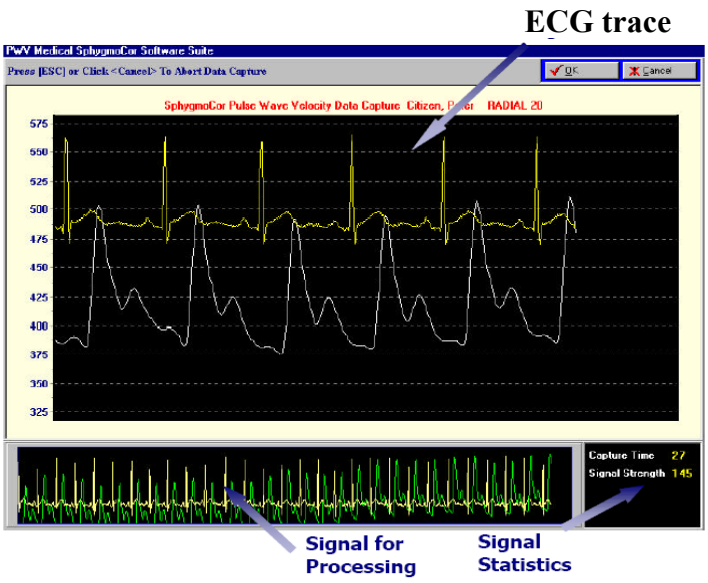


Figure 2.6 PWV acquisition page

Analysis of PWA measurements

The SphygmoCor analysis software automatically averages approximately 10 waveforms and calculates AIx on the averaged waveform. From the measurements in the radial and carotid arteries both the aortic AIx and the peripheral AIx are automatically calculated; Figure 2.7 *(Millasseau et al, 2003)*. Reasonable confidence is gained about the quality of the measurement if the pressure waves are highly consistent beat to beat, if amplitude is the greatest that can be achieved and if the pulse wave measured has the same character as one would expect in the artery i.e. sharp upstroke, straight rise to the first systolic peak, a definite sharp incisura, and near-exponential pressure decay in late diastole. The software has an inbuilt control system to ensure optimal quality in the waveform as defined by the manufacturer, ie mean pulse height >100mV, pulse height variability and variability of diastolic points as a percentage of the pulse height <5%.

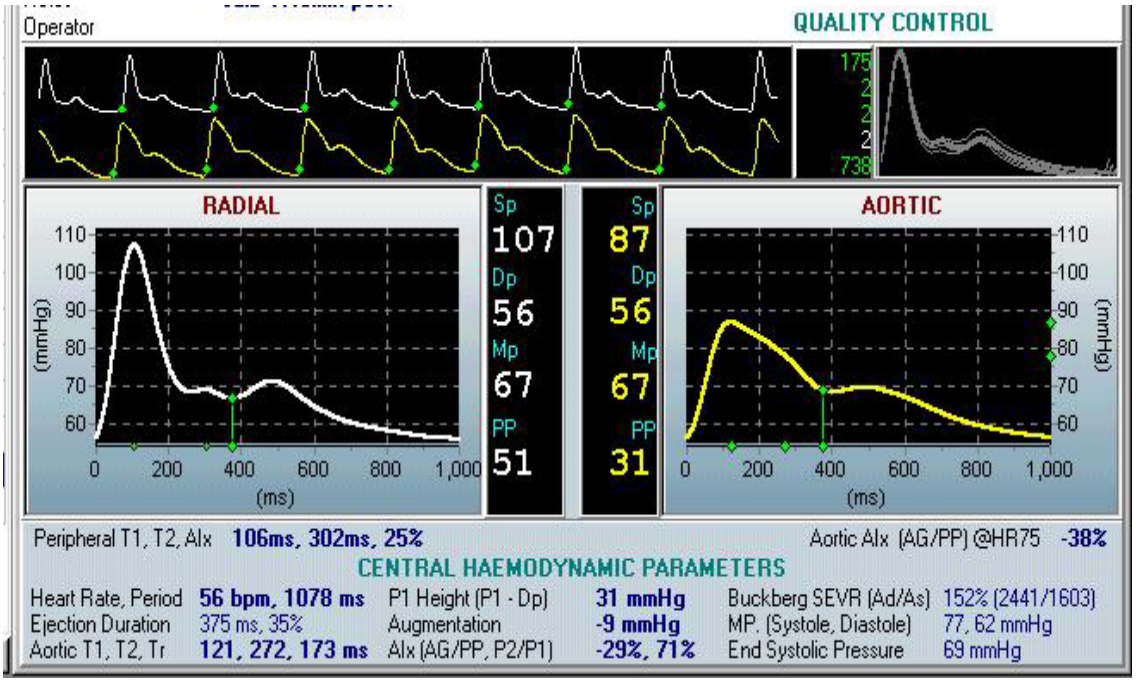


Figure 2.7 PWA analysis report

2.3 Radiographic assessment of vascular calcification

Plain x-ray

Plain radiology of pelvis, thigh and hands has been utilized in a number of studies of vascular calcification. Progressive calcification has been reported using this approach, and associated with cardiovascular mortality *(London et al, 2003)*. The pattern of calcification on plain radiographs (linear vs irregular calcification) may yield some information concerning the balance between medial and intimal vascular calcification *(Goldsmith et al, 1997)*; the presence of predominantly linear calcification, implying medial calcification, has been associated with a significant increase in cardiovascular risk. The low sensitivity of plain radiography in the detection of early calcification makes this method unsuitable for use in children or to accurately track changes in calcification status.

Electron beam CT

Electron Beam Computerised Tomography (EBCT) was introduced in the early 1980s, as a method of improving the temporal resolution of CT scanners (due to the rapid motion of the heart and relatively long acquisition times, conventional CT scanners cannot be used to visualise the coronary vessels). Instead of rotating a conventional X-ray tube around the patient, the EBCT machine houses a large vacuum tube in which an electron beam is electro-magnetically steered around circularly arranged tungsten X-ray anodes. The lack of moving parts allows very quick scanning, making the technique ideal for capturing images of the heart *(Bellasi et al, 2006)*.

The very high cost of EBCT equipment, and its poor flexibility (EBCT scanners are essentially single-purpose cardiac scanners), has led to poor uptake with less than 150 of these scanners worldwide. EBCT's role in cardiac imaging has been rapidly replaced by high-speed multi-detector CTs.

Multislice CT scans

To minimize motion artifacts in cardiac images, an increased speed of volume coverage is essential. Modern multislice CT scanners, with 16, 32 and now 64 detector rings, allow for increasing rotation speeds and spatial resolution. A 16-slice CT scanner - acquires 16 slices per rotation of the gantry around the patient, with each gantry rotation taking <500 milliseconds (Figure 2.8) - was used for the studies in this thesis. The technique has been used to generate coronary artery calcification (CAC) scores analogous to those from EBCT *(Raggi et al, 2003; Becker et al 2001; McIntyre C, 2006)*.

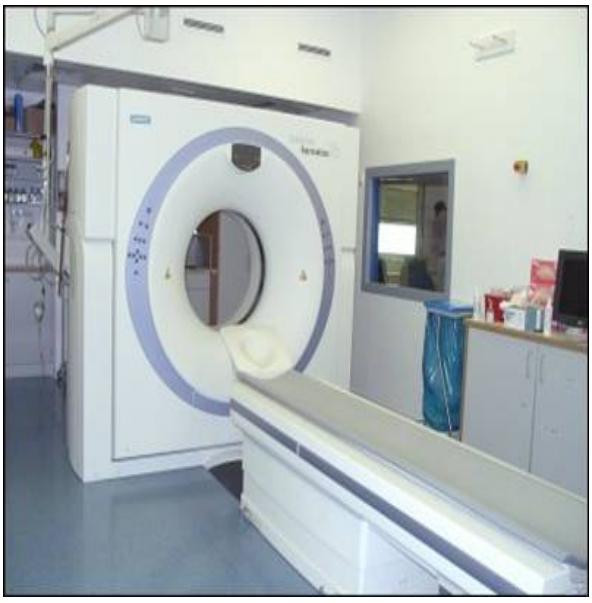


Figure 2.8 A 16- slice CT scanner (*Somatom Sensation 16, Siemens*)

Technical approaches to cardiac CT scanning

There are two different technical approaches for cardiac CT acquisition:

- Prospective ECG triggered sequential scanning
- Retrospective ECG gated spiral scanning

In both cases, an ECG is recorded and used to either initiate prospective image acquisition (ECG triggering), or to perform retrospective image reconstruction (ECG gating). In both cases, only diastolic images (taken at ~ 60 - 70% of the RR interval) are selected for image reconstructions, as these images have the least amount of motion. However, with retrospective ECG gating each portion of the heart is imaged more than once while an ECG trace is recorded. The ECG is then used to correlate the CT data with their corresponding phases of cardiac contraction. Once this correlation is complete, only the images acquired in diastole (while the heart was at rest) are used, while most of the images recorded in systole are discarded. Thus, although retrospective ECG gating gives an even higher temporal resolution, this comes at the cost of a very high radiation dose of 10-15mSv. Prospective ECG gating reduces the radiation exposure to <1.2mSv, and this was used for all of the scanning performed in our studies.

Imaging protocol

i. The child, along with a parent, is taken to the CT scan room and the procedure, including breath-holding, explained.
ii. The child is positioned on the CT table and correct placement within the gantry confirmed.

iii. A 3-lead ECG is obtained and continuously recorded so as to assess the child's cardiac rhythm and heart rate and decide the optimal time for prospective ECG triggering (usually 60% of the RR interval).

iv. The chest is imaged from the level of the carina to the bottom of the heart. A sequence of images through the diastolic phase of the cardiac cycle is acquired using subsecond CT scanning. The protocol we have used for calcium scoring with prospective ECG-triggered cardiac imaging is as follows:

- 12 slice acquisition
- 120 kV
- 30 mAs
- nominal slice width 3.0 mm
- slice collimation 1.5 mm
- gantry rotation time 0.42 sec
- table feed 5.6 mm/rotation

Radiation exposure

Using the CT EXPO dose estimation software, the calculated effective radiation dose depending on the child's weight was as follows:

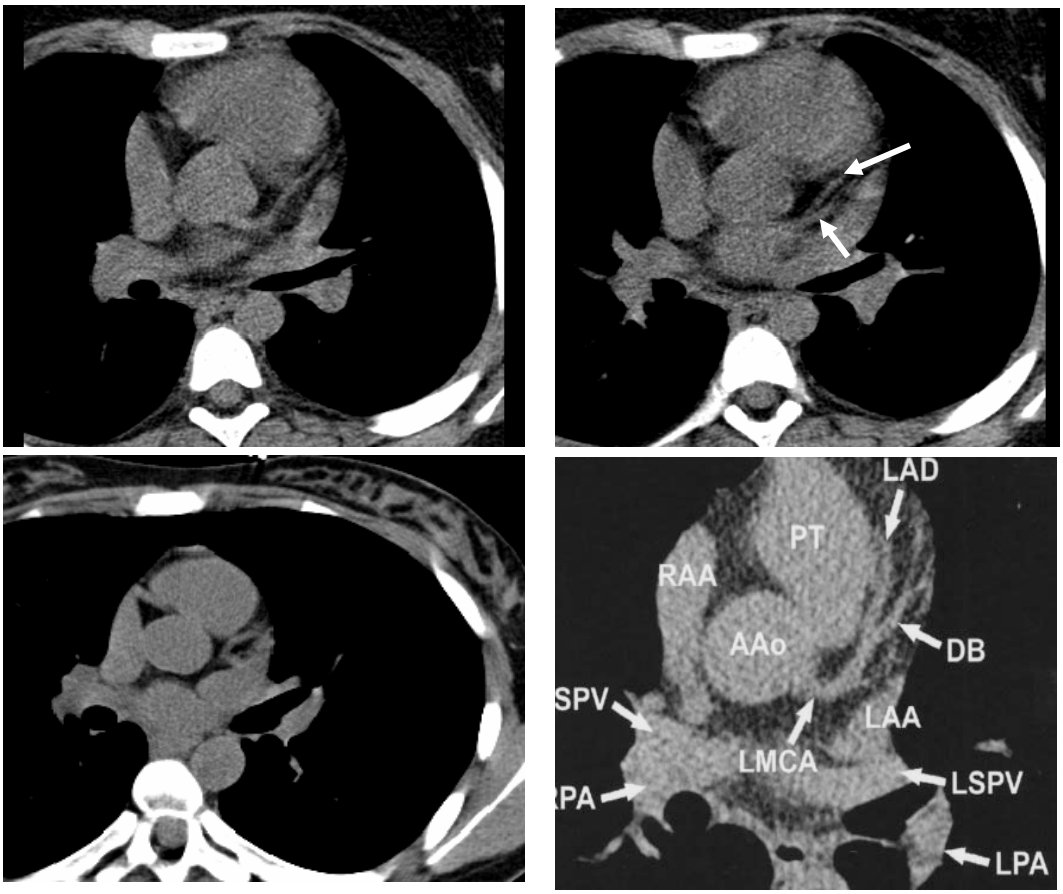
- 22kg − 0.68mSv
- 30kg − 0.61mSv
- 40kg − 0.52mSv
- 50kg − 0.43mSv
- 60kg − 0.34mSv
- 70kg − 0.25mSv

For purposes of comparison, the background radiation in the UK is 2.2mSv per year (range for regional averages is 1.5 − 7.5mSv), a chest x-ray delivers a radiation dose of 0.1mSv and a standard CT chest or abdomen 8 – 10mSv. For this reason, CT scans were not performed in any of the healthy controls in my studies.

Normal cardiac anatomy

The interpretation of cardiac CT scans requires a thorough knowledge of normal coronary artery anatomy. The non-opacified coronary arteries can be readily identified on CT because the lower CT density of periarterial fat produces marked contrast to blood in the coronary vessels, whereas the mural calcium is evident because of its high CT density relative to blood. Figure 2.9 shows the origin and course of the main coronary arteries in relation to important landmarks on a CT scan.

Figure 2.9 Multi-slice CT scan images showing normal cardiac anatomy



A. Origin of the left main coronary artery (LMCA) from the coronary sinus.
B. Bifurcation of the LMCA into the left anterior descending (LAD) artery (top arrow) and the circumflex artery (bottom arrow).
C. Origin of the right coronary artery (arrow on right). The circumflex artery (left arrow) is also seen in the same plane.

D. The LAD is seen passing between the pulmonary trunk and left atrial appendage. The proximal LAD, which travels horizontally, is seen in longitudinal section and the diagonal branch (DB) comes into view at this level. This branch traverses anterolateral aspect of left ventricle. Main pulmonary vessels are also seen.

LMCA - left main coronary artery
AAo - ascending aorta
PT - pulmonary trunk
LAA - left atrial appendage
RSPV- right superior pulmonary vein
LSPV - left superior pulmonary vein
RPA - right pulmonary artery
LPA - left pulmonary artery
RAA - right atrial appendage

Calcium scoring protocol

Calcium scoring is based on the Agatston scoring algorithm *(Agatston et al, 1990)*, which was originally developed for EBCT scoring. A CT threshold score of 130 Hounsfield units (HU) is selected and a coronary score for each of four main epicardial coronary arteries (left main coronary artery, left anterior descending artery, circumflex artery and right coronary artery) is obtained. The score is generated by measuring the volume of coronary calcification (mm^3) and multiplying it by a factor (between 1 and 4) based on the HU peak attenuation value of the lesion. The scores are stratified to a scale with 4 categories as defined by Rumberger et al *(Rumberger et al, 1999)* and shown in Table 2.1 below. A lack of calcification correlates with a lack of coronary artery disease (with a 95% negative predictive value), while a higher score can give a prognosis concerning risk from a myocardial event.

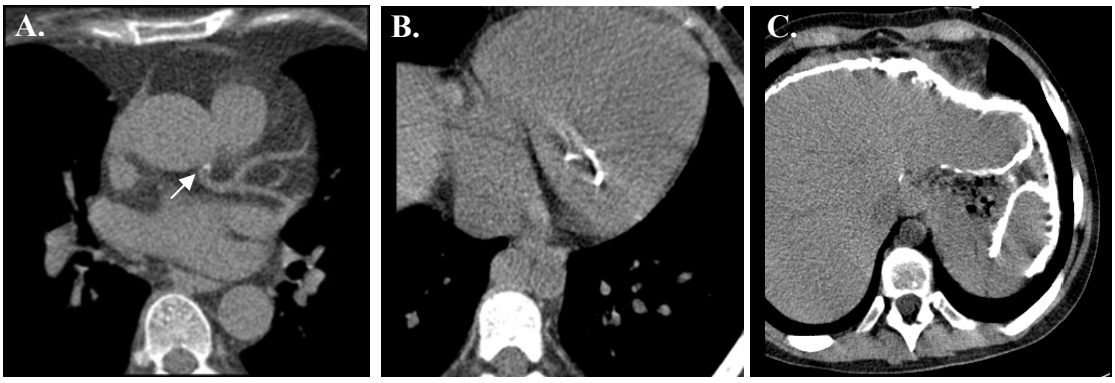
Table 2.1 Agatston scoring of Coronary Artery Calcification and its clinical significance

Calcium Score	Evaluation	Clinical Significance
no calcification	Normal	No risk of CHD
1-10	minimal calcification	stenosis unlikely
11-100	moderate calcification	CHD possible
101-400	intermediate calcification	CHD with stenosis
> 400	extensive calcification	high probability of significant stenosis

I received training from Professor Mike Rubens, Department of Medical Imaging, Royal Brompton & Harefield Hospital NHS Trust, in interpreting cardiac CT scans. All CT images were stored on videotape and analysed in a blinded fashion by myself. Intraobserver and interobserver reproducibility of measurements were determined on 10 randomly selected scans that were re-analysed in a blinded fashion by myself and Dr Melanie Hiorns, Radiology Consultant at Great Ormond Street Hospital. Intraobserver and interobserver variability was 2.8 and 4.9% respectively.

Some images of coronary artery calcification that I found in the course of my studies are shown in Figure 2.10.

Figure 2.10 Coronary and valvular calcification on 16-slice CT scans from children in my studies



A. Calcification at the origin of the left main coronary artery (arrow).
B. Calcification of the aortic valve.
C. An incidental finding of sclerosing peritonitis.

Limitations of cardiac CT scans in evaluating coronary artery calcification

Some limitations on the significance and reliability of calcium scoring by multi-slice CT include:

- motion artefacts due to breathing – breath holding was tried in all patients but was not possible in the very young or in those with learning difficulties
- cardiac motion related artefacts – current recommendations suggest that multi-slice CT scans should not be performed if the heart rate is >90/min, but this is not possible with paediatric patients. This was overcome by using a shorter fraction of the R-R interval for image acquisition.
- poor ECG gating – ECG gating is not possible in patients with arrhythmias. This was not an issue with any patients in this study.
- poor visibility of coronary arteries – children, particularly if malnourished, have less epicardial fat, and so the vessels are less distinctly visible than in adults.
- correlation with disease outcome - The role of coronary calcium scoring in children has not been studied against disease outcome nor correlated with findings on coronary angiography.
- distinction between intimal and medial calcification is not possible.

Coronary calcium scoring in CKD and dialysis patients

While some earlier studies in dialysis patients hav developed a composite 'calcium score' from plain x-rays of multiple arteries *(Blacher et al, 2001; London et al, 2003)*, subsequent studies have used EBCT to study prevalent and progressive vascular calcification *(Raggi et al, 2007)*, as well as the impact of therapy, sevelamer vs

calcium-based phosphate binder medication, *(Chertow et al, 2002; Block et al, 2004; Spiegel et al, 2007)*. Calcification scores have been correlated with mortality *(Raggi et al, 2008; Blacher et al, 2001; London et al, 2003),* with arterial compliance as measured by aortic PWV *(Haydar et al, 2004; Raggi et al, 2007),* and with bone mineral density *(Raggi et al, 2007)*. However, controversy exists concerning the correlation of coronary artery calcification scores and the angiographic appearances of the vessels *(Haydar et al, 2004; Sharples et al, 2004; Haberl et al, 2001)*. The functional cardiovascular consequences of coronary artery calcification, including impaired microcirculatory function in chronic haemodialysis patients, have been described by McIntyre et al *(McIntyre et al, 2007; Sigrist et al, 2008)*. Studies in children *(Goodman et al, 2000; Eiffinger et al, 2000; Civilibal et al, 2006)* and young adults *(Goodman et al, 2000; Oh et al 2002; Briese et al 2006)* have utilized EBCT and multislice CT scans to describe the prevalence and associations of coronary artey calcification with clinical and biochemical markers, and these have been described at length in chapter 1.

Section II – Biochemical assays

Serum samples were collected from all patients and controls immediately prior to performing the vascular scans. In all patients blood samples were taken before a mid-week session of haemodialysis or at a routine clinic visit for those on peritoneal dialysis. All serum samples were separated immediately upon collection and frozen at -80°C until used.

I have received help from Mrs Vanita Shah, Senior Technician at the Institute of Child Health with the fetuin-A and hs-CRP assays. Both the vitamin D assays were performed in the Chemical Pathology Department at Epsom General Hospital by Mrs Michala Bridal, Technician. We have collaborated with Dr Gerd Hawa, *Biomedica*, Vienna, Austria and Dr Leon Schurgers, *CARIM and VitaK*, University of Maastricht, Maastricht, The Netherlands who have kindly performed the Osteoprotegerin and Matrix-Gla protein assays respectively using novel in-house antibodies. I am very grateful for all the help that I have received with these.

2.1 Vitamin D assays

25-hydroxyvitamin D assay

25(OH)D levels were measured by enzymeimmunoassay (EIA) using the *Immunodiagnostics Systems* commercially available kit.

The IDS 25-Hydroxy Vitamin D EIA kit allows for the quantitation of 25-OH D and other hydroxylated metabolites in serum or plasma. Calibrators, controls and samples are diluted with biotin labelled 25OHD. The diluted samples are incubated in

microtitre wells which are coated with a highly specific sheep 25OHD antibody for 2 hours at room temperature before aspiration and washing. Enzyme (horseradish peroxidase) labelled avidin, is added and binds selectively to complexed biotin and, following a further wash step, colour is developed using a chromogenic substrate. The absorbance of the stopped reaction mixtures are read in a microtitre plate reader, colour intensity developed being inversely proportional to the concentration of 25OHD.

1,25-dihydroxyvitamin D [1,25(OH)$_2$D] assay

1,25(OH)$_2$D levels were measured by radioimmunoassay (RIA) using the *Diasorin* commercially available kit. The assay involves a two-step procedure: a preliminary extraction followed by a subsequent purification of vitamin D metabolites from serum or EDTA plasma using C18OH cartridges. Following extraction, the treated sample is then assayed using a competitive RIA procedure. The RIA method is based on a polyclonal antibody that is specific for both 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$.

The sample, antibody and tracer are incubated for 2 hours at 20-25°C. Phase separation is accomplished after 20-minute incubation at 20-25°C with a second antibody precipitating complex. After centrifugation and decantation, the bound fraction remaining in the pellet is counted in a gamma counter. Values are calculated directly from a calibrator curve of known concentrations. The final concentration of the 1,25-(OH)$_2$D is expressed as pg/ml.

2.2 High Sensitivity C-Reactive Protein (hs-CRP)

Hs-CRP levels were measured by Enzyme Linked ImmunoSorbent Assay (ELISA) using a commercially available kit from Biomerica, Inc. CA, USA. The kit was used according to the protocol provided by the manufacturer. In brief, microtitre wells coated with mouse monoclonal anti-CRP antibody incubated with serum samples from each of the study groups diluted 1:100 and CRP standards supplied with the kit followed by CRP enzyme conjugated antibody. After incubation for 45 minutes at room temperature, the wells were washed and substrate was added. A standard curve was generated by plotting logarithmic absorbance versus logarithmic human CRP concentration and the best fit line was determined by regression analysis.

2.3 Fetuin-A assay

Serum fetuin-A was measured by enzyme linked immunosorbent assay (ELISA) using a commercially available kit from *Epitope Diagnostics, Inc. San Diego CA,* as per the manufacturer's instructions. Microtitre wells coated with a high affinity polyclonal goat anti-human fetuin-A antibody were incubated with serum samples from each of the study groups diluted 1:10 000; and commercial fetuin-A standards supplied with the kit. After incubation for 2 hours at room temperature, wells were washed and followed by peroxidase conjugated polyclonal anti-human fetuin-A antibody and substrate was added. A standard curve was generated by plotting logarithmic absorbance versus logarithmic human fetuin-A concentration and the best fit line was determined by regression analysis.

The intra-assay and inter-assay coefficient of variation were < 5.5% and < 6.8% respectively. The reference range for healthy adults quoted by the manufacturer was 0.5 – 1.0 g/l g, and the minimum sensitivity of the assay was 5.0 ng/ml.

2.4 Osteoprotegerin (OPG) assay

OPG serum concentrations were analysed using an ELISA system from *Biomedica* (Vienna, Austria). In brief, a monoclonal IgG antibody was used as capture antibody and a biotin-labeled polyclonal antihuman OPG antibody as detection antibody. The immunoassay detects both free and complexed OPG, with an intra-assay and inter-assay variability of 9% and 10%, respectively.

2.5 Receptor Activator of Nuclear Factor κß Ligand (RANKL)

RANKL levels were determined by ELISA *(Biomedica)* based on microtiter plates coated with OPG. Soluble RANKL (sRANKL) from the sample binds to the coated OPG and is detected by a biotin-labeled polyclonal anti-human sRANKL antibody. Intra-assay and inter-assay variations ranged from 3-5% and 6-9 % respectively.

2.6 Undercarboxylated matrix γcarboxyglutamic acid protein (uc-MGP)

Uc-MGP was measured according to the home-made ELISA developed at the vitamin K-research institute (*VitaK BV*, Maastricht, The Netherlands). In brief, the moAb uc-MGP was coupled to the microtiter plate via R-M IgG (*Dako, Heeverlee,* Belgium) and the remaining sites blocked with 2% BSA in hepes buffer. After stringent washing, samples and standard were diluted in 2% HNBSA and supplemented with tracer (biotinylated 35-54 uc-MGP).One hundred µL of this solution were transferred to the microtiter plate and incubated overnight at 4°C. The plate was incubated with 100 µL Streptavidine-peroxidase (*Zymed, Breda,* The Netherlands), and after washing stained with 100 µL TMB (*KPL, Gennep*, The Netherlands). The process was stopped

by adding 50 μL of 1.0 mol/L H2SO4, and the plate was read at 450 nm. The uc-MGP concentration was calculated with the aid of a calibration curve of synthetic full-length uc-MGP.

Section III – Laboratory techniques

2.1 Collection of human vessels

Medium sized muscular arteries routinely removed and discarded in the course of planned intra-abdominal surgery were collected.

Intact human arteries were obtained from the following 3 groups of patients:

1. children in CKD stages IV and V just prior to initiating dialysis or at the time of performing a pre-emptive renal transplant (pre-dialysis group)
2. children receiving dialysis
3. age-matched healthy controls without underlying inflammatory disease who were undergoing routine intra-abdominal surgery

In order to keep the patient and control groups free of confounding pro-atherosclerotic risk factors, children with underlying inflammatory disorders, vasculitis, diabetes, dyslipidaemia or smokers were excluded. Informed written consent was obtained from all parents or caregivers and children, where appropriate. The study was approved by the local research ethics committee.

The types of vessels used and the surgical procedures performed to obtain them are as follows:

1. **Omental arteries**

At the time of insertion of a peritoneal dialysis catheter, omentectomy is routinely performed in children. The omentum is a highly vascular structure, and has numerous medium-sized muscular arteries. Also, a small piece of omentum was removed from disease-free controls at planned intra-abdominal surgery.

2. **Inferior epigastric artery**

The inferior epigastric artery runs in the subcutaneous fat in the right iliac fossa just beneath and perpendicular to the surgical incision for a renal transplant. This vessel has to be dissected out and discarded so that the surgeon can reach deeper planes in the abdomen.

3. **Mesenteric arteries**

A small (2-3cm) piece of mesenteric vessel was removed at planned intra-abdominal surgery (e.g. colostomy closure) in disease-free age-matched controls.

Figure 2.11 A and B shows a sample of inferior epigastric artery and omentum obtained for this work.

For the *ex vivo* and *in vitro* studies in chapters 7 and 8 in this thesis, I have obtained:

- 24 inferior epigastric arteries (18 from dialysis patients and 6 from CKD Stage V pre-dialysis patients [GFR <15 ml/min/1.73m^2])
- 14 omental arteries (6 dialysis, 4 pre-dialysis and 4 normal controls)
- 2 mesenteric arteries (normal controls) were studied.

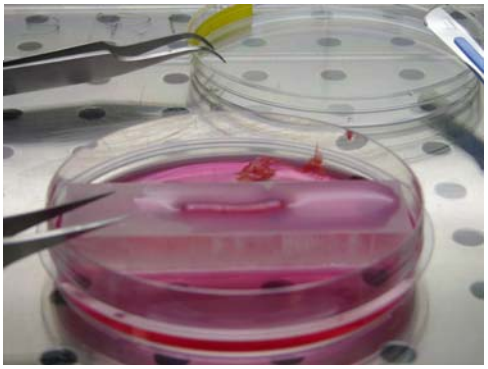
The vessels were collected and transported in tissue culture medium (M199) that was stored at 4°C. Samples were dissected under sterile conditions in a tissue culture hood with laminar flow as follows:

- surrounding fat and fibrous tissue were removed
- the adventitia was gently stripped off
- the vessel was cut into 1-2 mm rings so as to allow perfusion of the vessel *in vitro*

Figure 2.11 C and D shows the dissection and incubation of vessels *in vitro* media.

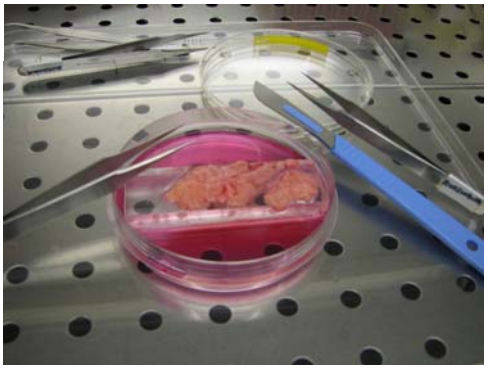
Figure 2.11 Sample types, processing and *in vitro* culture conditions

A.



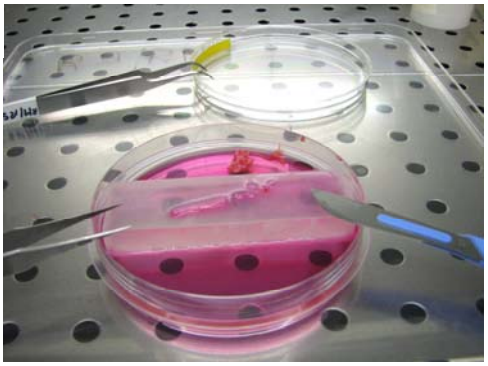
A. An inferior epigastric artery obtained at the time of renal transplantation.

B.



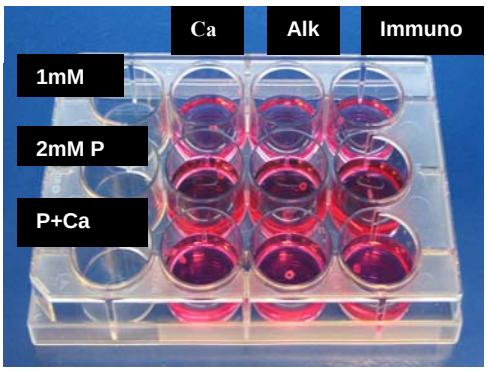
B. Omentum obtained at the time of insertion of a peritoneal dialysis catheter.

C.



C. The vessel is gently stripped of adventitia and cut into 1-2 mm rings. Typically 12 – 20 rings were obtained from each vessel.

D.



D. Culture in *in vitro* calcifying media with graded concentrations of Ca and P. Vessel rings were typically cultured for 14 days and then harvested for measurement of Ca load in the vessel wall, ALK activity and histology and immunohistochemistry.

Clinical and biochemical data

The patients' age, time in CKD stages IV and V and modality of dialysis were recorded. Cumulative biochemical parameters (serum Ca, P and intact parathyroid hormone [iPTH] levels) and the dosage of elemental calcium intake from phosphate binders and alphacalcidol (1-α hydroxycholecalciferol) therapy were recorded at monthly intervals over a 3-year period and expressed as mean time-averaged levels. For controls, results of a single blood test at the time of the study were used.

2.2 *In vitro* culture of vessels

Under sterile conditions, vessels were gently stripped of excess adventitia and cut into 1mm rings. Approximately 12 – 20 rings were typically obtained from a single vessel. The vessel rings were placed in serum-free tissue culture medium with graded concentrations of Ca and P and incubated at 37°C in a 5% CO_2 atmosphere. Serum was not added to the medium because it causes proliferation of smooth muscle cells. Using $CaCl_2$ and NaH_2PO_4, graded concentrations of ionic Ca and P were added to the standard culture medium M199 to give 4 in vitro conditions: control medium (1mM P + 1.8mM Ca), high P medium (2mM P + 1.8mM Ca) and a high Ca + P medium (2mM P + 2.7mM Ca). Vessel rings were incubated for 14 days in the above culture media for all experiments except the time-course studies that were performed at 7, 14 and 21 days.

2.3 Measurement of calcium load in the vessel wall

Vessel rings were washed in HBSS and decalcified in 0.1M HCl for 2 hours, with homogenisation of the tissue using a homogenizer. The Ca content of the supernatant was determined colorimetrically by the cresolphthalein method that was adapted from the method by Gitelman (1967). Briefly, a calcium chloride standard curve was diluted in 0.1M HCl. Water (30µL), ammonia buffer (200µL), and cresolphthalein solution (10µL) were added to the standard and samples (50µL) and the absorbance read immediately at 562nM. The linear line generated from the standard curve was used to calculate an equation in order to obtain an accurate value for the calcium load in each vessel ring. The final Ca load in the vessel ring was standardized for the protein content in that ring.

2.4 Measurement of alkaline phosphatase activity in the vessel wall

Vessel rings were washed in HBSS and lysed in 10% SDS for 1 hour with homogenisation of the tissue using a homogenizer. The entire experiment was carried out on ice, as ALK is a heat labile enzyme. Dilutions of p-nitrophenol for the calibration curve were prepared using 0.02M NaOH. The stock substrate was prepared with a 100mg capsule diluted in 25ml H_20. 50µL of supernatant of the 10%SDS was mixed with 50µL of Alkaline buffer solution (*Sigma*) and 50µL of the prepared stock substrate solution and incubated at 37˚C for exactly 15 mins. The reaction was stopped by neutralising with 0.5ml 0.5M NaOH. 100µL of each reaction and the standard curve was aliquoted onto a 96 well plate and absorbance read at 420nM. The linear line generated from the standard curve was used to calculate an equation in

order to obtain an accurate value for the ALK activity in each vessel ring. The final ALK activity in the vessel ring was standardized for the protein content in that ring.

Measurement of the protein content in the vessel wall

Using the vessel rings after lysis with HCl or NaOH in the Ca and ALK experiments described above, were washed and then homogenised with 100μL of 0.1M NaOH in 1% SDS for 1 hour. Using commercially available standard solution (*BioRad*) a standard curve was prepared. The supernatant from each reaction was plated onto a 96-well plate and the absorbance read at 710nM.

The final Ca load and ALK activity in the vessel ring was standardized for the protein content in that ring.

2.5 Histology and Immunohistochemistry

A vessel ring from each patient was snap frozen at baseline and after incubation in the various tissue culture media for varying lengths of time as described above. Detailed procedures for each histology and immunohistochemical preperation are described below. Slides were viewed on an Olympus BX51 light microscope and images were captured digitally using an Olympus TV1-X digital camera and analySIS software (Soft Imaging System GmBH).

A. Harris' Haematoxylin. Sigma HHS-32

1. De-wax sections and rehydrate through graded alcohols to Milli-Q.
2. Place slides in haematoxylin solution for 5 minutes.
3. Briefly dip slides 10 times in destain solution.
4. Place slides in Scott's solution to "blue" for 5 minutes.
5. Place slides in Eosin solution for 6 minutes.

6. Quickly rinse in Elix-10.
7. Rapidly dehydrate through graded alcohols to two changes of xylene.
8. Coverslip and mount with DEPX.

B. Von Kossa Stain for Calcium Salts

1. Place sections in water.
2. Incubate sections in 2% Silver Nitrate Aqueous Solution for 1 hour under a bright light.
3. Wash in three changes of distilled water.
4. Incubate sections in 3% aqueous Sodium thiosulphate solution for 3 mins.
5. Wash in two changes of tap water for 5 mins each.
6. Counterstain with 1% Neutral Red Aqueous solution for 3 – 4 mins.
7. Quickly dehydrate through graded alcohols and clear in two changes of xylene.
8. Mount in DEPX.

C. Ki-67

1. Bring sections to water.
2. Place slides in 0.5% H2O2/methanol for 10 minutes.
3. Wash in running tap water for 10 minutes.
4. Pre-boil 1mM EDTA, pH8 in the microwave.
5. Place slides in heated EDTA and maintain sub-boil for 10 minutes.
6. Carefully remove slides and plunge into tap water.
7. Wash in PBS 2 x 5 minutes.
8. Block sections with 5% Goat/PBS for 10 minutes.
9. Tip off excess serum.
10. Incubate sections with 1° @ 1:100 @ 25°C for 1 hour.
11. Repeat step 7.
12. Incubate slides with Dako 2° Goat anti mouse biotin @ 1:400/PBS for 30 mins.
13. Repeat step 7.
14. Incubate slides with RTU ABC Reagent for 30 minutes.
15. Repeat step 7.
16. Visualize with DAB Solution, monitor closely with a microscope.
17. Wash well in water.
18. Counter stain and mount as required.

D. TUNEL (TdT-mediated dUTP-biotin nick end labelling)

1. Tissue samples are fixed in 4% buffered formaldehyde pH 7.5.
2. Specimens are embedded in paraffin wax.
3. 4 – 6 micrometre paraffin sections are adhered to subbed slides.
4. Samples are deparaffinised in xylene.
5. Rehydrate in descending series of xylene solutions (100%, 70%).
6. Rinse in tap water, then in DDW.
7. Incubate with 50µg/ml Proteinase-K (PK), 3-5min/RT, pH 7.5
8. Wash in DDW for 2 minutes x 4 times.
9. Immerse in TdT buffer (potassium cacodylate, 200mmol/l, Tris-HCl 25mmol/l, bovin serum albumin 0.25mg/ml, pH 6.6 at 25˚C, cobalt chloride 5mmol/l for 5 mins.
10. Tip off excess TdT buffer.
11. Incubate with TdT enzyme (0.05 – 0.2U/µl) + Digoxigenin-dUTP in TdT buffer in humid atmosphere at 37˚C for 30 mins.
12. Terminate the reaction by transferring the slides to TB buffer. Rinse in DDW.

13. Place slides in 0.1M Tris buffer, pH = 7.6 for 5 mins.
14. Cover sections with 10% FCS or BSA in TBS (blocking buffer) for 10 mins.
15. Incubate with anti-digoxigenin-alkaline phosphatise (anti-sheep F'ab fragments) in blocking buffer, 1:100 for 1 hour.
16. Rinse with TBS for 5 mins.
17. Incubate with chromogenic substrate solutions for alkaline phosphatise either BCIP/NBT (bluish purple) or Fast Red.
18. Rinse in DDW.
19. Counterstain if necessary.
20. Mount sections using aqueous mounting medium.

E. CD68

1. Sections to water.
2. Microwave in pre-boiled citrate buffer pH6, 2 x 10 minutes on med/high.
3. Cool to room temperature.
4. Wash in two changes of PBS for 5 minutes each.
5. Block with 1% hydrogen peroxide in methanol for 30 minutes.
6. Wash in running tap water for 5 minutes.
7. Repeat step 4.
8. Incubate sections with Proteinase K solution for 10 minutes at room temp.
9. Repeat step 4.
10. Incubate sections with 5% horse serum for 30 minutes.
11. Tip off excess and blot carefully around the sections.
12. Incubate sections with the primary antibody @ 1:50 in 5% horse serum for 2 hours at room temperature.
13. Repeat step 4.
14. Incubate sections with the secondary antibody, Vector horse anti mouse, @ 1:400 in PBS for 30 minutes.
15. Repeat step 4.
16. Incubate sections with ABComplex for 30 minutes.
17. Repeat step 4.
18. Incubate sections with DAB solution (Vector Kit SK-4100). Monitor reaction closely using the microscope.
19. Wash in two changes of Milli-Q for 5 minutes each.
20. Counterstain as required.
21. Dehydrate, clear in two changes of xylene.
22. Coverslip and mount with DEPX.

F. Alpha Smooth Muscle Actin

1. Sections to water.
2. Microwave in preheated citrate buffer pH6 for 2 x 10 minutes on med/high.
3. Place in running tap water for 10 minutes.
4. Wash twice for 5 minutes each in PBS.
5. Block with 1 % hydrogen peroxide in methanol for 15 minutes.
6. Wash in running tap water for 5 minutes.
7. Repeat step 4.
8. Incubate sections with 5% horse serum in PBS for 30 minutes.
9. Tip off excess serum and carefully blot around the sections.
10. Incubate sections with primary antibody @ 1:500 in 5% horse serum for 1 hour.
11. Repeat step 4.

12. Incubate sections with secondary antibody, Vector biotinylated horse anti mouse @ 1:400 in PBS for 30 minutes.
13. Repeat step 4.
14. Incubate with ABComplex for 30 minutes.
15. Repeat step 4.
16. Incubate sections with DAB solution, Vector Kit SK-4100. Monitor reaction closely using the microscope.
17. Wash in two changes of Milli-Q for 5 minutes each.
18. Counterstain as required, dehydrate and clear in two changes of xylene.
19. Coverslip and mount with DEPX.

G. Cbfa 1 / runx2

1. Sections to water.
2. Microwave in citrate buffer pH6 until boiling, then for 10 minutes on med/high.
3. Cool to room temp.
4. Wash in PBS buffer 2 x 5mins.
5. Block with .3% hydrogen peroxide in H_2O_2 for 15mins.
6. Wash in PBS buffer 2 x 5mins.
7. Block with 10% Goat serum/PBS for 30 mins.
8. Incubate with 1º @ 1:100 in 10% Goat serum/PBS overnight @ 4°C
9. Wash in PBS 2 x 5mins.
10. Incubate with 2º biotinylated Goat anti Rabbit (Dako) @ 1: 200
11. Wash in PBS 2 x 5mins.
12. Incubate with ABC as for 30 mins
13. Wash in PBS 2 x 5mins.
14. Incubate with DAB solution and monitor reaction under the microscope.
15. Wash in distilled water.
16. Counterstain and mount as required.

H. Osterix

1. Sections to water.
2. Microwave in citrate buffer pH6 until boiling, then for 10 minutes on med/high.
3. Cool to room temp.
4. Wash in PBS buffer 2 x 5mins.
5. Block with .3% hydrogen peroxide in H_2O_2 for 15mins.
6. Wash in PBS buffer 2 x 5mins.
7. Block with 3% BSA for 30 mins.
8. Incubate with 1º @ 1:100 in 3% BSA/PBS overnight @ 4°C
9. Wash in PBS 2 x 5mins.
10. Incubate with 2º biotinylated Goat anti Rabbit (Dako) @ 1: 400
11. Wash in PBS 2 x 5mins.
12. Incubate with ABC as for 30 mins
13. Wash in PBS 2 x 5mins.
14. Incubate with DAB solution and monitor reaction under the microscope.
15. Wash in distilled water.
16. Counterstain and mount as required.

I. Fetuin

1. Take sections to water.
2. Place slides in Citrate buffer pH6 and microwave on high for 12 minutes

3. Remove from the microwave and leave to cool for 20 minutes.
4. Wash in dH2O for 5 minutes.
5. Wash in two changes of PBS for 5 minutes each.
6. Place slides in 3% Hydrogen peroxide/dH2O for 10 minutes.
7. Repeat steps 4 and 5.
8. Incubate slides with 5% Goat serum in PBS for 1 hour at room temperature.
9. Tip off excess serum and incubate with 1° @ 1:200/5% serum @ 4°C overnight.
10. Repeat step 5.
11. Incubate sections with Dako 2° Goat anti Rabbit @ 1:400 for 30 minutes.
12. Repeat step 5.
13. Incubate sections with ABC reagent for 30 minutes.
14. Repeat step 5.
15. Visualize with DAB solution, monitor reaction with a microscope.
16. Wash well in water.
17. Counter stain and mount as required.

J. Annexin 6

1. Sections to water
2. Microwave in citrate buffer pH6 until boiling, then for 10 minutes on med/high.
3. Cool to room temp.
4. Wash in PBS buffer 2 x 5mins.
5. Block with .3% hydrogen peroxide in H2O2 for 15mins.
6. Wash in PBS buffer 2 x 5mins.
7. Block with 5% Goat serum/PBS for 30 mins.
8. Incubate with 1º @ 1:500 in 5% Goat serum/PBS overnight @ 4°C
9. Wash in PBS 2 x 5mins.
10. Incubate with 2º biotinylated Goat anti Mouse (Dako) @ 1: 400
11. Wash in PBS 2 x 5mins.
12. Incubate with ABC as for 30 mins
13. Wash in PBS 2 x 5mins.
14. Incubate with DAB solution and monitor reaction under the microscope.
15. Wash in distilled water.
16. Counterstain and mount as required.

2.6 Transmission Electron microscopy (TEM)

TEM was performed to examine cell morphology, localization of calcification, vesicle release and mineral deposition. Blood vessels were fixed by immersion in 4% glutaraldehyde containing 2mmol/l $CaCl_2$ in 0.1M PIPES buffer at pH 7.4. 100µl 33% H_2O_2 was added to each 10 ml aliquot immediately before use. They were fixed for 4 hours at 4°C, washed twice in buffer (0.1M PIPES) and stored at 4°C. After buffer washes they were post-fixed in 1% osmium ferricyanide for 1 hour, rinsed 3 time in water and bulk stained in 2% uranyl acetate for 1 hour. They were rinsed in water and

dehydrated in an ascending series of ethanol solutions to 100% ethanol, rinsed twice in acetonitrile and embedded in Quetol epoxy resin. 9.0g Quetol 651, 11.6g nonenylsuccinic anhydride (NSA), 5.0g methylnadic anhydride (MNA) and 0.5g benzyl dimethylamine (BDMA). Fifty nanometre sections were cut on a Leica Ultracut UCT, stained with saturated uranyl acetate in 50% ethanol and lead citrate and viewed in a FEI Philips CM100 operated at 80kv.

2.7 RNA extraction and PCR for cDNA

To study the relative amounts of osteogenic transcription factor cbfa-1 / runx2 expression in pre-dialysis and dialysis vessels and to correlate it with the veseel Ca load, total RNA was extracted from all the vessels harvested. The vessel rings of 1-2mm thickness were frozen at -80˚C until used. The median weight of the vessel was 5.4mg (range 1.6 to 31mg).

Techniques for RNA extraction

RNA extraction was initially attempted using the *RNeasy fibrous tissue kit* from *Qiagen*, that utilizes a proteinase K digest to remove protein and is recommended for fibrous tissue such as aorta, heart and skeletal muscle. A very low yield of RNA was obtained and I then tried the TRIZOL method from *Invitrogen*. Both of these methods recommend using 10 – 50 mg of tissue for the RNA extraction, and the very small sample size made the techniques extremely difficult.

TRIZOL (Invitrogen) technique for RNA extraction

TRIZOL is a monophasic solution of phenol and guanidine isothyocyanate. During the homogenization and lysis phases, TRIZOL maintains the integrity of the RNA,

while at the same time disrupting cells and dissolving cell components. Briefly, the vessel ring was homogenized in 1ml of TRIZOL reagent using repeated cycles with a power homogenizer until the tissue was well homogenized. The sample was left on ice for 40 mins, and then the supernatant carefully transferred to a separate container and incubated at 15 - 30˚C for 5 mins to complete dissociation of nucleoprotein complexes. Chloroform (0.2ml) was then added and the samples centrifuged to separate the solution into an aqueous phase that contains RNA and an organic phase. RNA was recovered from the aqueous phase by precipitation with 1ml of isopropyl alcohol. The RNA pellet was washed with 75% ethanol and then resuspended in 10µl of RNase free water.

Sample purity and DNA extraction

Purity of the RNA extracted was checked on a 'nanodrop', and this showed significant contamination with genomic DNA and protein (A260/A280 ratio <2). Hence DNase treatment (*Promega*) of the RNA was performed prior to performing RT-PCR. Briefly, DNA was digested with 5U DNase, 20U RNase inhibitor, 50mM Tris-HCl, 1mM MgCl2 at 37˚C for 1 hour to remove any contaminating genomic DNA.

RNA yield and attempts at optimization

The RNA yield after DNAse treatment was very low, and every attmpt was made to optimise this by:

- Using fresh tissue (within 2 hrs of harvesting the sample)
- Taking every care to prevent RNase contamination

- Homogenisation and lysis of the rings using more cycles of high-speed centrifugation with magnetic beads, so as to increase extraction of RNA
- Dissolving the final RNA pellet in a minimal amount of RNase free water

Despite all of these measures, the RNA yield was only a median of 1.2 (0.02 to 2.1) μgm. RNase samples were stored at -80˚C until further use.

Pooling of RNA samples before cDNA synthesis

Given the very low yield of RNA, we decided to pool samples from normal controls, pre-dialysis patients, dialysis patients with von kossa positivity and dialysis patients without von kossa positivity. After pooling, the total amounts of RNA in each of the 4 groups was 10.7, 81.7, 17.9 and 65.5 μgm respectively. However, despite the DNase treatment, the samples were of very low purity (A260 / A280 ratios on nanodrop = 1.2 to 1.6) indicating that contamination with proteins was still present.

Synthesis of cDNA

The *SuperScript III First-Strand Synthesis System (Invitrogen)* was used for reverse transcription to yield cDNA. The total RNA from the pooled samples above was reverse transcribed in a reaction mixture containing 100ng oligo dT primer, 10U RNase inhibitor, 0.1mM each of dNTPs (ATP, CTP, GTP and TTP) and 400U reverse transcriptase (Promega) in a 1x commercial enzyme reaction buffer at 42˚C for 1 hour. Sample cDNAs within an experiment were standardized using non-saturated amplification of a control house-keeping gene β-microglobulin that remains the same between samples.

Amplification of cDNA – the polymerase chain reaction

PCR amplification was used to investigate the relative expression of mRNA transcripts for the osteochondrocytic transcription factor cbfa-1 / runx2 using the Platinum Taq DNA Polymerase High Fidelity kit from *Promega*.

Each PCR reaction mix contained 2µl of 10x PCR buffer, 1µl of 50mM MgCl2, 2.5µl of 1.25mM solutions of dATP, dCTP, dGTP and dTTP, 2.5µl each of 10 µM solutions of forward and reverse primers, 0.5µl og Taq polymerase, DNA template and milliQ water to a final volume of 20 µl. The reactions were set up under PCR conditions and the final component added was Taq polymerase, which was added on ice. The PCR reaction was run in a PCR block (*Biometra*). For the cbfa-1 gene amplification the PCR cycling conditions used were 60˚C annealing temperature and 35 cycles. The PCR readouts showed that there was a high contamination with genomic DNA, and to try and improve the yield, cDNA was amplified to 50 cycles as well. Given difficulties with a very low yield, PCR or 18S were also run on the pooled samples.

PCR products were electrophoresed on a 1.5% agarose gel containing ethedium bromide and visualised using a UV transilluminator.

Section IV - <u>Statistical analysis</u>

Detailed statistical analysis performed in each study in this thesis is described in the relevant chapters.

Section V - <u>Ethical approval</u>

All studies presented in this thesis were approved by the Institute of Child Health and Great Ormond Street Hospital research ethics committee *(documents attached in Appendix C)*.

Chapter 3

The Long-Term Outcome of Chronic Dialysis at Great Ormond Street Hospital

3.1 Abstract

In this chapter I have described the outcome data since 1984 of children receiving chronic dialysis at Great Ormond Street Hospital for ≥ 3 months with a minimum follow-up of 5 (median 7.2) years.

There were 98 children (61 male), with a median age at start of dialysis of 4.2 (range birth to 16.2) years. Twenty-one children started dialysis when ≤1 year age while 54 were ≤5 years age at start of renal replacement therapy. The median time on dialysis was 1.4 (0.3 – 14.4) years. In 80 children the initial mode of dialysis was PD. 53 children received a renal transplant, but 21 (39%) returned to dialysis. An improvement in PTH control was seen with 80% of patients having PTH levels within twice the upper limit of normal range at follow-up as compared to 15% at start of dialysis. There was a trend towards improvement in weight and height standard deviation scores on dialysis.

There were 17 deaths over the 20-year study period; of these 10 died on dialysis. The overall patient survival was 83 %. Amongst the survivors, 84% have a functioning graft while 14% remain on dialysis. The mortality rate was 2.7 times greater in children who required renal replacement therapy under the age of 1 year. Only one death was from cardiovascular causes (congestive cardiac failure).

In conclusion, the very low incidence of cardiovascular mortality in our population highlights the importance of maintaining a tight control on PTH levels throughout the course of CKD and on dialysis.

3.2 Introduction

Paediatric patients have been taken onto dialysis treatment for the last 30 years and comprise approximately 2% of any national dialysis programme *(USRDS, 2003)*. Despite this, long-term outcome data for this group are scarce, reflecting the relatively small numbers involved and the difficulties in tracking these patients beyond adolescence. There is an increasing awareness that cardiovascular disease begins early in the course of CKD *(Kari et al, 2004, Groothoff et al 2005)* and is present even in children on dialysis *(Shroff et al, 2007, Litwin et al, 2005, Goodman et al, 2000)*. Moreover, CKD patients now seldom die of uraemic complications, but far more commonly from cardiovascular disease *(Foley et al, 1998),* and this is true of paediatric dialysis patients as well *(Parekh et al, 2002; Chavers et al, 2002; Oh et al, 2000)*.

In this chapter I have reviewed our experience of 20 years of paediatric dialysis and described the long-term outcome, with particular emphasis on survival and causes of death in this group. Also, PTH control and growth, a possible surrogate marker of adynamic bone disease, has been evaluated.

3.3 Methods

A dialysis programme for children under 5 years of age was started at Great Ormond Street Hospital in 1984, with the first patients starting peritoneal dialysis (PD) and haemodialysis (HD) in 1984 and 1985 respectively. In this chapter I have described the outcome of all children under 18 years of age with CKD who have received chronic dialysis (defined for the purpose of this study as dialysis for a continuous period of ≥ 3 months), with a minimum follow-up of 5 years.

Patients

Between January 1984 and December 1998, 98 children (61 boys) received chronic peritoneal or haemodialysis at Great Ormond Street Hospital for Children. The primary diagnoses were as follows: renal dysplasia (32), posterior urethral valves (17), congenital nephrotic syndrome (12), focal segmental glomerulonephritis (9), bilateral Wilm's tumour (4), cystinosis (4), cortical necrosis (3), Alport's syndrome (3), nephronophthisis (3), autosomal recessive polycystic kidney disease, post-streptococcal acute glomerulonephritis, atypical haemolytic uraemic syndrome and glomerulosclerosis in 2 cases each, nephrocalcinosis of unknown etiology in one case and metabolic disorders with end-stage renal involvement (Methylmalonicacidaemia and Lesch-Nyhan syndrome) in one case each.

Data collection

Through a retrospective review of case-notes I collected the following information:

1) *Demographic data:* diagnosis, associated comorbid factors and age at the start of renal replacement therapy (RRT).
2) *Details of the dialysis regimen used:* the index course of dialysis, switches between dialysis modalities, duration of dialysis therapy before transplantation and return to dialysis after a failed transplant.
3) *Outcome measures on dialysis:* anthropometric data, PTH levels, and developmental outcome were reviewed in all children. Changes in standard deviation scores (SDS) for weight and height were calculated. As part of a larger study, the developmental progress of all the children was determined during the pre-transplant work-up, but will not be discussed further in this chapter.

4) *Follow-up:* All children were followed-up for a minimum period of 5 years from initiation of dialysis with final follow-up up to 31st December 2003. Details regarding current dialysis modality or transplantation after transfer to adult units were obtained through telephonic queries. Mortality data, including the cause of death and any association with comorbidity was reviewed through death certificates wherever available.

Statistical analyses

Statistical analysis for growth variables (expressed as mean and standard deviations) was performed in all children, comparing values at the start of dialysis treatment with final levels (measured at the time of renal transplantation or last follow-up) using the paired t test. Kaplan-Meyer survival analysis was performed for the entire cohort as well as for those starting dialysis under the age of five years.

3.4 Results

Incidence

Ninety-eight children (61 males) have received chronic peritoneal or haemodialysis at Great Ormond Street Hospital between January 1984 to December 1998. Thus, on average 5.7 new patients required RRT each year. With the merging of children's renal services in 1996 there has been a considerable expansion in our dialysis programme with 45 new cases requiring dialysis between January 1996 to December 1998: 11.2 new cases per year.

Co-morbidity

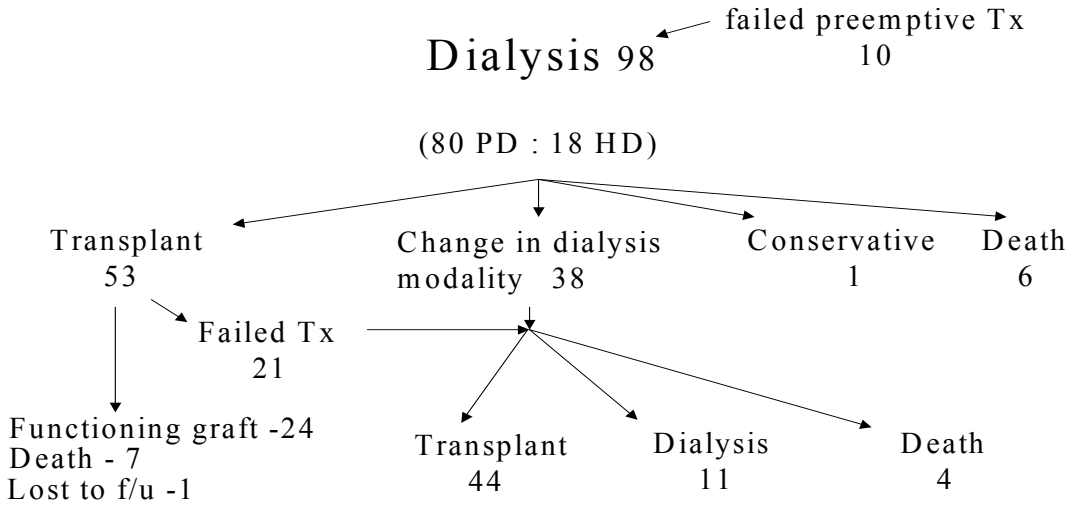
Significant co-morbidity was present in 30 children. Five infants were born prematurely at ≤ 32 weeks gestation. None of the premature babies had pulmonary hypoplasia. An antenatal diagnosis of obstructive uropathy was made in 28 fetuses; an intra-uterine vesicostomy was attempted twice in one fetus. Details of the co-morbid conditions and their impact on patient outcome are not directly relevant to this thesis and have not been discussed further, but have been presented in the publication 'Long-term outcome of chronic dialysis in children' attached in Appendix A.

Details of RRT

The median age at the start of dialysis was 4.2 (range birth to 16.2) years, with the youngest patients starting PD and HD from day 1 and day 16 of life respectively. Twenty-one children were under 1 year, and 33 were between 1-5 years age. Of the 98 patient cohort, 80 received PD as the index course of dialysis – only 3 of 21 children (14.3%) under 1 year of age were started on HD, and 9 of 54 under 5s (16.6%) started HD as the index course of dialysis. There were a total of 54 switches between dialysis modalities with a maximum of 5 switches in one patient. HD was principally used as a backup for PD in most cases, with HD used only transiently in the majority. The median duration on PD before peritoneal membrane failure was 3.9 (1.9 – 7.3) years. The median time on dialysis was 1.4 (0.3 – 14.4) years, giving the centre a total dialysis experience of 296 patient-years. Figure 3.1 shows the incident RRT modality, switches between RRT and the outcome at final follow-up.

Of the 98 children, 88 (90%) received a renal transplant; 29 (33%) live related. Twenty-one of the 68 transplanted patients (31%) returned to dialysis. The median transplant survival was 10.1 (0.1 – 18+) years: 14.8 (0.1 – 18+) years for a live-related transplant and 7.2 (0.1 – 18+) years with a cadaveric transplant. In total there were 115 transplants in 88 patients, with 19 patients having received their second grafts, and 4 their third grafts before transfer to an adult unit.

Figure 3.1



At final follow-up

Transplant	Dialysis	Deaths	Conservative / lost to f/u
68	11	17	2

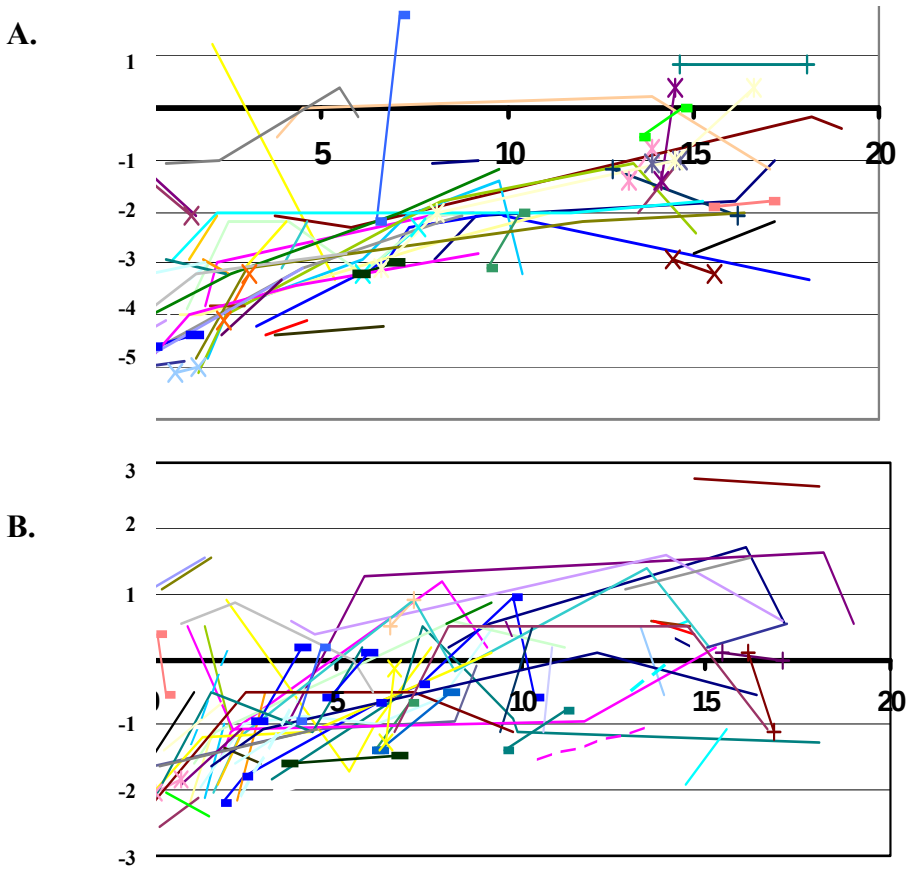
Long-term outcome of chronic dialysis in children

Outcome Measures on Dialysis

Growth

The changes in median weight and height SDS (SDS at the start of dialysis treatment - final SDS [measured at the time of renal transplantation or final follow-up]) are shown in Figure 3.1. The median weight SDS was –2.8 (1.0 to – 4.2) at the start of dialysis and –1.7 (3.6 to – 4.0) pre-transplant or at final follow-up. The median height SDS (analysed only for children who received dialysis for ≥6 months, n = 78) improved from – 2.8 (1.9 to – 5.0) at the start of dialysis to –1.9 (range 0.8 to – 4.8). Predictably the height deficit was most severe in the youngest patients - the baseline height SDS was –3.6 (1.1 to –5.0), but linear growth was maintained with final height SDS of – 2.6 (0.2 to –4.2). The change from baseline in height deficit (ΔZ-score) was 0.5 (0.9 to –1.1) for the overall population. For patients with the most severe height deficit at the onset of dialysis (SDS ≥-2), greater catch-up growth was seen (median ΔZ-score = 0.8, 1.1 to –1.4). None of the patients received growth hormone.

Figure 3.2 Change in height (A) and weight (B) standard deviation scores
ΔSDS = SDS at the start of dialysis treatment - final SDS (measured at the time of renal transplantation or final follow-up). Values on dialysis are shown by solid lines and those post-transplant by dotted lines. ΔSDS for height has been calculated for those on dialysis for a minimum period of 6 months, n = 78.



PTH

Intact PTH assay was performed serially in 91 patients (available from April 1989). As the PTH assay has changed during the study period, the levels have been expressed as within twice the upper limit of the normal range (ULN). The measured intact PTH was less than twice the ULN in 15 children when dialysis was started and in 80 children at final follow-up or pre-transplantation. PTH levels were better controlled in the children who predominantly received PD than in the group who were on haemodialysis for the majority of their renal replacement therapy: 58 of the 69 (84%) of the PD population had a PTH level within twice ULN at final follow-up or pre-transplantation while 15 of the 22 (70%) on haemodialysis achieved PTH levels within twice ULN.

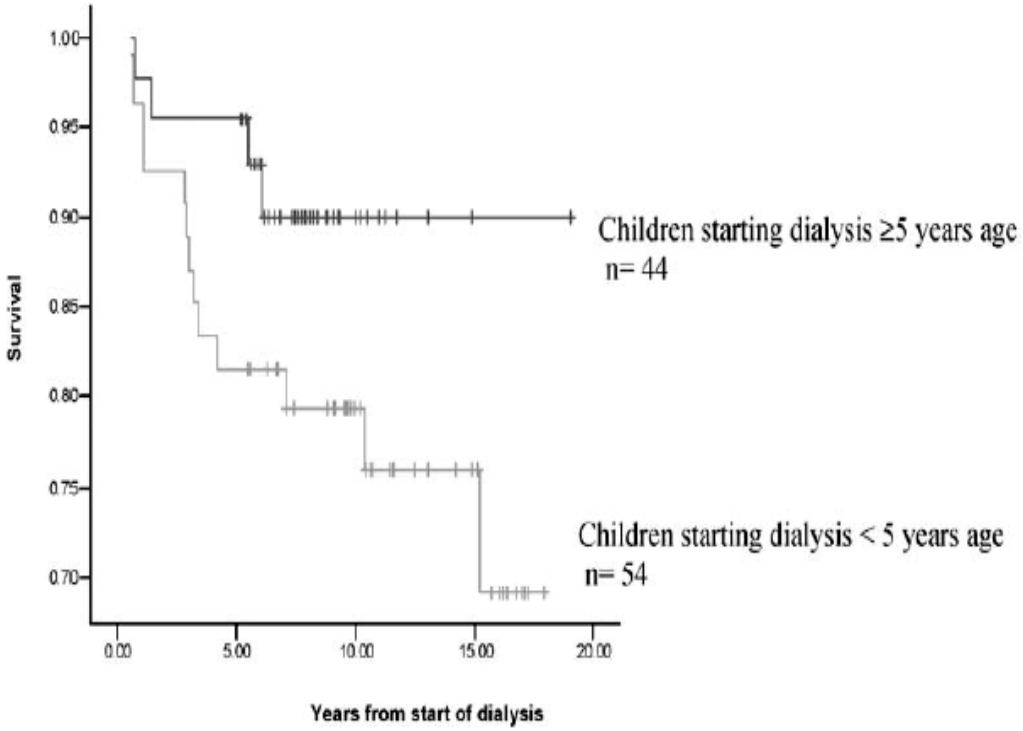
Mortality

There were 17 deaths over the study period. The overall patient survival was 92% at 1 year, 88% at 5 years and 84% at 10 years from the index case of dialysis. Ten deaths (59%) occurred on dialysis (7 on PD) and 7 following transplant. Six (35%) had never been transplanted.

The median age at death was 5.9 (0.8 – 18.6) years. Six (35%) of these children had started dialysis before their first birthday, and 13 of the 17 (76%) started dialysis before the age of 5 years (Figure 3.3). In those patients starting dialysis ≤1 year of age, the 5-year survival was 72%; a 2.7 times higher relative risk of death than in the overall population.

Table 3.1 describes the causes of death and associations with comorbidity, age at start of dialysis and age at transplant.

Figure 3.3



Long-term survival of children based on age at start of RRT

Follow-up

The median follow-up time was 7.2 (5 – 18.6) years from initiation of dialysis. One patient returned to his native country following transplantation and has been lost to follow-up. During this study period 27 patients were transferred to adult units. Following transfer 10 received a renal transplant. One patient died soon after transfer.

At final follow-up the overall survival was 83%. Of the survivors, 68 (84%) have a functioning graft, 11 (14%) remain on dialysis, 1 patient is conservatively managed and 1 has been lost to follow-up.

Table 3.1 Causes of death (in ascending age order of starting dialysis)

Patient no.	Diagnosis	Comorbidity	Age at start of dialysis (yrs)	Age at previous transplant (yrs)	Age at death (yrs)	Cause of death	Duration of dialysis (yrs)
Deaths on dialysis							
1	Dysplasia	Severe developmental delay, blindness, hypothyroidism	0.0	-	1.1	Mesenteric infarction with bowel necrosis	1.1
2	Congenital Nephrotic Synd	Developmental delay	0.1	-	1.8	Encephalopathy with ischaemic infarction	1.7
3	Posterior urethral valves	-	0.3	1.7	4.5	Peritonitis	0.6
4	Dysplasia	Alagille's syndrome with portal hypertension	0.4	-	1.5	Sepsis/pneumonia	1.3
5	Glomerular sclerosis	Developmental delay	1.1	-	1.7	Pulmonary oedema	0.6
6	Posterior urethral valves	-	1.2	1.8 and 4	8.3	Cardiac failure	1.3
7	Dysplasia	-	3.2	11.9	18.6	Intracranial bleed	13.2
8	Focal segmental glomerulosclerosis	Spondyloepiphyseal dysplasia	3.7	-	6.6	Ischaemic encephalopathy	2.8
9	Glomerular sclerosis	Hepatosplenomegaly ? storage disorder	4.1	-	7.2	Sepsis	3.1
10	Autosomal recessive polycystic kidney disease	Hepatic fibrosis with oesophageal varices	9.7	10	15.2	Hepatic encephalopathy and sepsis	5.5
Deaths post renal transplant							
11	Posterior urethral valves	Immunodeficiency	0.2	1.5	3.6	Epstein Barr viraemia	
12	Congenital Nephrotic Synd	Developmental delay	2.0	3.0	3.1	Gastric haemorrhage	
13	Wilms	Chemotherapy	2.0	5.1	5.2	Sepsis	
14	Congenital Nephrotic Synd	Microcephaly, cerebral palsy	2.3	3.9	12.7	Aspiration pneumonia	
15	Focal segmental glomerulosclerosis	-	13.6	14.7	15	Recurrent FSGS, varicella encephalopathy, cerebral h'hage	
16	Wilms	Chemotherapy	14.1	14.7	14.9	Metastatic Wilms	
17	Methylmalonic acidemia	Multisystem disorder	15.4	17.1 (liver + kidney)	17.5	Hepatic failure	

3.5 Discussion

In this single-centre 20-year longitudinal outcome study I have shown three important points: a younger age at the start of renal replacement therapy is related to poor outcome, that there is a trend towards improved height and weight SDS on dialysis and most importantly, that maintaining a tight control on PTH levels throughout the course of CKD and on dialysis may be associated with the low cardiovascular mortality seen in our population.

Despite the improved survival of children and adolescents with end-stage renal disease, there are few long-term outcome data available. Longitudinal outcome studies on large numbers of patients are required to allow informed decision making by clinicians and the involved families. The UK Renal Registry (6^{th} annual report, 2003) *(UK Renal Registry, 2003)* has 6 years of continuous data collection from the 13 paediatric renal units in the UK and gives detailed information on patient demographics, causes of ESRD, growth and mortality data. The Australia and New Zealand Dialysis and Transplant (ANZDATA) Registry has recently published its 20-year data *(McDonald et al, 2004)* discussing long-term survival data and demonstrating the improvement in survival over 4 decades of RRT. The largest available database comes from the North American Pediatric Renal Transplant Co-operative Study (NAPRTCS) involving 134 centres from North America. Its recent 2003 annual report *(NAPRTCS 2003)* offers extensive demographic, anthropometric and mortality data. I have compared our results with these three registry reports. Our study offers a longer period of follow-up and growth data and associations with comorbidity that are not discussed in any of the above registries.

As demonstrated in several registry reports, a younger age at the start of RRT is a strong predictor of mortality *(USRDS annual report, 2003; UK Renal Registry 2003; McDonald et al, 2004)*. Despite the overall younger age of our study group, the mortality was 2.7 times greater in children who started RRT ≤ 1 year of age than in the overall population; the five-year survival was 72% in this group. This compares favourably with the UK Renal Registry report wherein the 5-year survival rate for children starting RRT at ≤ 1 year of age was 66% *(UK Renal Registry 2003)*. NAPRTCS report a 66% 3-year survival *(USRDS annual report, 2003)* and ANZDATA a 73% 5-year survival in the ≤ 1s (*McDonald et al, 2004),* (the overall population survival rates were 89% at 3 years and 86% at 5 years in the two series respectively). Also, ANZDATA have shown a 4 fold higher mortality in the ≤ 1s as compared to patients between 15-19 years of age at initiation of dialysis (*McDonald et al, 2004)*. A selection bias reflecting the ethical views of the referral centres or the nephrologists *(Geary et al, 1998)* may well have resulted in the apparent difference in outcome of the ≤ 1s starting RRT. Unfortunately there was limited statistical power in this study to enable determination of the relative risk of death between the two modalities of dialysis or any association between the duration of dialysis pre-transplantation and overall survival.

Although statistical significance was not reached, there was a trend towards improvement in weight and height SDS while on dialysis. Also, children with comorbidity have been included in the analysis, keeping in mind that they will negatively influence the statistics. As shown in other registry reports *(UK Renal Registry, 2003 and NAPRTCS 2003)*, most of the children with poor linear growth presented in the 1st year of life. This study spans 20-years of data and includes

patients starting dialysis in the mid-1980s when most series showed deterioration in height SDS on dialysis and when aggressive nutritional management was not pursued. However, as shown in more recent reports *(Groothoff et al, 2002 and Rees L, 2002)* and in infant dialysis series from our hospital *(Shroff et al, 2003; Coulthard et al, 2002; Kari et al, 2000)* increase in height on both PD and HD can take place on dialysis. Although impressive growth rates with the use of growth hormone in infants have been reported in many studies, in this institution we have shown that the change in height SDS is matched by a rigorous feeding programme in children under 2.5 years age *(Ledermann et al, 2000 and Ledermann et al, 2002)*. While an improvement in height SDS intuitively suggests that adynamic bone disease could not be present, there are no studies examining bone histology and height velocity to support or refute this possibility. However, studies from my colleagues at Great Ormond Street Hospital have shown that pre-dialysis patients in CKD stages 2 - 4 show catch-up growth when medical therapy is aimed at normalizing PTH levels *(Waller et al, 2003)* and that keeping PTH within the normal range prevents further loss of height SDS in short children on dialysis *(Cansick et al, 2007)*.

While no single most common cause of death could be identified in our study, NAPRTCS, ANZDATA and the Dutch cohort studies *(Groothoff et al, 2005)* report 'cardiac/cardiopulmonary' as the most common cause of death. NAPRTCS reports that 21.8% of all deaths were from cardiopulmonary causes, both for the overall population and for each age group *(NAPRTCS 2003)*, ANZDATA reports that 43% of deaths in the PD group and 57% in the HD group were from cardiac causes *(McDonald et al, 2004)*, and the Dutch cohort *(Groothoff et al, 2002)* reports cardiac deaths in 33% of the children who received RRT for >10 years. In our study there was

only one death from a cardiac cause (congestive cardiac failure): this child had received dialysis for 1.3 years and had two failed grafts. The follow-up period and completeness of long-term data available in this study is comparable to that of the large registry reports, but this is the only study that has examined PTH levels over a long period of time in dialysis patients. The PTH control, with 80% of children on dialysis achieveing the target range of PTH levels at < 2-fold ULN suggests that this, along with the ensuing control in mineral metabolism, may have contributed to the favourable cardiovascular outcome in our patients.

3.6 Conclusions

In conclusion, I have shown in this chapter that maintaining a tight control on PTH levels throughout the course of CKD and on dialysis may be associated with lower cardiovascular mortality in children on dialysis. Also, keeping PTH levels at < 2-fold ULN allows for a trend towards improvement height SDS on dialysis. The effects of PTH levels on the vasculature will be explored in subsequent chapters.

Chapter 4

The Effects of Parathyroid Hormone Levels on the Vasculature

4.1 Abstract

Cardiovascular disease is increasingly recognised as a life-limiting problem in young patients with chronic kidney disease (CKD), but there are few studies in children that describe its determinants. In this chapter I have described the impact of intact parathyroid hormone (iPTH) levels on vascular structure and function in children on dialysis.

Children aged 5–18 years who had received dialysis for ≥6 months and had an eGFR<30ml/min/1.73m^2 for ≥3 years were recruited from 4 paediatric Nephrology centre in the UK. Mean time-averaged levels of calcium, phosphate (PO_4) and iPTH levels, and doses of phosphate-binders and Vitamin D were recorded from the start of CKD stage IV, giving us at least 3½ years of cumulative data on all patients. Patients were divided into two well-matched groups based on mean time-integrated iPTH levels: Group I– iPTH levels <2 upper limit of normal (ULN) [n = 41] and Group II– iPTH >2 ULN [n = 44], and compared to age-matched controls [n = 40]. Carotid intima-media thickness (cIMT), aortic and brachio-radial pulse-wave velocity (PWV) and coronary and valvular calcification on CT scan were measured.

Dialysis patients had increased cIMT and PWV as compared to controls. All vascular measures positively correlated with PO_4 levels, and the cIMT and calcification also correlated with iPTH levels. All vascular measures in Group I were comparable to controls, but group II had thicker cIMT ($p < 0.0001$, RR 3.7), stiffer vessels ($p = 0.03$) and increased calcification ($p = 0.004$, RR 2.3). Patients with increased cIMT had stiffer vessels and a greater prevalence of cardiac calcification. The dose of Vitamin D strongly influenced all vascular measures while cIMT showed a weak correlation with calcium intake from PO_4-binders.

In conclusion, I have shown that children on dialysis with iPTH levels > 2ULN were more likely to have vascular damage and calcification as compared to those with iPTH levels <2ULN. Also, increased cIMT and calcification were associated with a higher activated vitamin D dosage.

4.2 Introduction

Ca, PO_4, and PTH are important and potentially modifiable risk factors in the development and progression of vascular disease *(Block et al, 1998, Goldsmith et al 2004)*. The K/DOQI, Clinical Practice Guidelines recommend maintaining intact PTH levels (iPTH) at 3-5 times the ULN in patients on dialysis *(K/DOQI, clinical practice guidelines, 2003)*, while the European best practise guidelines suggest a more conservative levels of 2-3 times ULN *(Klaus et al, 2006)*. Both sets of guidelines are based on bone histomorphometry studies in children and adults *(Mathias et al, 1993; Salusky et al, 1994; Goodman et al, 1994)* but as evidence based studies are scarce, most of these recommendations are based on expert opinion. Higher than normal PTH levels are recommended because, as CKD progresses, continued stimulation of the parathyroid glands by high plasma PO_4 and low Ca levels leads to parathyroid gland hypertrophy and re-setting of the calcium sensing receptor *(Lewin et al, 1997)* so that higher than normal levels of ionised Ca are required to alter PTH secretion *(Rostand et al, 2003)*. This 'skeletal resistance' to PTH thought to result in low turnover (adynamic) bone disease, and an inability of bone to buffer fluxes in serum Ca resulting in ectopic soft tissue calcification. However, it has to be remembered that high iPTH levels are *per se* a risk factor for vascular disease and soft tissue calcification *(Rees L, 2008; Rostand et al, 2003)*.

At Great Ormond Street Hospital we aim to prevent the escape of the parathyroid glands from normal control mechanisms by early dietary and therapeutic intervention. We have shown that keeping iPTH levels in the normal range in CKD stages I - IV, and <2 ULN in children on dialysis maintains normal growth velocity *(Waller et al, 2003; Cansick et al, 2007)* and bone mineral density *(Waller et al, 2007)*, but the presence of renal osteodystrophy was ubiquitous in children with CKD irrespective of iPTH levels (*Waller et al, 2008)*. However, some other centres in the UK continue to follow the K/DOQI guidelines and aim for PTH levels at 3 - 5 times the ULN. These long-standing differences in management of secondary hyperparathyroidism amongst paediatric renal units in the UK have given me the opportunity to study the impact of a wide range of iPTH levels and their management on vascular structure and function.

In this chapter I have examined the hypothesis that maintaining iPTH levels at <2 ULN throughout the course of CKD will prevent vascular damage and calcification in children on dialysis.

4.3 Methods

Patients and controls

In this multicentre study we recruited children from 4 paediatric renal units in the UK (Great Ormond Street Hospital n = 56, Birmingham Children's Hospital n = 12, St James's University Hospital, Leeds n = 12 and Nottinhgam City Hospital n = 5). These 4 hospitals provide approximately 60% of paediatric dialysis services in the

UK. Of 103 eligible patients 97 agreed to participate. Seven were excluded because of criteria identified at the first visit or unwillingness to undergo scans. Informed written consent was obtained from all parents or caregivers and children, where appropriate. The study was approved by a multi-centre research ethics committee.

Inclusion and exclusion criteria

All children aged 5 to 18 years who had received dialysis for at least the preceding 6 months, and had been in CKD Stage IV (GFR≤30 ml/min/1.73m^2) for ≥3 years were included. The age criteria (>5 years) was selected on the basis that vascular measures are very difficult to perform in younger children, and that very young children may not stay still during a CT scan; the use of any form of sedation or anaesthesia for a research study would not have been ethical. More importantly, the seminal paper by Goodman et al *(Goodman et al, 2000)*, the only available study in children at the time, has described calcification only in those with a dialysis vintage of >4 years.

In order to keep the study groups free of confounding pro-atherosclerotic risk factors, we excluded patients with underlying vasculitis, diabetes, uncontrolled hyperlipidemia (defined for the purpose of this study as a serum cholesterol ≥5mMol/L despite statin therapy), uncontrolled hypertension (defined for the purpose of this study as systolic BP ≥ 95thcentile for age despite anti-hypertensive therapy), and smokers.

In all centres calcium-based phosphate binders were used for management of hyperphosphataemia, with sevelamer introduced only in those with hypercalcaemia and persistently high iPTH levels. Alphacalcidol (1-α hydroxy-Vitamin D_3) was used

in all patients, titrating the dose against iPTH levels, with temporary discontinuation if hypercalcaemia resulted. All children received regular dietary advice.

As vascular measures are age-dependent, and normative data is available only for children above 10 years of age, we compared our patient group with 40 age- and gender-matched healthy controls.

Data collection

CKD-specific cardiovascular risk factors were measured from the start of CKD Stage IV, giving a minimum of 3½ years cumulative data in all children. Serum Ca, PO_4 and iPTH levels and the doses of elemental calcium intake from phosphate binders and Vitamin D were recorded at monthly intervals. As the iPTH assay varied between different centres, iPTH was expressed as multiples of the upper limit of normal (ULN). In control subjects, biochemical values from a single blood test at the time of the scans were used.

Study design

Patients were divided into 2 groups based on mean time-integrated serum iPTH levels: Group I with serum iPTH <2 ULN and Group II with iPTH >2 ULN. Patients in Group II included those in whom the unit policy was to aim for iPTH levels >2 ULN and also those with non-compliance to treatment despite an intention to keep iPTH levels <2 ULN.

Vascular measures

Vascular measures were performed on 90 dialysis patients and 40 controls (5 patient's scans were of poor quality and excluded from analysis). All patients underwent a carotid artery ultrasound scan to measure intima-media thickness (cIMT), applanation tonometry for aortic and brachio-radial pulse wave velocity (PWV), and a multi-slice CT scan to look for coronary artery and valvular calcification as described in detail in Chapter 2, section 2.1. In the control group, aortic PWV was not measured as permission was not granted for femoral artery measures and CT was not undertaken because of radiation concerns.

All vascular scans were performed at Great Ormond Street Hospital after a mid-week session of haemodialysis or overnight cycling peritoneal dialysis. Scans were performed simultaneously by myself and a second blinded operator and analysis was performed by myself after blinding to all patient details.

Statistical analyses

Results are presented as mean ± SD. All data was analysed in a linear fashion and then between the 2 groups of dialysis patients. The student t-test, Mann-Whitney U test or Fisher exact t-test were used as appropriate. From univariate analysis variables associated with vascular measures with $p < 0.15$ were entered into a stepwise multiple regression analysis. $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS, version 12.0.1 (*SPSS Inc, Chicago, IL, USA*).

4.4 Results

Dialysis and control groups

Diagnoses of the 85 study children (45 boys) were dysplasia (n = 50), inherited nephropathies (n = 13), cystic kidney disease (n = 6), primary tubular disorders (n = 6), renovascular disorders (n = 4), malignancies (n = 3) and metabolic disorders (n = 3). Comparisons between controls and Groups I and II are shown in Table 4.1.

Carotid IMT and brachio-radial PWV were significantly greater in the dialysis population than the control group (Table 4.2). The duration of dialysis was associated with an increasing cIMT ($r = 0.31$, $p = 0.04$), but not with PWV or the presence of cardiac calcification. The time spent in CKD Stage IV, age at initiation of dialysis, dialysis modality and preservation of residual renal function did not correlate with any vascular measures.

Table 4.1 Demographic, clinical, anthropometric and biochemical characteristic of patients and controls

	Controls (n=40)	Group I (n=41)	Group II (n=44)	p (Group I vs II)
Age (yr)	13.2 ± 4.8	12.6 ± 3.9	13.5 ± 3.2	0.30
Gender (males / females)	21/19	24/17	21/23	0.89
Estimated GFR (ml/min/1.73m^2)	111 ± 8.8	9.1 ± 8.0	7.6 ± 3.7	0.25
Time in CKD Stage IV (yr)	-	5.8 ± 3.5	5.0 ± 4.3	0.26
% with residual renal function	-	39	42	0.82
Age at start of dialysis (yr)	-	9.8 ± 5.1	8.8 ± 3.9	0.34
Time on dialysis (yr)	-	2.2 ± 1.7	2. 4 ± 1.9	0.09
Dialysis modality at point of study (PD / HD)	-	32/9	32/12	0.09
% CKD time spent on dialysis	-	39.4 ± 31.2	44.2 ± 36.3	0.08
Height SDS	0.5 ± 2.6	-1.4 ± 1.7	-1.6 ± 2.1	0.75
Body Mass Index SDS	0. 9 ± 0.9	-0.5 ± 1.4	-0. 4 ± 1.9	0.06
Systolic BP Index*	0.9 ± 0.1	1.2 ± 0.4	1.2 ± 1.2	0.09
Number of anti-hypertensive medications	0	1 (0 – 2)	1 (0 – 3)	0.07
Haemoglobin (gm/dl)	13.3 ± 1.1	11.7 ± 1.5	10.9 ± 2.4	0.61
Albumin (g/L)	41 ± 0.6	39 ± 3.8	37 ± 3.7	0.92
Total Cholesterol (mMol/L)	3.3 ± 1.3	4.4 ± 0.8	3.9 ± 1.3	0.75
Triglycerides (mMol/L)	0.9 ± 2.2	1.3 ± 1.3	1.6 ± 1.0	0.67
Serum PO_4 level (mMol/L)	0.9± 0.3	1.4 ± 0.3	2.1 ± 0.8	<0.0001
Serum Ca (albumin adjusted) (mMol/L)	2.2± 0.2	2.4 ± 0.1	2.4 ± 0.3	0.15
% of episodes with Ca ≥2.5mMol/L per patient	0	5	11	0.08
Ca-PO_4 product (mMol^2/L^2)	3.3± 0.3	3.5 ± 0.6	4.9 ± 0.9	<0.0001
Serum iPTH (fold ULN)	n/d	0.7 ± 0.6	6.0 ± 5.2	<0.00001
Parathyroidectomy	0	0	1 (partial)	-
PO_4 binders				
Number on Ca-based PO_4 binders	-	36 (88%)	26 (59%)	0.08
Sevelamer +/- Ca-based PO_4 binder	-	5 (12%)	18 (41%)	
Cumulative intake of elemental Ca from PO_4 binders gm/kg	-	119 ± 71	131 ± 112	0.07
Calcitriol (Vitamin D_3) µgm/kg	-	49.6 ± 14.6	85.7 ± 29.9	<0.0001

Group I - mean time-integrated serum iPTH ≤ twice ULN (upper limit of normal)
Group II - mean time-integrated serum iPTH> twice ULN
All values expressed as mean ± SD. *BP Index = measured BP/95th centile BP for age, gender and height.

Table 4.2 Comparison of carotid artery structure, vascular stiffness and calcification scores between the study groups

	Controls	Dialysis	p	Dialysis Patients		
	n = 40	n = 85		Group I n = 41	Group II n = 44	p
Carotid IMT [mm]	0.38 ±0.01	0.46 ±0.12	0.002	0.39 ± 0.01	0.58 ± 0.02	<0.0001
Aortic PWV [m/sec]	n/d	7.14 ± 1.2	-	5.81 ± 1.2	8.63 ± 2.3	0.03
Brachio-radial PWV [m/sec]	5.1 ± 1.0	8.89 ± 1.9	0.03	9.06 ± 2.1	8.57 ± 1.8	0.82
Number with Cardiac calcification	n/d	17 (20%)	-	5 (12%)	12 (27%)	0.004
Agatston score	-	21.3 ± 30.1	-	11.9 ± 10.3	65.6 ± 278 Median 85.3 (0 – 2039)	0.01
Coronary arteries	-	13	-	3 (7%)	10 (22%)	0.02
Valves	-	5	-	2	3	-
Aorta	-	7	-	1	6	-

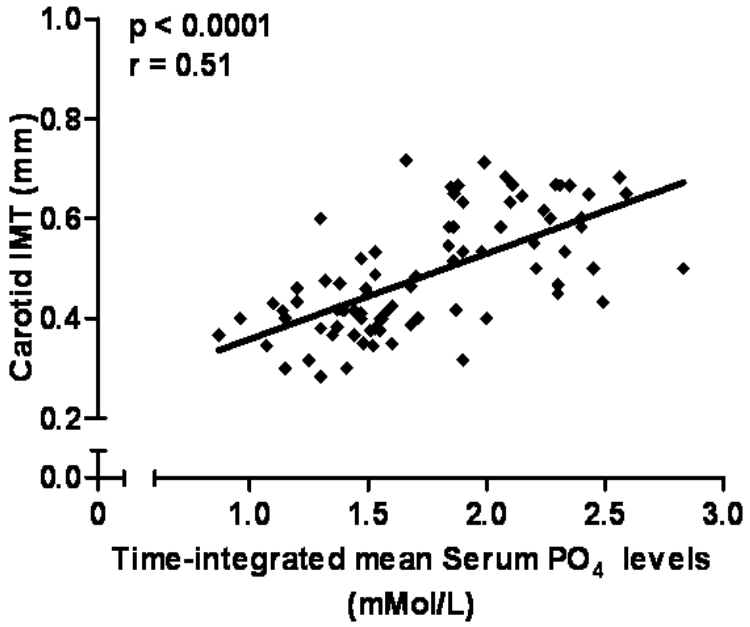
cIMT – carotid artery intima-media thickness, PWV – pulse wave velocity, n/d – not done.
All values expressed as mean ± sd unless otherwise stated.

Vascular measures and calcification score in Groups I and II

Carotid Intima-media thickness

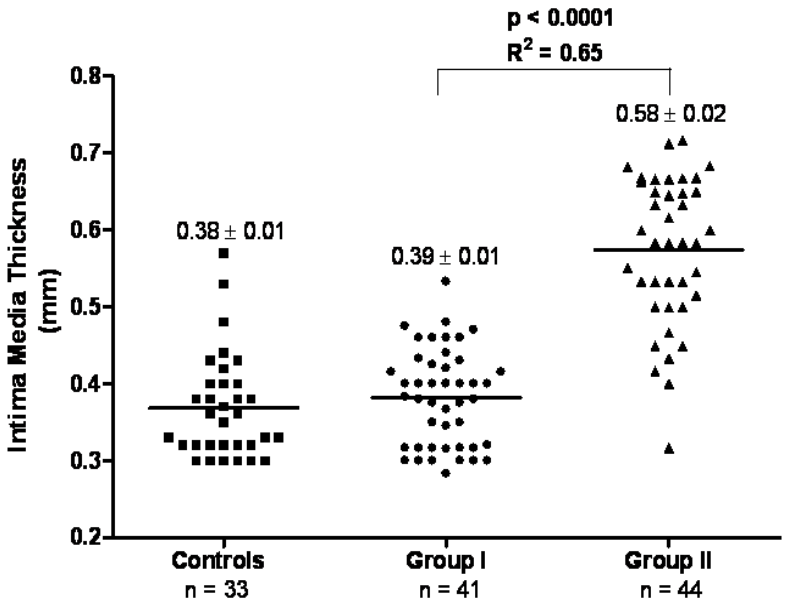
cIMT showed a strong linear correlation with iPTH ($r = 0.71$, $p = 0.0001$), PO_4 ($r = 0.51$, $p < 0.0001$; Figure 4.1) and Ca x PO_4 product ($r = 0.65$, $p < 0.0001$). The cIMT in Group I was comparable to the control group (0.39 ± 0.01 vs 0.38 ± 0.01 mm, $p = 0.44$), and significantly lower than in Group II (0.58 ± 0.02, $p < 0.0001$); relative risk = 3.7 (Figure 4.2).

Figure 4.1



Correlation of carotid artery intima-media thickness (cIMT) with mean time-integrated serum PO_4 level.

Figure 4.2



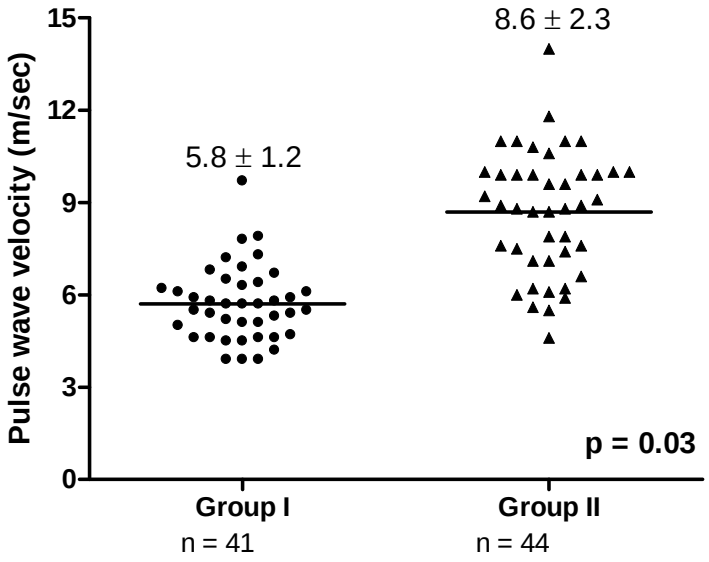
Comparison of carotid IMT levels between controls, Group I and Group II

Group I - mean time-integrated serum iPTH ≤ twice ULN
Group II - mean time-integrated serum iPTH > twice ULN

Pulse wave velocity

Aortic PWV also showed a positive correlation with PO_4 levels (r = 0.39, p = 0.03) and Ca x PO_4 product (r = 0.37, p = 0.018). Aortic PWV was greater in Group II than in Group I (8.63 ± 2.3 vs 5.81 ± 1.2 m/sec, p = 0.03, Figure 4.3), Table 4.2. However, the brachio-radial PWV did not correlate with any demographic or biochemical parameters.

Figure 4.3



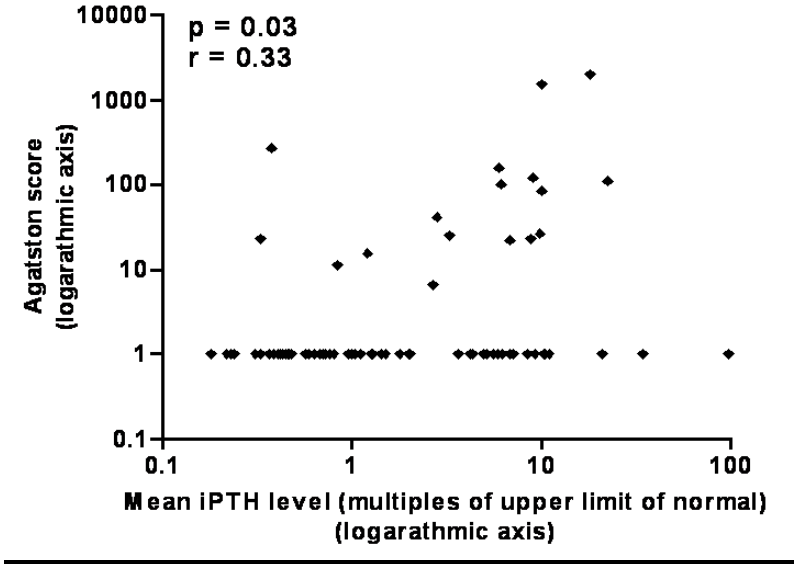
Comparison of aortic PWV levels between Group I and Group II

Cardiac calcification scores

The calcification score was associated with iPTH levels (r = 0.39, p = 0.03, Figure 4.4) and serum PO_4 (r = 0.34, p = 0.03), but did not correlate with age, duration of CKD or time on dialysis. Five (12%) patients in Group I and 12 (27%) in Group II (p = 0.004) had calcification (Table 4.2); relative risk = 2.3. Calcification was graded as minimal (Agatston score <10) in 4, mild (score 11 – 100) in 6, moderate (score 101 –

400) in 5 and severe (score > 400) in 2. Moderate and severe grades of calcification were only seen in Group II. There was no definite anatomical pattern of calcium deposition in the vessels or valves. Three of 17 (17%) patients under 10 years had calcification as compared to 14/68 (20%) above the age of 10.

Figure 4.4



Correlation of Agatston score for cardiac calcification with mean time-integrated intact PTH levels

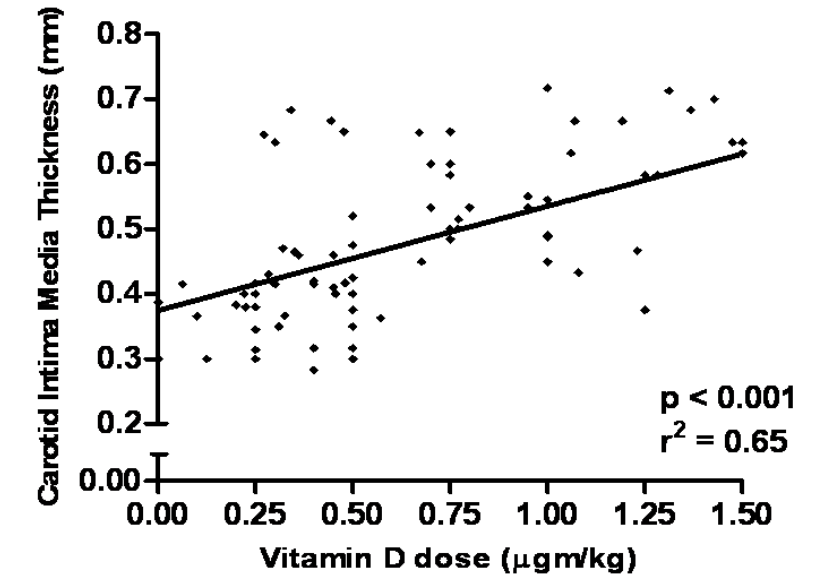
Effect of PO_4 binders and Vitamin D therapy on vascular measures

Eighteen (41%) patients in Group II received sevelamer ± calcium-based PO_4 binders compared to only 5 (12%) in Group I (Table 4.1). The group of patients on sevelamer were older (15 ± 4.1 years), had a longer dialysis vintage (3.0 ± 1.9 years) and were predominantly on haemodialysis (73%) as compared to the overall cohort. The mean elemental Ca intake from the prescribed dose of PO_4 binders was marginally greater in Group II than Group I (Table 4.1). Although the elemental Ca intake from PO_4 binders did not correlate with any of the vascular measures in the overall cohort, after

excluding patients who received sevelamer, the IMT did show a weak correlation with calcium containing PO_4 binder dose ($p = 0.054$, $r = 0.19$).

The Vitamin D (alphacalcidol) dose showed a strong dose-dependent correlation with cIMT ($r = 0.65$, $p < 0.001$), aortic PWV ($r = 0.17$, $p = 0.03$) and calcification score ($r = 0.28$, $p = 0.02$); Table 4.3, Figure 4.5. Patients with calcification ($n = 17$) received a 2.8 fold higher Vitamin D dose than those without calcification (21.9 ± 8.9 vs 53.7 ± 13.4 μgm/kg, $p = 0.0001$).

Figure 4.5

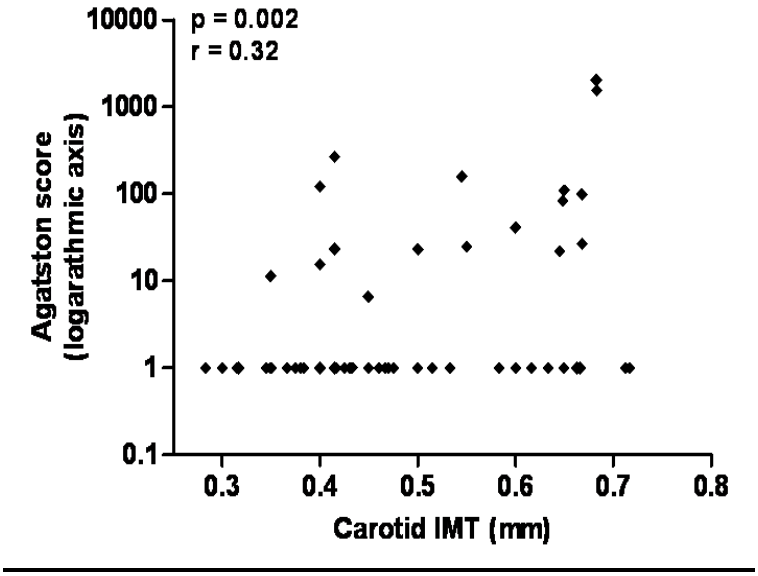


Correlation of carotid artery intima-media thickness (cIMT) with the mean alphacalcidol dosage

Correlations between vascular measures

cIMT was associated with aortic PWV (r = 0.41, p <0.0001) and the calcification score (r = 0.32, p = 0.002): patients with calcification (n = 17) had higher cIMT than those without calcification (0.55 ± 0.11 vs 0.45 ± 0.12mm, p = 0.004), Figure 4.6.

Figure 4.6



Correlation of Agatston score for cardiac calcification with cIMT

Predictors of cIMT, aortic PWV and calcification score

On multiple regression analysis, the Vitamin D dose was the strongest predictor of cIMT, aortic PWV and calcification, whereas iPTH levels were an independent predictor of cIMT and calcification (Table 4.3).

Table 4. 3 Multiple regression analysis for independent predictors of cIMT, aortic PWV and calcification score

Variable	Standardized coefficient (ß)	P	Model R2
cIMT			0.74
- Vitamin D dose	0.65	<0.001	
- iPTH level	0.72	<0.0001	
- PO_4 level	0.51	0.001	
Aortic PWV			0.23
- Vitamin D dose	0.17	0.03	
Cardiac Calcification (Agatston) score			0.54
- Vitamin D dose	0.28	0.02	
- iPTH level	0.53	<0.001	

4.5 Discussion

This is the largest paediatric study in the youngest cohort of CKD patients on dialysis that describes vascular changes and the impact of iPTH control and Vitamin D treatment on these. I have shown that both hyperparathyroidism and its management with Vitamin D impact on structural and functional vascular changes that begin as early as the first decade of life in children on dialysis.

Uraemia is a vasculopathic process. Children provide an ideal opportunity to study uraemic influences on the arterial wall as they rarely have risk factors such as diabetes, dyslipidaemia and hypertension that are prevalent in adults *(Goldsmith et al, 2004; London et al 2005)*. The patients in our study were carefully selected so that they were free of such confounders, and capitalizing on detailed serial biochemistry over ≥3½ years, we were able to demonstrate the impact of iPTH and its management

on the vascular phenotype. While PO_4 has been shown to be an independent risk factor for cardiovascular disease *(Block et al, 1998)*, including increased IMT *(Litwin et al, 2005; Mitsnefes et al 2005; Oh et al, 2002)* vessel stiffness *(Blacher et al; 2003; Covic et al, 2006)* and left ventricular hypertrophy *(Mitsnefes et al, 2005)*, PTH *per se* may contribute to vascular injury via mechanisms other than its effect on Ca – PO_4 homeostasis. It would be impossible to extricate the individual effects of PO_4, PTH and the medications used in their regulation. PTH may mediate vascular damage by playing a permissive role in arteriolar wall thickening and myocardial interstitial fibrosis *(Rostrand et al, 1999)*, increasing triglycerides and LDL cholesterol and contributing to chronic hypertension *(Massry et al, 1997)*; progression of these vascular changes is reduced after parathyroidectomy *(Massry et al, 1970)*. Thus, while it is now widely accepted practise to aim for PO_4 levels within the normal range, the optimal level for iPTH is as yet unclear. Results of this hypothesis-generating study will allow for a prospective randomised trial to evaluate the cardiovascular benefits of maintaining iPTH levels < 2ULN using the lowest possible dose of Vitamin D.

The long-term consequences of vascular damage are particularly important in children, who have a life-time of renal replacement therapy ahead of them. Studies in adults with CKD have shown that ~65% have coronary calcification at the start of dialysis *(Block et al, 2005)*, suggesting that prevention of secondary hyperparathyroidism is in fact key to the prevention of vascular damage and calcification. We and others have shown that endothelial dysfunction *(Kari et al, 1997)* and vascular damage *(Saygili et al, 2002; Litwin et al, 2005; Covic et al, 2006; Mitsnefes et al, 2005)* begins early in the course of CKD. The vascular damage is only partially reversible following transplantation *(de Lima et al, 2002, Litwin et al, 2008)*

and use of lipid-lowering agents *(Wanner et al, 2004)*, folate *(Bennett-Richards et al, 2002)* or arginine *(Bennett-Richards et al, 2002)* supplementation have little effect. Goodman and others have shown that once a nidus of calcification forms in the soft tissues, "calcium begets calcium" *(Goodman et al, 2000)* so that patients with pre-existing calcification are at greatest risk of accelerated calcification. Thus, the prevention of secondary hyperparathyroidism from the earliest stages of CKD is key to preventing the development and progression of vascular calcification.

In young adults with childhood onset CKD, Groothoff et al have reported a significant increase in arterial stiffness but normal IMT *(Groothoff et al, 2002)*, whereas a similar study by Oh et al has shown increased IMT and calcification in 92% of his cohort *(Oh et al, 2002)*. However, these studies in young adults can only support speculations of the potential changes in children on dialysis since it is possible that uraemia multiplies the natural age-related vascular damage. Evidence of vascular changes in children on dialysis has come from observational studies that have shown increased IMT damage *(Saygili et al, 2002; Litwin et al, 2005; Mitsnefes et al, 2005)*, stiffer vessels *(Covic et al, 2006)* and calcification *(Goodman et al, 2000; Civilibal et al, 2006; Eifinger et al, 2000)*, and linked these with PO_4 levels and Ca-PO_4 product. However, patients in these studies were older than in our cohort, often had comorbidity, and the small patient numbers and widely variable duration of CKD and time on dialysis may have resulted in confounders in their analyses.

The management of secondary hyperphosphataemia with use of calcium based PO_4 binders has been under considerable debate with concerns that the Ca intake from PO_4 binders results in calcium overload and ectopic soft tissue calcification *(Goodman et*

al, 2000; Litwin et al, 2005; Klaus et al, 2006; K/DOQI clinical practise guidelines, 2003; Goldsmith et al, 2004) . This study was not designed to determine the vascular effects of different PO_4 binders as sevelamer was used only as a second line agent in our patients with persistently high iPTH levels and hypercalcaemia. Goodman, Litwin and Briese *(Goodman et al, 2000; Litwin et al, 2005; Briese et al, 2006)* have shown a positive correlation between the cumulative PO_4 binder dose and coronary calcification or cIMT, and in our study IMT showed a weak correlation with PO_4 binder dose approaching statistical significance. However, adolescents and young adults are notoriously non-compliant with PO_4 binder medication, making it difficult to assume that the prescribed dose of PO_4 binder is indeed what the patient consistently receives.

In our study, the Vitamin D dose was the most important predictor of increased arterial thickness, stiffness and calcification. Although the prescribed dose of Vitamin D is also a surrogate marker of the severity of hyperparathyroidism, the Vitamin D dose predicted vascular damage independent of iPTH levels. The role of Vitamin D in the pathogenesis of vascular calcification has been shown in other observational studies *(Litwin et al, 2005; Mitsnefes et al, 2005)*, as well as in ex vivo *(Milliner et al, 1990)* and in vitro models *(Jono et al, 1998)*. While the primary role of Vitamin D is to increase the gastrointestinal absorption of calcium, it also significantly increases phosphate absorption. Moreover, Vitamin D acts on the vascular smooth muscle cells via the Vitamin D receptor and can induce proliferation and osteoblastic differentiation of these cells *(Jono et al, 1998)*.

We found a significant positive correlation between carotid IMT, aortic stiffness and presence of cardiac calcification, suggesting that the vascular damage is widespread,

involving both large muscular arteries like the carotids and elastic vessels like the aorta. Unlike aortic PWV, the brachio-radial PWV although increased, did not correlate with any demographic or biochemical parameter or with other vascular measures. It is known that vascular damage in the aorta begins earlier than in the brachio-radial vasculature *(Blacher et al, 2003)*, suggesting that the lack of correlations with brachio-radial changes may in fact be the result of dissociation in time rather than a difference in underlying pathology as discussed in one other study *(Tillin et al, 2007)*. A positive correlation between cIMT and calcification may imply that deposition of Ca- PO_4 crystals in the arterial media may be at least partly responsible for the increased cIMT. Carotid artery ultrasound, a cheap, easily available, highly reproducible and non-invasive test to measure cIMT may reliably substitute other methods for detection, monitoring and prognostication of vascular damage in dialysis patients.

On multiple regression analysis, the age at study or time spent in CKD or on dialysis did not show any significant correlation with the vascular measures. While increased vascular damage with age and dialysis vintage that has been reported *(Goodman et al, 2000; Litwin et al, 2005; Oh et al, 2002)*, this may in fact be the result of prolonged hyperparathyroidism and its consequences on the vasculature. Goodman et al showed calcification only in patients above 20 years age *(Goodman et al, 2000)*, but the group with calcification had significantly higher PO_4 and Ca x PO_4 products than those without calcification. Subsequent studies by Eifinger *(Eifinger et al, 2000)* and Civilibal *(Civilibal et al, 2006)* have documented coronary calcification in paediatric dialysis patients. In our cohort, age did not correlate with any vascular measure: the youngest patient with calcification was 5.8 years old.

Although we were unable to perform a randomised study, the groups were well matched for cardiovascular risk factors. The iPTH assay varied between different participating centres, and the inter-assay variability of different PTH assays must be recognised *(Souberbielle et al, 2006)*. Due to small patient numbers our study lacked the power to show any potential differences in vascular measures based on dialysis modality. Paediatric dialysis populations are currently limited due to a high transplantation rate; in the largest comparable study there were only 37 children on dialysis *(Litwin et al, 2005)*.

4.6 Conclusions

In conclusion, in this chapter I have shown that both the iPTH level and the Vitamin D dose are significant and independent predictors of vascular damage and calcification in children on dialysis. Maintaining the iPTH level at <2 ULN is associated with the lowest risk of structural and functional arterial wall damage and calcification. Future prospectively longitudinal studies, to simultaneously evaluate both the vascular benefits as well as the effect on bone histology, of keeping PTH levels at <2 ULN in a large cohort of children on dialysis are required in order to make evidence based recommendations for changes to the existing guidelines.

Chapter 5

The Effects of Vitamin D

on the Vasculature

5.1 Abstract

In addition to its classical role in calcium-phosphate homeostasis, vitamin D has anti-inflammatory effects that may influence vascular disease. I have studied the impact of vitamin D levels on the vascular phenotype in children on dialysis.

I studied 61 children (5-18 years) on dialysis for ≥3months and 40 age-matched controls. All patients received daily oral 1-α hydroxyvitaminD_3. The relation between 25-hydroxyvitaminD [25(OH)D], 1,25-dihydroxyvitaminD [1,25$(OH)_2$D], and high-sensitivity CRP [hs-CRP] levels and carotid intima-media thickness, pulse-wave velocity and coronary calcification on CT scan were examined.

92% of patients had 25(OH)D deficiency. 1,25$(OH)_2$D levels were low in 36% and high in 11%, and showed a weak dose – level concordance. Both cIMT and calcification scores showed a 'U-shaped' distribution for 1,25$(OH)_2$D: patients with both low and high 1,25$(OH)_2$D had greater cIMT ($p < 0.0001$) and calcification ($p = 0.0002$) than those with normal levels. Low 1,25$(OH)_2$D levels were associated with higher hs-CRP ($p < 0.0001$). Calcification was most frequently seen in patients with the lowest 1,25$(OH)_2$D and the highest hs-CRP. No correlation was seen between 25(OH)D levels and any vascular measure.

In conclusion, I have shown that both low and high 1,25$(OH)_2$D levels are associated with adverse morphological changes in large arteries, and that this may be mediated through the effects of 1,25$(OH)_2$D on Ca-PO_4 homeostasis and inflammation. Optimal vascular protective strategies in dialysis patients may therefore require careful monitoring of not only the vitamin D dose, but also 1,25$(OH)_2$D levels.

5.2 Introduction

Disorders in mineral metabolism associated with secondary hyperparathyroidism and vitamin D deficiency are the most important causes of vascular damage and calcification in CKD patients, and are also associated with abnormal bone turnover states: this is called Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD) *(Moe et al, 2006)*. Vitamin D deficiency is widely prevalent in CKD patients *(Levin et al, 2007)*, and thus, vitamin D analogues are routinely used, but both vitamin D deficiency and supplementation with vitamin D analogues have been implicated as potential risk factors in the development and progression of vascular disease *(Chertow et al, 2004; Goldsmith et al, 2004; Teng et al, 2003; Tentori et al, 2006)*. In the previous chapter, I have shown that higher doses of activated vitamin D compounds are associated with greater structural damage and calcification in children on dialysis *(Shroff et al, 2007)*.

In addition to its endocrine effects in regulating the Ca – PO_4 - PTH axis and bone turnover, vitamin D also has important autocrine / paracrine actions, especially on the cardiovascular system *(Zitterman A, 2006; Carthy et al, 1989; Xiang et al, 2005)* and also has anti-inflammatory and immunomodulatory effects *(Towler D, 2007; Tabata et al, 1988; Tokuda et al, 2000)* as discussed in chapter 2. Thus, although Vitamin D exerts potentially deleterious pro-calcific effects on the vasculature, its anti-inflammatory properties may confer a significant cardioprotective benefit. The effects of vitamin D on left ventricular function are well described *(Zitterman A, 2006)*, but there is little data on its role in the vasculature.

In this chapter, I have examined the hypothesise that $1,25(OH)_2D$ levels in the normal range are associated with less vascular damage and calcification in children on dialysis.

5.3 Methods

Patients and controls

From January 2005 to December 2006, I recruited 61 consecutive children (aged 5 – 18 years) who had been on dialysis for ≥ 3 months from the Great Ormond Street Hospital Renal Unit. Fourteen patients in this study also participated in the study described in chapter 4. All patients were prescribed daily oral 1-α hydroxycholecalciferol (alphacalcidol), titrating the dose so as to keep the iPTH level <2 upper limit of normal (ULN) as per our unit policy. Hyperphosphataemia was managed by dietary PO_4 restriction and calcium-based PO_4 binders. Sevelamer was only used in patients with hypercalcaemia and persistently high iPTH levels.

Patients were compared with healthy age- and gender-matched school children (Table 5.1) who underwent vascular scans in our department as part of a parallel study investigating normal levels of cIMT and PWV in healthy children. From this cohort, we included 40 consecutive children aged 5 to 18 years with no known medical illnesses or family history of heart disease or diabetes to serve as controls in our study, in a 1.5:1 patient:control ratio. Surprisingly, the majority of children in the control group were overweight, although no intentional selection bias was involved.

Informed written consent was obtained from all parents or caregivers, and children where appropriate. The study was approved by the Great Ormond Street Hospital research ethics committee.

Table 5.1. Demographic, clinical, anthropometric and biochemical characteristics of patients and controls

	Patients n = 61	**Controls n = 40**	**p**
Age (yr)	13.4 ± 4.1	14.4 ± 3.8	0.29
Gender (males / females)	37/24	22/18	0.19
Race (Caucasian/Asian/Black/Others)	37 / 12 / 9 / 3	27 / 11 / 2 / 0	-
Estimated GFR (ml/min/1.73m^2)	8.9 ± 8.0	121 ± 5.8	< 0.0001
Time in CKD Stage IV (yr)	median 4.9 (range 0.2 – 6.8)	-	-
Time on dialysis (yr)	median 1.1 (range 0.25 – 8.7)	-	-
Dialysis modality (PD / HD)	43 / 18	-	-
Body Mass Index SDS	-0.5 ± 1.6	0. 9 ± 0.9	< 0.0001
Systolic BP Index*	1.3 ± 0.3	0.9 ± 0.1	0.02
Number on anti-hypertensive medications	11	0	-
Number on ACEi or AIIRB	2	0	-
Haemoglobin (gm/dl)	11.7 ± 1.5	13.3 ± 1.1	0.07
Albumin (g/L)	38 ± 3.0	41 ± 0.6	0.22
Total Cholesterol (mMol/L)	4.0 ± 1.1	3.3 ± 1.3	0.10
Triglycerides (mMol/L)	1.2 ± 1.2	0.9 ± 2.2	0.67
Number on statins	2	0	-
Diabetes mellitus	0	0	-
Smokers	0	1	-

All values expressed as mean ± SD unless indicated
SDS – Standard Deviation Score
*BP Index = measured BP/95th centile BP for age, gender and height
ACEi – Angiotensin Converting Enzyme inhibitor, AIIRB – Angiotensin II Receptor Blocker, n/d – not done

Data collection

Serum Ca, PO_4 and iPTH levels and the doses of elemental calcium intake from phosphate binders and alphacalcidol were recorded at monthly intervals from the start of CKD Stage IV, and mean time-averaged values calculated: (the sum total of the monthly values for each variable was divided by the number of months of exposure to provide a mean time-averaged value). Given the variable amounts of time spent in CKD IV, the above biochemical values and medication dosages at the time of the study have been expressed separately in Table 5.2. The number of hypercalcaemic episodes (defined as albumin-adjusted serum Ca levels >2.5mMol/L, and expressed as a percentage of the total number of measurements in each patient) was calculated. In controls a single blood test at the time of the scans was performed.

Table 5.2 Serum Ca, PO_4, and PTH levels and doses of PO_4-binders and alphacalcidol in patients and controls

		Patients n = 61		Controls n = 40	p#
		Exposure from the onset of CKD Stage IV	Values at the time of study		
Serum PO_4 level	(mMol/L)	1.5 ± 0.7*	1.4 ± 0.5	0.9 ± 0.3	0.007
Serum Ca (albumin adjusted)	(mMol/L)	2.4 ± 0.1*	2.4 ± 0.0	2.3 ± 0.2	0.19
Serum Ca-PO_4 product	($mMol^2/L^2$)	4.2 ± 0.9*	4.3 ± 0.4	3.3 ± 0.3	0.002
Serum iPTH (fold ULN)		1.8 ± 1.3*	1.6 ± 0.9	n/d	-
PO_4 binders					
Number on Ca-based PO_4 binders		n/a	52 (88%)	-	-
Sevelamer +/- Ca-based PO_4 binders			9 (12%)	-	
Intake of elemental Ca from PO_4 binders					
	(gm/kg/year)	31.9 ± 11.8**	n/a	0	-
	Mean daily dosage (mg/day)	n/a	1828 ± 52	0	-
Alphacalcidol	(μ/kg/year)	19.2 ± 7.3**	n/a	0	-
	Mean daily dosage (μgm/day)	n/a	0.89 ± 0.3	0	-
Parathyroidectomy		0	0	0	-

All values are expressed as mean ± standard deviation
* All biochemical values in this column are expressed as mean time-averaged values from the onset of CKD Stage IV.
** The dosage of elemental calcium intake from PO_4 binders and the alphacalcidol dosage are expressed as the cumulative intake from CKD IV, and standardized per year of exposure to account for the varied duration since onset of CKD IV.
p# - Compares values at the time of the study between patients and controls
n/d – not done
n/a – not applicable

Vitamin D levels and hs-CRP

All biochemical data was determined on blood samples taken prior to the vascular scans and before a mid-week session of haemodialysis, but at varying times throughout the year. There was an interval of 10 – 12 hours between alphacalcidol intake and blood sampling. Serum 25(OH)D and $1,25(OH)_2D$ levels were measured by Mrs Michala Bridel, Senior Pathology Technician at the West Park Hospital, Epsom.

The enzyme immunoassay (IDS-OCTEIA 25-HydroxyvitaminD EIA kit, *Immunodiagnostics Systems Ltd*) and radioimmunoassay (1,25 dihydroxyvitaminD ^{125}I RIA kit, *DiaSorin*) were used for 25(OH)D and 1,25$(OH)_2$D respectively as per the methods described in Chapter 2, section 2.2. 1,25$(OH)_2$D levels in 120 healthy normal children have been measured and validated in our lab (unpublished data), and given cost constraints, we measured 25(OH)D levels only in the 40 controls.

hs-CRP levels were measured by Mrs Vanita Shah, Senior Technician in all patients and controls using the ELISA (EIA test kit, *MP Biomedicals*) as described in Chapter 2, section 2.2.

Vascular measures

The carotid intima media thickness, aortic and brachioradial pulse wave velocity, carotid augmentation index and coronary calcification on CT scan were measured as described in Chapter 2, section 2.1. For ethical reasons, CT scans were not performed in the controls.

Statistics

Results are presented as mean ± SD or median and range depending upon the distribution. Comparisons between groups were made using Student's t test or the Mann-Whitney U test as appropriate, and correlations tested using Pearson's or Spearman correlation tests for parametric and nonparametric data respectively.

The two main outcome variables were cIMT and calcification score (transformed to $\log_{10}(CS + 1)$ to adjust for skewness). Factors affecting the two outcome variables

were explored using multiple regression analysis. From univariate analyses variables with $p < 0.15$ were entered into the stepwise multiple regression analyses. The non-linear effect of vitamin D levels was fitted by including a quadratic term: the outcome variables were regressed on $1,25(OH)_2D$ and the square of $1,25(OH)_2D$, and the significance of the squared (quadratic) term noted. A third outcome measure, hs-CRP, was analysed after log transformation to account for skewness, and its correlation with $1,25(OH)_2D$ was strengthened when that too was log-transformed. The interaction between $1,25(OH)_2D$ and hs-CRP was tested by two way analysis of variance (ANOVA).

5.4 Results

Of the 61 patients (37 boys), 39 had renal dysplasia, 9 inherited nephropathies, 5 cystic kidney disease, 4 primary renal tubular disorders, 3 renovascular disorders and one Wilm's tumour. The clinical and biochemical characteristics of the patient and control groups are described in Tables 5.1 and 5.2.

Vitamin D levels in patients and controls

Levels of 25(OH)D were low in the majority of patients as well as controls. Despite all the patients being prescribed daily oral alphacalcidol, $1,25(OH)_2D$ levels were low (<40 pmol/L) in 22 (36%) and high (>150 pmol/L) in 11 (18%) patients. These are described in Table 5.3 below.

Table 5.3 Vitamin D levels in dialysis and control groups

		Dialysis N = 61	Controls n = 40	p
25(OH)D (ngm/ml)		13.3 ± 10.9	28.2 ± 9.9	<0.0001
	<10	30 (50%)	0	
	10 – 30	26 (42%)	29 (72%)	
	>30	5 (8%)	11 (27%)	
1,25(OH)$_2$D (pmol/L)		Median 10 (range 10 – 216)	n/d	
	<40	22 (36%)		
	40 – 150	28 (46%)		
	>150	11 (18%)		

25(OH)D - 25-hydroxy Vitamin D; 1,25(OH)$_2$D - 1,25-dihydroxy Vitamin D; n/d – not done.
To convert 25(OH)D levels to nmol/L multiply by 2.5.
All values expressed as mean ± sd.

1,25(OH)$_2$D levels showed a weak correlation with the alphacalcidol dose at the time of the study (p = 0.06, r = 0.25). No correlation was found between 25(OH)D and 1,25(OH)$_2$D levels (p = 0.4, r = 0.11).

Vitamin D levels and their clinical and biochemical correlations

Patients on peritoneal dialysis [PD] (n = 43, 70%) had significantly lower 1,25(OH)$_2$D levels than those on haemodialysis (HD) (46.7 ± 18.8 vs 68.2 ± 35.7 pmol/L, p = 0.02, r = 0.31); 53% of PD and 24% of HD patients had low 1,25(OH)$_2$D levels. Comparisons between clinical and biochemical measures between the dialysis patients and the control group is shown in Tables 5.1 and 5.2 above.

There was no correlation with serum albumin levels in the overall cohort, but PD patients with low 1,25(OH)$_2$D had lower serum albumin at the time of the study (35.2 ± 8.0 vs 41.1 ± 3.1, p = 0.04) than those with 1,25(OH)$_2$D in the normal or high range.

Unfortunately, we did not measure the urinary or peritoneal losses of vitamin D and D binding protein in this study, but these may have resulted in lower $1,25(OH)_2D$ levels in PD patients and also contributed to the poor concordance between alphacalcidol dosage and $1,25(OH)_2D$ levels.

$1,25(OH)_2D$ showed a linear correlation with both the Ca x PO_4 product at the time of the study ($p = 0.02$, $r = 0.28$) as well as the mean time-averaged Ca x PO_4 product ($p = 0.03$, $r = 0.22$). Patients with high $1,25(OH)_2D$ had more hypercalcaemic episodes (15% vs 6%, $p = 0.02$). As classically observed, the mean time-averaged iPTH level inversely correlated with $1,25(OH)_2D$ ($p = 0.02$, $r = 0.36$ for mean time-averaged iPTH vs $1,25(OH)_2D$ levels and $p = 0.003$, $r = 0.41$ for iPTH levels at the point of study vs $1,25(OH)_2D$ levels). No correlation was found between 25(OH)D or $1,25(OH)_2D$ levels and the serum Ca, PO_4 or alkaline phosphatase measured at any time point. Neither 25(OH)D nor $1,25(OH)_2D$ showed any correlation with the patients' age, gender, race, dialysis vintage, blood pressure or body mass index. There was no seasonal variation in the levels.

Vascular measures in dialysis and control groups

A comparison between vascular measures in the dialysis and control groups is shown in Table 5.4.

Table 5.4 Comparison of vascular measures between dialysis and control groups

	Dialysis n = 61	Controls n = 40	p
Carotid IMT [mm]	0.48 ± 0.17	0.38 ± 0.01	0.001
Aortic PWV [m/sec]	7.7 ± 1.0	n/d	-
Number with cardiac calcification	13 (21%)	n/d	
Agatston score	median 141.2 (range 0 – 2039)	-	-
Coronary arteries	12	-	
Valves	3	-	
Aorta	2	-	

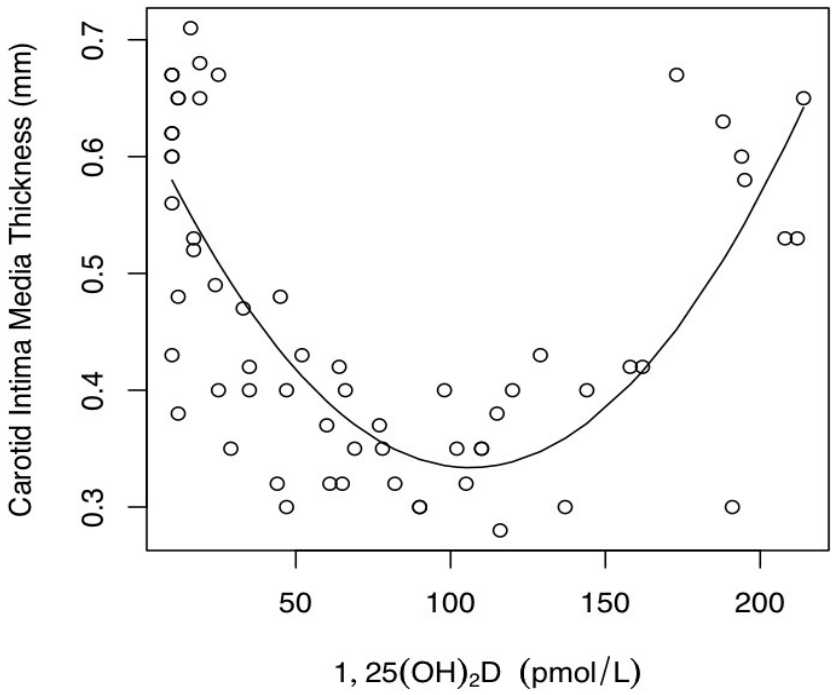
cIMT – carotid artery intima-media thickness; PWV – pulse wave velocity; n/d – not done.
All values expressed as mean ± sd unless indicated otherwise.

Vitamin D levels and correlations with vascular measures

Both low and high levels of $1,25(OH)_2D$ were associated with abnormal vascular measures: patients with $1,25(OH)_2D$ in the normal range had cIMT levels comparable to those in the controls, but those with $1,25(OH)_2D$ <40 or >150 pmol/L had significantly higher cIMT ($p < 0.0001$ for quadratic term, Figure 5.1). The cardiac calcification score showed a similar relationship to $1,25(OH)_2D$: calcification was seen in 8 of 22 (36%) patients with $1,25(OH)_2D$ levels <40 pmol/L, 5 of 11 (45%) patients with levels >150 pmol/L, and only 1 of 28 (3.6%) patient with levels in the normal range ($p = 0.0002$ for quadratic term, Figure 5.2).

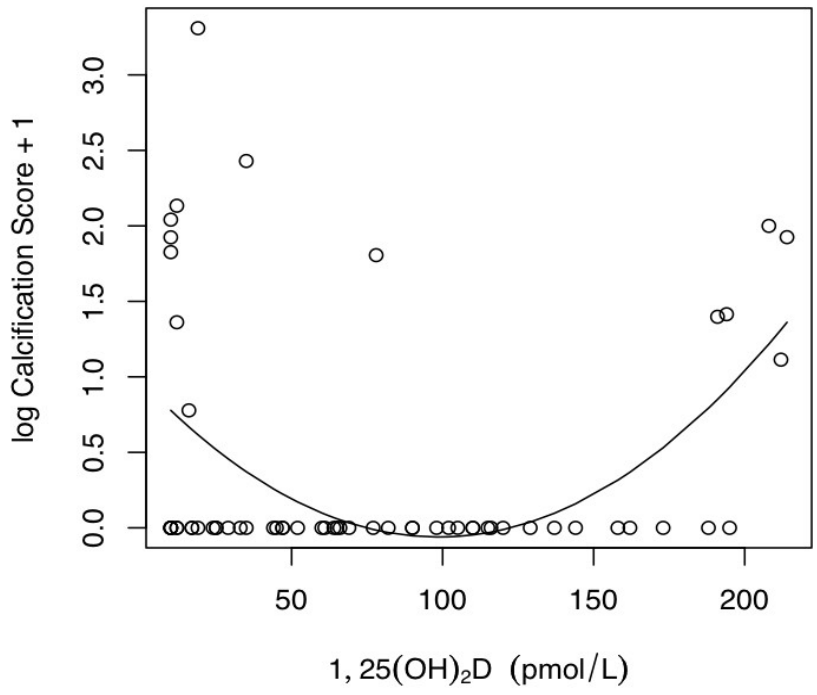
No association was found between $1,25(OH)_2D$ and PWV or between 25(OH)D and any of the vascular measures.

Figure 5.1



The bimodal relationship between cIMT and $1,25(OH)_2D$ levels ($p < 0.0001$). These non-linear effect of vitamin D levels was fitted by including a quadratic term: the outcome variable (cIMT) were regressed on $1,25(OH)_2D$ and the square of $1,25(OH)_2D$, and the significance of the squared (quadratic) term noted.

Figure 5.2

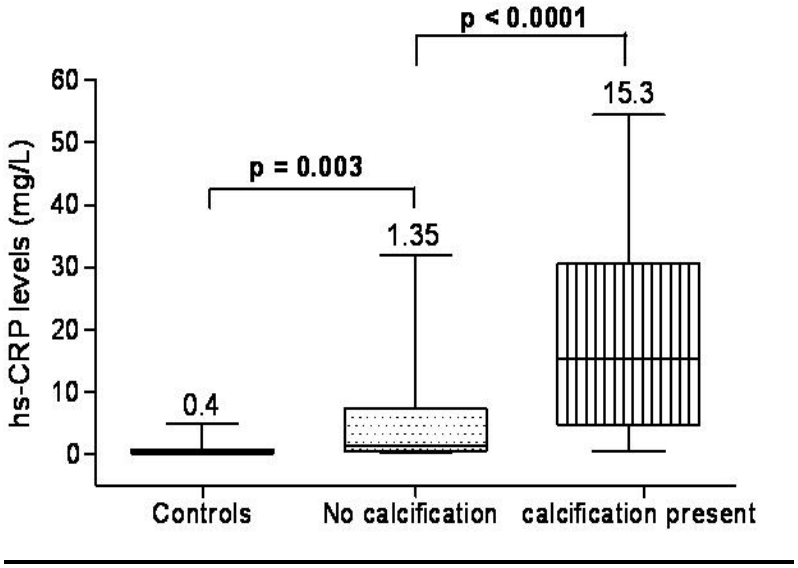


The cardiac calcification score showed a similar significant quadratic (bimodal) relationship ($p = 0.0002$). The calcification score was transformed to $\log_{10}(CS + 1)$ to adjust for skewness and the quadratic association calculated as described for cIMT above.

Vitamin D levels and correlations with inflammation

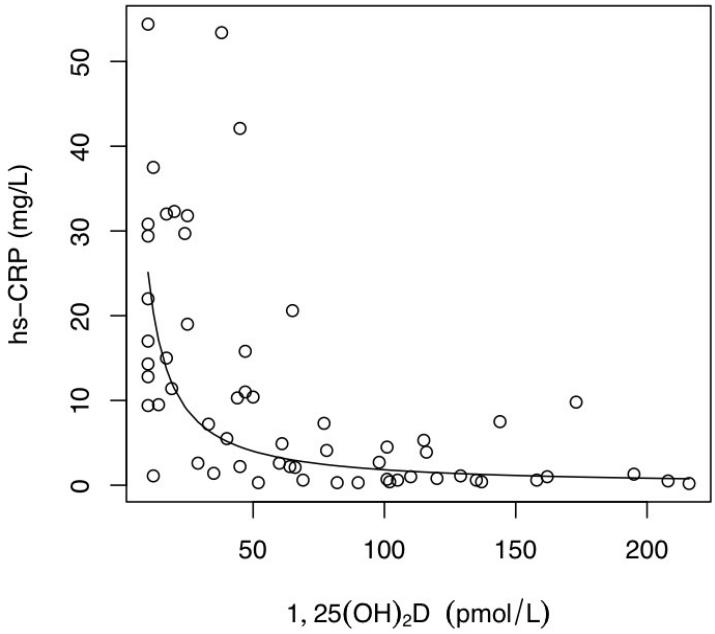
The hs-CRP was higher in patients than controls (median 9.3 (range 1.2 – 53) vs 0.43 (0.1 – 5.02) mg/L, p<0.00001, r = 0.73), and higher in HD than PD patients (17.9 (1.7 – 54.4) vs 3.2 (0.3 – 23) mg/L, p = 0.01, r = 0.52). None of the patients had active infections at the time of the study. The hs-CRP was significantly higher in patients with calcification (15.3 (0.5 – 54.4) vs 1.35 (0.3 – 32) mg/L, p < 0.0001, r = 0.25, Figure 5.3) and was independent of age, dialysis vintage, BP, body mass index, lipid profile, cIMT or PWV. An inverse correlation was seen between $1,25(OH)_2D$ and hs-CRP (p<0.0001, r = -0.29, Figure 5.4). Both the prevalence of calcification as well as the highest calcification scores were found in patients with a combination of low $1,25(OH)_2D$ levels and high hs-CRP, whereas patients with $1,25(OH)_2D$ levels in the normal range had the lowest incidence of calcification (Figure 5.5). There was no interaction between $1,25(OH)_2D$ and hs-CRP (p = 0.11). There was no association between 25(OH)D and hs-CRP.

Figure 5.3



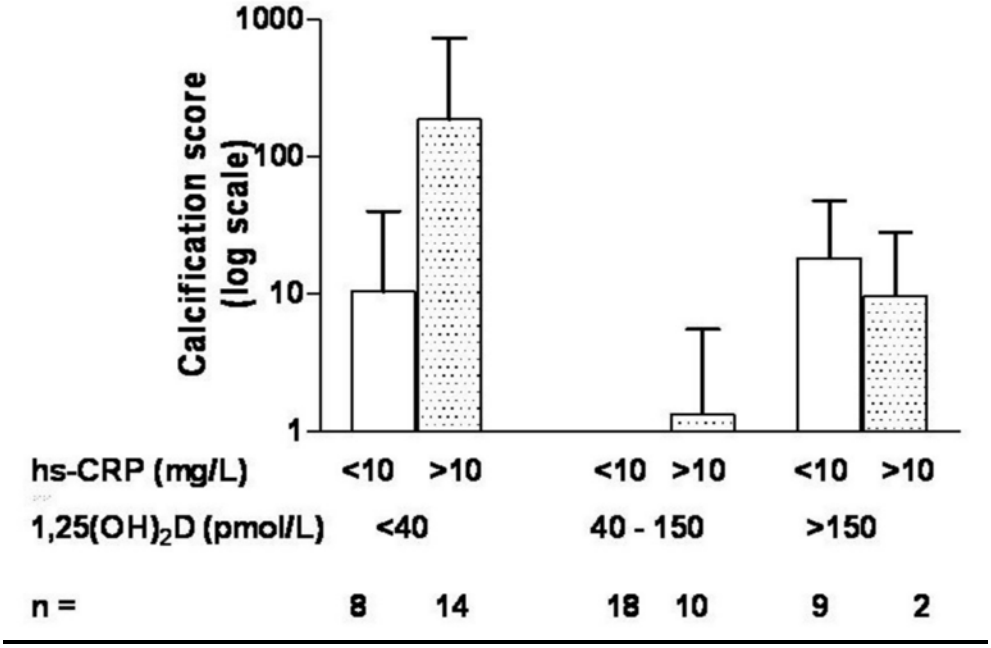
The hs-CRP was higher in patients than controls (p<0.00001), and significantly higher in patients with calcification compared to patients without calcification on CT scan (p<0.0001).

Figure 5.4



There was an inverse correlation between $1,25(OH)_2D$ and hs-CRP levels ($p<0.0001$, $r = -0.29$). The regression line was fitted on the log-log scale and is shown back-transformed.

Figure 5.5



When patients were divided into groups based on both their $1,25(OH)_2D$ levels and hs-CRP, the prevalence of calcification as well as the highest calcification scores were found in patients with a low $1,25(OH)_2D$ levels and high hs-CRP, whereas patients with $1,25(OH)_2D$ levels in the normal range had the lowest incidence of calcification.

Predictors of cIMT and calcification

In addition to the strong quadratic relationship between cIMT and $1,25(OH)_2D$ and between the calcification score and $1,25(OH)_2D$, we found univariate associations between cIMT and the mean time-averaged Ca x PO_4 product ($r = 0.38$, $p < 0.001$), calcification score and the mean time-averaged Ca x PO_4 product ($r = 0.27$, $p = 0.02$) and calcification score and intact PTH levels ($r = 0.18$, $p = 0.04$). Significant predictors of the outcome variables, cIMT and calcification score, from univariate analyses were entered into stepwise multiple linear regression analyses (Table 5.5). $1,25(OH)_2D$ was an independent predictor of cIMT whereas both $1,25(OH)_2D$ and hs-CRP were significant predictors of the calcification score.

Table 5.5 Multivariate analysis for predictors of cIMT and calcification score

Variables	ß	SE	p	Model R^2
cIMT				57%
$1,25(OH)_2D$ level	-2.49	0.001	<0.0001	
Square of $1,25(OH)_2D$ level (quadratic co-efficient)		<0.001	<0.0001	
Mean time-integrated $CaxPO_4$	0.32	0.09	0.073	
Calcification score *				16%
$1,25(OH)_2D$ level	-9.1	4.2	0.04	
Square of $1,25(OH)_2D$ level (quadratic co-efficient)	0.04	0.02	0.04	
hs-CRP	11.6	4.7	0.02	
Mean time-integrated $CaxPO_4$	10.2	1.7	0.16	
Intact PTH levels	5.2	2.6	0.48	

ß – (unstandardized) regression coefficient (indicates the difference in the outcome variable [cIMT or calcification score] per unit change in the independent variables.

SE – standard error

Model R^2 – indicates the amount of variance in the dependent variable that can be explained by the model.

* Analysed as $\log_{10}$(Calcification score + 1)

5.5 Discussion

In this study I have shown, for the first time, that both low and high levels of $1,25(OH)_2D$ are associated with abnormal vascular structure and calcification, possibly through a dual effect on Ca-PO_4 homeostasis and inflammation. A significant number of children on dialysis have low levels of $1,25(OH)_2D$ despite daily alphacalcidol supplements, and given the narrow therapeutic window for vitamin D analogues on vascular health, careful monitoring of $1,25(OH)_2D$ levels is recommended in the dialysis population.

92% of our patients had 25(OH)D deficiency, and studies have shown that the majority of CKD patients have low 25(OH)D levels *(Zittermann A, 2006; Teng et al, 2005)*. However, unlike other studies where the patients were naïve to vitamin D sterols, all of our patients were prescribed daily alphacalcidol. The 'nutritional' form, 25(OH)D, is unaffected by alphacalcidol treatment, and so we also measured $1,25(OH)_2D$, the 'hormonal' or 'active' form of Vitamin D produced by 25-hydroxylation of alphacalcidol in the liver *(Dusso et al, 2005; Feldman et al, 2005)*, and found that 36% of the children had low $1,25(OH)_2D$. Reduced levels of $1,25(OH)_2D$ despite treatment may be due to a loss of albumin-bound Vitamin D in the peritoneum as suspected in our patients, or non-concordance with treatment. None of our patients had malabsorption syndromes or liver disorders to suggest poor absorption or reduced enzymatic conversion of alphacalcidol. Despite the strong inverse correlation between inflammation and vitamin D, and the higher inflammatory status of HD patients, interestingly we found lower vitamin D levels in the PD cohort, suggesting that a loss of albumin-bound vitamin D did indeed play a major role. In the previous chapter I showed that in children on dialysis the dose of alphacalcidol

influences cIMT and calcification *(Shroff et al, 2007)*, but the lack of a consistent dose-response relationship as shown in this study, suggests that adjustments in the alphacalcidol dose alone may not be sufficient for optimal management.

The effects of high vitamin D doses on vascular calcification are well recognised in clinical studies *(Shroff et al, 2007; Civilibal et al, 2006; Litwin et al, 2005)* and in vitro work *(Carthy et al, 1989; Inoue et al, 1988; Jono et al, 1998)* and this study confirms an increased cIMT and calcification in patients with high $1,25(OH)_2D$ levels. Vitamin D analogues may induce calcification by a number of mechanisms that include enhancing the gastrointestinal absorption of Ca and PO_4, over-suppression of PTH leading to adynamic bone disease *(Brown et al, 2002; Dusso et al 2005)* and a direct effect on VSMCs *(Carthy et al, 1989; Jono et al, 1998)*. An early study by Milliner showed that, at autopsy, 60% of children with CKD had soft-tissue calcification and 36% had systemic calcinosis, and use of any vitamin D analogue showed the strongest independent association with calcinosis *(Milliner et al, 1990)*. In addition, both 25(OH)D and $1,25(OH)_2D$ can have a direct effect on the VSMC: $1,25(OH)_2D$ upregulates the vitamin D receptor and induces cellular calcium uptake *(Inoue et al, 1988)*, decreases VSMC proliferation *(Carthy et al, 1989)*, induces VSMC migration and osteoblastic conversion of the VSMCs *(Jono et al, 1997; Jono et al, 1998)*. Recent observational studies by Litwin *(Litwin et al, 2005)*, Civilibal *(Civilibal et al, 2007)*, Mitsnefes *(Mitsnefes et al, 2005)* and my previous work *(Shroff et al, 2007)* have shown that a high vitamin D dose adversely affects cIMT and calcification. Newer vitamin D analogues, such as paricalcitriol and doxercalciferol, are shown to be less calcaemic *(Brown et al, 2003; Sprague et al, 2003)* but have only

a marginal survival advantage over calcitriol *(Teng et al, 2003; Tentori et al, 2006)*, and their effects on the vasculature have not as yet been studied.

Despite the clearly deleterious calciotropic effects of vitamin D, in this study, low levels of $1,25(OH)_2D$ were also associated with increased cIMT and calcification. Low $1,25(OH)_2D$ results in an increase in PTH levels that can promote soft-tissue calcification through its effect on calcium absorption and an efflux of Ca and PO_4 from a high turn-over bone state *(Brown et al, 2003; Dusso et al, 2005)*. Our findings are supported by 2 recent studies in adult HD patients that have demonstrated a ~20% survival advantage of any vitamin D formulation over no vitamin D treatment *(Teng et al, 2003; Tentori et al, 2006)*. In a small cohort of Japanese HD patients, Shoji et al showed that the use of 1-α hydroxyvitamin D_3 was associated with a 28% lower risk of death from cardiovascular disease as compared to a group not on any vitamin D supplements *(Shoji et al, 2004)*. London recently showed that in a cohort of adult dialysis patients who were all naïve to Vitamin D analogues, low 25(OH)D and $1,25(OH)_2D$ were associated with greater vessel stiffness and reduced brachial artery distensibility *(London et al, 2007)*. In my earlier study (chapter 4) I have shown that children on dialysis who have PTH levels >2-fold ULN have higher cIMT, PWV and calcification than those with PTH levels <2-fold ULN *(Shroff et al, 2007)*. However, as seen in this study, $1,25(OH)_2D$ levels are associated with cIMT and calcification independent of PTH, suggesting that the biological consequences of $1,25(OH)_2D$ extend beyond the regulation of Ca-PO_4 homeostasis alone.

Recently described anti-inflammatory actions of vitamin D *(Mathieu et al, 2002)* may also contribute to its effects on the vasculature but have not been explored in clinical

studies. Interestingly, we found a strong inverse correlation between hs-CRP and $1,25(OH)_2D$ levels that was associated with vascular calcification, but no association between 25(OH)D and hs-CRP. 1-alpha hydroxylase is expressed on many cell types such as macrophages, endothelial cells and dendritic cells where it acts in an autocine / paracrine manner independently of the PTH-bone axis and is unaffected by renal failure, but may be regulated by immune stimuli. In vitro studies as well as studies in other inflammatory disease states such as rheumatoid arthritis *(Patel et al, 2007)* have shown that vitamin D can influence various aspects of inflammation *(Levin et al, 2005)* including inhibition of antigen-presenting cell maturation, downregulation of nuclear factor-κß, and modulation of cytokine production to create an anti-inflammatory environment (increased IL-10 and decreased IL-6, IL-12 and TNF-α) *(Mathieu et al, 2002; Schleithoff et al, 2006)*, but this is the first clinical study that has found an association between $1,25(OH)_2D$ levels and inflammation. However, it is also possible that inflammation leads to low vitamin D levels. In a cross-sectional study it would be impossible to discern a cause – effect relationship between inflammation-malnutrition and vitamin D levels, but given the results of in vitro studies that have shown a causal effect of vitamin D on inflammation, it is likely that low vitamin D levels contribute to the pro-inflammatory milieu in the dialysis patients. Studies in non-renal failure patients have shown that vitamin D supplementation can suppress serum TNF-α and increase IL-10 levels *(Muller et al, 1992)*: TNF-α promotes atherosclerosis and IL-10 has anti-atherogenic properties. Vitamin D also has direct cardioprotective effects such as an anti-proliferative effect on cardiomyocytes *(Xiang et al, 2005)* and negative endocrine regulation of the renin-angiotensin system *(Li et al, 2002)*.

In this cross-sectional study an association between vitamin D levels and vascular phenotype does not necessarily indicate a cause – effect relationship. However, this study serves as a starting point to stimulate cell-biology work and generate hypotheses for randomised controlled studies of the effects of vitamin D analogues on cardiovascular health. We were unable to find a correlation between hs-CRP levels and cIMT, suggesting that either the study population is too small to demonstrate an effect, or that inflammation has a greater influence on calcification than on the mechanisms involved in vessel thickening. Unlike the study by London et al, we did not find a correlation between vitamin D levels and vessel stiffness *(London et al, 2007)*. The greater plasticity of children's vessels and their shorter dialysis vintage may allow for compensatory mechanisms that can maintain normal vessel function in the face of early structural damage to the vessel. Vessels from children provide an ideal model to study uraemic influences on the arterial wall, as they do not have the confounding pro-atherosclerotic risk factors that are prevalent in the adult CKD population.

5.5 Conclusions

In conclusion, I have shown, for the first time, that both low and high levels of $1,25(OH)_2D$ are associated with adverse morphological changes in the large arteries, and that the vascular damage may be determined by the effects of Vitamin D on Ca-PO_4 homeostasis and inflammation. Given the narrow therapeutic window for vitamin D analogues on vascular health, optimal vascular protective strategies in dialysis patients may require careful monitoring of not only the vitamin D dose, but also $1,25(OH)_2D$ levels.

Chapter 6

Role of Calcification Inhibitors in Children on Dialysis

6.1 Abstract

Vascular calcification occurs in the majority of patients with chronic kidney disease, but a subset of patients do not develop calcification despite exposure to a similar uraemic environment. Physiological inhibitors of calcification, fetuin-A, osteoprotegerin (OPG), and undercarboxylated-matrix Gla protein (uc-MGP) may play a role in preventing the development and progression of ectopic calcification, but there are scarce and conflicting data from clinical studies. In this chapter, I measured fetuin-A, OPG, and uc-MGP in 61 children on dialysis, and studied their relationships with clinical, biochemical and vascular measures.

Fetuin-A and OPG were higher and uc-MGP lower in dialysis patients than controls. In controls, fetuin-A and OPG increased with age. Fetuin-A showed an inverse correlation with dialysis vintage ($p = 0.0013$), time-averaged serum phosphate ($p = 0.03$), and hs-CRP ($p = 0.001$). Aortic pulse wave velocity and augmentation index showed a negative correlation with fetuin-A, while a positive correlation was seen with pulse wave velocity and OPG. Patients with calcification had lower fetuin-A and higher OPG than those without calcification. On multiple linear regression analysis Fetuin-A independently predicted aortic PWV ($p = 0.004$, ß = -0.45, model $R^2 = 48\%$) and fetuin-A and OPG predicted cardiac calcification ($p = 0.02$, ß = -0.29 and $p = 0.014$, ß = 0.33 respectively, model $R^2 = 32\%$).

In this study, I have defined normal levels of the calcification inhibitors in healthy children, and shown, for the first time, that fetuin-A and OPG are associated with increased vascular stiffness and calcification in children on dialysis. Higher levels of

fetuin-A in children suggests a possible protective upregulation of in the early stages of exposure to the pro-calcific and pro-inflammatory uraemic environment.

6.2 Introduction

In CKD pro-calcific stimuli such as increased Ca, PO_4 and iPTH levels *(Goodman et al, 2000; Litwin et al, 2005; Shroff et al, 2007)*, and, potentially, treatment with Ca-based PO_4 binders and vitamin D can promote vascular and soft-tissue calcification *(Goodman et al, 2000; Block et al, 2005; Shroff et al, 2007; Shroff et al, 2008)*. Yet, some patients with CKD do not develop calcification despite exposure to the same uraemic milieu *(Block et al, 2005)*. *In vitro* studies and animal experiments have shown that ectopic calcification is a highly regulated, cell-mediated process that involves a balance between inducers and inhibitors of calcification *(Shanahan C, 2007; Moe et al, 2005)*.

Circulating fetuin-A, OPG and MGP levels have been linked with cardiovascular mortality in adults with CKD *(Ketteler et al, 2003; Kiechl et al, 2004; O'Donnell et al, 2006)*, but there is a complex and poorly understood relationship between these physiological calcification inhibitors at different stages of CKD and conflicting data on their impact on vascular measures. Moreover, circulating inhibitors of calcification have not been studied in children with CKD, nor have their normal levels been defined in the healthy childhood population.

In this chapter I have describe serum fetuin-A, OPG and uc-MGP levels in a cohort of healthy children and dialysis patients, and examined the hypothesis that normal levels

of the circulating calcification inhibitors will protect against vessel stiffness and calcification in children on dialysis.

6.3 Methods

Patients and controls

From January 2005 to December 2006, 61 consecutive children (5 – 18 years) who had been on dialysis for ≥ 3 months were recruited. This is the same cohort of patients who participated in the vitamin D study described in Chapter 5.

As the levels of calcification inhibitors have not been described in the healthy childhood population, levels of fetuin-A, OPG and uc-MGP were first defined in an age- and gendre-matched control group before comparisons with the dialysis cohort could be made. 75 healthy children participated in the study: 55 children formed part of a larger study investigating nutritional parameters in healthy children and 20 underwent routine corrective surgery for external auricular malformations or squints at Great Ormond Street Hospital. Controls were confirmed to have no known medical illnesses, family history of heart disease, or active infections at the time of the study.

Informed written consent was obtained from all parents or caregivers, and children where appropriate. The study was approved by the Great Ormond Street Hospital research ethics committee.

Biochemical measures

Blood samples were taken before a mid-week session of haemodialysis or immediately after a peritoneal dialysis session. Serum Ca, PO_4 and iPTH levels and

the dosage of elemental calcium intake from phosphate binders and vitamin D therapy were recorded at monthly intervals from the start of CKD stage IV and expressed as mean time-integrated values. In controls, a single blood test at the time of the scans was performed.

Fetuin-A, OPG and uc-MGP levels were measured as described in detail in Chapter 2, section 2.2. High sensitivity CRP levels that were measured as part of the vitamin D study described in Chapter 5 were also used for this study.

Vascular measures

The carotid intima media thickness, aortic and brachioradial pulse wave velocity, carotid augmentation index and coronary calcification on CT scan were measured as described in Chapter 2, section 2.1.

As vascular scans could only be obtained in 18 of the healthy children, the vascular measures in our dialysis cohort were compared with 40 age-matched controls who participated in the PTH and vascular outcome study described in Chapter 4. For ethical reasons, CT scans were not performed in the controls.

Statistical analyses

Results are presented as mean ± SD unless otherwise indicated. Spearman's (non-parametric) correlations were used to test for associations between the calcification inhibitor levels and selected clinical, anthropometric, biochemical and vascular measures. Comparisons between patient and control groups were made using one-way analysis of variance (ANOVA).

Two separate stepwise multiple linear regression analyses were performed to test the associations between the calcification inhibitors against the outcome variables PWV

and calcification score. Variables with $p < 0.1$ on univariate analyses (age, time in CKD stage IV, dialysis vintage, mean time-integrated Ca x PO_4 product PTH and serum albumin and dosage of alphacalcidol) were entered into the multiple regression models. Given the interaction between serum PO_4 and Ca x PO_4 ($p = 0.02$), I have excluded serum PO_4 from the regression models, despite its significant association with fetuin-A levels on univariate analysis so as to avoid collinearity. All the 3 calcification inhibitors under study (fetuin-A, OPG and uc-MGP) were entered into both the regression models, even though uc-MGP did not show any significant associations with vascular measures on univariate analyses. Also, given the known fluctuations in biochemical measures and calcification inhibitor levels following a single haemodialysis session, and to address the issue of pooling all dialysis (HD and PD) patients for analyses, the dialysis modality was entered into both regression models despite non-significance on univariate analyses.

6.4 Results

The clinical and biochemical characteristics of the patient and control groups are shown in Table 6.1. Of the 61 patients (37 boys), 39 had renal dysplasia, 9 inherited nephropathies, 5 cystic kidney disease, 4 primary renal tubular disorders, 3 renovascular disorders and one Wilm's tumour. None of the patients had diabetes, dyslipidaemias, or active infections at the time of the study, and none were on warfarin therapy.

Levels of Fetuin-A, OPG and uc-MGP in healthy children

The mean fetuin-A levels in the healthy controls were 0.41 ± 0.13 g/L, and lower than that reported in adults (0.5 – 1.0 g/L). Fetuin-A showed a linear increase with age ($p < 0.0001$, $r^2 = 0.55$, Figure 6.1A), but was independent of gender. In children aged 12 to 18 years, fetuin-A levels were lower in those ≥ 50^{th} percentile for age-appropriate height as compared to children below this percentile (0.45 ± 0.1 vs 0.6 ± 0.2 g/L, $p = 0.03$). In this cohort with a normal biochemical profile and no evidence of inflammation, fetuin-A did not show any associations with serum Ca, PO_4 or PTH levels, fasting glucose, triglyceride or hs-CRP.

The mean OPG levels in healthy children were 5.2 ± 1.2 pmol/L. OPG levels also showed a linear increase with age ($p = 0.004$, $r^2 = 0.34$, Figure 6.1B), but no correlation was found between OPG and any anthropometric, biochemical or vascular measure.

The mean uc-MGP levels in the healthy controls were 527 ± 185μM, and were independent of age and unrelated to any other measured parameters.

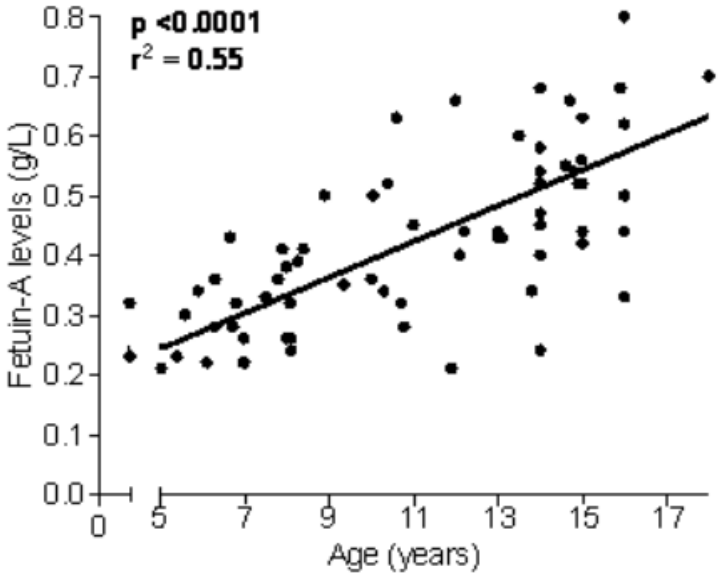
In the 18 controls who underwent vascular scans, no associations were seen with calcification inhibitor levels.

Table 6.1 Demographic, clinical, anthropometric and biochemical characteristics of patients and controls

	Patients n = 61	Controls n = 75	p
Age (yr)	13.4 ± 4.1	12.4 ± 4.1	0.33
Gender (males / females)	37/24	42/33	0.19
Race (Caucasian/Asian/Black/Others)	37 / 12 / 9 / 3	39 / 19 / 12 / 5	-
Estimated GFR (ml/min/1.73m^2)	8.9 ± 8.0	118 ± 3.4	< 0.0001
Time in CKD Stage IV (yr)	4.0 ± 2.2	-	-
Time on dialysis (yr)	0.9 ± 1.9 (median 1.1 [0.25 – 8.7)	-	-
Dialysis modality (PD / HD)	43 / 18	-	-
Body mass index SDS	-0.5 ± 1.6	0. 9 ± 0.6	< 0.0001
Systolic BP index*	1.3 ± 0.3	0.9 ± 0.3	0.03
Patients on anti-hypertensive medications	11	0	-
Patients on ACEi or AIIRB	2	0	-
Haemoglobin (g/dl)	11.7 ± 1.5	13.7 ± 2.1	0.08
Albumin (g/L)	38 ± 3.0	41 ± 0.6	0.22
Total cholesterol (mmol/L)	4.0 ± 1.1	3.4 ± 1.0	0.16
Triglycerides (mmol/L)	1.2 ± 1.2	0.8 ± 1.7	0.74
Patients on statins	2	0	-
Diabetes mellitus	0	0	-
Smokers	0	0	-
Serum PO_4 level (mmol/L) [#]	1.5 ± 0.7	0.9 ± 0.4	0.005
Serum Ca (albumin adjusted) (mmol/L) [#]	2.4 ± 0.1	2.4 ± 0.4	0.22
Ca-PO_4 product (mmol^2/L^2) [#]	4.2 ± 0.9	3.7 ± 0.2	0.001
Serum iPTH[#] - pmol/L[##]	10.8 ± 2.9	n/d	-
- fold ULN	1.8 ± 1.3	n/d	-
Parathyroidectomy	0	0	-
PO_4 binders			
Number on Ca-based PO_4 binders	52 (88%)	-	-
Sevelamer +/- Ca-based PO_4 binders	9 (12%)	-	
Cumulative intake of elemental Ca from PO_4 binders gm/kg[#]	124 ± 81	-	-
Alphacalcidol (1-α hydroxy Vit D_3) μ/kg	33.1 ± 20.3	-	-

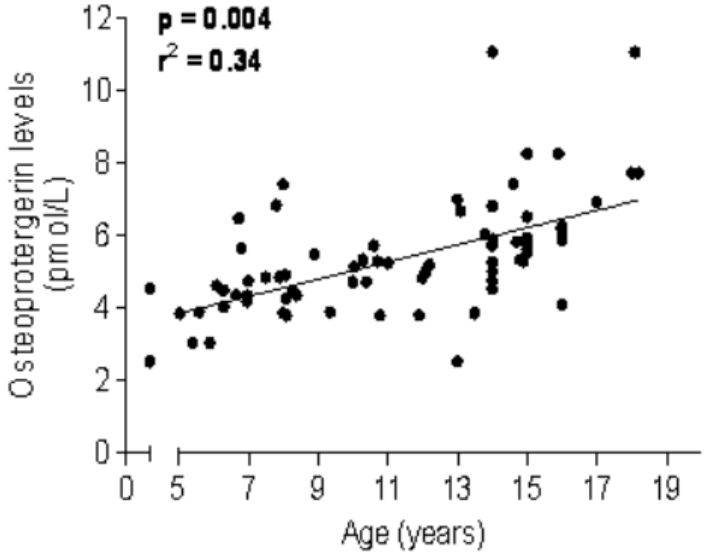
All values expressed as mean ± SD. *BP index = measured BP/95th centile BP for age, sex, and height [##] For PTH values in pg/mL multiply by 10.5 [#]Expressed as mean time-integrated values from the onset of CKD stage IV

Figure 6.1A



Circulating serum fetuin-A levels vs age in healthy controls

Figure 6.1B

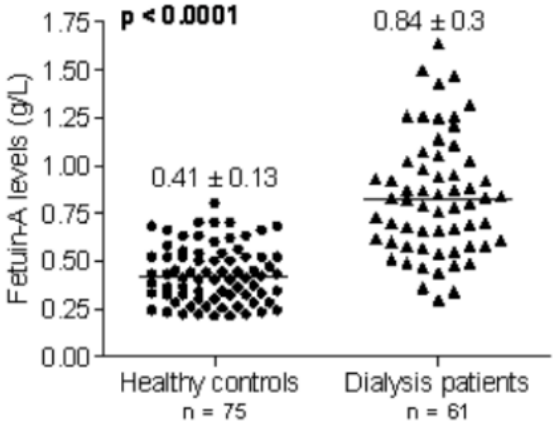


Circulating osteoprotegerin (OPG) levels vs age in healthy controls

Fetuin-A levels in dialysis patients

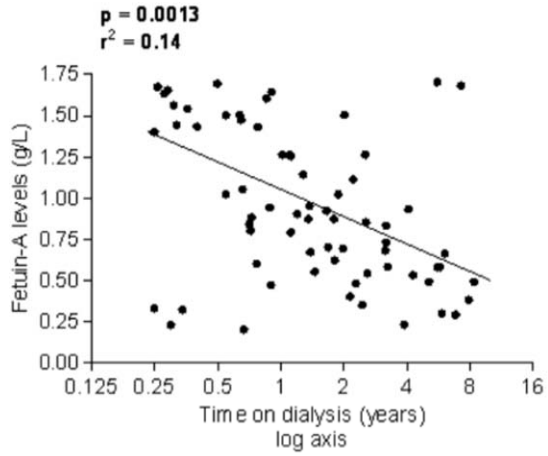
Unlike the levels seen in adult CKD patients, fetuin-A levels in children on dialysis were significantly higher as compared to controls (0.84 ± 0.3 vs 0.41 ± 0.13 g/L, $p < 0.0001$, Table 6.2 and Figure 6.2A). The correlation between age and fetuin-A in healthy controls was not seen in the dialysis population ($p = 0.33$). Fetuin-A showed an inverse correlation with time on dialysis ($p = 0.0013$, $r^2 = 0.14$, Figure 6.2B). Fetuin-A was lower in haemodialysis [HD] (n = 18) as compared with peritoneal dialysis [PD] patients (0.69 ± 0.4 vs 1.11 ± 0.2 g/L, $p = 0.03$), but this significance was lost after correction for the time on dialysis.

Figure 6.2A



Serum fetuin-A levels in children on dialysis and healthy controls

Figure 6.2 B



Serum fetuin-A levels vs time on dialysis

Table 6.2 Associations between the calcification inhibitors and clinical, anthropometric, biochemical and vascular measures*

Variables	Circulation calcification inhibitor levels		
	Fetuin-A	**Osteoprotegerin**	**Undercarboxylated Matrix Gla-protein**
Clinical measures			
Age	$p = 0.33$	$p = 0.07$	$p = 0.22$
Gender	$p = 0.64$	$p = 0.88$	$p = 0.66$
Time in CKD stage IV	$p = 0.09$	$p = 0.14$	$p = 0.37$
Time on dialysis	$p = 0.0013$, $r^2 = 0.14$	$p = 0.19$	$p = 0.36$
Anthropometric measures			
Height	$p = 0.28$	$p = 0.08$	$p = 0.78$
Body mass index	$p = 0.16$	$p = 0.15$	$p = 0.56$
Biochemical levels and dosage of medications[#]			
Serum Ca	$p = 0.16$	$p = 0.12$	$p = 0.15$
Serum PO_4	$p = 0.03$, $r^2 = 0.19$	$p = 0.07$	$p = 0.11$
Serum Ca x PO_4	$p < 0.0001$, $r^2 = 0.24$	$p = 0.09$	$p = 0.18$
Serum PTH	$p = 0.08$	$p = 0.01$ $r^2 = 0.35$	$p = 0.34$
High sensitivity CRP	$p = 0.001$, $r^2 = 0.42$	$p = 0.24$	$p = 0.56$
Serum albumin	$p = 0.07$	$p = 0.52$	$p = 0.47$
Dosage of elemental Ca intake from PO_4 binders	$p = 0.11$	$p = 0.62$	$p = 0.51$
Dosage of alphacalcidol	$p = 0.07$	$p = 0.30$	$p = 0.39$
Vascular Measures			
Carotid Intima-media thickness	$p = 0.08$	$p = 0.14$	$p = 0.09$
Aortic pulse wave velocity	$p = 0.016$, $r^2 = 0.19$	$p = 0.03$ $r^2 = 0.18$	$p = 0.22$
Aortic augmentation index	$p = 0.03$, $r^2 = 0.11$	$p = 0.09$	$p = 0.18$
Coronary calcification score on CT scan	$p = 0.007$, $r^2 = 0.20$	$p = 0.005$ $r^2 = 0.23$	$p = 0.61$

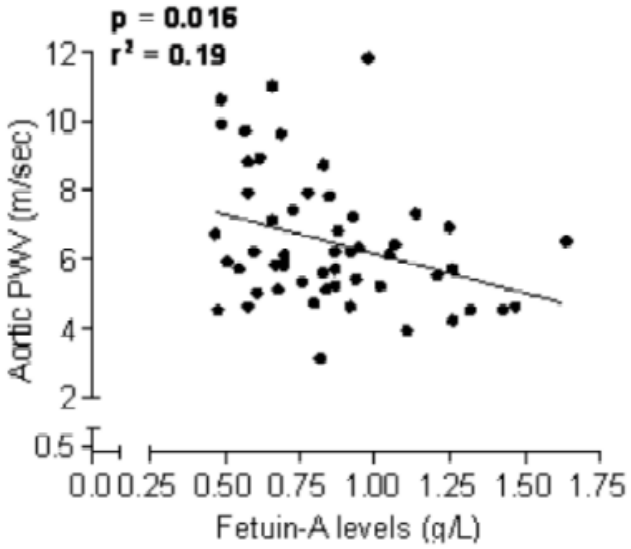
*All associations have been tested by Spearmans non-parametric correlations. An r value has only been given for correlations with a p value <0.05.

#Expressed as mean time-integrated values from the onset of CKD stage IV

Association of Fetuin-A levels and vascular measures

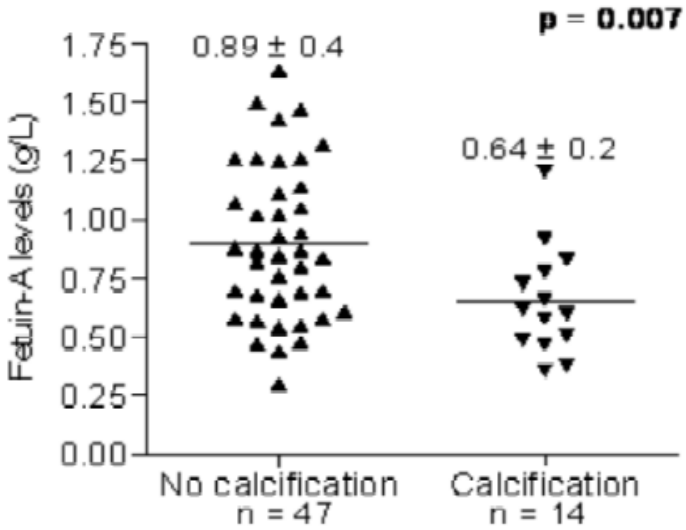
Fetuin-A showed an inverse correlation with the mean time-integrated serum PO_4 levels ($p = 0.03$, $r^2 = 0.19$) and Ca x PO_4 product ($p < 0.0001$, $r^2 = 0.24$). Fetuin-A levels showed a strong negative correlation with hs-CRP ($p = 0.001$, $r^2 = 0.42$). Fetuin-A was associated with vessel stiffness: both the aortic PWV and the aortic augmentation index showed an inverse correlation with fetuin-A levels ($p = 0.016$, $r^2 = 0.19$ [Figure 6.3A] and $p = 0.03$, $r^2 = 0.11$ respectively). Fetuin-A levels were significantly lower in children with coronary or valvular calcification (n = 14) on CT scan than in those without calcification (0.64 ± 0.2 vs 0.89 ± 0.4g/L, p = 0.007, Figure 6.3B). For every 0.1 g/L increase in serum fetuin-A, there was a 5% decrease in risk of calcification (95% CI 0.84 to 0.91, p = 0.013).

Figure 6.3A



The aortic pulse wave velocity correlate with serum fetuin-A levels

Figure 6.3B

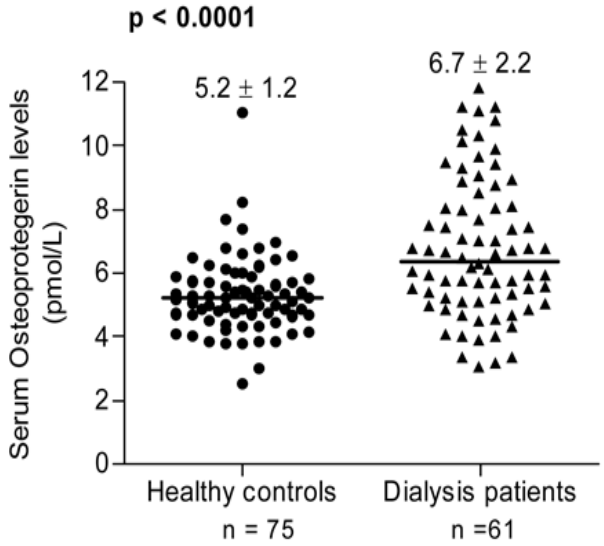


Fetuin-A levels are lower in children with coronary or valvular calcification

OPG levels in dialysis patients

OPG levels were significantly higher in dialysis patients as compared to healthy controls (6.7 ± 2.2 vs 5.2 ± 1.2 pmol/L, $p < 0.0001$, Figure 6.4). A linear relationship was seen between OPG and intact PTH levels ($p = 0.01$, $r^2 = 0.35$). OPG levels were higher in HD compared to PD patients (8.9 ± 1.6 vs 6.0 ± 0.9 pmol/L, $p = 0.02$), but this reflected the higher PTH levels in the HD patients. There was no correlation between RANKL or OPG/RANKL and any clinical, biochemical or vascular measure.

Figure 6.4



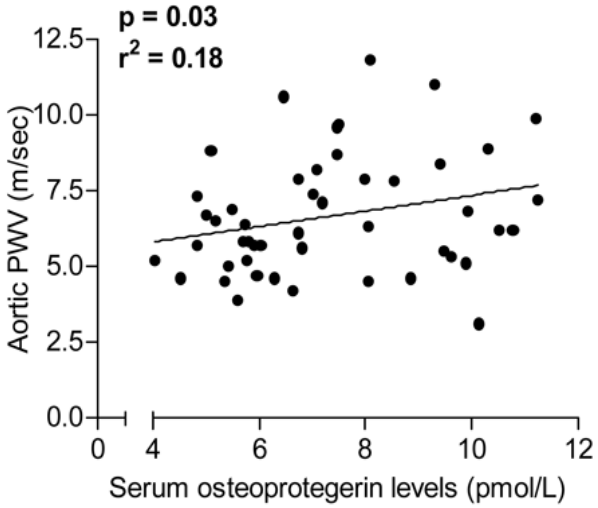
Serum osteoprotegerin levels were higher in dialysis patients compared to age-matched controls

Association of OPG levels and vascular measures

OPG levels were associated with vessel stiffness and calcification. The aortic PWV increased with increasing OPG levels ($p = 0.03$, $r^2 = 0.18$, Figure 6.5A), and children

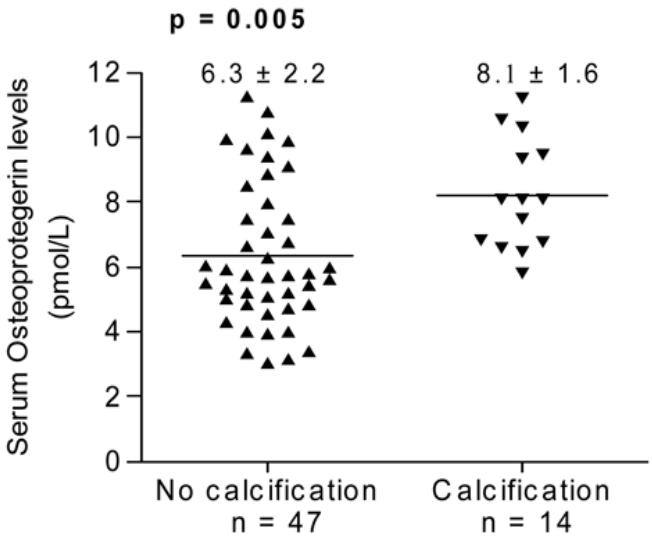
with calcification had significantly higher OPG levels than those without calcification (8.1 ± 1.6 vs 6.3 ± 2.2 pmol/L, p = 0.005, Figure 6.5B).

Figure 6.5A



The aortic pulse wave velocity correlated with serum osteoprotegerin levels

Figure 6.5B

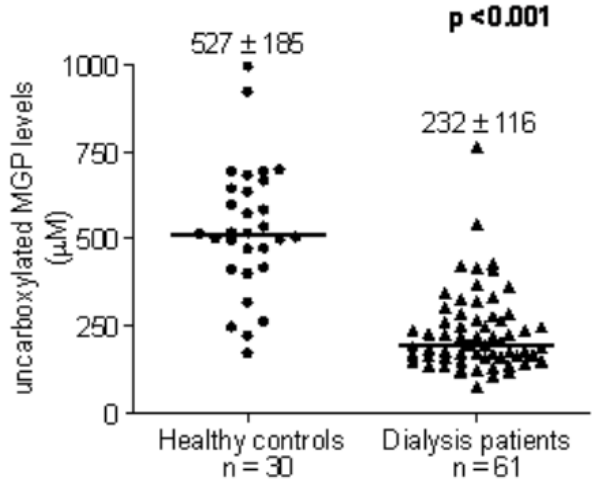


Children with calcification had higher OPG levels than those without calcification

uc-MGP levels in dialysis patients

uc-MGP levels in children on dialysis were significantly lower as compared to controls (232 ± 116 vs 527 ± 185 μM, $p < 0.001$, Figure 6.6), but no further associations with uc-MGP levels were found (Table 6.2).

Figure 6.6



Serum levels of undercarboxylated matrix–Gla protein were lower in children on dialysis compared to controls

Correlations between the calcification inhibitors

I was unable to find any correlations between serum levels of fetuin-A, OPG and uc-MGP in the overall cohort, but in the patients with calcification, an association was seen between fetuin-A and OPG levels ($p = 0.04$, $r = 0.09$). There was no correlation with uc-MGP and fetuin-A or OPG.

Associations between the calcification inhibitor levels and determinants of vascular measures

On stepwise multiple linear regression analyses fetuin-A levels independently predicted aortic PWV ($p = 0.004$, ß = - 0.45, model $R^2 = 48\%$) and fetuin-A and OPG predicted cardiac calcification ($p = 0.02$, ß = - 0.29 and $p = 0.014$, ß = 0.33, model $R^2 = 32\%$ respectively).

6.5 Discussion

This is the first study to describe circulating levels of the calcification inhibitors fetuin-A, OPG, and uc-MGP in a paediatric population and show that children on dialysis have a significant perturbation of these levels that is associated with increased vascular stiffness and calcification.

In healthy children, both fetuin-A and OPG levels increased with age, presumably because both these proteins are expressed in bone and play a role in skeletal mineralization. Also, in healthy controls, fetuin-A levels were lower in taller children as compared to their age-related peers, suggesting that fetuin-A may be consumed in the course of active skeletal mineralization, as shown in an animal model. Fetuin-A is located in mineralising areas of normal human bone matrix *(Schinke et al., 1996)*, suggesting an increased consumption of fetuin-A during periods of active skeletal growth. Fetuin-A deficient mice have impaired growth plate chondroyte maturation and retardation in the longitudinal growth of femurs *(Szweras at al., 2002)* along with extensive calcification of the heart, lungs, and kidneys on a mineral and vitamin D-rich diet or on a calcification-prone genetic background on normal diet, and similar to $OPG^{-/-}$ mice, this calcification can be seen without apparent or only a moderate

increase in serum Ca x PO_4 levels *(Schafer et al, 2003; Bucay et al., 1998)*. We were unable to find a correlation between fetuin-A and features of bone turnover including height, alkaline phosphatase, or PTH levels in dialysis patients; however, as the PTH levels in our cohort were lower than that recommended by the K/DOQI guidelines *(K/DOQI, clinical practise guidelines, 2003)*, a high-turnover bone state is unlikely.

Although several studies have reported that adults on dialysis have significantly lower fetuin-A levels than controls *(Ketteler et al, 2003; Wang et al, 2005; Cozzolino et al, 2007; Coen et al, 2006; Moe et al, 2005; Stenvinkel et al, 2005)*, we found higher levels of fetuin-A in paediatric dialysis patients as compared to healthy age-matched controls. Only children with evidence of calcification on CT scan had reduced fetuin-A, but even this group had higher levels than the controls. Nevertheless, with increasing dialysis vintage and hs-CRP, fetuin-A levels decreased. Our findings are supported by a reports showing that adults with early CKD do not have a reduction in fetuin-A *(Ix et al, 2006)* and that there is no change in fetuin-A levels in dialysis patients with low levels of inflammatory activity *(Hermans et al, 2006)*. Fetuin-A is a negative acute phase reactant *(Leberton et al, 1979)*, and in the pro-inflammatory dialysis milieu its production may be reduced. In addition, the pro-calcific uraemic milieu may consume circulating fetuin-A: in vitro studies have shown that fetuin-A contributes to almost 50% of the calcification inhibitory capacity of human plasma *(Price et al, 2003)*, and by 'shielding' mechanisms prevents further crystal growth *(Reynolds et al, 2005)*. Taken together, this suggests that a protective mechanism allows an upregulation of fetuin-A in the early stages of CKD and dialysis, and only severe or prolonged exposure to a pro-inflammatory and/or pro-calcific environment

eventually leads to low levels due to reduced production and/or increased consumption.

However, it may also be that patients with calcification have genetically lower fetuin-A levels that predispose them to calcification. Genetic polymorphism studies have given conflicting results *(Cozzolino et al, 2007)*, but the association between reduced fetuin-A levels and cardiovascular *(Herman et al, 2007; Ketteler et al, 2003; Stenvinkel et al, 2005; Wang et al, 2005)* and even all-cause *(Herman et al, 2007)* mortality in 4 large studies suggest that fetuin-A is likely to have a causal effect on vascular calcification. Moreover, low circulating fetuin-A levels are associated with high serum PO_4 levels even in the general population *(Osawa et al, 2005)* and have been associated with valvular calcification in patients with normal renal function *(Ix et al, 2007)*. In our study, fetuin-A was a significant and independent predictor of vascular stiffness and calcification irrespective of dialysis vintage, Ca x PO_4 levels, or hs-CRP, implying that genetic polymorphisms may indeed play a role in an individual patients' susceptibility to calcify, possibly by modulating the magnitude of change in fetuin-A production in response to a pro-inflammatory or pro-calcific environment.

We found an independent association between fetuin-A levels and aortic PWV and calcification, and there are few and conflicting reports in the literature on this. While a Japanese study has shown that fetuin-A levels predict carotid artery stiffness even in healthy subjects *(Mori et al, 2007)*, Hermans et al could not find an independent association between PWV or augmentation index and fetuin-A in dialysis patients *(Herman et al, 2006)*. The above dialysis cohort in fact had normal fetuin-A levels and a low level of inflammatory activity as compared to controls, and may not be a

representative population of dialysis patients. Animal studies have shown that fetuin-A knock-out mice develop widespread soft-tissue and myocardial calcification whereas their large arteries are spared *(Merx et al, 2005)*. In these animals the myocardial Ca content can increase upto 60-fold, initiating a profound pro-fibrotic response with the 'myocardial stiffness' leading to cardiac fibrosis, diastolic dysfunction, reduced cardiac output and an impaired tolerance to ischaemia *(Merx et al, 2005)*. Indeed, fetuin-A levels are an independent predictor of death in non-renal failure patients following electrocardiogram changes of ST-elevation and acute myocardial infarction *(Lim et al, 2007)*.

So far, no studies have addressed OPG levels and its associations with vascular measures in children on dialysis. Several lines of evidence from mouse genetics suggest a vasculoprotective role for OPG: (I) Targeted deletion of the OPG gene leads to vascular calcification of the aorta and renal arteries *(Bucay et al., 1998)*, (II) transgenic overexpression in $OPG^{-/-}$ mice leads to rescue of the phenotype of vascular calcification *(Min et al., 2000)*, and (III) vascular calcification induced by warfarin or toxic doses of vitamin D is inhibited by simultanous application of OPG in a rat model *(Price et al., 2001)*. Since OPG is a decoy receptor for RANKL, the principal regulator of osteoclast function, it is unclear if OPG inhibits vascular calcification by inhibiting mineral release from bone, by vascular wall dependent mechanisms, or by effects in the circulation.

A number of studies have shown that OPG is elevated in vascular disease and that OPG seems to be a biomarker for increased vascular mortality and an increased risk for cardiovascular disease, especially in adult populations of renal failure patients

(Browner et al., 2001; Kiechl et al., 2004; Hjelmesaeth et al., 2006). Serum OPG was related to severity and progression of carotid atherosclerosis *(Kiechl et al., 2004)*, associated with cIMT *(Erdogan et al., 2004)* and coronary artery calcification *(Abedin et al., 2007)*. OPG may be either cause or consequence of vascular calcification, and high circulating OPG levels in patients with cardiovascular disease could also mean that raised OPG levels detrimentally affect vascular homeostasis.

Although uc-MGP levels were significantly lower in dialysis patients than healthy controls, we were unable to find any correlations with uc-MGP and clinical or vascular measures. *In vitro* work has shown that vessels with intimal and/or medial calcification, uncarboxylated MGP (uc-MGP) is localized around areas of calcification, whereas in healthy arteries active or γ–carboxylated MGP is present, with no uc-MGP *(Schurgers et al, 2005; Murshed et al, 2004)*. It is possible that uc-MGP levels were so low that correlations with biochemical or vascular parameters is no longer possible. Also, we have not measured total MGP levels, and the low circulating uc-MGP may represent a lack of MGP production or reduced vitamin-K dependent γ–carboxylation of the uc-MGP to its active form. As MGP levels can potentially be modulated by dietary supplementation of vitamin K *(Schurgers et al, 2001)*, the role of circulating MGP, if any, needs to be further explored.

There is clearly a complex relationship between the calcification inhibitory proteins and vascular measures in different clinical settings and even at the different stages of uraemic vasculopathy that are impossible to discern given the cross-sectional nature of this study and indeed all the other published work in this field. The results of the

present study warrant further investigations of circulating inhibitor levels as well as their genetic polymorphisms to elucidate the potential clinical utility of these biomarkers. As cardiovascular morbidity is known to begin in the early stages of CKD, future studies should assess serial measures of the circulating inhibitors from CKD stages II - III.

6.6 Conclusions

In conclusion, in this study I have defined the normal levels of the calcification inhibitors in the healthy childhood population and shown, for the first time, that fetuin-A and OPG impact on vascular stiffness and calcification in children on dialysis. Paediatric dialysis patients have an upregulation of fetuin-A, possibly as a protective response to the pro-calcific and pro-inflammatory uraemic environment. While further longitudinal studies in both adult and paediatric CKD patients are required to fully characterise these circulating biomarkers, they may prove to be a useful and convenient measure of an individual patients' susceptibility to calcify.

Chapter 7

Evidence of *In Vivo* Vascular Damage and Calcification: Clinical and Laboratory Correlations

7.1 Abstract

Vascular calcification is associated with increased morbidity and mortality in stage 5 chronic kidney disease, yet its early pathogenesis and initiating mechanisms *in vivo* remain poorly understood. To address this we quantified the calcium (Ca) load in arteries from children (10 pre-dialysis, 24 dialysis) and correlated it with clinical, biochemical and vascular measures. Vessel Ca load was significantly elevated in both pre-dialysis and dialysis and correlated with the patients' mean serum Ca x P product. However, only dialysis patients showed increased carotid intima-media thickness and increased aortic stiffness and calcification on CT was present in only the 2 patients with the highest Ca loads. Importantly, pre-dialysis vessels appeared histologically intact, whereas dialysis vessels exhibited evidence for extensive vascular smooth muscle cell (VSMC) loss due to apoptosis. Dialysis vessels also showed increased alkaline phosphatase activity and Runx2 and osterix expression indicative of VSMC osteogenic transformation. Deposition of the vesicle membrane marker, annexin VI and vesicle component mineralization inhibitors, fetuin-A and matrix Gla-protein increased in dialysis vessels and preceded von Kossa positive overt calcification. Electron microscopy showed hydroxyapatite nanocrystals within vesicles released from damaged/dead VSMCs, indicative of their role in initiating calcification. Taken together, this study shows that Ca accumulation begins pre-dialysis, but it is the induction of VSMC apoptosis in dialysis that is the key event in disabling VSMC defence mechanisms and leading to overt calcification, eventually with clinically detectable vascular damage. Thus, the identification of factors that lead to VSMC death in dialysis will be of prime importance in preventing vascular calcification.

7.2 Introduction

Evidence of vascular changes from imaging studies in adult and paediatric CKD patients have shown indirect evidence of vascular damage such as increased carotid IMT *(Oh et al, 2002; Mitsnefes et al, 2006; Shroff et al, 2007)* and arterial stiffness *(London et al, 2002; Covic et al, 2006; Shroff et al, 2007)* and direct evidence of arterial calcification on CT scans *(Goodman et al, 2000; Shroff et al, 2007)* and autopsy *(Milliner et al, 1990)*. Also, two small studies have used arteries removed at the time of transplantation to describe the histological changes in the vessel wall *(Moe et al, 2002; Nayir et al, 2001)*. However, studies in adults are complicated by the presence of multiple pro-atherosclerotic risk factors such as diabetes, dyslipidaemia, hypertension and often the presence of pre-existing vascular calcification, and few studies have been able to characterize the earliest events or the natural history of progression of uraemic calcification *in vivo*.

In vitro studies using human vascular smooth muscle cells [VSMCs] have been instrumental in defining the cell biological mechanisms of vascular calcification and have demonstrated that it is a highly regulated process with many similarities to bone formation, and have been described in Chapter 1, section V. Briefly, when exposed to high Ca – PO_4 media, VSMC undergoes apoptosis *(Proudfoot et al, 2000)* as well as cellular adaptation and vesicle release *(Reynolds et al, 2004)*, with these small membrane-bound bodies forming a nidus for the deposition of basic Ca-PO_4 in the form of hydroxyapatite *(Reynolds et al, 2004)*. In the healthy vessel wall these vesicles are loaded with physiological inhibitors of calcification such as fetuin-A and MGP *(Reynolds et al, 2005)*, but evidence suggests that these proteins may be deficient or non-functional in CKD patients. In addition, as part of the mineralization

process VSMCs lose their normal contractile phenotype and change to an osteo/chondrocytic phenotype *(Shanahan et al, 1990; Tyson et al, 2003)* that is characterized by the upregulation of bone-specific transcription factors and matrix proteins including Runx2/Cbfa1, osterix and alkaline phosphatase [Alk] *(Shanahan et al, 1990; Tyson et al, 2003; Moe et al, 2002).* In the uraemic milieu, a reduction or perturbation in the physiological calcification inhibitors *(Ketteler et al, 2003; Schurgers et al, 2007; Lou et al 1997)*, leads to an increased expression of osteogenic markers by VSMCs that further enhances the pro-calcific environment.

However, the series of events that lead to the initiation and progression of vascular calcification in the context of an intact vessel wall are not known. To investigate these processes *in vivo* I have studied the natural history of vascular calcification in children with CKD and linked the findings from vessel histology to the patients' clinical, biochemical and vascular measures. Children provide a good opportunity to study uraemic influences on the arterial wall as they have fewer pro-atherosclerotic risk factors that are major confounders in similar adult studies.

7.3 Methods

Subjects

Medium sized muscular arteries routinely removed and discarded in the course of planned intra-abdominal surgery were used for this work. The detailed method of sample removal, collection and transportation are described in chapter 2, section III.

24 inferior epigastric arteries (18 from dialysis patients and 6 from CKD Stage V pre-

dialysis patients [GFR <15 ml/min/1.73m^2]), 14 omental arteries (6 dialysis, 4 pre-dialysis and 4 normal controls) and 2 mesenteric arteries (normal controls) were studied.

The primary diagnoses in the 34 CKD patients (20 boys) were dysplasia (n = 18), inherited nephropathies (n = 6), cystic kidney disease (n = 3), primary tubular disorders (n = 3), malignancies (n = 3) and renovascular disorders (n = 1). Patient details are described in Table 7.1 below. In order to keep the patient and control groups free of confounding pro-atherosclerotic risk factors, children with inflammatory disorders, vasculitis, diabetes, dyslipidaemia or smokers were excluded. Informed written consent was obtained from all parents or caregivers and children, where appropriate. The study was approved by the local research ethics committee.

Table 7.1 Clinical and biochemical features of the patient and control groups

	Normal controls N = 6	**Pre-dialysis** n = 10	**Dialysis** n = 24
Age	12.3 ± 3.1	13.1 ± 2.6	13.0 ± 2.5
Gender (% ♂)	66	56	68
Body mass index SDS	0.5 ± 1.1	-0.2 ± 1.1	-0.3 ± 1.4
Systolic Blood Pressure (mmHg)	100 ± 7.1	109 ± 14.0	111 ± 12.1
Systolic Blood Pressure Index	0.9 ± 0.1	1.0 ± 0.6	1.1 ± 1.2
Number of patients on anti-hypertensive medications (mean number of anti-hypertensives per patient)	0 (0)	2 (1)	3 (1)
Dialysis modality (PD / HD)	-	-	17 / 7
Serum Ca (mMol/L)	2.4 ± 0.3	2.4 ± 0.1	2.3 ± 0.2
Serum PO_4 (mMol/L)	1.1 ± 0.4	1.2 ± 0.2	1.6 ± 0.7 *
Serum iPTH (pMol/L)	Not done	7.3 ± 1.9	12.8 ± 7.5 *
Fasting blood glucose (mMol/L)	Not done	4.3 ± 1.4	4.6 ± 0.5
Serum cholesterol (mMol/L)	3.5 ± 0.3	3.4 ± 0.2	3.9 ± 0.2
Serum triglycerides (mMol/L)	1.0 ± 0.3	1.3 ± 0.1	1.2 ± 0.4
High-sensitivity CRP (mg/L)#	Not done	Not done	2.89 ± 1.3
Ca intake from PO_4 binders (g/day)	-	1.4 ± 1.0	1.6 ± 1.3*
Alphacalcidol dose (μgm/day)	-	0.4 ± 0.02	0.5 ± 0.02

Results are expressed as mean ± standard deviation.
Systolic Blood Pressure Index = measured BP/95th centile BP for age, gender and height.
* significant difference ($p<0.05$) between the dialysis and CKD groups.
# - performed in 15 patients only.

Clinical and biochemical data

The patients' age, time in CKD stages IV and V pre-dialysis, time on dialysis and dialysis modality were recorded. Biochemical parameters (serum Ca, P and intact parathyroid hormone [iPTH] levels) and the dosage of elemental calcium intake from phosphate binders and alphacalcidol (1-α hydroxycholecalciferol) therapy were recorded at monthly intervals over a 3-year period and expressed as mean time-averaged levels. For controls, results of a single blood test at the time of the study were used. The characteristics of the vessel on histology and the quantification of the vessel Ca load were correlated with the patients' clinical and biochemical parameters and vascular scans as described in Chapter 2, section III.

Vascular scans

To characterise fully the clinical vascular phenotype, all patients and controls above the age of 5 years (n = 31) underwent a high-resolution ultrasound scan to measure the carotid IMT, applanation tonometry for PWV and multi-slice cardiac CT scan to identify coronary artery and valvular calcification. Methods for these have been described in detail in chapter 2, section I. Given the small number of healthy controls in this study, the cIMT and PWV were compared with 40 healthy age and gender-matched children who participated in the study described in chapter 4.

Calcification assay and Alkaline Phosphatase levels

Vessels rings immediately after harvest were used to quantify the Ca load in the vessel wall and the Alkaline phosphatase (ALK) activity as described in Chapter 2, section III. Both the vessel Ca load and the ALK activity were standardized to the protein content in the vessel wall.

Histological analyses and Immunohistochemistry

Detailed methods for histology and immunohistochemistry are descried in chapter 2, section III. Immunohistochemistry preperations and staining were all performed by Mrs Nichola Figg, Senior Technician, Department of Medicine, Addenbrooke's Hospital, Cambridge, and I am very grateful for her help with this work.

Viability of VSMCs in the vessel rings was confirmed by trypan blue (0.5%) staining as standard. Hematoxylin/eosin (*Sigma HHS-32 and E8017*) staining was performed for vessel integrity and cell counting: the number of VSMC nuclei and the percentage of apoptotic cells were counted in a $0.25m^2$ area and expressed as cells per unit area. Each sample was analysed twice and the mean of the readings used. Vessel rings were stained with Von Kossa for calcification, Mason's trichrome stain for collagen and Verhoeff van Geison (VVG) for integrity of the elastic laminae, and immunohistochemistry performed for α-smooth muscle cell actin (*DAKO M0851*, 1:500 dilution) for smooth muscle cells, KI67 (*Vector Biotechnology, VP-K452*, 1:100) for proliferation and CD68 (*DAKO M0814*, 1:100) for macrophage infiltration using 3% peroxidase in water to block endogenous peroxidase activity. Apoptosis was examined by immunohistochemistry using a cleaved caspase-3 antibody and transferase-mediated dUTP nick-end labelling (TUNEL) staining visualized by a rhodamine-labelled anti-digoxigenin antibody. Immunohistochemistry for known vesicle components and osteogenic factors was performed using annexin VI (*BD Bioscience 610300*, 1:500 dilution) and Fetuin-A (AS237 antibodies, 1:200 dilution). The carboxylated and undercarboxylated (Gla and Glu) forms of Matrix Gla Protein (MGP), Runx2 (*Santa Cruz SC10758*, 1:100 dilution) and osterix (*Abcam 22552*)

were examined. Using ImageJ software on the Olympus BX51 microscope, a region of interest was marked around the von kossa positive areas, and this was expressed as a percentage of the total area of the tunica media of the vessel. The percentage of TUNEL, Runx2, fetuin-A and annexin VI positive areas were expressed in a similar manner, analysing each sample in duplicate. The relative area of Gla and Glu MGP positivity was described as a ratio in normal controls and dialysis vessels.

Transmission Electron Microscopy (TEM)

TEM was performed to examine cell morphology, localization of calcification, vesicle release and mineral deposition and is descried in chapter 2, section III. Electron microscopy was performed by Dr Jeremy Skepper at the Multi-Imaging Centre, Department of Anatomy, Cambridge, and I am very grateful for his help.

RNA extraction and Reverse Transcription-Polymerase Chain Reaction

A 1 – 2 mm sized ring from each vessel was frozen at harvest and used for mRNA isolation, cDNA production and real-time quantitative polymerase chain reaction (qRT-PCR) for the osteogenic marker cbfa-1. Deatiled methods are described in chapter 2, section III.

Statistical analyses

Data are presented as mean ± standard deviation or median and range. Correlations between groups were performed using the paired or unpaired t test as appropriate. One-way ANOVA (Kruskal-Wallis test) was used to compare non-parametric data between groups, and the Mann-Whitney U test (non-parametric) performed to compare values between two groups. Linear regression analyses were performed to

test the associations between vessel Ca load and the time in pre-dialysis CKD stages IV and V, time on dialysis, mean time-averaged serum Ca x P levels and the carotid artery intima-media thickness respectively. Raw data for the Ca load in the vessel and its correlation with clinical, biochemical and vascular measures is shown in Table 7.2. Significance was defined as $p < 0.05$. Statistical analyses were performed using SPSS, version 14.0 (*SPSS Inc, Chicago, IL, USA*).

Table 7.2 Patient and vessel characteristics (arranged in asscending order of vessel Ca load)

Pt	Age yrs	Status	eGFR ml/min/ 1.73m^2	Time Pre-D yrs	Time on dialy-sis yrS	Mean Ca x P mMol^2/L^2	cIMT mm	Vessel type	Ca load in the vessel wall µg/µL	Von Kossa posit-ivity	ALK
1	13.5	Control	129	-	-	3.8	0.34	mesenteric	6.1	-	7.9
2	1.9	Control	122	-	-	2.1	0.36	omental	8.3	-	4.6
3	14.7	Control	134	-	-	3.6	0.38	mesenteric	8.4	-	10.5
4	8.3	Control	130	-	-	2.7	0.30	omental	10.6	-	2.8
5	16.0	Control	118	-	-	2.4	0.34	omental	11.0	-	5.2
6	12.0	Control	124	-	-	3.0	0.36	omental	12.3	-	3.6
7	16.6	Pre-d	15.9	0.4	-	4.8	0.38	omental	12.6	-	8.2
8	14.0	Pre-d	12.7	3.7	-	4.1	0.28	Inf epi	13.8	-	12.0
9	2.8	Pre-d	9.6	1.3	-	2.9	nd	Inf epi	15.0	-	7.4
10	14.4	Pre-d	8.2	3.2	-	3.8	0.38	Inf epi	15.9	-	3.7
11	9.1	Pre-d	6.8	2.9	-	4.4	0.36	omental	16.1	-	5.3
12	13.1	Pre-d	10.4	3.6	-	5.2	0.30	Inf epi	22.0	-	8.0
13	10.0	Pre-d	8.2	2.3	-	3.9	0.38	Inf epi	24.7	-	4.1
14	15.7	Pre-d	11.2	0.9	-	4.8	0.40	omental	25.6	-	7.2
15	15.4	Pre-d	7.7	1.3	-	4.2	0.34	omental	25.9	-	7.0
16	9.8	Dialysis	D	3.1	0.6	4.8	0.38	Inf epi	29.3	-	13.7
17	16.3	Dialysis	D	1.2	0.7	5.1	0.42	Inf epi	29.6	-	16.2
18	12.4	Dialysis	D	0.7	2.1	4.5	0.42	Inf epi	29.7	-	10.4
19	12.8	Dialysis	D	2.9	1.0	4.2	0.46	Inf epi	31.9	-	12.9
20	12.8	Pre-d	10.3	0.7	-	4.7	0.40	Inf epi	32.0	-	5.8
21	3.3	Dialysis	D	2.8	0.7	4.0	nd	Inf epi	33.0	-	11.0
22	11.7	Dialysis	D	3.1	1.0	4.0	0.44	omental	35.3	-	16.2
23	14.0	Dialysis	D	1.7	2.3	4.1	0.56	omental	36.2	-	10.9
34	13.8	Dialysis	D	0.8	0.2	3.4	0.30	Inf epi	36.2	+	17
25	7.2	Dialysis	D	4.4	0.62	4.0	0.42	Inf epi	37.1	-	17.3
26	10.7	Dialysis	D	2.2	0.5	3.8	0.46	Inf epi	38.0	-	8.8
27	14.1	Dialysis	D	2.0	3.7	5.4	0.60	Inf epi	38.4	-	17.1
28	14.9	Dialysis	D	0.8	0.6	4.9	0.54	Inf epi	38.5	-	13.2
29	4.7	Dialysis	D	3.1	0.8	3.6	nd	Inf epi	38.5	-	8.6
30	16.6	Dialysis	D	2.9	1.8	5.9	0.56	omental	39.1	-	17.9
31	8.8	Dialysis	D	5.3	2.2	3.5	0.34	omental	41.1	++	14.6
32	15.3	Dialysis	D	0.9	0.4	3.6	0.32	Inf epi	41.6	-	18.8
33	11.0	Dialysis	D	1.0	2.8	5.6	0.62	omental	42.0	-	12.3
34	14.6	Dialysis	D	1.2	4.2	5.4	0.50	Inf epi	43.0	-	13.7
35	9.5	Dialysis	D	1.6	5.5	5.2	0.54 © p,i	Inf epi	43.0	+++	16.9
36	12.8	Dialysis	D	4.3	3.8	6.1	0.56	Inf epi	45.9	-	16.2
37	16.6	Dialysis	D	0.6	3.0	5.0	0.46	omental	47.1	+	19.4
38	14.0	Dialysis	D	4.1	3.6	6.1	0.65	Inf epi	48.4	-	15.4
39	16.3	Dialysis	D	5.5	1.2	5.9	0.66 © p,i	Inf epi	48.8	+++	20.1
40	14.9	Dialysis	D	3.3	1.9	5.2	0.54	Inf epi	49.1	+	16.9

cIMT = carotid Intima Media Thickness. Normal cIMT in an age-matched population was 0.40mm.
© = coronary artery calcification on CT scan, Agatston scores 1221 and 631 in patients 35 and 39 respectively
nd - not done
^{p} = Increased Pulse Wave Velocity above control levels ^{i}= presence of neointima on histology
Von kossa positivity graded semi-quantitatively as + (speckled calcification) to +++ (diffuse punctuate calcification)

7.4 Results

Pre-dialysis and dialysis vessels have an increased Ca load

The vessel Ca load was significantly higher in pre-dialysis compared to control vessels (20.4 ± 6.5 vs 9.4 ± 2.3μg/μL), but was highest in dialysis vessels (39.2 ± 6.0μg/μL) where levels were 2-fold greater than pre-dialysis and 3- to 4-fold greater than in normal vessels ($p < 0.0001$ [ANOVA]; Figure 7.1A, Table 7.2). The Ca load was significantly and consistently higher in dialysis vessels despite a similar age and level of renal deterioration in the pre-dialysis and dialysis patients, and was independent of preservation of residual renal function in dialysis patients. The Ca load was higher in the vessels of HD as compared to PD patients, but this significance was lost after correcting for time on dialysis.

Calcification was not detectable by von kossa staining in control or pre-dialysis vessels. In contrast, diffuse speckled calcification in the media and along the internal elastic lamina was present in 6 of 24 (25%) dialysis vessels (Figure 7.1B), suggesting that Ca accumulation/loading within the tunica media begins before overt calcification can be detected by von Kossa. There was no evidence of intimal calcification or a plaque in any of the vessels.

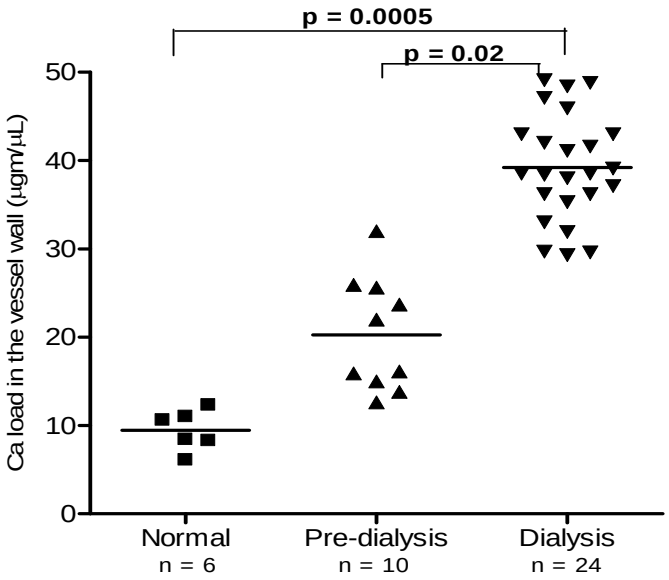


Figure 7.1 A Quantifying the vessel Ca load in different vessel types

The Ca load in vessel rings was quantified by the cresolphthalein complexone method after hydrolysing the Ca out of the vessel using HCl.
Dialysis vessels had a significantly higher Ca load compared to pre-dialysis or normal vessels ($p < 0.0001$, ANOVA).

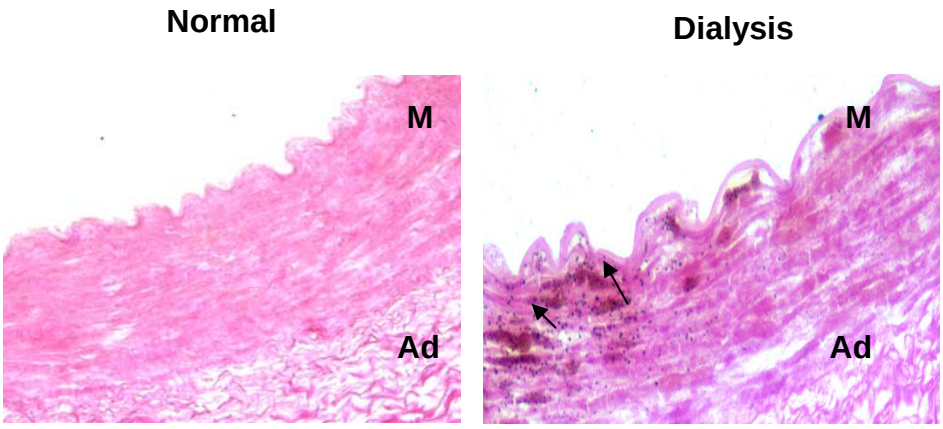


Figure 7.1 B Histology for vessel calcification

Von kossa staining to demonstrate calcification in dialysis but not pre-dialysis or control vessels. Diffuse speckled calcification (arrows) in the media and along the internal elastic lamina was seen in the dialysis vessel.
M, media; Ad, adventitia.

Clinical and biochemical correlations with vessel Ca load

To investigate the clinical and biochemical determinants of vessel Ca load I correlated data on demographic and biochemical parameters (Table 7.1) with the Ca load in the vessel wall. The Ca load did not increase with the time spent in CKD stages IV and V before dialysis was commenced ($p = 0.29$, $r^2 = 0.03$; Figure 7.2A), but there was an increase in Ca load with increasing time on dialysis ($p = 0.017$, $r^2 = 0.22$; Figure 7.2B) that was independent of age, gender and dialysis modality.

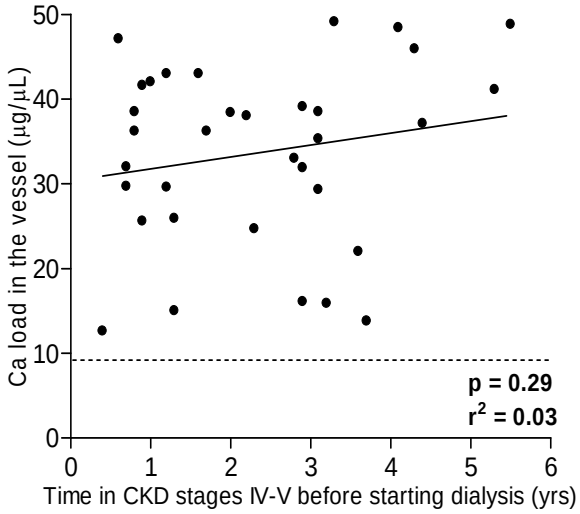


Figure 7.2A Vessel Ca load vs time in CKD stage IV-V pre-dialysis
There was no correlation between Ca load and time spent in CKD stages IV-V before the initiation of dialysis (n = 34).

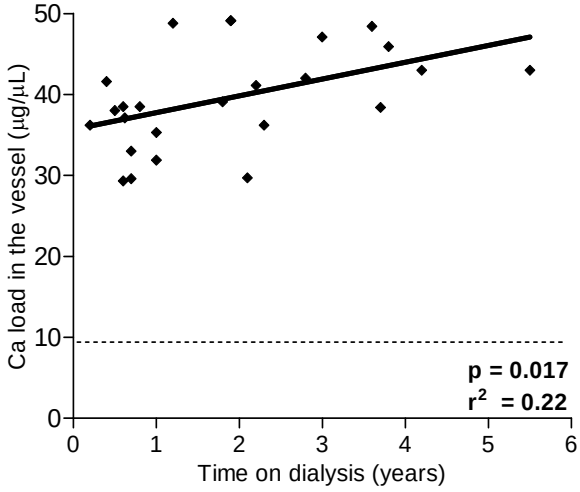


Figure 7.2B Vessel Ca load vs time on dialysis
There was a strong linear correlation between the vessel Ca load and the time on dialysis (n= 24).

The mean time-averaged serum Ca x P product showed a strong linear relation with the vessel Ca load ($p = 0.008$, $r^2 = 0.20$; Figure 7.2C). However, no correlation was seen with mean time-averaged serum Ca, PO_4, PTH, high-sensitivity CRP or albumin levels. Also, there was no association between the mean time-averaged doses, cumulative doses or doage at the time of study of alphacalcidol or phosphate binders and the vessel Ca load.

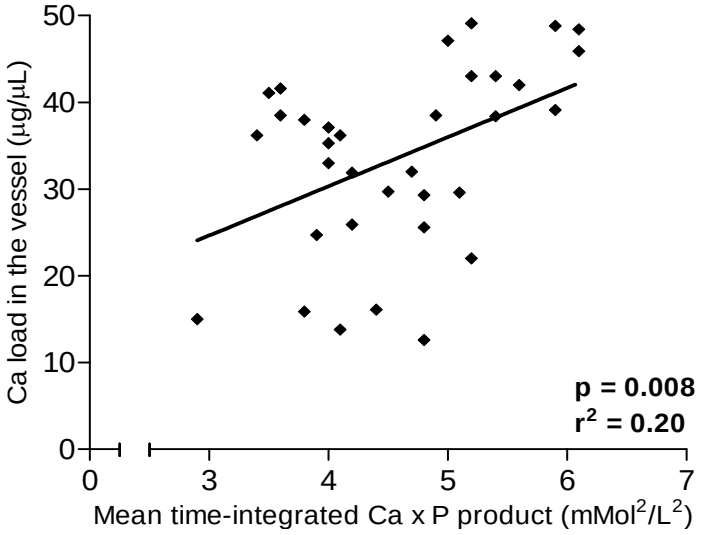


Figure 7.2C Vessel Ca load vs mean time-averaged Ca x PO_4 product

Vessel Ca load correlates with the carotid intima-media thickness

The carotid IMT, showed a strong independent correlation with Ca load ($p = 0.01$, $r^2 = 0.28$; Figure 7.3). Despite the increased Ca load in all pre-dialysis and dialysis patients, the IMT was increased in only 18 (75%) dialysis patients, and remained within age-related normal limits (= 0.40mm) in all the pre-dialysis patients. On cardiac CT scan, calcification was seen only in the 2 patients who had the highest Ca loads and macrocalcification visible by von Kossa (Agatston score 1221 and 631; patients 35 and 39 in Table 7.1). The PWV did not correlate with the Ca load

in the overall cohort (not shown), and was increased in only the 2 patients with coronary calcification on CT.

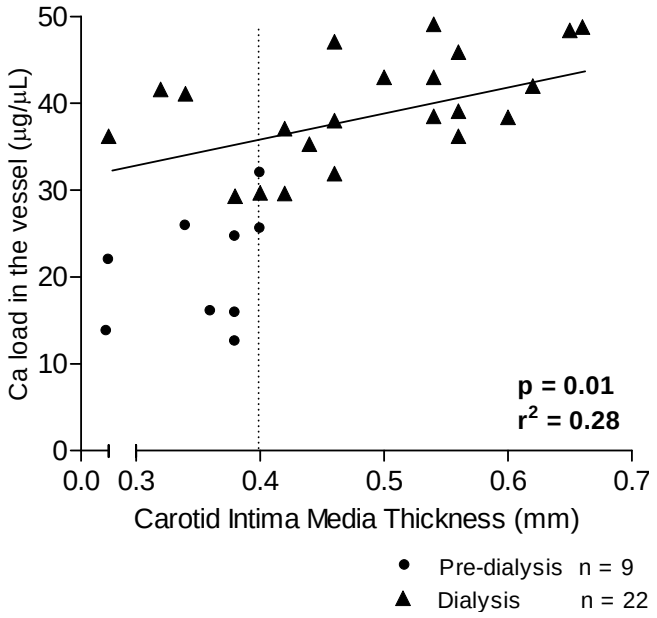


Figure 7.3 Correlation of vessel Ca load against carotid IMT
n = 22 dialysis and 9 pre-dialysis patients (includes all children above 5 yrs age).
The dotted line in the figure represents the normal value of cIMT (= 0.40mm) in a population of healthy age-matched controls.

Dialysis vessels have fewer VSMCs per unit area

To determine if the increased Ca load in the dialysis vessels was associated with any phenotypic changes to the VSMCs, I performed detailed histology on all the vessels as described in chapter 2.

First I looked for any reduction in the number of VSMCs between the three vessel types by counting the number of VSMC nuclei per unit area of vessel on a hemotoxylin-eosin stained sample to determine the number of VSMCs in different vessel types. Despite the increased Ca load in all the pre-dialysis vessels, there was no difference in the number of VSMCs between pre-dialysis and control vessels (median 122 [range 112 – 129] vs 126 [118 – 144] cells per unit area, p = 0.08;

Figure 7.4A). However, there was a significant reduction in VSMC numbers in dialysis vessels (80 [58 – 106] VSMCs / unit area; Fig 7.4A).

It was not clear if the reduced cell numbers was a result of a significantly greater Ca load in the dialysis vessels or was induced by exposure to specific 'damage-inducing' agents in the dialysis milieu. To try and elucidate this I compared pre-dialysis and dialysis vessels with a similar Ca load of 25-35 μg/μL (patients 13 to 22 in supplemental Table 7.2; n = 4 pre-dialysis and n = 6 dialysis vessels). Significantly fewer VSMCs were present in dialysis compared to pre-dialysis vessels (88 [67 – 110] vs 120 [114 – 126] cells /unit area; p = 0.004) (Figure 7.4B).

The reduction in VSMC number was also demonstrated by alpha-smooth muscle cell actin staining (Figure 7.4C). In addition, cystic areas were observed in von Kossa positive regions suggesting that lost VSMCs were not replaced. Ki67 staining showed that few cells were proliferating (< 0.5% positivity; results not shown), with no difference between control and dialysis vessels, implying that VSMC proliferation was not induced in response to cell loss.

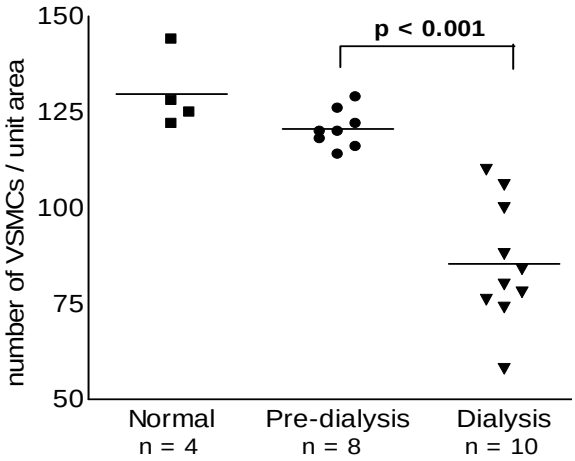


Figure 7.4A Vascular smooth muscle cell numbers in different vessel types
VSMC numbers were significantly reduced in dialysis vessels.

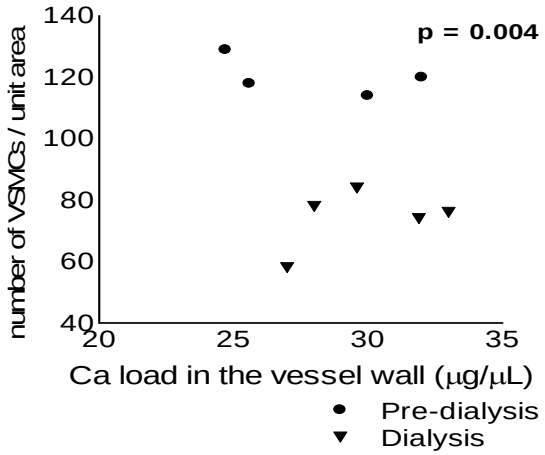


Figure 7.4B Vascular smooth muscle cell numbers
The number of VSMCs was independent of the vessel Ca load.

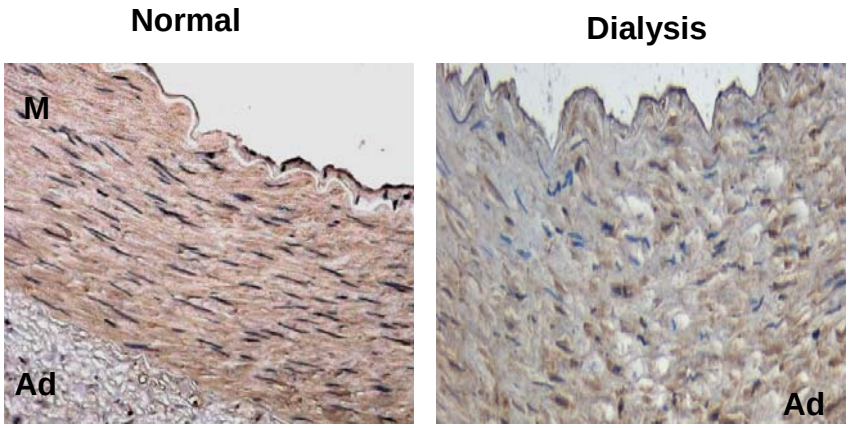


Figure 7.4C Immunohistochemistry for alpha-smooth muscle cell actin
Arrows indicate cystic areas of cell loss in dialysis vessels.

Apoptosis of VSMCs occurs in dialysis vessels

To determine if apoptosis was contributing to VSMC loss, TUNEL staining was performed. There were significantly more TUNEL positive cells in dialysis as compared to pre-dialysis or normal vessels (0.4 ± 0.4%, 0.27 ± 05% and 3.16 ± 1.0% in normal, pre-dialysis and dialysis vessels respectively, p = 0.008); Figures 7.5 A and B. Vessels with fewer VSMCs had the greatest percentage of TUNEL positive areas. Importantly, areas of apoptosis were seen in the same region of the vessel as von Kossa positive calcified areas in adjacent sections (Figure 7.5 C).

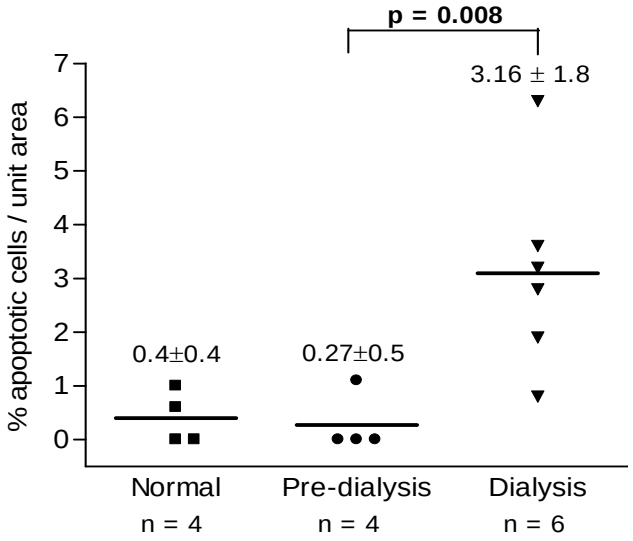


Figure 7.5A TUNEL staining for apoptosis

The percentage of TUNEL positive cells in a unit area of vessel were counted in control, pre-dialysis and dialysis vessels.

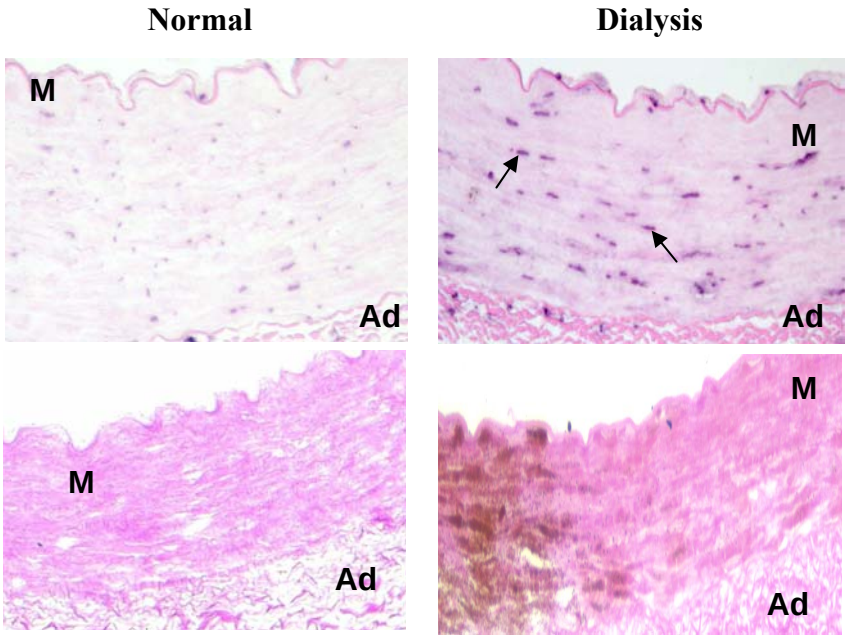
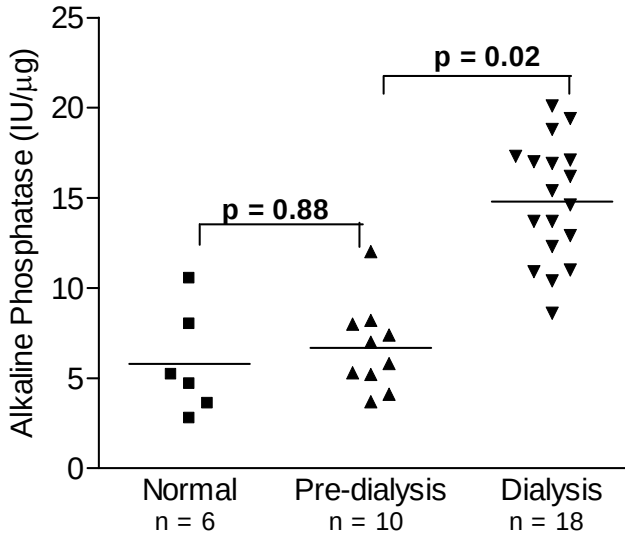


Figure 7.5 B and C TUNEL staining
TUNEL staining was present in dialysis vessels but absent in controls (top panel).
Von Kossa staining showed that areas of medial calcification in dialysis vessels localized to regions that were also TUNEL positive in adjacent sections (bottom panel).

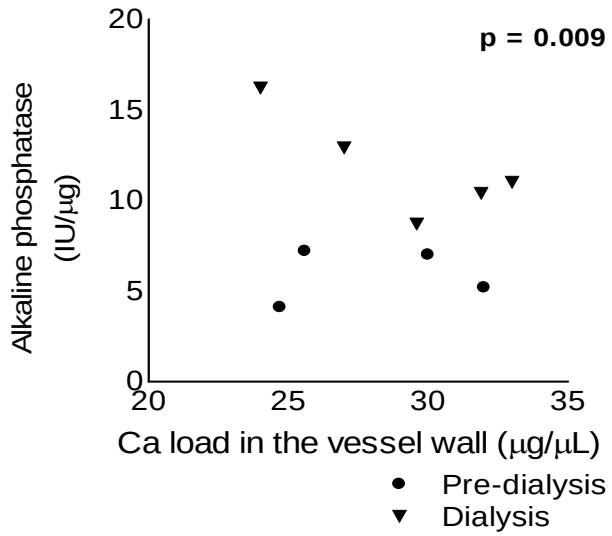
To further explore the nature of dialysis induced vascular damage, detailed histology was performed on all 3 vessel types. A neointima was seen only in the 2 most severely affected dialysis patients (Table 7.2) who also had evidence of abnormal vascular structure stiff vessels and coronary calcification. Calcification was exclusively medial on von Kossa staining. There was no evidence of an inflammatory response or macrophage infiltration in any vessel, the internal and external elastic laminae were intact and there was no evidence for increased collagen deposition suggesting that the vascular changes in dialysis patients were not atherosclerotic.

VSMCs undergo osteogenic differentiation in dialysis vessels

Osteoblastic conversion of VSMCs, measured by ALK activity, is an early event in vascular calcification. Despite an increased Ca load there was no increase in ALK activity in pre-dialysis (6.4 [3.7 – 12.0]IU/μL) compared to control vessels (4.9 [2.8 – 10.5] IU/μL), but dialysis vessels had ~2-fold higher ALK levels (15.0 [8.6 – 20.1] IU/μL; Figure 7.6A). Again, it was not clear if the greater Ca load in dialysis vessels or factors specific to dialysis per se were responsible for triggering an osteoblastic phenotypic change. There was no correlation between the Ca load and ALK in the overall cohort (p= 0.08), but dialysis vessels with comparable Ca loads to pre-dialysis vessels (= 25-35 μg/μL, patients 13 to 22 in Table 7.2; n = 4 pre-dialysis and n = 6 dialysis vessels) had greater ALK levels (13.3 [10.4 – 16.2] IU/μL vs 6.4 [4.1 – 7.2] IU/μL, p = 0.009); Figure 7.6B. However, ALK levels did not increase with time on dialysis (p = 0.39).



Figures 7.6A Alkaline Phosphatase activity in the three vessel types
Dialysis vessels showed significantly higher ALK levels than pre-dialysis or normal vessels.



Figures 7.6B Alkaline Phosphatase activity in the three vessel types
Dialysis vessels with comparable Ca loads to pre-dialysis vessels had higher ALK levels. n = 4 pre-dialysis vessels, n = 5 dialysis vessels

Upregulation of osteogenic transcription factors in dialysis vessels

In order to demonstrate that increased ALK levels were indeed the result of osteo/chondrocytic conversion of VSMCs, immunohistochemistry for the osteogenic transcription factors Runx2 and osterix was performed. All vessel types had some positivity for Runx2 and osterix, but the levels and distribution varied. Control and pre-dialysis vessels showed diffuse staining for Runx2 in a predominantly nuclear distribution (2% and 4.4% /unit area respectively) whereas dialysis vessels showed an upregulation of Runx2 staining (13.4%/unit area, $p < 0.0001$), with a marked cytoplasmic distribution (Figures 7.7 A and B). Staining for osterix showed a similar distribution (Figure 7.7 C).

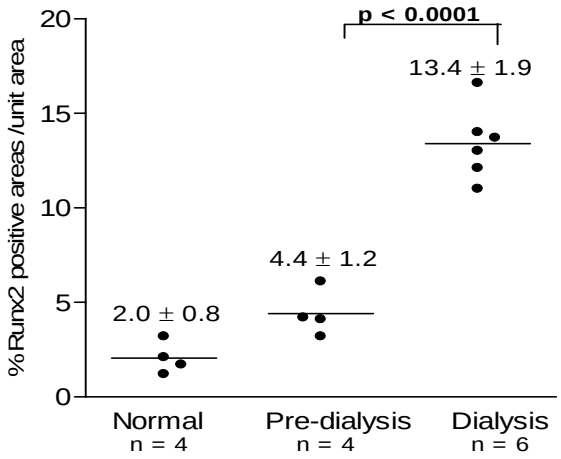


Figure 7.7A Immunohistochemistry for osteogenic markers
There was significantly greater Runx2 positivity in dialysis vessels.

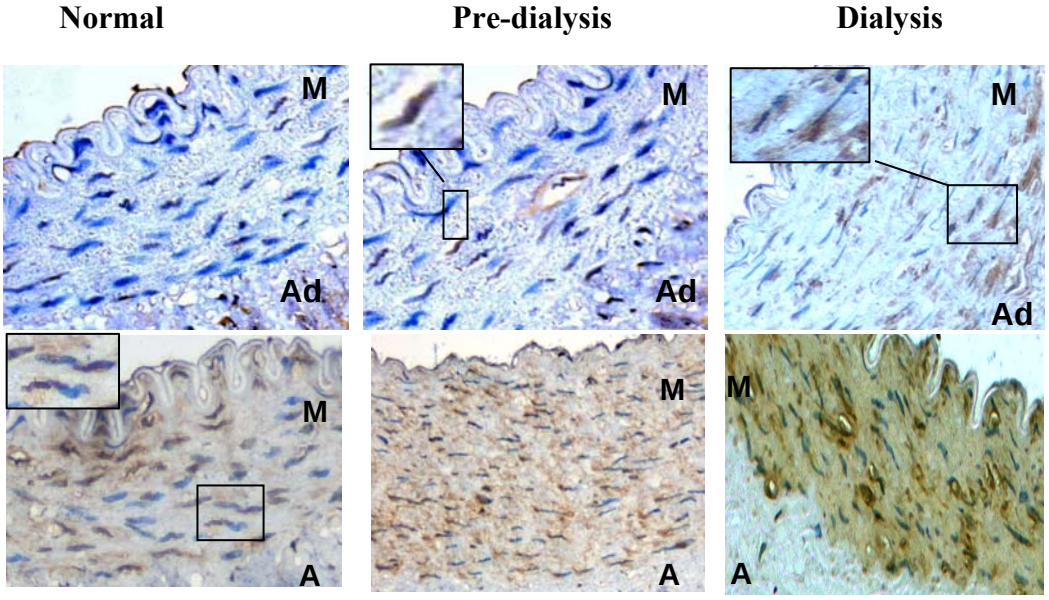


Figure 7.7B & C Immunohistochemistry for Runx2 and osterix
Immunohistochemistry for Runx2 showed a predominantly cytoplasmic distribution pattern (enlarged in inset) in dialysis compared to pre-dialysis and normal vessels that had lower levels of Runx2 that was predominantly nuclear (enlarged in inset) – top panel.
Osterix showed a similar pattern of distribution as Runx2 – bottom panel.

mRNA of osteogenic transcription factors

Estimation of mRNA expression of cbfa-1 / runx2 was attempted, but as described in chapter 2, section III. The very low yield of RNA and its contamination with genomic DNA made qRT-PCR impossible as shown in Figure 7.8A. Figure 7.8B confirms this on an agarose gel.

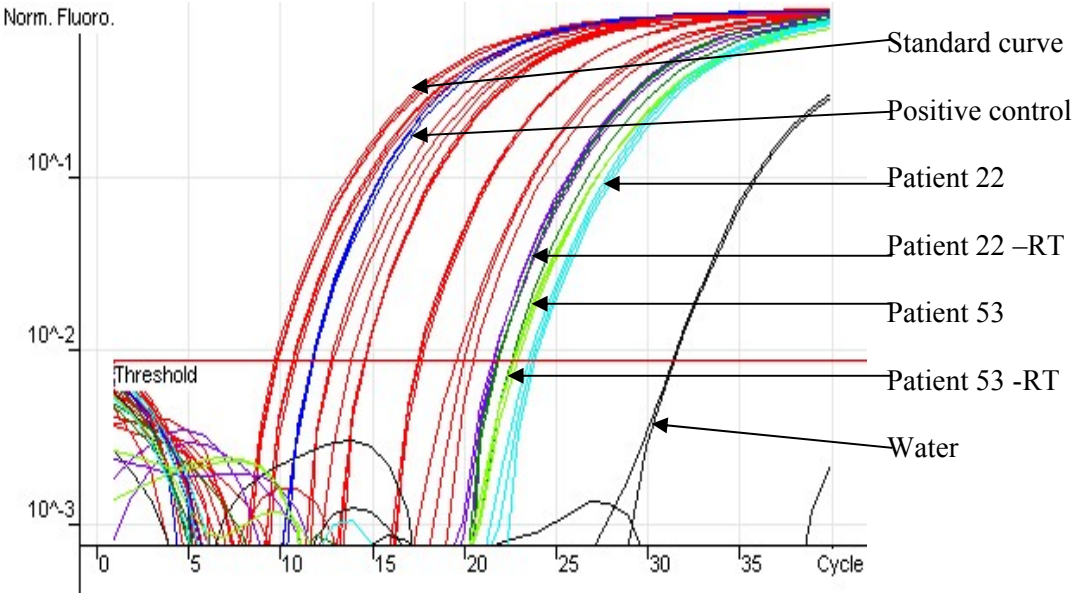


Figure 7.8A qRT-PCR for Runx2.
No amplification of DNA was seen until cycle 22 suggesting minimal if any pure RNA in the sample. The negative controls for each patient sample (patient 22-RT and patient 53-RT) show amplification at the same time as or even before the corresponding patient sample, confirming absence of RNA in the sample. An appropriate amplification with positive control and no response with the negative control (water) confirm that the technique was appropriate.

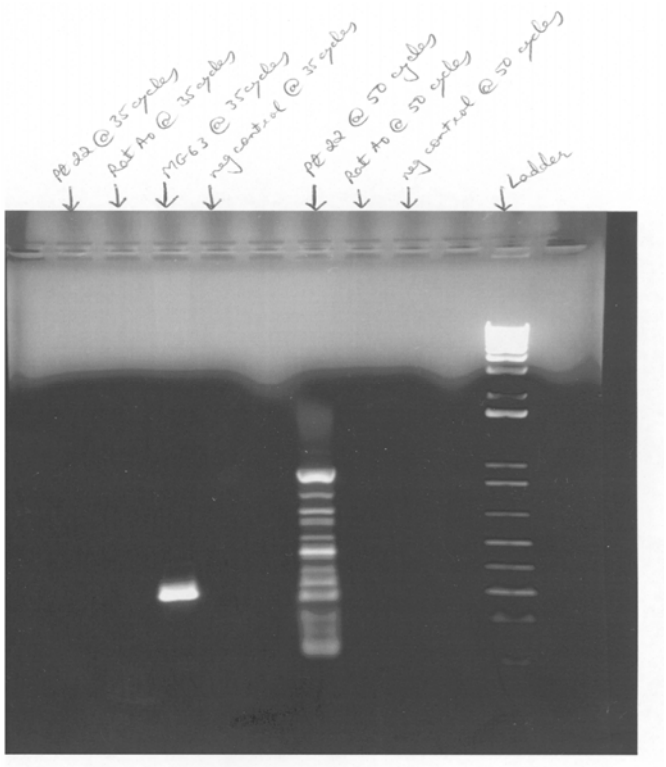


Figure 7.8 B Agarose gel for 18S DNA shows contamination with genomic DNA

Vessel calcification is associated with Fetuin-A deposition

MGP and Fetuin-A are inhibitors of calcification released by VSMCs in vesicles and apoptotic bodies. Fetuin-A positive staining was minimal (1.2%/unit area) in controls, but progressively increased in pre-dialysis (8.5%/unit area) and dialysis (16.2%/unit area) vessels. (Figures 7.9 A and B). Also, there was increased fetuin-A staining in the von Kossa positive dialysis vessels (19.3 vs 8.2%/unit area, p = 0.04).

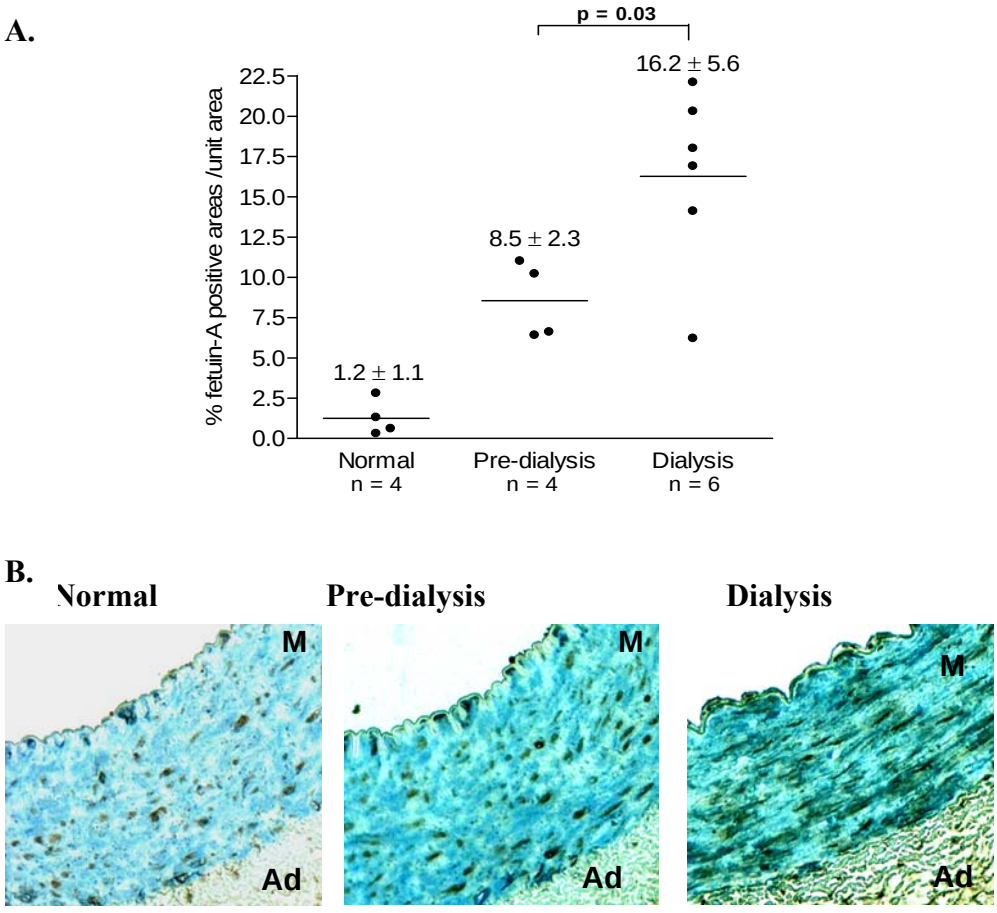


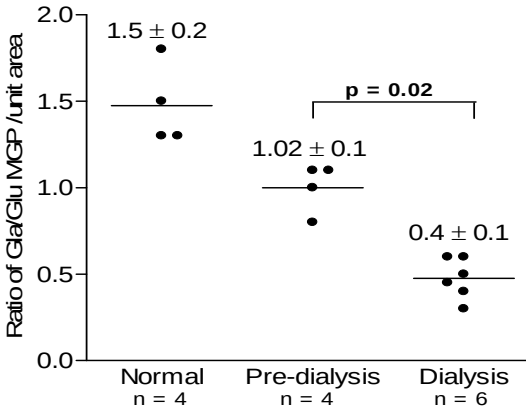
Figure 7.9 Staining for Fetuin-A positivity

A. Fetuin-A positive staining was minimal in controls, but progressively increased in pre-dialysis and dialysis vessels.
B. Maximum fetuin-A positivity was present in dialysis vessels, especially those with significant calcification.

Vessel calcification is associated with ucMGP deposition

Vessels from all groups were positive for both undercarboxylated and carboxylated MGP (Glu-MGP and Gla-MGP respectively), however the relative amounts varied. While normal vessels showed predominantly Gla-MGP (ratio Gla/Glu MGP = 1.5) and pre-dialysis vessels maintained a balance between the Gla- and Glu-MGP fractions (ratio Gla/Glu MGP=1.02), dialysis vessels had more Glu-MGP (ratio Gla/Glu MGP = 0.4, p = 0.02; Figures 7.10A, B). Glu-MGP staining was localized in the same region as von Kossa positive areas on adjacent sections (not shown).

A.



B.

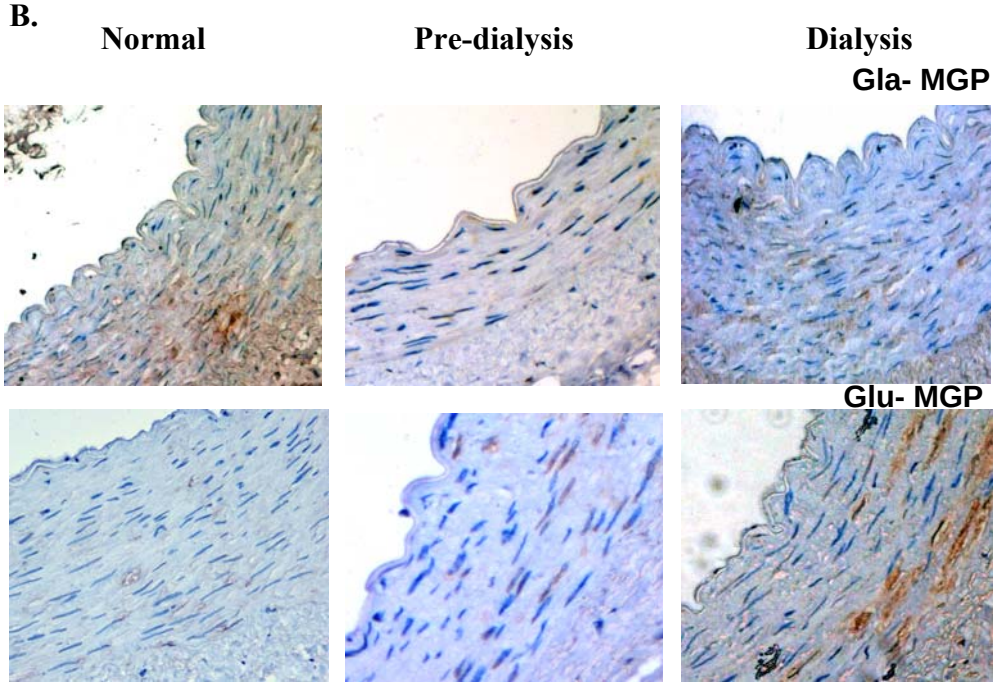
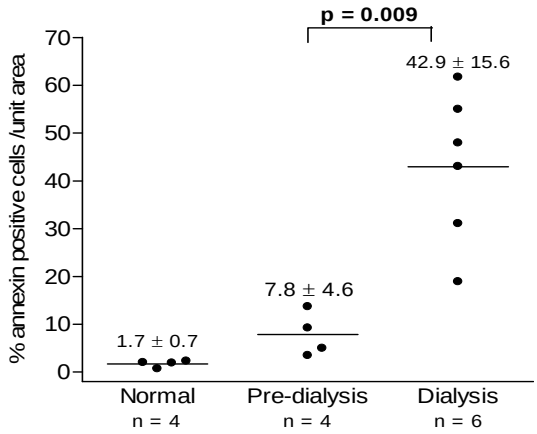


Figure 7.10 Immunohistochemistry for Gla and Glu forms of MGP

Calcification in vivo is a vesicle-mediated process

The presence of MGP and fetuin-A at sites of calcification is highly suggestive of a vesicle-mediated calcification process and this was confirmed by staining for annexin VI, a Ca binding protein concentrated in both chondrocyte and VSMC (our unpublished data) matrix vesicles. Annexin VI staining was punctate and minimal in control and pre-dialysis vessels (1.7 ± 0.7% and 7.8 ± 4.6% per unit area respectively) and was clearly vesicular in nature (Figures 7.11 A and B). Dialysis vessels had greater Annexin VI positivity (43 ± 15.6 % per unit area; p = 0.009) with a diffuse punctate distribution consistent with vesicle induced microcalcifications.

A.



B.

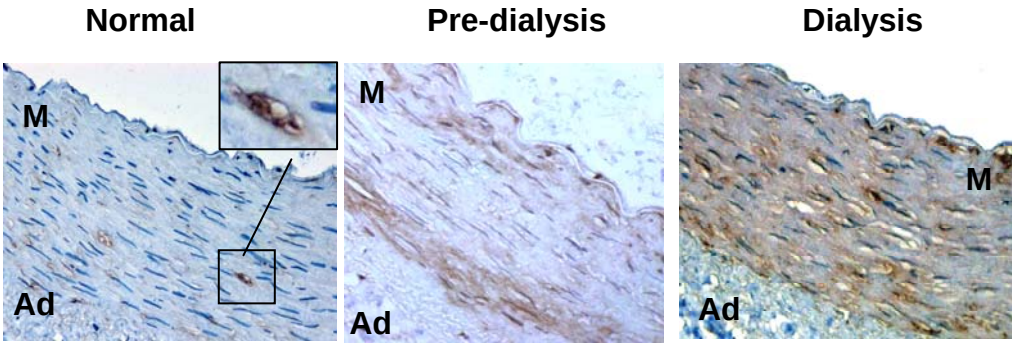


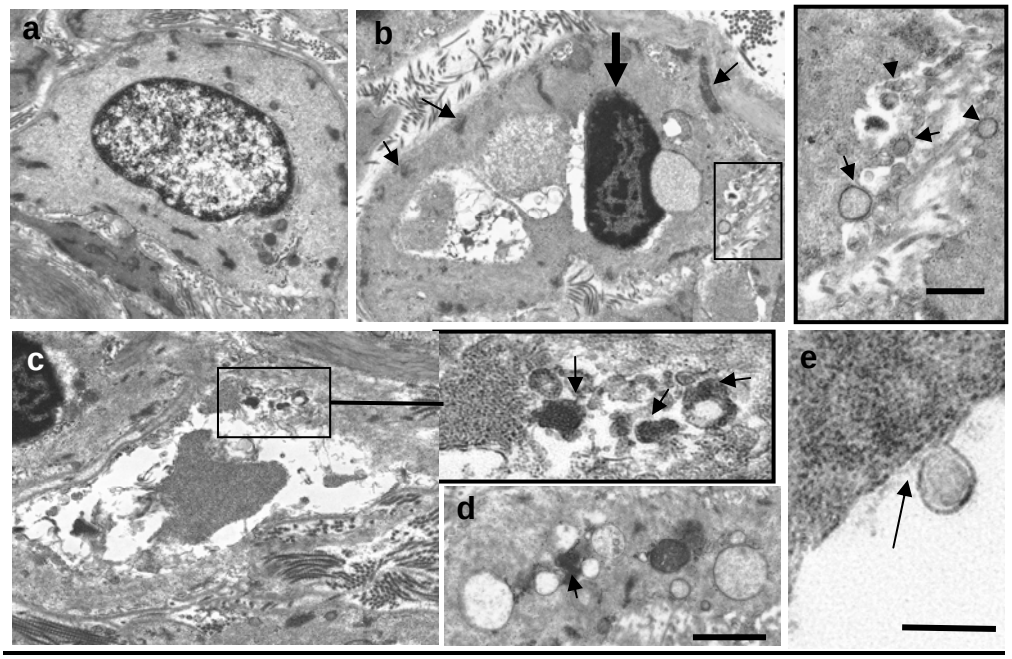
Figure 7.11 Annexin VI staining

A. Dialysis vessels had significantly more annexin-6 positive areas (42.9 ± 15.6%) compared to pre-dialysis (7.8 ± 4.6%) or normal controls (1.7 ± 0.7%), p = 0.009.

B. Immunohistochemistry for annexin-6 was minimal in the normal and pre-dialysis vessels but widespread in the matrix and VSMCs in dialysis vessels.

Electron Microscopy for vessel ultrastructure

TEM ultrastructural analysis revealed that in the normal vessel wall, VSMCs were morphologically contractile, there was no evidence of extracellular vesicles and the nuclei showed normal appearance and distribution of heterochromatin (Figure 7.12 A). However VSMCs in dialysis vessels showed apoptosis and damage characterized by increased electron density of nuclear heterochromatin, cell shrinkage and/or vesicle release (Figure 7.12 C-b). This damage was evident in >60% of VSMCs in one patient with a Ca load of 33μg/μL (patient 21, supplemental Table 7.2) and no evidence of calcification on von Kossa, suggesting that it is an early event preceding overt calcification. Interestingly, VSMCs with heteropycnotic nuclei did not have all the hallmarks of typical apoptosis or necrosis nor did they exhibit characteristics of 'synthetic' VSMCs consistent with the absence of proliferation and suggesting that apoptosis of contractile VSMCs may have unique features. In patients without overt calcification, extracellular vesicles did not contain any evidence of hydroxyapatite nanocrystals, but vessels with calcification had vesicles containing microcrystalline structures consistent with hydroxyapaptite. Vesicles deposited in the extracellular matrix were in the size range of 0.1-1.0μm consistent with their derivation from both apoptotic bodies and plasma membrane budding of matrix vesicles (Figure 7.12 C-E).



Figures 7.12 Vessel ultrastructure on electron microscopy

(a) A normal contractile VSMC showing a normal nucleus with heterochromatic areas localized predominantly around the nuclear envelope. The euchromatin is interspersed with heterochromatin in the deeper regions of the nucleus. Arrowheads indicate dense bodies indicative of a contractile cell.
(b) Interspersed with normal VSMCs were contractile cells with evidence of damage showing heteropycnotic nuclei (large arrow), with highly increased electron density of heterochromatin and much less euchromatin. Cytoplasmic vacuoles and matrix vesicles were deposited in the extracellular matrix adjacent to the plasma membrane. Boxed area is enlarged to show vesicles.
(c) Many cells had undergone cell death leaving cellular debris including vesicular debris (boxed area enlarged).
(d) In some areas vesicles stained with high contrast indicating the presence of mineral (arrowed).
(e) The origin of vesicles from budding of the plasma membrane is shown (arrowed). Bar =1μm

7.5 Discussion

In this study I have provided quantitative evidence that Ca accumulation in the vessel wall begins pre-dialysis, and that factors specific to the dialysis milieu trigger accelerated calcification. We hypothesise that Ca accumulation in the vessel begins in response to increased Ca and PO_4, but protective mechanisms such as adequate mineralization inhibitor levels and extrusion of intracellular Ca via vesicle release, preserves normal VSMC function. In the dialysis milieu, damage-inducing agents that include continued exposure to high, and possibly worsening, Ca and PO_4, lead to apoptosis. This in turn increases local Ca levels and reduces local levels of VSMC-derived mineralization inhibitors, that potentiates osteo/chondocytic differentiation of smooth muscle cells and the release of pro-calcific vesicles that form a nidus for calcification (Figure 7.13).

Importantly, the clinical detection of VSMC damage and calcification was only possible in the most severely calcified patients. Calcification was inexorable and extremely rapid on dialysis, with a dialysis vintage of even 2 months sufficient to induce histologically overt calcification and VSMC damage, emphasising the need to avoid dialysis and perform pre-emptive renal transplantation wherever possible. The identification of factors specific to dialysis that trigger the accelerated wave of VSMC death will be key in minimizing the detrimental effects of arterial calcification in renal failure.

The distribution and histological correlation with vessel Ca load

Arterial damage and calcification in dialysis patients was widespread and involved multiple vascular beds including the carotid, coronary, omental and inferior epigastric vessels. Histology showed that calcification was entirely medial in

distribution and there was no evidence for inflammation, suggesting that calcification, at least in its early stages and in young CKD patients, is an arteriosclerotic rather than an atherosclerotic process. Interestingly, although an increased Ca load was present in all pre-dialysis and dialysis vessels, histological evidence for calcification by von kossa staining was only found in 6 of the 24 dialysis vessels that had the highest Ca loads. This shows that histology is not a sensitive tool for detecting the early stages of calcification. Earlier studies that relied on histology may have underestimated the extent of calcification *(Moe et al, 2003; Saygili et al, 1997)*. However, it may be that as von kossa stains inorganic phosphorous, and as the earliest phases of mineralization are relatively poor in phosphorous, we may not have detected early calcification. Using alizarin red staining for calcium may have helped, but this does not give well defined pictures of calcified regions as does von kossa, and also it is unlikely to be a more sensitive method than direct quantification of the Ca load.

The Ca load in CKD vessels is medial and correlates with dysregulated mineral metabolism

Ca loading was evident in both pre-dialysis and dialysis, but evidence for vascular remodelling (ie. increased cIMT and neointima formation) was observed only in dialysis vessels with the highest Ca loads. Potentially this implies that there may be a causal relationship between Ca loading and increased susceptibility to vessel wall damage and remodelling however, currently available clinical tools are not sensitive enough to detect what may be functionally significant vascular damage in CKD.

The Ca load showed a strong correlation with the patients' serum Ca x P product in all CKD vessels. Numerous adult studies have previously shown an association between the serum Ca x P product and an increased cIMT or vascular calcification in dialysis patients *(Block et al, 1998; Goldsmith et al, 2004)*, but there are conflicting reports in pre-dialysis patients *(Block et al, 2005; Toussaint et al, 2007)*. However, these adult studies were complicated by pre-existing vascular disease as well as other risk factors for calcification, whereas, the children in our study were free of the major confounders for cardiovascular disease, diabetes, dyslipidaemia and uncontrolled hypertension. Also, unlike adult studies, the increased Ca load was independent of the patients' age and related only to their time on dialysis. There was no increase in Ca load with increasing time spent in CKD stages IV or V before initiating dialysis, but significantly greater calcification was seen with increasing time on dialysis and correlated with the induction of apoptosis.

Dialysis patients had more severe dysregulation of mineral metabolism as compared to pre-dialysis patients. There was no significant difference in the mean serum Ca, PO_4 or Ca x PO_4 products between the PD and HD patients, but the vessel Ca load was significantly higher in HD than PD patients, suggesting that transient fluctuations in serum Ca, as often seen after HD *(McIntyre et al, 2005)*, but are not reflected in serum Ca levels may contribute to calcification. *In vitro* work has shown that VSMC calcification increases markedly when in addition to phosphate, Ca is added to the culture medium as it triggers apoptosis and vesicle release *(Reynolds et al, 2004),* lending further support to this observation. Also,

serum Ca is a poor marker of total body Ca and may account for this discrepancy. When corrected for dialysis vintage, the vessel Ca load was not significantly different between PD and HD patients. Treatment regimens such as calcium-based phosphate binders *(Goodman et al, 2000; Litwin et al, 2005)* and vitamin D *(Milliner et al, 1990; Shroff et al, 2008)* have also been shown to increase hypercalcemic episodes and Ca load however, we did not find any association between these and the vessel Ca load in this study.

Animal studies suggest roles for oxidative stress, inflammatory mediators *(Soriano et al, 2007)* and advanced glycation end-products *(Shanahan et al, 2006)* in promoting VSMC injury but these have yet to be explored in a human *in vivo* model. However, although inflammation plays a key role in intimal calcification and in medial calcification in diabetic patients' *(Ishimura et al, 2002; Byon et al, 2008)* its role in uraemic medial calcification, at least in our cohort of paediatric patients free of diabetes, appeared minimal given the lack of inflammatory cell infiltrate in the vessel wall and the absence of any correlation between calcium load and hs-CRP. Thus, other, factor(s) specific to the dialysis milieu that may be responsible for VSMC death remain to be identified.

Dialysis induces VSMC apoptosis and osteo/chondrocytic differentiation

Previous *in vitro* studies have shown that apoptosis precedes the development of VSMC calcification. Apoptotic bodies form a nidus for the deposition of hydroxyapatite *(Proudfoot et al, 2000; Reynolds et al, 2004)* and apoptosis has been shown to increase the local concentrations of Ca to >30mM, with this elevation potentially inducing further VSMC death, vesicle release and calcification *(Olszak et*

al, 2000). A reduction in the number of VSMCs would also reduce local production of mineralization inhibitors such as MGP, resulting not only in accelerated calcification but enhanced osteo/chondrocytic differentiation *(Reynolds et al, 2005; Proudfoot et al, 2000; Rogers et al, 2007)*. Further evidence for this comes from the incremental increases in the deposition of vesicle proteins annexin VI, MGP and fetuin-A through pre-dialysis to dialysis as well as the presence of vesicles and dying VSMCs within dialysis vessels that had not yet developed overt calcification as shown by EM. Studies have shown that VSMCs release Ca-loaded vesicles in response to Ca overload potentially to prevent apoptosis and that these vesicles are loaded with calcification inhibitors including fetuin-A and MGP that act to limit their calcification potential *(Reynolds et al, 2005; Proudfoot et al, 2000)*. However, with time in the dialysis milieu, vesicle release and VSMC damage increases, resulting in a reduced capacity of the VSMCs to handle Ca overload and to produce or incorporate inhibitors. The circulating protein fetuin-A is greatly reduced in dialysis *(Ketteler et al, 2003)* and is protective against VSMC apoptosis *(Reynolds et al, 2005)*, while the form of MGP in the calcified dialysis vessels was the unmodified Glu form that has a much reduced capacity to inhibit calcification *(Shanahan et al, 1998; Schurgers et al, 2005; Schurgers et al, 2007)* its lack of modification may be due to progressive VSMC loss or dysfunction resulting in reduced γ–carboxylase enzymatic activity in the cells. However, although evidence supports a causal relationship between apoptosis and the calcification cascade, we cannot rule out the possibility that some VSMCs may have undergone hypertrophy and thus cell loss may be overestimated. Hypertrophy has been shown to occur as a response to injury and is associated with an irreversible modulation of VSMC phenotype which may also contribute to vessel remodelling in dialysis *(Walsh K, 1999)*. Further studies to investigate this possibility

would be difficult, but include genomic DNA estimation, accurate measures of cell size and ploidy.

Is osteoblastic conversion protective or detrimental?

Dialysis vessels with the highest Ca loads also had the highest levels of ALK, suggesting that ALK levels were enhanced by dialysis-induced VSMC injury despite the reduction in VSMC number. The importance of vascular damage in inducing calcification has been suggested in a previous *in vitro* study where rat aortic rings were induced to calcify only if subjected to mechanical damage *(Lomashvili et al, 2004)*, implying that in injured vessels an upregulation of ALK plays a key role in inducing calcification. ALK can promote calcification by hydrolysis of pyrophosphate *(Lomashvili et al, 2004)* and this may be an additional mechanism for accelerated calcification in dialysis. In humans elevated serum ALK levels have been associated with calcific uraemic arteriopathy *(Rogers et al, 2007)* and a genetic deficiency in pyrophosphate levels causes infantile idiopathic arterial calcification *(Rutch et al, 2003)*.

We also found significantly greater Runx2 and osterix positivity in dialysis vessels implying *in vivo* osteoblastic conversion of VSMCs. The expression of Runx2 and osterix in the control vessels was unexpected but may reflect a developmental pattern of expression for these proteins in the still immature vasculature of children *(Minguell et al, 2005)*. The cytoplasmic distribution of Runx2 in dialysis vessels was also striking and may reflect VSMC damage *(Liberman et al, 2008)*, or the expression of alternate isoforms of this protein in response to calcium/calcification and this observation requires further analysis *(Harrington et al, 2002; Terry et al,*

2004). Expression of osteoblastic markers by VSMCs preceded calcification and it remains unclear whether the expression of osteo/chondrocytic proteins by VSMCs is an adaptive response aimed at regulating mineralization, or whether these changes, which interestingly in this study occurred in contractile VSMCs, act to enhance the mineralization process.

7.6 Conclusions

In conclusion, in this chapter I have shown direct evidence for the presence of vascular damage and calcification in vessels from pre-dialysis and dialysis patients. A summary figure to explain the proposed mechanisms leading to vascular damage and calcification at different stages of CKD is shown below (Figure 7.13).

There are some important clinical implications of this study.

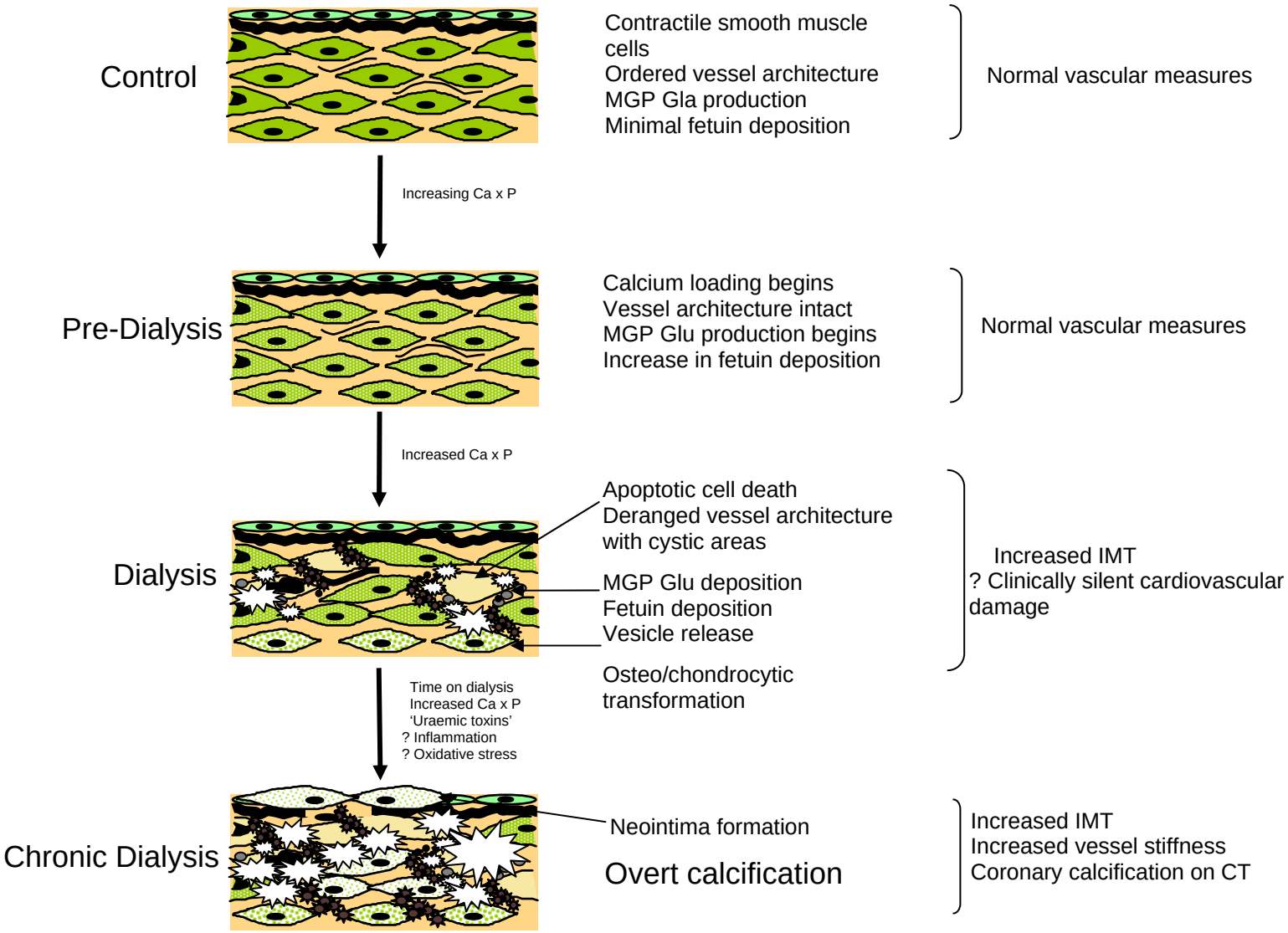
1. An increased Ca load was present from pre-dialysis stages, and correlated with the mean Ca x PO_4 product, stringent measures to control the serum PO_4 levels and also to limit the Ca load to patients from binders and dialysate should be practised.

2. As even a short dialysis vintage was associated with calcification and significant VSMC damage, this study further adds support for avoiding dialysis and performing pre-emptive renal transplantation wherever possible.

3. The currently available clinical measures of vascular damage and calcification (cIMT, PWV and CT scan) are not sensitive enough to detect the early stages of calcification, and a normal / negative test should be interpreted with caution.

The sequence of events that leads to VSMC injury, osteo/chondrocytic transformation and ALK upregulation, as well as the factors that initiate VSMC apoptosis will be crucial to our further understanding of the calcification process.

Figure 7.13 Mechanisms involved in VSMC calcification



Chapter 8

An *In Vitro* Model of Intact Human Vessels to Study the Role of Mineral Ion Induced Vascular Calcification

8.1 Abstract

The mechanisms of early vascular calcification in chronic kidney disease are poorly understood. Our previous study showed that vessels from dialysis patients have higher Ca loads, apoptotic cell death and osteochondrocytic differentiation of VSMCs compared with pre-dialysis or control vessels. I have developed the first *in vitro* model of intact human vessels and used this to explore the role of Ca and P in calcification.

Vessels from healthy controls did not accumulate Ca even in highly calcifying media, whereas pre-dialysis and dialysis vessels showed an increased Ca load in high P and high Ca+P media. Calcification was approximately 10-fold greater in dialysis compared to pre-dialysis vessels in all *in vitro* conditions. In the presence of increased P, even a small increase in Ca significantly increased calcification ($p = 0.02$). Dialysis vessels showed apoptotic cell death in the high Ca+P media, with loss of ~30% of VSMCs. The pan-caspase inhibitor ZVAD reduced apoptosis and inhibited calcification ($p = 0.04$). Both pre-dialysis and dialysis vessels had increased ALK activity associated with an upregulation of the bone transcription factor runx2. The ALK inhibitor levamisole reduced ALK production in the vessels, but was unable to reduce calcification. Electron microscopy revealed extensive mitochondrial calcification in normal vessels incubated in high Ca+P, whereas dialysis vessels showed extensive extracellular calcification associated with vesicle release and no intracellular calcification.

In conclusion, I have shown that both factors specific to the vessel as well as exposure to high Ca-P levels are required to induce calcification in intact vessels. As a result of prolonged exposure to slowly increasing Ca levels in its extracellular milieu, dialysis vessels have potentially developed an adaptive response to extrude

Ca from the cell by a vesicle mediated process, whereas healthy vessels are overwhelmed by a sudden increase in Ca load and undergo mitochondrial calcification and necrotic cell death.

8.2 Introduction

In the previous chapter I have shown that vessels from dialysis patients have significantly greater calcification, and this may, at least in part, be triggered by VSMC apoptosis that is caused by damage inducing agents specific to the dialysis milieu. The Ca load in all the CKD vessels correlated with Ca x P product suggesting that one of the major causes of vascular calcification is dysregulated mineral metabolism. However, the significantly greater apoptosis and osteo/chondrocytic differentiation in dialysis compared to pre-dialysis vessels could not be explained by mineral dysregulation alone.

Studies into the pathophysiology of medial calcification have been hampered by the lack of an appropriate *in vitro* model as cultures of VSMCs lack the matrix and architecture of a normal vessel. In intact vessels it is believed that medial calcification begins at the level of the elastic lamellae *(Vyavahere et al, 1999)*, and circulating macrophages and endothelial-derived stem cells contribute to both the progression and repair of calcification *(Shanahan et al, 2006; Shanahan et al, 2007)*. Moreover, VSMCs in culture undergo a rapid conversion to a proliferative and secretory phenotype, losing their smooth muscle specific properties, making this a poor model for studying the VSMC response to injury.

To address this, I have developed an *in vitro* model of intact human arteries from children, providing a novel and highly versatile tool to study medial calcification in CKD. I have used this model to test the susceptibility of vessels *ex vivo* from dialysis, pre-dialysis and control patients to graded concentrations of Ca and or P to further dissect the mechanisms of VSMC calcification in response to dysregulated mineral metabolism and dialysis.

8.3 Methods

Patients and sample collection

Vessels removed at routine surgery that were used in chapter 7 have been used for the *in vitro* experiments. The methods of sample collection, transport and preparation are described in chapter 2, section III.

Media and in vitro treatments

Vessel rings were incubated in calcifying conditions with graded concentrations of Ca and P to mimic conditions seen in uraemic patients. This is described in detail in chapter 2, section III.

Calcification and Alkaline Phosphatase assays

Vessel rings at baseline and after incubation in control and test media were harvested on days 7, 14 and 21 to measure the calcium content and ALK levels as described in chapter 2, section II and in the previous chapter.

Role of apoptosis and ALK

To determine the contribution of apoptosis to calcification, 100μM of ZVAD.fmk, an inhibitor of caspase cleavage, was added to vessel rings that were cultured in the above calcifying conditions, using more rings from the same vessel as internal controls. Similarly, to study the potential role of alkaline phosphatase (ALK) in the osteogenic differentiation of VSMCs, 10μM levamisole, an inhibitor of ALK, was added to the vessel rings as above.

Vessel histology and Electron Microscopy (EM)

After incubation in *in vitro* conditions, histology for vessel integrity, cell number, calcification and apoptosis were performed using hematoxylin/eosin, von kossa and TUNEL stains respectively as described in chapter 2, section II and in the previous chapter. To examine the extent of osteogenic differentiation of VSMCs after exposure to *in vitro* calcifying conditions, immunohistochemistry was performed for Runx2.

EM was performed to examine cell morphology, localization of calcification, vesicle release and mineral content as described in the previous chapter.

Statistical analyses

Data are presented as mean ± standard deviation or median and range. The paired or unpaired t test was used as appropriate. ANOVA was used for multiple comparisons. Significance was defined as $p < 0.05$. Statistical analyses were performed using SPSS, version 12.0.1 (*SPSS Inc, Chicago, IL, USA*).

8.4 Results

Dialysis vessels undergo time-dependent Ca accumulation in in vitro calcifying conditions

To study the responses of vessels from controls and pre-dialysis patients to calcification inducing conditions, vessel rings were exposed to high Ca and/or P for 7, 14 and 21 days (Figures 8.1A and B). Normal vessels did not increase their Ca load in any of the conditions tested (10.0 ± 2.4 vs 8.4 ± 3.6 vs 11.4 ± 1.6 μg/μL in high P media at 7, 14 and 21 days respectively, p = 0.40 and 11.2 ± 1.9 vs 12.9 ± 3.5 vs 17.0 ± 6.0 μg/μL in high Ca + P media at 7, 14 and 21 days respectively, p = 0.16). Pre-dialysis vessels showed a small increase in Ca load only in high Ca + P media (43.2 ± 13.8 vs 115.1 ± 49.0 vs 170.8 ± 35.5 μg/μL at 7, 14 and 21 days respectively, p = 0.01), but not in high P media alone (20.2 ± 5.8 vs 30.1 ± 11.9 vs 41.7 ± 7.2 μg/μL at 7, 14 and 21 days respectively, p = 0.07). In contrast, dialysis vessels showed progressively increasing Ca loads in all the *in vitro* conditions with the greatest response in the presence of high Ca and P. Ca loading in the dialysis vessels was time-dependent, with maximal calcification at day 21 (176.3 ± 17.9 vs 284.2 ± 23.7 vs 726.7 ± 103.9 μg/μL in high P media at 7, 14 and 21 days respectively, p = 0.07 and 325.7 ± 137 vs 1120 ± 306 vs 1857 ± 390 μg/μL in high Ca + P media at 7, 14 and 21 days respectively, p = 0.0007). At each time-point, the Ca load was significantly higher in dialysis > pre-dialysis > control vessels in comparable *in vitro* conditions.

The Ca load in the vessel rings was further analysed by von Kossa staining (Figure 8.1C). Normal vessels did not show any calcification at baseline or in high P media, but after incubation for 14 days in Ca + P medium, 2 of 6 vessels (33%) developed a patchy rim of punctate calcification along the internal elastic lamina. Similarly, pre-dialysis vessels did not show any von kossa positivity at baseline or in the high P media, but 2 of the 10 (20%) vessels showed patchy von kossa positivity throughout the tunica media when exposed to high Ca and P. On the other hand, dialysis vessels developed histologically overt calcification after incubation in both the high P and Ca + P media, with the most pronounced effect in the Ca + P media: 4 of 24 (16%) vessels showed diffuse punctate calcification in the tunica media on exposure to high P medium and 8 of 24 (33%) vessels showed extensive von kossa positivity, often with large confluent areas of speckled calcification, in the high Ca + P media.

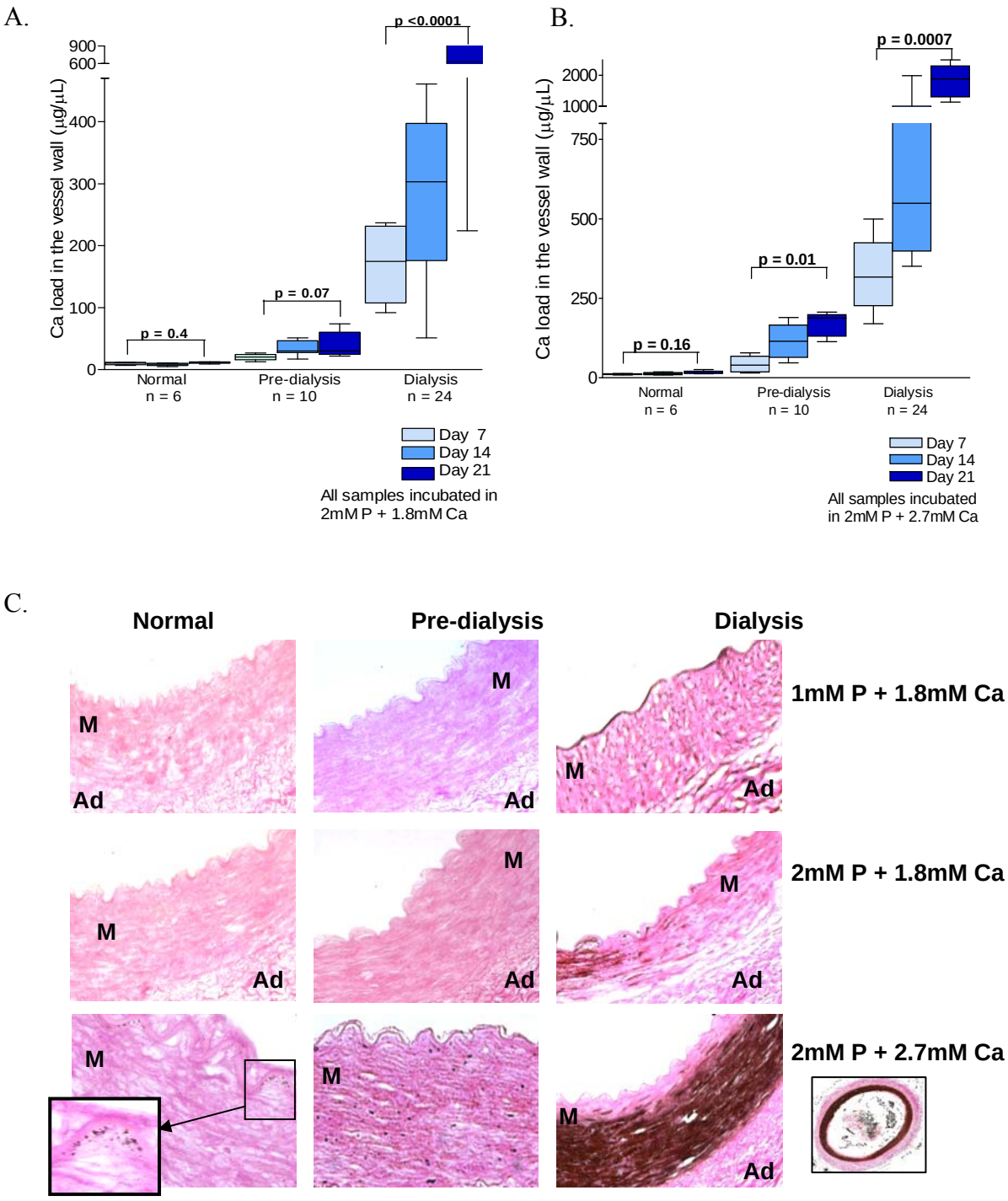


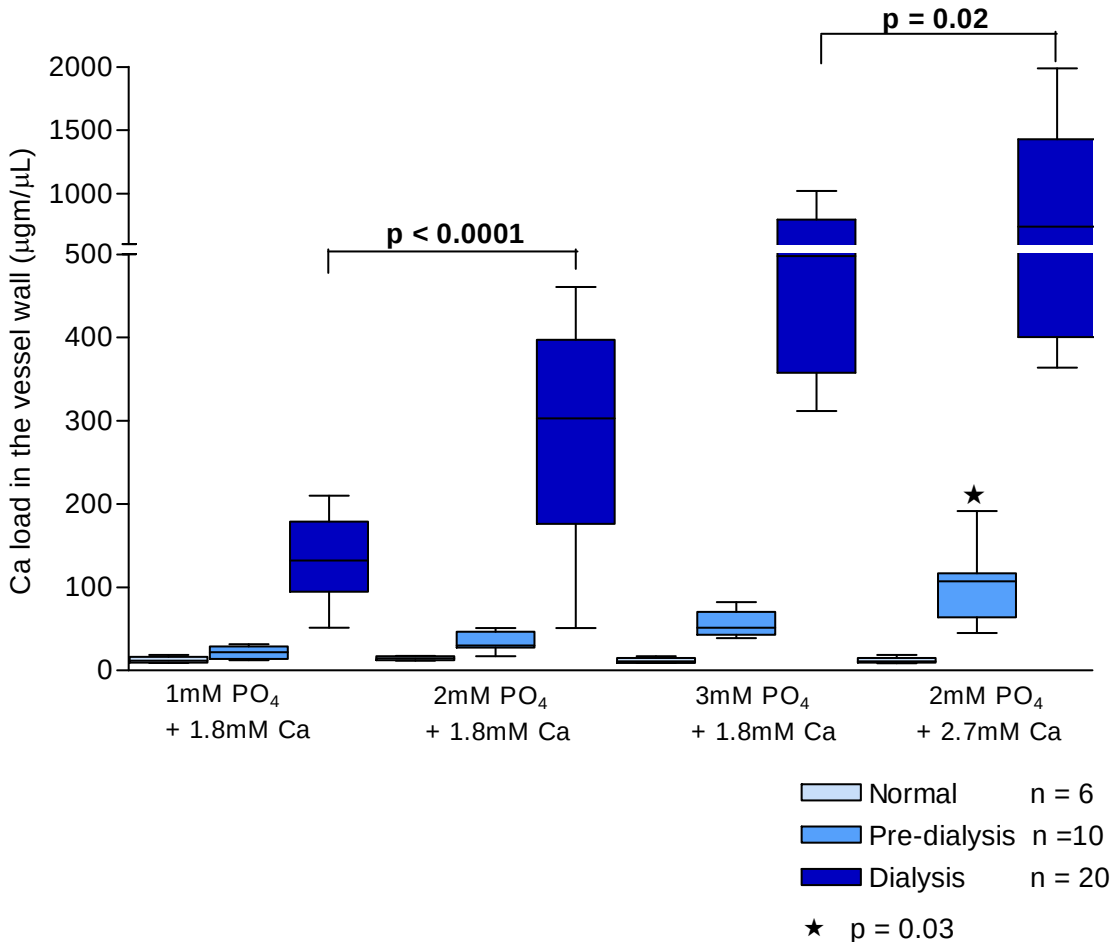
Figure 8.1 Time-dependent increase in vessel Ca load after exposure to calcifying media

Figures A and B – Dialysis and pre-dialysis vessels show a time-dependent increase in Ca accumulation in in vitro calcifying media (2mM P + 1.8mM Ca in figure A and 2mM P + 2.7mM Ca in figure B), whereas no Ca accumulation occurs in the normal control vessels. C – Von kossa staining demonstrates a patchy rim of punctate calcification along the internal elastic lamina in normal vessels after exposure to high Ca + P media, whereas dialysis vessels have developed dense calcification throughout the tunica media.

Ca is a more potent inducer of calcification than P at equivalent Ca x P products

The above studies are in agreement with our previous work showing greater VSMC calcification when VSMCs or vessel rings are exposed to high Ca and P media than in high Ca or high P media alone. To determine the potencies of Ca and P at inducing calcification, we compared media with equivalent Ca x P products (1.8mM Ca + 3mM P vs 2.7mM Ca + 2mM P; Ca x P = 5.4 mM^2 in both media). Dialysis vessels showed approximately ~2-fold greater calcification in the Ca + P medium than in the high P medium alone (1120 ± 115 vs 553 ± 247 μg/μL, p = 0.02; Figure 8.3). Similarly, vessels from healthy controls did not show any increase in Ca load even in the 3mM P or in the high Ca + P media (8.4 ± 3.6 vs 12.9 ± 3.5 vs 11.8 ± 2.9 vs 12.8 ± 4.0 μg/μL, p = 0.3 in 1mM P + 1.8 mM Ca, 2mM P + 1.8 mM Ca, 3mM P + 1.8 mM Ca and 2mM P + 2.7 mM Ca respectively). Pre-dialysis vessels showed a graded increase in calcification in higher P media, but the highest Ca load was found in P + Ca media (19.7 ± 1.1 vs 30.1 ± 11.9 vs 47.9 ± 8.3 vs 115.1 ± 49.0 μg/μL, p = 0.03 in 1mM P + 1.8 mM Ca, 2mM P + 1.8 mM Ca, 3mM P + 1.8 mM Ca and 2mM P + 2.7 mM Ca respectively). These findings confirm our previous data on VSMC cultures showing that in the presence of high P even a small increase in Ca will significantly increase calcification.

Figure 8.2



The effects of Ca and P in inducing calcification

Dialysis vessels are primed for rapid Ca accumulation

Accelerated calcification was observed under all conditions for dialysis vessels ex vivo. However, dialysis vessels generally had higher baseline Ca loads than pre-dialysis vessels, and this might have explained their increased propensity to calcify. To determine if the baseline Ca load influenced further *in vitro* Ca accumulation, the calcification responses of pre-dialysis and dialysis vessels with similar baseline Ca loads were compared (25-35 µg/µL, n = 4 pre-dialysis and n = 6 dialysis

vessels; Table 7.2 from previous chapter). Dialysis vessels accumulated significantly more Ca than pre-dialysis vessels under identical *in vitro* conditions (27.0 ± 3.3, 48.7 ± 13.2 vs 96.8 ± 14.4 μg/μL at baseline and after 14 days of culture in high P and high Ca + P media respectively, p = 0.03 for pre-dialysis vessels and 31.5 ± 2.4, 278.2 ± 105.0 vs 1077.7 ± 657 μg/μL at baseline and after 14 days of culture in high P and high Ca + P media respectively for dialysis vessels, p = 0.0008; Figure 8.3 A). Thus, even with moderate Ca loads calcification was more prevalent in dialysis than pre-dialysis vessels, suggesting that dialysis vessels may have exhausted their protective mechanisms or undergone a phenotypic transformation that encourages rapid Ca accumulation.

However, within the dialysis vessel group there were some vessels that calcified much more aggressively than others. Further analysis demonstrated that dialysis vessels with histologically overt calcification (von kossa positive, n = 6) at baseline had a greater increase in *in vitro* Ca accumulation (44.2 ± 5.1, 370.0 ± 55.1 and 1254 ± 406.2 μg/μL at baseline, and after culture in high P and high Ca + P media respectively, p< 0.001) than dialysis vessels that did not show von kossa positivity (n = 18; (37.5 ± 5.4, 208 ± 78.6 and 476.3 ± 143.7 μg/μL at baseline, and after 14 days of culture in high P and high Ca + P media respectively, p = 0.0005; Figure 8.3 B). This suggests that once a nidus for calcification is formed this can act to accelerate calcification further.

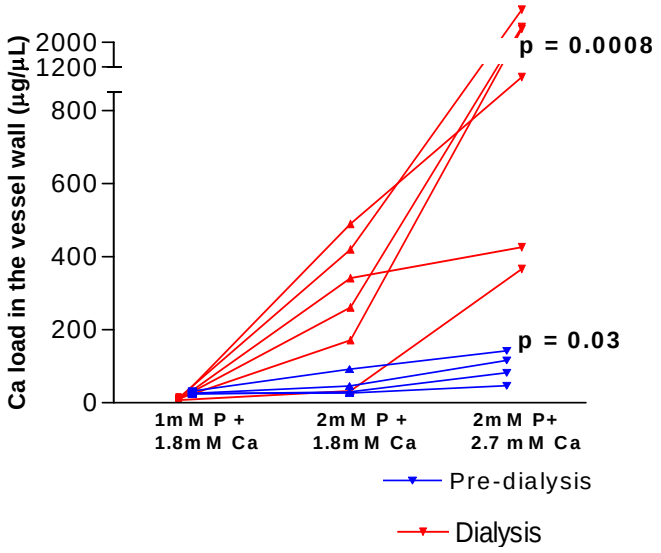


Figure 8.3A Dialysis vessels are primed to undergo rapid Ca accumulation.

Dialysis vessels showed significantly greater Ca accumulation than pre-dialysis vessels with comparable baseline Ca loads after culture in identical calcifying media.

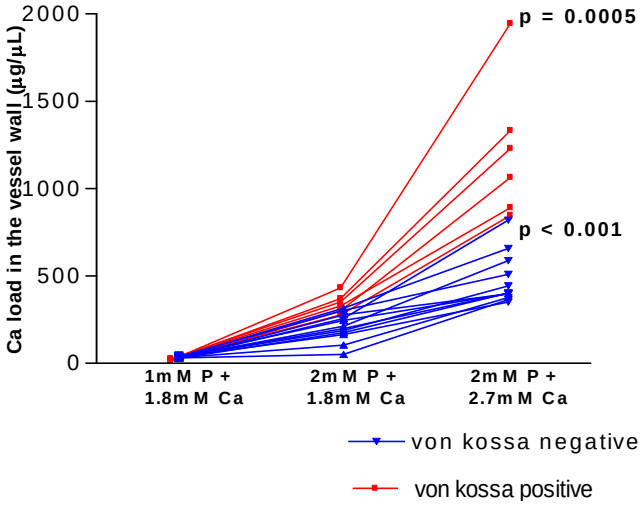


Figure 8.3B Dialysis vessels with baseline von kossa positivity undergo rapid Ca accumulation as compared to dialysis vessels without baseline von kossa positivity.

Dialysis vessels undergo VSMC loss due to apoptotic cell death

We have previously shown that dialysis vessels *in vivo* contain significantly fewer VSMCs than control or pre-dialysis vessels. In response to calcifying conditions there was no significant change in cell number in normal vessels (122 ± 3.9 vs 120 ± 5.8 vs 115 ± 4.7 cells per unit area, p = 0.09, in control, high P and Ca + P media respectively; Figure 8.4 A). In contrast, although both pre-dialysis and dialysis vessels could maintain their cell number on incubation in the control and high P media (118 ± 9.1 vs 118 ± 9.8 cells per unit area for pre-dialysis vessels and 85 ± 17.9 vs 82 ± 14.6 cells per unit area for dialysis vessels respectively), they showed a reduction in VSMC number in the high Ca + P medium (108 ± 7.7 cells per unit area, p = 0.047 for pre-dialysis and 59 ± 10.7 cells per unit area, p = 0.03 for dialysis vessels), and this was most striking in the dialysis vessels. As compared to their *in vivo* cell number, dialysis vessels had a 30% decrease in VSMC number (80 ± 6.2 vs 59 ± 10.7 cells per unit area, p <0.001) in the high Ca + P medium.

The above findings suggest that exposure to increased P alone does not induce cell loss, whereas the addition of increased Ca is a potent stimulus for VSMC loss. To determine whether the *in vivo* Ca load in the vessel or factors specific to the dialysis vessels contributed to a greater cell loss, we again compared pre-dialysis and dialysis vessels with similar *in vivo* Ca loads as described above. There was no change in cell number in the pre-dialysis vessels in any *in vitro* conditions (120.5 ± 8.4 vs 114.3 ± 11.3 vs 107 ± 8.6 cells per unit area, p = 0.16, in control, high P and Ca + P media respectively; Figure 8.4 B), but dialysis vessels showed a significant reduction in cell number (87.8 ± 9.1 vs 76 ± 9.9 vs 54 ± 6.7 cells per unit area, p =

0.003), suggesting that factors specific to dialysis or the loss of defence mechanisms allow for rapid cell loss in dialysis vessels.

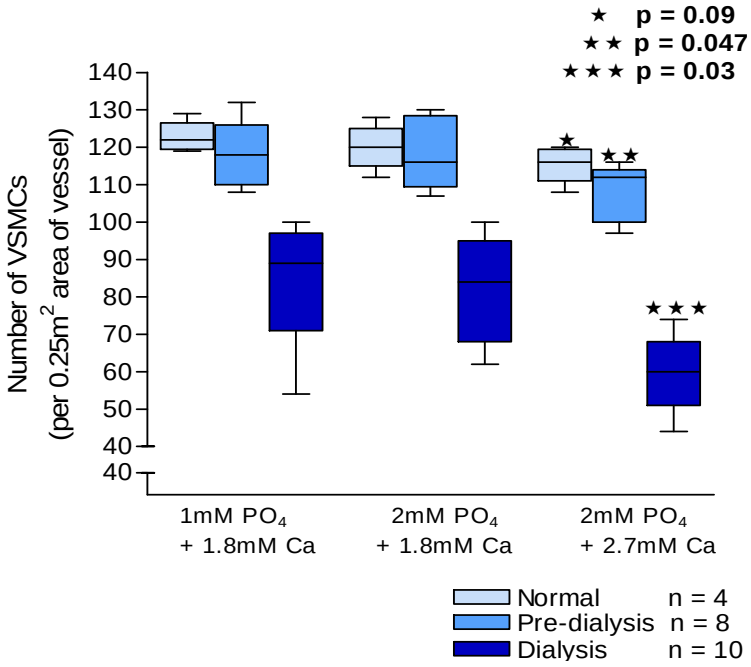


Figure 8.4A VSMC numbers after culture in *in vitro* calcifying media

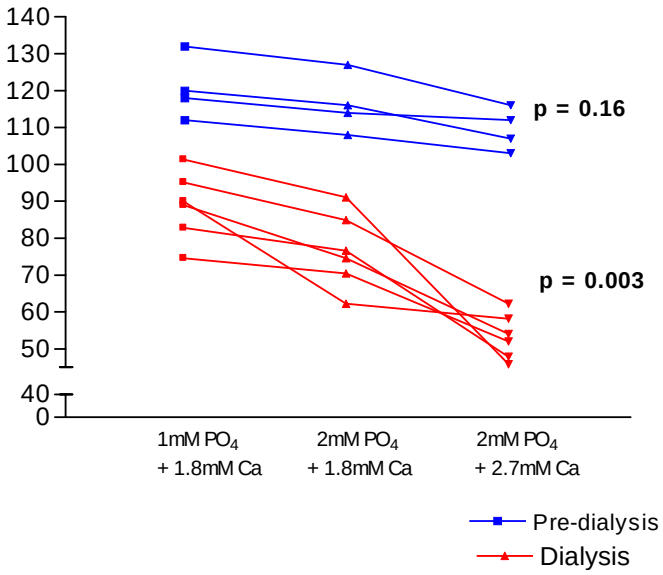


Figure 8.4B There was a significantly greater cell loss in dialysis as compared to pre-dialysis vessels with a similar Ca load when cultured in identical *in vitro* conditions.

The reduction in VSMC numbers was confirmed by α-smooth muscle cell actin staining in all the vessel types (Figure 8.5A, top panel), and TUNEL staining confirmed that apoptosis was contributing to this VSMC loss (Figure 8.5B, bottom panel). After incubation in high Ca + P media the percentage of TUNEL positive areas was significantly higher in dialysis (7.7 [0 – 37.9] %) as compared to pre-dialysis (0.25 [0 – 1.2]%) or normal vessels (0.3 [0 – 1.0]%)).

Also, while dialysis vessels showed TUNEL positivity in all *in vitro* conditions, pre-dialysis and normal vessels did not show any increase in the percentage of TUNEL positive areas after incubation in calcifying media. Moreover, TUNEL positive areas in dialysis vessels co-localised with von kossa positive areas in adjacent sections (Figure 8.5C).

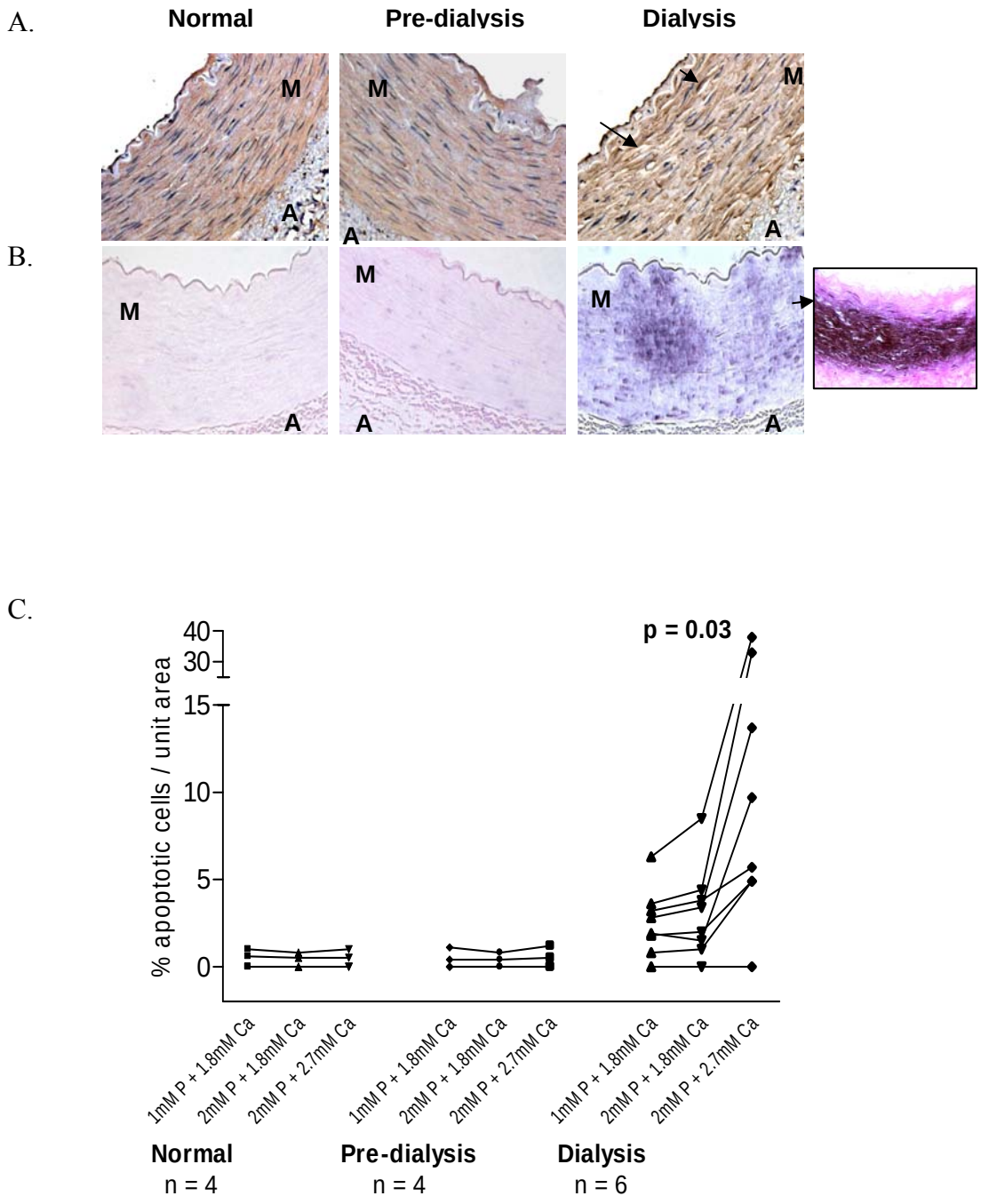


Figure 8.5 Reduction in VSMC numbers is due to apoptosis.

Alpha-smooth muscle cell actin staining shows fewer VSMCs in dialysis vessels (top panel), and this co-localises with areas of apoptosis on TUNEL staining (bottom panel). The number of apoptotic cells per unit area is quantified (figure C).

To experimentally confirm a role for apoptosis in increased calcification of dialysis vessels, these were cultured in the presence of the pan-caspase inhibitor ZVAD.fmk. Dialysis vessels cultured in high Ca + P media that had significant apoptosis were compared with vessel rings from the same patient cultured in high P media that did not show apoptosis. The addition of ZVAD reduced calcification (363.7 ± 28 vs 278 ± 34 μg/μL, p = 0.04); Figure 8.6) in Ca + P treated dialysis vessels that showed an increased rate of apoptosis but had no effect on calcification in vessels treated in high P media alone (279.2 ± 24.1 vs 265.3 ± 30.3 μg/μL, p = 0.12), in keeping with the absence of apoptosis under these conditions.

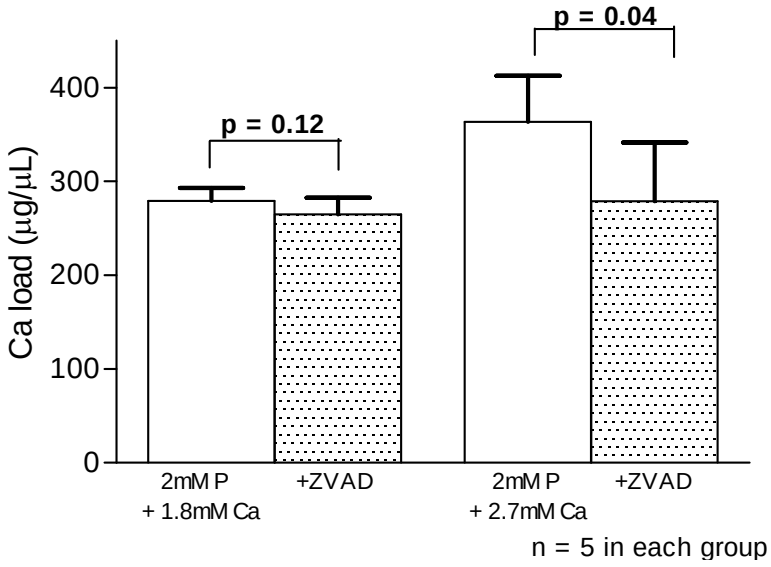


Figure 8.6 Calcification was reduced by inhibiting apoptosis with ZVAD.

Osteogenic conversion of VSMCs occurs in pre-dialysis and dialysis vessels

Osteogenic conversion of VSMCs associated with ALK and Runx2 expression has been associated with increased calcification. In addition, ALK has been shown to decrease pyrophosphate, a natural inhibitor of calcification in rat vessel *ex vivo* studies. ALK activity in human vessels was higher at baseline in dialysis than pre-dialysis or controls. In *in vitro* conditions, normal vessels did not show any increase in ALK in the high P media compared to control medium (5.7 [2.3 – 7.5] vs 4.2 [3.2 – 4.8] IU/μL, p = 0.4; Figure 8.7A), but both pre-dialysis and dialysis vessels showed an ~2-3-fold increase in ALK in the high P medium (14.4 [4.2 – 27.1] and 30.5 [16.0 – 42.1] IU/μL respectively) as compared to control medium (3.9 [2.4 – 15.1] and 15.0 [5.7 – 19.3] IU/μL, p = 0.03 and p = 0.001 for pre-dialysis and dialysis vessels respectively). However, on incubation in high Ca + P medium, ALK was decreased to below baseline in all the vessel types: 2.1 [0.9 – 3.1], 2.2 [1.0 – 2.9] and 5.7 [3.8 – 8.9] IU/μL in normal, pre-dialysis and dialysis vessels respectively. The reduction in ALK activity in P + Ca media was not attributable to VSMC loss, as it was observed in all vessel types.

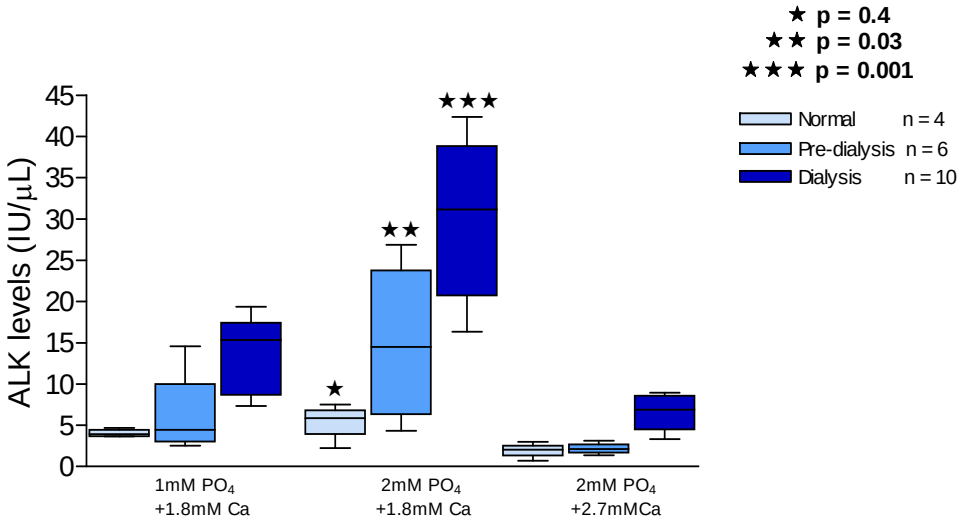


Figure 8.7A Osteogenic conversion of VSMCs in calcifying media

To explore a possible link between ALK levels and calcification, vessel rings from dialysis patients were incubated in high P medium with the addition of levamisole, an inhibitor of ALK activity. The addition of levamisole caused an ~ 50% decrease in ALK levels (31.1 [16.3 – 42.3] vs 19.1 [6.2 – 22.3], p = 0.03). However, despite a reduction in ALK, the vessels incubated with levamisole showed a similar Ca load as those incubated without levamisole (387.2 ± 22 vs 356 ± 37 μg/μL, p = 0.62, Figure 8.7B).

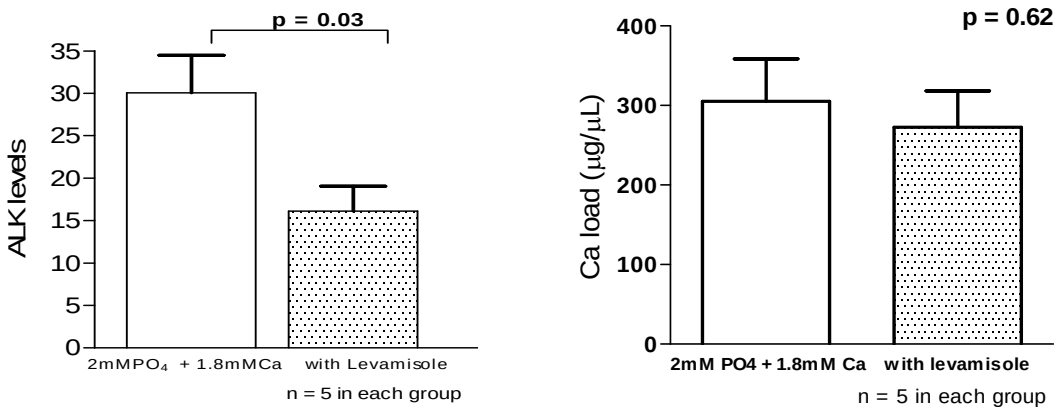
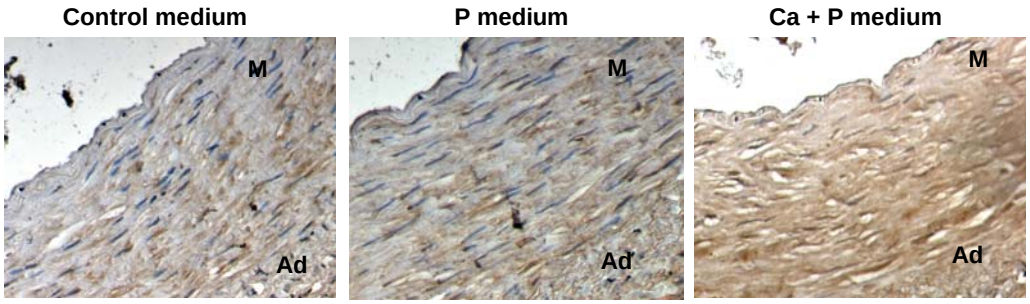


Figure 8.7B Reduction in ALK levels with levamisole is not associated with a reduction in Ca accumulation

Immunohistochemistry for runx2, an osteogenic transcription factor, showed that all vessel types had runx2 positivity after in vitro culture for 14 days in high P media, but this was greatest in the dialysis vessels (figure not shown). Also, the runx2 was predominantly cytoplasmic and concentrated at the media – adventitia interface in all vessels. To examine the effects of the different calcifying media on

runx2 levels, we cultured vessel rings from dialysis patients in the different calcifying media for 14 days. Dialysis vessels cultured in the high Ca + P medium showed the greatest runx2 positivity (the percentage of runx2 positive areas 16.6 ± 4.1, 24.9 ± 7.8 and 61.5 ± 10.9 in control, high P and high Ca + P media respectively, p = 0.002; Figure 8.7D and E). Of note, runx2 positivity was greater in the vessels in high Ca + P medium compared to high P medium alone, but ALK levels were significantly lower in the high Ca + P medium.

D.



E.

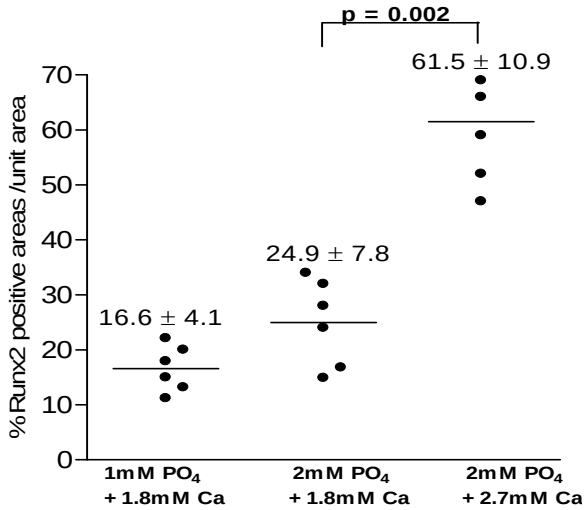


Figure 8.7D and E Immunohistochemistry for runx2 shows maximum positivity in dialysis vessels.

Calcification in intact vessels is a vesicle mediated process

Our ex vivo data showed that calcification in the dialysis vessels was associated with increased annexin VI positivity, suggesting a vesicle-mediated calcification process. To determine if the increased in vitro calcification in dialysis vessels was associated with greater vesicle release, we performed immunohistochemistry for annexin VI in dialysis vessels cultured in calcifying media for 14 days. Annexin VI positivity was seen in all the *in vitro* conditions, predominantly distributed along the elastic lamellae, and was greatest in the high Ca + P medium (Figure 8.8).

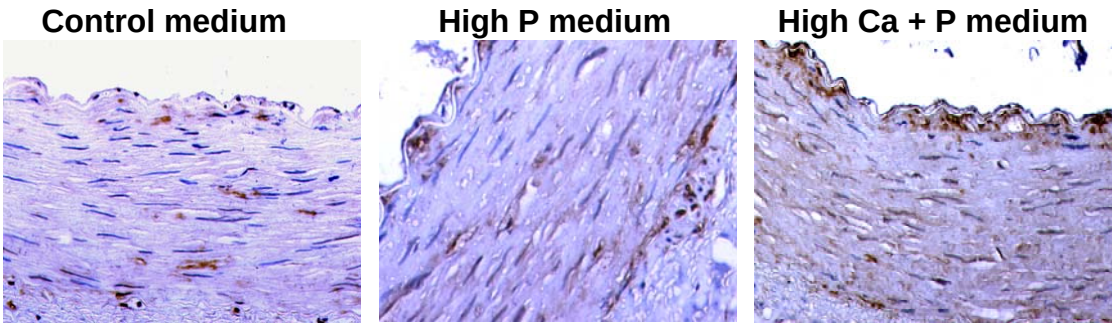


Figure 8.8 Annexin VI positivity in dialysis vessels.

EM was used to investigate mechanisms of calcification at the cellular level. Remarkable differences in the localization and mechanisms of calcification were observed between normal and dialysis arteries (Figure 8.9). In control arteries cultured in P media there was very little evidence of calcification in terms of increased Ca load or von kossa positivity. However, there was an increase in nuclear heterochromatin as well as evidence of cell death in a subset of nuclei and associated with membraneous debris. Moreover, in Ca +P media calcification was seen within the mitochondria of VSMCs of control vessels. In contrast, in dialysis

vessels treated with Ca + P media there was evidence of extensive extracellular calcification and vesicle release but no intracellular calcification. The nuclei were generally normal in appearance as were the mitochondria. Calcification was extensive in some areas and clearly associated with the extracellular release of membranous vesicles some of which had crystalline apatite present.

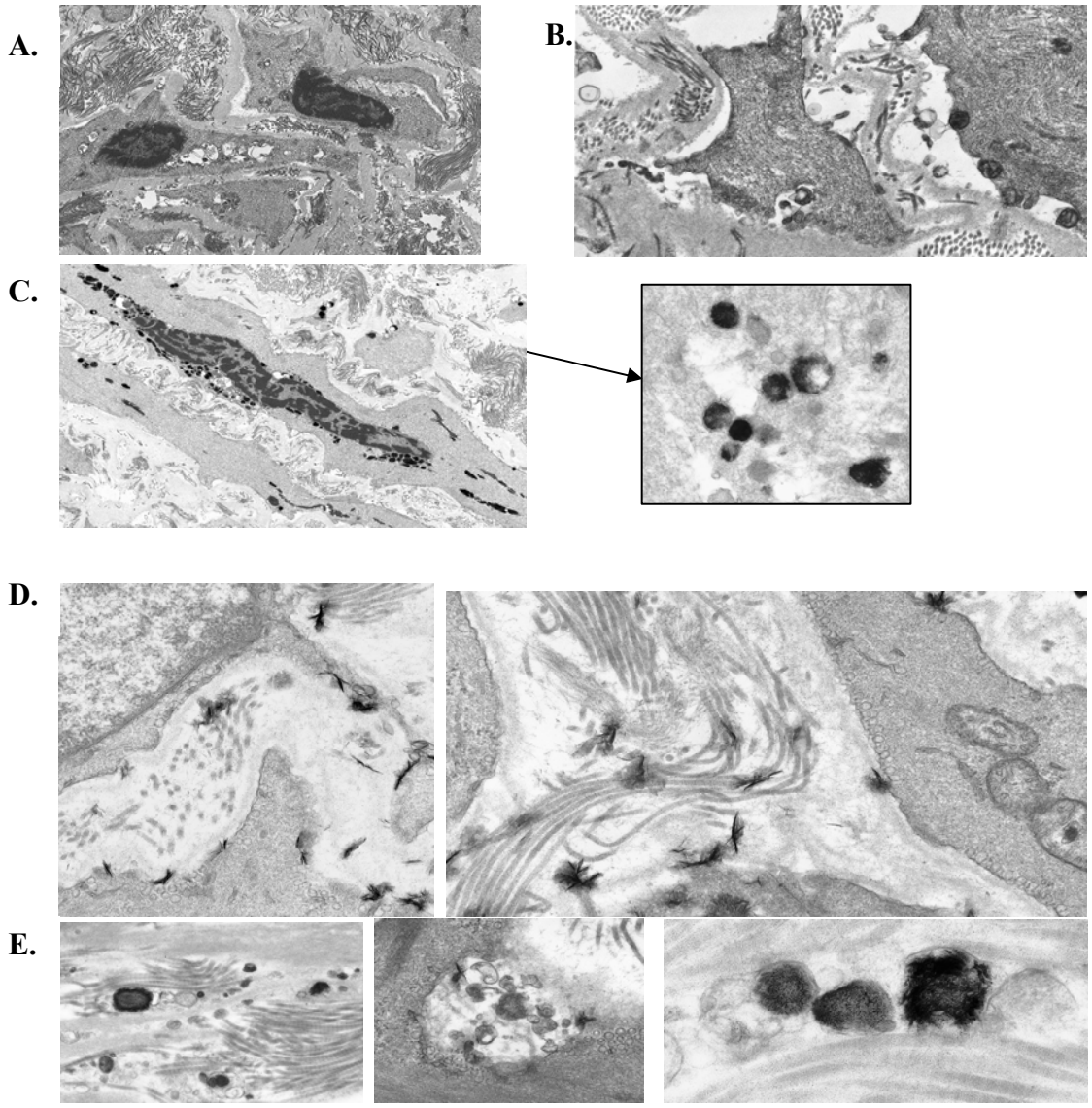


Figure 8.9 Mitochondrial vs vesicular calcification in control and dialysis vessels

Figure 8.9 A, B and C Control vessel in high Ca + P medium showing dense heterochromatic nuclei not typical of apoptosis. Figure C shows mitochondrial calcification associated with necrosis
D and E Dialysis vessel in high Ca + P medium showing vesicular calcification with hydroxyapatite crystals in the extracellular matrix.

8.4 Discussion

This is the first time that mechanisms involved in vascular calcification have been demonstrated in intact human vessels. This study shows that factors specific to the vessel as well as exposure to high Ca-P levels are required to induce calcification. Dialysis vessels undergo calcification through a vesicle mediated process by mechanisms involving apoptotic cell death and osteo/chondrocytic differentiation, and this may be an adaptive response of the VSMC to prolonged exposure to progressively increasing Ca levels. In contrast, vessels from healthy controls could not be induced to calcify even on prolonged incubation in highly calcifying media, but nevertheless showed extensive mitochondrial calcification on electron microscopy, suggesting that these vessels have not been able to undergo adaptive changes to a high Ca-P environment and have undergone a necrotic response *in vitro*.

Previous *in vitro* studies have used VSMC cultures, and while these models of calcification have provided important mechanistic insights into the pathophysiology of calcification, they cannot explain the complex cellular interactions that take place in intact vessels. Vessels from children provide an ideal model to study uraemic influences on the arterial wall as they are not subjected to confounding pro-atherosclerotic risk factors that are prevalent in adults with CKD. Moreover, vessels from adults are unlikely to lend themselves to *in vitro* studies as the perfusion and viability of the considerably larger adult vessel in culture medium may not be possible.

Pre-dialysis and dialysis vessels are 'primed' to calcify

In this study vessels from healthy controls did not calcify even after long-term culture at supraphysiological levels of Ca + P, up to or exceeding levels seen in uraemic patients. This suggests that exposure to very high Ca + P alone is not sufficient to produce medial calcification. Indeed, medial calcification is commonly seen in diabetes *(Everhart et al, 1988)* and aging, and in several genetic defects *(Bucay et al, 1998; Luo et al, 1997; Rutch et al, 2003)* in the presence of normal Ca and P levels. The only other study that has looked at intact vessels *in vitro* has shown similar findings: vessel rings from non-uraemic rats could only be induced to calcify by the addition of ALK to the culture medium *(Lomashvili et al, 2006)*, suggesting that the presence of physiological inhibitors of calcification prevent healthy vessels from calcifying. On the other hand, pre-dialysis and dialysis vessels calcified in the presence of increased P or increased Ca + P, suggesting that these vessels have reduced levels or perturbation of calcification inhibitors, or have undergone a phenotypic change that predisposes them to calcify. When dialysis and pre-dialysis vessels with a similar in vivo Ca load were compared, dialysis vessels had greater Ca accumulation under identical *in vitro* conditions, implying that factors other than mineral homeostasis were involved. In all *in vitro* conditions, dialysis vessels had an ~ 10-fold increased Ca load compared to pre-dialysis vessels under similar conditions, implying significantly greater VSMC damage or that factors specific to the dialysis milieu had 'primed' these vessels for further calcification.

Ca is a more potent stimulus to induce calcification than P

In our previous in vitro studies using human VSMC explants we have shown that Ca and P have a synergistic effect on inducing calcification, such that in the presence of increased P, even a small increase in Ca could substantially increase calcification *(Reynolds et al, 2004)*. This study provides direct evidence for the first time that when intact vessels are exposed to increased Ca, calcification is increased. For a fixed Ca x P product the increase in serum Ca was a more potent stimulus to induce calcification than an increase in serum P. This finding has also been confirmed in cultures of rat aortic vessels rings *(Lomashvili et al, 2006)*. This finding has important clinical implications, as the changes in Ca levels in our study were within the range seen in haemodialysis patients. A study has shown that the transient increase in serum Ca levels after haemodialysis are correlated with the rate of progression of coronary calcification *(Yamada et al, 2007)*. The cyclical changes in serum Ca levels (that do not occur with peritoneal dialysis) may play an important role in the frequent development of vascular calcification in haemodialysis patients.

Elevated Ca and P in the culture medium have pro-mineralising effects that extend beyond simply raising the Ca x P product, and regulate signalling systems in VSMCs that promote a susceptibility to matrix mineralization *(Jono et al, 2000; Giachelli et al, 2001; Giachelli et al, 2003)*. Extracellular P is taken up by the cell by the sodium-dependent phosphate transporter (Pit-1), and increases intracellular P, that in turn upregulates bone-regulatory proteins Runx2 and osteocalcin, and downregulates smooth muscle lineage markers *(Giachelli et al, 2001; Giachelli et al, 2003)*. Prolonged exposure of cultured VSMCs to elevated Ca induces Pit-1 mRNA levels, so that Ca levels can regulate the P sensitivity of VSMCs. Our own work has shown

that Ca stimulated the release of mineralization-competent vesicles from human VSMCs *(Reynolds et al, 2004)*. Moreover, vesicles released in the presence of elevated Ca and P are more likely to be 'mineralization competent' and nucleate preformed crystalline apatite that can rapidly proliferate on a permissive vascular matrix *(Reynolds et al, 2004)*.

Extracellular Ca induces apoptosis that in turn accelerates calcification

Our previous in vitro work has shown that apoptosis precedes calcification, and that apoptotic bodies are capable of initiating vascular calcification *(Proudfoot et al, 2000)*. Furthermore, we have shown that intact vessels from dialysis patients have undergone VSMC loss as a result of apoptotic cell death *in vivo*, but the cause of this remained unclear. In this study we found that *in vitro* exposure to increased Ca and / or P alone was not sufficient to cause apoptosis, as normal vessels did not undergo any apoptotic cell death even in highly calcifying media, whereas dialysis vessels lost ~30% of the VSMCs from apoptosis in high Ca + P media. Apoptosis increases the local concentration of Ca to >30mM, and this potentially induces further VSMC death, vesicle release and calcification *(Olszak et al, 2000)* Also, it may be possible that after induction of calcification only healthy/resistant cells survive.

To test if apoptosis was actually required for calcification to occur in intact vessels, we inhibited apoptosis with the pan-caspase inhibitor ZVAD. Ca quantitation and von Kossa staining both confirmed reduced calcification, and areas of calcification co-localised with areas of apoptosis. Caspase inhibition can also inhibit the release of apoptotic bodies from cells *(Kim et al, 1995; Zhang et al, 1999)* , and inefficient

phagocytosis of these in dialysis vessels may be another mechanism of calcification *(Shanahan et al, 2006)* .

Upregulation of ALK occurs in high P media

A study using intact rat aortic rings in culture has shown that medial calcification can be induced by causing acute mechanical injury to the vessel *(Lomashvili et al, 2004)*, and increased ALK production that inhibited pyrophosphate was shown to lead to calcification. Although CKD, and in particular dialysis, causes mechanical and chemical damage to the vessels, this is a slow, chronic process that is distinct from the acute mechanical injury caused to the vessels in the above studies, and animal experiments have shown that mechanical injury *per se* can induce calcification *(Gadeau et al, 2001)*.

Although ALK levels increase in uraemic and injured vessels, a definitive link between raised ALK levels and vascular calcification has not been made. In our previous work we have shown that vessels from pre-dialysis and dialysis patients, that were documented to have prolonged exposure to high Ca and / or P in vivo, had an upregulation of ALK, but this did not correlate with the Ca load in the vessel wall. Similarly, when vessels from uraemic rats are cultured in high Ca and / or P media, they have increased production of ALK *(Lomashvili et al, 2004)*, but the correlation, if any, with calcification has not been explored. Tissue non-specific ALK is upregulated during phenotypic transdifferentiation of VSMCs into osteogenic cells induced by β-glycerophosphate, which in turn is linked with the downregulation of smooth muscle lineage markers *(Steitz et al, 2001)*. In this study we found that vessels from healthy controls were unable to increase ALK

production even in highly calcifying media, whereas both pre-dialysis and dialysis vessels showed a significant increase in ALK, suggesting that these vessels have been 'primed' in vivo, possibly as a result of uraemic vascular damage. Also, it is known that P induces osteoblastic differentiation factors such as runx2 and osteocalcin *(Giachelli et al, 2003; Moe et al, 2003)* , and ALK has been found in calcified vessels of patients, colocalising with these transcription factors *(Doherty et al, 2004)* . However, after incubation in high Ca + P media wherein significant VSMC apoptosis was seen, ALK levels were significantly lower in all vessel types, but were nevertheless associated with markedly greater calcification. Finally, although levamisole was able to inhibit ALK, this did not translate to a reduction in calcification.

Pyrophosphate is a circulating calcification inhibitor that binds to nascent hydroxyapatite crystals and prevents further incorporation of inorganic P into these *(Addison et al, 2007)* . ALK has dual functions, acting to catalyse the hydrolysis of phosphomonoesterases with the release of P as well as acting as pyrophosphatases to hydrolyse pyrophosphate *(Schoppet et al, 2008)*. Pyrophosphate hydrolysis by ALK allows for regulated extracellular matrix mineralization as seen in bones *(Addison et al, 2007; Moreno et al, 1987)*, and on prolonged exposure to high circulating Ca and P in uraemic patients, as osteo/chondrocytic differentiation of VSMCs occurs, they may adapt and produce increased ALK. In osteoblasts, hydrolysis of pyrophosphate by ALK produces additional inorganic P *(Moss et al, 1967)* that is potentially available for hydroxyapatite mineral deposition. Pyrophosphate not only inhibits hydroxyapatite crystal growth *(Moreno et al, 1987)* but also upregulates osteopontin production , and downregulates tissue non-

specific ALK *(Addison et al, 2007)* to regulate mineralization. Similarly, in VSMCs ALK production may regulate the fine balance between pyrophosphate – inorganic P levels, but exposure to very high Ca or P or when there is insufficient time for the VSMCs to adapt to their milieu and upregulate ALK production, overwhelming calcification occurs that can potentially result in cell death. Thus, it is unclear if ALK production by the VSMC is an adaptive response, or whether it perpetuates a vicious cycle of pyrophosphate degradation and accelerated calcification.

Different mechanisms of calcification in normal and dialysis vessels

The different mechanism of calcification in dialysis and control vessels is an exciting new finding that needs further exploration. Mitochondrial calcification suggests that on acute exposure to high Ca and P levels, the cell is unable to 'contain' these crystals within vesicles and extrude them from the cell, resulting in necrotic cell death. On the other hand, when the cell is exposed to increasing concentrations of Ca and / or P over a prolonged period, the cell has a chance to adapt to its changing milieu and can extrude the Ca/P crystals into the extracellular matrix in order to ensure its survival. Thus vesicle release by dialysis vessels may be an adaptive response of the dialysis vessels.

8.5 Conclusions

In conclusion, I have shown that both factors specific to the vessel as well as exposure to high Ca-P levels are required to induce calcification in intact vessels. As a result of prolonged exposure to slowly increasing Ca levels in its extracellular milieu, dialysis vessels have developed an adaptive response to extrude Ca from

the cell by a vesicle mediated process but they are also more susceptible to apoptosis, whereas healthy vessels are overwhelmed by a sudden increase in Ca load and eventually undergo mitochondrial calcification and necrotic cell death *in vitro*.

Chapter 9

Conclusions and Future Directions

The translational research in this thesis has shown some important associations between modifiable risk factors and vascular damage and calcification that may translate to improved care of patients with CKD-MBD. My key findings are:

1. Children on dialysis in whom the mean PTH levels are within twice the upper limit of the normal range have less vacular damage, more compliant vessels and less coronary calcification than those with PTH levels above twice the upper limit of normal. In fact, the group of children with PTH levels less than twice upper limit of normal have carotid IMT and aortic PWV that was comparable to an age-matched control group. Also, the significantly lower cardiovascular mortality amongst our patients as compared to the published data on paediatric dialysis populations may be attributable to a strict control of PTH levels from the earliest stages of CKD through to dialysis.

2. Children on dialysis who have normal $1,25(OH)_2D$ levels have less vascular damage and calcification than those with either low or high levels. Moreover, children with low $1,25(OH)_2D$ levels had high levels of hs-CRP, suggesting that the beneficial effects of $1,25(OH)_2D$ extends beyond its role in the hormonal regulation of Ca – PO_4 – PTH and involves a significant anti-inflammatory effect. In this study, there was a poor correlation between $1,25(OH)_2D$ levels and the dose of alphacalcidol, suggesting that optimal vascular protective strategies should include careful monitoring of the vitamin D levels.

3. The physiological calcification inhibitors, in particular fetuin-A, showed a strong correlation with vascular stiffness and calcification, suggesting that it

may prove to be a useful indicator of an individual patient's susceptibility to calcify. Also, the observation that there may be a protective upregulation of fetuin-A in the earliest stages of exposure to a pro-calcific and pro-inflammatory uraemic milieu requires further exploration. Fetuin-A's role in inhibiting apoptosis and inducing phagocytosis offers intriguing possibilities of its use as a future therapeutic agent.

4. The correlation of vascular measures against vessel histology has provided some very exciting findings. This study has shown direct evidence of calcium accumulation even in pre-dialysis patients, with significantly greater calcium loads in dialysis vessels. Our currently available vascular measures, including multi-slice CT scan, are not sensitive enough to detect the earliest stages of calcification, possibly leading to a false reassurance in patients with negative scans. Most importantly, calcification was inexorable and extremely rapid on dialysis, with a dialysis vintage of even 2 months sufficient to induce histologically overt calcification and VSMC damage, emphasising the need to avoid dialysis and perform pre-emptive renal transplantation wherever possible.

5. The vessel Ca load in all CKD (pre-dialysis and dialysis) vessels strongly correlated with the mean time-averaged Ca x PO_4 product, suggesting that mineral dysregulation is central to ectopic vascular calcification. Furthermore, in the *in vitro* studies I found that in the presence of increased PO_4 even a small increase in Ca could significantly increase calcification. In the dialysis patient transient increases in serum Ca levels are frequently seen after haemodialysis or

with medications, and while these may escape detection on routine blood tests, they may contribute to an increased risk of calcification.

6. Vessels from dialysis patients show vascular smooth muscle cell loss that is a result of apoptotic cell death. Apoptosis, along with osteogenic transformation of VSMCs and loss of the protective calcification inhibitors, may trigger accelerated calcification in these vessels. From my studies it appears that factors other than the Ca load in the vessel induce apoptosis. The identification of factors specific to dialysis that trigger the accelerated wave of VSMC death will be key in minimizing the detrimental effects of arterial calcification in renal failure.

7. The role of alkaline phosphatase in vascular calcification requires further study. Although osteochondrocytic transformation of VSMCs is alleged to play a role in accelerating calcification, my studies have shown that it may in fact be protective. Osteochondrocytic transformation may allow for some form of regularized and controlled mineralisation that protects the cell from overwhelming calcification and cell death. Further mechanistic insights into this process are required.

8. In the *in vitro* studies I have found a consistent and highly significant increase in calcification in the dialysis vessels that is time-dependent and is a graded response to the Ca and P in the culture media. I have shown that chronic exposure to high Ca and P leads to adaptive changes in CKD vessels that manifests as low-grade Ca accumulation, but dialysis overwhelms these

adaptive mechanisms, possibly through VSMC apoptosis and vesicle release, leading to accelerated calcification.

The above associations between various potential risk factors and vascular damage are drawn from cross-sectional observational studies and cannot be taken to imply causality. The complex interplay of these factors at different stages of uraemia is poorly understood. Whether the various factors truly contribute to the calcification process, or are innocent bystanders that simply behave as markers of vascular damage also needs to be elucidated. Therefore, my studies can at best serve as hypothesis-generating work that paves the way for a future prospective longitudinal study in a much larger cohort of paediatric CKD patients.

I have designed the following studies to continue my work in this field, and have already obtained funding and ethical approval for some.

Clinical Studies

1. Prospective long-term monitoring of cardiovascular disease risk factors and measurement of surrogate markers of vascular damage in children with CKD. Data on clinical and biochemical parameters, medication dosage and annual vascular scans will be performed starting from CKD stage III through dialysis to transplantation. Additional blood tests will include 25(OH)D and $1,25(OH)_2D$ levels, fetuin-A levels, hs-CRP, FGF-23 and genetic studies. We have obtained ethical approval to recall our original patient cohort for repeat vascular scans including the multi-slice CT scan.

2. Given the multiple beneficial effects of vitamin D on cardiovascular health and the survival advantage from vitamin D therapy that has been demonstrated in numerous studies, I have focussed my attention on future prospective studies on the role of vitamin D in cardiovascular health. My study has demonstrated that there is a narrow therapeutic window for vitamin D analogues on vascular health, and we would like to extend this work further by prospectively monitoring vitamin D [25(OH)D and $1,25(OH)_2D$] levels in all CKD 5 and dialysis patients. Also, the role of PTH as a marker of bone health has been questioned. We propose that monitoring patients on their vitamin D levels rather than their PTH levels will correlate with a better cardiovascular outcome.

In a cohort of chronic dialysis patients at Great Ormond Street Hospital we will measure regular vitamin D [25(OH)D and $1,25(OH)_2D$] levels and titrate the alphacalcidol dosage so as to keep $1,25(OH)_2D$ levels in the normal range. We will perform 6-monthly cIMT and PWV measures and annual CT scans on these patients. An initial pilot study is required to assess the feasibility of this protocol in achieving adequate Ca, P and PTH control and also to assess the rate of progression of vascular abnormalities in children (from the study described in 1). We have obtained ethical approval and funding and will begin this work shortly. If feasible, this pilot study will lead on to a prospective randomized controlled study across several Paediatric Nephrology units in the UK. Titrating medications against the vitamin D level as opposed to the current regimen where PTH is the key target will lead to a paradigm shift in out management of mineral dysregulation in CKD.

3. I have shown that high PTH is associated with increased cIMT, stiffer vessels and a higher prevalence of calcification. Hyperparathyroidism begins early in CKD when the eGFR is 40 – 45 ml/min/1.73m^2 *(Levin et al, 2006)* and is associated with low 25(OH)D and 1,25$(OH)_2$D levels. If patients who are 25(OH)D deficient were supplemented with vitamin D (ergocalciferol or cholecalciferol) early in the course of their CKD and before hyperparathyroidism becomes manifest, this may increase their time to development of secondary hyperparathyroidism, and protect them from the ensuing mineral dysregulation. We will set up a randomised double-blinded placebo controlled study to determine the effects of ergocalciferol in delaying the onset of secondary hyperparathyroidism in children in early CKD. We have ethical approval and funding to begin this study at Great Ormond Street, and are applying for funding in order to extend it to other Paediatric centres.

Laboratory Studies

The *in vitro* model of VSMC calcification is a novel and highly versatile tool, and is the best model to study accelerated calcification that is available to researchers. Neither VSMC explants nor animal studies can mimic the chronic changes in the vessel wall induced by the uraemic milieu and dialysis. This model will be an invaluable tool in further studies, and we have planned future experiments as detailed below.

1. Although vitamin D analogues are shown to increase Ca uptake into smooth muscle cells, they also have vasculoprotective properties by reducing smooth muscle cell proliferation and have anti-inflammatory and immunomodulatory properties. Also, the newer vitamin D analogues, paricalcitol and doxercalciferol, are thought to be less calcaemic than calcitriol. Studies have been performed on bovine and murine smooth muscle cell explants and given conflicting results.

   I have performed some preliminary studies using alphacalcidol and calcitriol in *in vitro* cultures of intact human arteries and have found that calcitriol causes significantly greater Ca uptake and also ALK upregulation than alphacalcidol under similar conditions. Furthermore, calcitriol but not alphacalcidol causes increased Ca uptake even in pre-dialysis vessels. Studies in this field are ongoing.

2. The role of oxidative stress and inflammation in the calcification pathway have been discussed in many clinical studies. From some preliminary data I have found that dialysis vessels have significantly more oxidative DNA damage [8-oxo-7,8-dihydro-2'-deoxyguanosine positivity] as compared to healthy controls, and after exposure to calcifying media virtually all nuclei in dialysis vessels show evidence of DNA damage whereas VSMCs from normal vessels remained intact. I will extend this work to study other markers and mechanisms of oxidative damage, as it is clear that VSMC damage is central to the calcification process.

Through long-term prospective clinical and *in vitro* studies that explore the mechanisms of vascular damage, I will continue my work in the field of cardiovascular disease in chronic kidney disease with the ultimate aim of modifying the evolution of cardiovascular disease in chronic kidney disease.

APPENDIX A

LIST of PUBLICATIONS

Original Research Articles

1. R Shroff, R McNair, N Figg, A Gupta, J Skeppers, L Schurgers, A Donald, M Hiorns, J Deanfield, L Rees, C Shanahan. Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis. **Circulation**. 2008;118(17):1748-57.

2. R Shroff, V Shah, M Schoppet, L Hofbauer, G Hawa, L Schurgers, A Singhal, I Merryweather, P Brogan, C Shanahan, J Deanfield, L Rees. The circulating calcification inhibitors, fetuin-A and osteoprotegerin, but not Matrix Gla protein, are associated with vascular stiffness and calcification in children on dialysis. **Nephrology Dialysis and Transplantation.** 2008;23(10):3263-71.

3. R Shroff, M Egerton, M Bridel, V Shah, A Donald, T Cole, M Hiorns, J Deanfield, L Rees. A bimodal association of vitamin D levels and vascular disease in children on dialysis. **Journal of the American Society of Nephrology.** 2008 (19):1239-1246.

4. S Waller, R Shroff, A Freemont, L Rees. Bone histomorphometry in children prior to commencing renal replacement therapy. **Pediatric Nephrology**. 2008;23:1523-1529.

5. G Marhaug, V Shah, R Shroff, H Varsani, L Wedderburn, C Pilkington, P Brogan. Age-dependent inhibition of ectopic calcification: a possible role for fetuin-A and osteopontin in patients with juvenile dermatomyositis with calcinosis. **Rheumatology**. 2008 (47):1031-37

6. R Shroff, A Donald, M Hiorns, A Watson, S Feather, D Milford, E Ellins, C Storry, D Ridout, J Deanfield, L Rees. Mineral metabolism and vascular damage in children on dialysis. **Journal of the American Society of Nephrology** 2007(18):2996-3003.

7. R Shroff, R Trompeter, C Hutchinson, L Rees, S Ledermann. The long-term outcome of peritoneal and haemodialysis in children with chronic renal failure. **Pediatric Nephrology** 2006 (21):257-264.

Refereed Review Articles

8. R Shroff, C Shanahan. The Vascular Biology of Calcification. Invited review for **Seminars in Dialysis** 2007;20(2):103-109

9. M Schoppet, R Shroff, L Hofbauer, C Shanahan. Exploring the Biology of Vascular Calcification in Chronic Kidney Disease: What's Circulating? **Kidney International**. 2007; Nov 28 [E-pub available]

APPENDIX B

GRANTS OBTAINED

I have been awarded a total of £280,000 from 4 research grants and 2 travel bursaries over the last 3 years. I have contributed significantly to writing my grant applications and presenting my work to grant bodies to secure this funding.

1. **British Association for Paediatric Nephrology -** Travel bursary for Dr R Shroff for oral and poster presentations at the American Society of Nephrology in November 2007.
Amount - £1000

2. **British Heart Foundation** - Dr R Shroff, Clinical Research Training Fellowship.
An *in vitro* model of intact human arteries to study the mechanisms of vascular calcification in chronic kidney disease: clinical and laboratory correlation.
Co-applicants – Dr Lesley Rees, Professor Catherine Shanahan and Professor John Deanfield.
Amount - £138,500 for 24 months.

3. **Eyck and Strutt scholarship (from the British Medical Association)**
An investigation into the presence and extent of coronary calcification in children with end stage renal failure (ESRF) and the role of hyperparathyroidism in these abnormalities.
PI – Dr R Shroff. Co-applicant – Dr L Rees.
Amount - £10,000 for cardiac CT scans.

4. **Kidney Research UK (KRUK)** – Project grant for named student Dr R Shroff.
The effects of calcium, phosphate and parathyroid hormone regulation on vascular smooth muscle cell calcification in children with End Stage Renal Failure.
Applicants – Dr Lesley Rees, Professor Deanfield and Professor Shanahan.
Amount - £65,800 for 12 months.

5. **Kidney Research Aid Fund (KRAF)** - Research Affiliate Studentship for Dr R Shroff
An investigation into the presence and extent of early markers of vascular calcification in children with chronic renal failure.
Co-applicants – Dr Lesley Rees and Professor John Deanfield.
Amount - £64,000 for 12 months.

6. **The Royal Society of Medicine, Section of Nephrology** – Travel bursary for Dr R Shroff for a presentation at the American Society of Nephrology in November 2006.
Amount - £750

Appendix C

Ethics Committee Approval Documents

Reference List

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