# The Role of Calcification Regulatory Proteins in the Arterial Stiffening of Chronic Kidney Disease Stages 3 & 4

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## Abstract

**Background:** Chronic Kidney Disease (CKD) is common and is associated with an increased cardiovascular morbidity and mortality which is not completely explained by traditional risk factors. Non-traditional risk factors include arterial stiffening and calcification. Several calcification regulatory proteins (CaRP) are implicated in arterial stiffening. Fetuin-A may be important in inhibiting mineralisation via physicochemical interaction with calcium and phosphate, and can form circulating CalciProtein Particles (CPP) in pro-calcific states. Osteoprotegerin (OPG) & Receptor Activator of Nuclear Factor κ-B Ligand (RANKL) control bone resorption and are also expressed in the vasculature. They may be important in vascular calcification. Fibroblast growth factor 23 (FGF-23) promotes urinary phosphate excretion and is also thought to be implicated in vascular disease. This study tests the hypotheses that these CaRP are associated with aortic stiffening in pre-dialysis CKD, and that CaRP and/or arterial stiffening are associated with outcomes.

**Method:** 200 CKD stage 3 & 4 subjects were enrolled in this study. Subjects underwent annual measurement of aortic stiffness with carotid-femoral pulse wave velocity (C-F PWV) and CaRP were measured. Outcomes measures were C-F PWV and high sensitivity troponin T (hs-cTnT), a recently introduced biomarker of adverse cardiovascular outcome assessed at baseline, rate of change of C-F PWV, rate of change of renal function and survival.

**Major Results:** OPG and CPP were independently associated with arterial stiffness. OPG, RANKL and FGF-23 were associated with hs-cTnT, independently of other recognised risk factors. Neither CaRP nor arterial stiffness were associated with rate of change of renal function. OPG:RANKL was associated with adverse survival in addition to hs-cTnT. C-F PWV increased across the follow up period and was independently associated with the combined end point of renal and patient survival in a time dependent manner.

**Conclusion:** This study provides epidemiological evidence of the association between CaRP and a variety of cardiovascular measurements and outcomes including arterial stiffness, stiffening and survival. Arterial stiffness was associated with the combined outcome of death or progression of renal disease. Evidence of an association between arterial stiffness and either progression of renal disease or survival alone could not be proven.

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## Preface

This work is dedicated to family members, current and future, who have supported me over the 3 years of study. Thank you for listening, thank you for your patience, and thank you for just being there.

## Acknowledgements

Many people have contributed to the work in this thesis. I am very grateful for the input from all of them. In October 2008 I took over the day to day running of the Arterial Compliance And oxiDant strEss as predictors of rate of loss of renal function, morbidity and Mortality In CKD (ACADEMIC) study from Dr. Laurie Tomlinson (LT). I inherited an organised and well administered project. The hard graft and determination that had been applied to reach this point are not, I hope, underestimated. Laurie remained a very valuable sounding block, guide, source of ideas and gentle critic throughout. Thank you.

Ed Smith (ES) was a crucial member of the team. His avid enthusiasm for all things calcification related and ability to turn around biochemical assays whilst juggling the work of an on call biochemist were greatly appreciated.

Within the supervisory team, I must thank Professor Rajkumar for his input, particularly with regard to the practicalities of measurement of arterial stiffness. Dr. Charley Chatterjee came on board the team in the final year and provided a most useful, accessible and friendly guide, especially in the process of thesis writing. Liz Cheek from the University of Brighton was a very willing guide through the intricacies of statistical analysis.

At the Royal Sussex County Hospital, I was very fortunate to be supported within the Clinical Investigation and Research Unit by an excellent team of nurses and administrative & laboratory staff. In particular I was very lucky to work alongside Allison Leslie (AL). Allison was a stalwart in the vascular laboratory, an assiduous colleague and when all else failed, a shoulder to cry on. Allison's nursing assistance with the practicalities behind the repeated patient visits, sample collections and administration was greatly appreciated. In addition, I would like to thank the Brighton nephrologists, in particular Dr. Chris Kingswood and Dr. Ed. Kingdon for their support and ideas.

Finally, I would like to thank my antipodean supervisor, Assoc. Professor Steve Holt. Steve worked in Brighton with Laurie to establish the ACADEMIC study, then recruited me to the study and supported me to my mark on it. He ensured there was funding for the study, and provided an invaluable source of enthusiasm, ideas and insightful appraisal. Without Steve's supervisory input there would have been no ACADEMIC study!

## ACADEMIC declaration

The ACADEMIC study was originally designed by LT and supervisors. I took over the running of the study on 1st October 2008. At this point, 133 patients had been recruited and baseline arterial stiffness, measurements, interpretation of ECGs and classification of cause of CKD had been performed for these patients. 12 month follow up visits had been performed for 94/141 patients, and 24 month follow up for 32/90 patients. Cross-sectional analysis of these patients (including analysis of the relationship of total fetuin-A with C-F PWV) had been performed and is included in the thesis of LT [2]. LT had also extracted renal function data for the 120 patients included in her analysis of the determinants of rate of decline in renal function.

Subsequent to 1st October 2008, I recruited a further 67 patients. I performed baseline and all follow up measurements of these patients included in this thesis, including ECG interpretation and classification of cause of CKD. In addition, I performed all subsequent follow up measurements in the 133 previously recruited patients. After discussion with my supervisors I made the decision to measure additional Calcification Regulatory Proteins (CaRP) and high sensitivity troponin T (hs-cTnT). Quantification of the titres of CaRP and hs-cTnT in all patients (except total fetuin-A in the 133 patients) was performed in the laboratory by ES after 1st October.

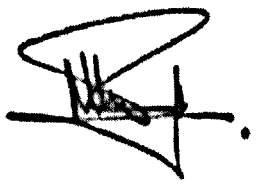
I extracted renal function measurements de novo for all patients used in the rate of renal function decline analysis included in this thesis. Patient and renal survival end points were gathered prospectively throughout the study. I performed a final exhaustive determination of end points in the summer of 2011.

I performed all statistical analyses presented in this thesis

## Author's Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this, or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed



Dated 12th March 2012

# Chapter 1

# Introduction

## 1.1 Introduction & Aims

Changes to arterial structure and function along with decline in renal function have all been associated with poor outcomes including cardiovascular morbidity and mortality. Although there is a link between vascular disease and renal dysfunction, the direction of causality remains a subject of debate [1]. However many believe that renal dysfunction may accelerate vascular dysfunction due to changes in the vessel wall, including those of vascular calcification, which occur commonly in renal disease.

Many proteins have been implicated in the pathophysiology of arterial stiffening. Renal dysfunction is associated with perturbation of both titres of these proteins and the arterial phenotype. There is therefore plausibility in the hypothesis that the two are linked.

### Study Hypotheses

The ACADEMIC (Arterial Compliance And oxiDant strEss as predictors of rate of loss of renal function, morbidity and Mortality In Chronic Kidney Disease) study was established in 2006 with the goal of exploring three hypotheses:

1. That renal function, as defined by eGFR, is an independent determinant of aortic stiffness in patients with chronic kidney disease (CKD) stages 3 & 4.

2. That aortic stiffness is an independent determinant of rate of decline of renal function in patients with CKD stages 3 & 4.

3. That aortic stiffness is independently associated with cardiac events and total mortality in patients with CKD stages 3 & 4.

Appraisal of the first two of these hypotheses, based on the interim analysis of the first 133 patients recruited is included in the doctoral study of LT [2].

My thesis aims to continue investigation of these hypotheses, but also to investigate the role of the CaRP fetuin-A, osteoprotegerin (OPG), Receptor Activator of Nuclear Factor κ-B Ligand (RANKL) and Fibroblast Growth Factor 23 (FGF-23) in the arterial changes and adverse outcomes associated with CKD. As will be illustrated in this introduction, this is a subject area which is fast moving, but in which many processes remain incompletely understood. The central hypothesis on which this

study is predicated is that the pathological processes important in the arteries of dialysis patients are also relevant and important in pre-dialysis CKD. Moreover, that these processes result in arterial stiffening and are associated with a variety of adverse outcomes – death, dialysis or decline in renal function.

Specifically, the hypotheses to be explored by this study are:

1. That the association between CaRP, aortic stiffness and mortality present in CKD-5 is also manifest at earlier stages of CKD.

2. That abnormal titres of CaRP are associated with increased arterial stiffness and increased progression of arterial stiffening.

3. That arterial stiffness and/or the CaRP are associated with rate of decline of renal function, troponin-T (a biomarker of cardiac risk) and with mortality.

## Aims of Study

The aims of this study were to investigate the association of four CaRP, fetuin A, OPG, RANKL and FGF-23, with cardiovascular and renal outcomes in a cohort of patients with CKD stages 3 & 4.

The specific aims of this study were:

1. To explore the relationship between CaRP and rates of aortic stiffening and change in renal function.

2. To explore the relationship of the CaRP with myocardial dysfunction.

3. To explore the relationship of plasma fetuin-A concentration with inflammatory markers and urinary fetuin-A loss.

4. To explore the relationship of the CaRP with hard clinical outcomes, i.e. mortality and end stage renal failure (ESRF).

In the introduction I will first set out some of the current evidence relating to the CaRP, renal function, arterial stiffening and the associated adverse outcomes.

## 1.2 Chronic Kidney Disease, Arterial Stiffness and Cardiovascular Disease

### A Complex Inter-relationship

Chronic Kidney Disease is a common condition that is associated with significantly increased cardiovascular morbidity and mortality. Approximately 8.5% of the UK population has a glomerular filtration rate (GFR) less than 60ml/min/1.73m$^2$ [2]. The cardiovascular system and the kidney are highly interdependent, in that dysfunction of either system appears to affect the other.

Associative studies are therefore complicated by a number of potential interactions which may be involved in the pathogenesis of problems in one or both organs. For example, pathophysiological processes such as hypertension, diabetes mellitus [3] and vasculitis [4] may affect both organ systems concurrently. The impact of intervention for disease in one organ may also affect the other organ system. For example, the prescription of certain phosphate binders for hyperphosphataemia in CKD may change lipid levels with a potential impact upon cardiovascular disease [5]. In addition, compensatory physiological changes caused by dysfunction of one organ system may also impact the other. For example, secondary hyperparathyroidism due to renal dysfunction is associated with changes to serum mineral concentrations that may lead to, or exacerbate, vascular calcification.

The increased cardiovascular mortality seen in CKD is partially attributable to exposure to traditional Framingham risk factors such as hypertension, diabetes and hyperlipidaemia [6]. However in many studies, renal functional decline is associated with an increased risk of cardiovascular events and death independently of traditional risk factors including cardiovascular disease, diabetes, hypertension and dyslipidaemia [7-9] (see fig. 1.1). Traditional risk factors are thus not sufficient to explain the excess vascular morbidity [7]. Interest has therefore focused increasingly on 'non-traditional' risk factors which include arterial stiffness, left ventricular hypertrophy (LVH) and renal function itself.

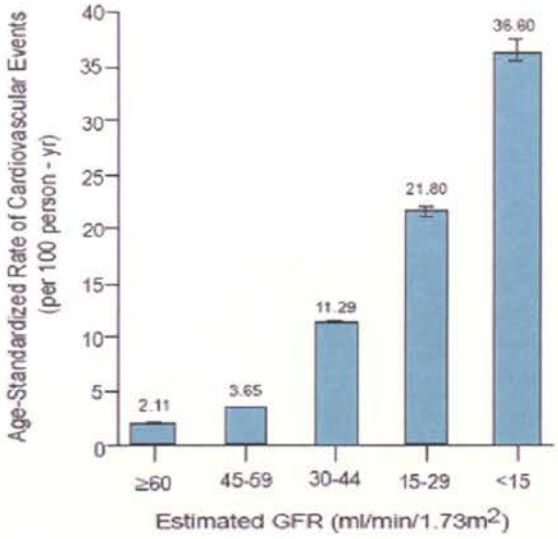



**Figure 1.1 - Histogram of age-standardised rate of cardiovascular events (per 100 person years) across different stages of CKD (eGFR >60ml/min/1.73m$^2$ - stages 1 & 2, eGFR 45 - 59ml/min/1.73m$^2$ - stage 3a, eGFR 30-44 ml/min/1.73m$^2$ - stage 3b, eGFR 15-29 ml/min/1.73m$^2$ - stage 4, eGFR <15 ml/min/1.73m$^2$ - stage 5)** [8]

### Arterial Structure and Function

Arteries have two distinct functions. They act as a conduit for delivery of blood to the tissues from the heart, but in addition perform a capacitance facility within the vasculature.

### Arterial Capacitance

Arterial capacitance allows the vessel to dampen the changes in arterial blood pressure and volume generated by ventricular systole and diastole. Integration of these forces reduces cardiac afterload, dampens potentially damaging pressure changes in the periphery and maintains diastolic blood pressure (DBP) which is particularly important in coronary perfusion.

The capacitance capability of the arterial tree is determined by the elasticity of the arterial wall which is, in turn, determined by the composition of the arterial wall. The arterial wall is composed of three tunicae. Structural strength is provided by the two outermost layers; the tunicae media and adventitia. These are composed of layers of vascular smooth muscle cells (VSMC) and collagen and elastin fibres supported by an extracellular matrix.

The elastin:collagen ratio varies with vessel calibre. Large vessels, such as the aorta, have a high elastin:collagen ratio providing greater elastic recoil. Conversely more

peripheral vessels, for example the radial artery, have a narrower calibre, are more muscular and have a lower elastin:collagen ratio [10].

## 1.3 Arterial Stiffness and Stiffening – Pulse Wave Velocity

Arterial stiffness is defined as the unit of pressure required to generate a change in volume of one unit and can be assessed non-invasively by measurement of pulse wave velocity (PWV).

The Moens-Korteweg [11] and Bramwell-Hill [12] equations show that PWV is related to the vessel properties.

Several parameters (h, r, ρ) can be effectively eliminated from these equations as constants [10], and distensibility is inversely related to stiffness. Thus we can show that PWV is proportional to stiffness.

$$PWV = \sqrt{V/\rho D} = \sqrt{Eh/2r\rho}$$

**D-distensibility; E-Young's modulus of elasticity of vessel wall; h-thickness of the vessel wall; r-inner radius of the vessel; ρ-density of blood; V-arterial volume**

### Choice of Arterial Segment

Different arteries have different compliances, are affected by different pathologies and stiffen at different rates [13]. Therefore, in order to compare stiffness both within and across cohorts, a section of the arterial tree must be selected for repeated measurement. This segment should be readily accessible, easily and reproducibly measurable and, in order for this measurement to be widely adopted, relevant such that changes in the PWV of this segment should have clinical value.

For these reasons the Association for Research into Arterial Structure and Physiology (Artery Society) recognise Carotid-Femoral PWV (C-F PWV) to be the 'gold standard' method of non-invasive assessment of arterial stiffness [14]. This is the method that has been used throughout this study. The benefit of C-F PWV over other measures is that the carotid-femoral section of the arterial tree is the segment of which the capacitance determines cardiac afterload. In addition, measurement of C-F PWV is relatively simple and operator independent.

Other methods used for measurement of arterial stiffness include PWV measurement over different arterial segments including the brachial ankle (B-A), heart femoral (H-F), radial dorsalis pedis (R-DP) and carotid radial (C-R) segments, and other forms of pulse wave analysis (PWA) such as augmentation index (AI). These are described

in appendix 2 in order to aid interpretation of referenced studies which used these methods.

Whilst H-F PWV is similar to C-F PWV, other measurements such as C-R and R-DP PWVs differ significantly due to incorporation of muscular arteries in the territory over which the pulse wave is recorded. B-A PWV use has been generally limited to Japanese and Korean studies where limited outcome data is available [15]. However concerns regarding the validity of the path measured (carotid-brachial plus carotid-tibial segments), the associated 'transit time' over this non-physiological pathway and the variation in properties of the arterial wall over this distance which includes both muscular and elastic arteries have limited wider uptake of this parameter [16].

## Determinants of Arterial Stiffness

Arterial stiffness increases with age [17] and blood pressure [18]. Histologically, age related changes to the arterial wall include progressive disorganisation of the elastin fibres with an increase in collagen and matrix. Hypertensive changes are thought to reflect an acceleration of this degeneration.

The stepwise association of PWV with stage of CKD was first shown by Wang *et al* [19]. The UREKA (United Kingdom REsearch alliance in Kidney disease and Arterial stiffness) study (a collaborative UK study which incorporated all baseline data from this study in addition to data from 11 other centres) again found C-F PWV was increased in patients with CKD compared to healthy controls, and reported a direct independent relationship between age and PWV in an analysis adjusted for mean blood pressure (MBP) and other variables (see fig. 1.2) [20].

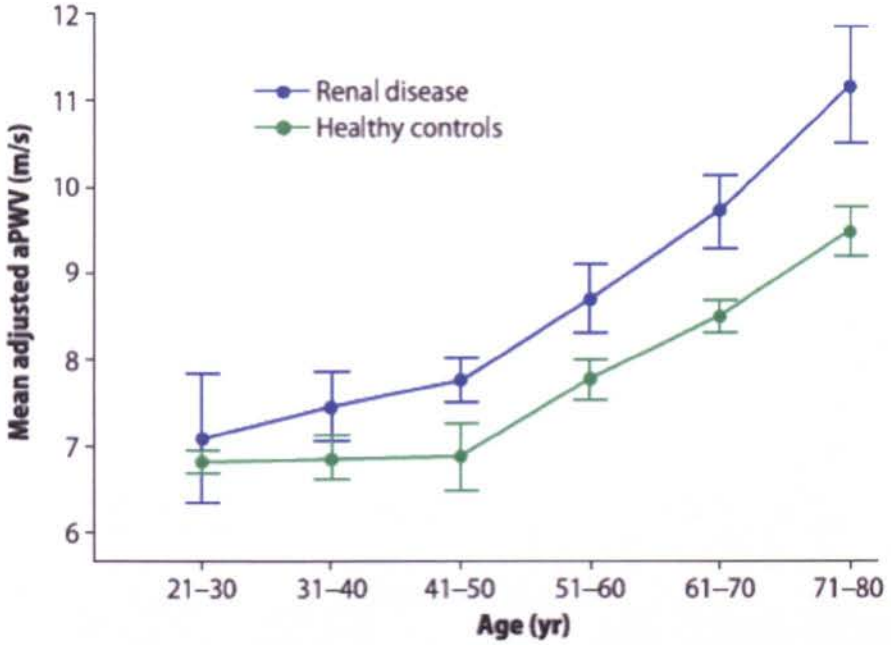



**Figure 1.2 - Relationship of C-F PWV (m/s) (adjusted for MBP, sex, cholesterol and smoking) with age (years) in patients with renal disease (blue) and healthy controls (green) [20]**

Townsend *et al* reported a large study of 2564 CKD stage 3 & 4 patients from the Unites States of America (USA). They reported that age, MBP, kidney function, diabetic status, blood glucose (non-diabetics only), ethnicity (increased in African-Americans), gender (higher in males) and waist circumference were all independently associated with C-F PWV (see fig. 1.3) [21]. Other cross-sectional studies investigating arterial stiffening have found association with dyslipidaemia [22], diabetes [23], tobacco smoking [24] and GFR [19]

Lilitkarntakul *et al* studied a cohort of patients with CKD stages 1-5 with minimal comorbidity. They found that age and MBP were the main determinants of C-F PWV, though asymmetrical dimethylarginine (ADMA) and high sensitivity C reactive protein (hsCRP) were also independently associated with C-F PWV in their final model [25].

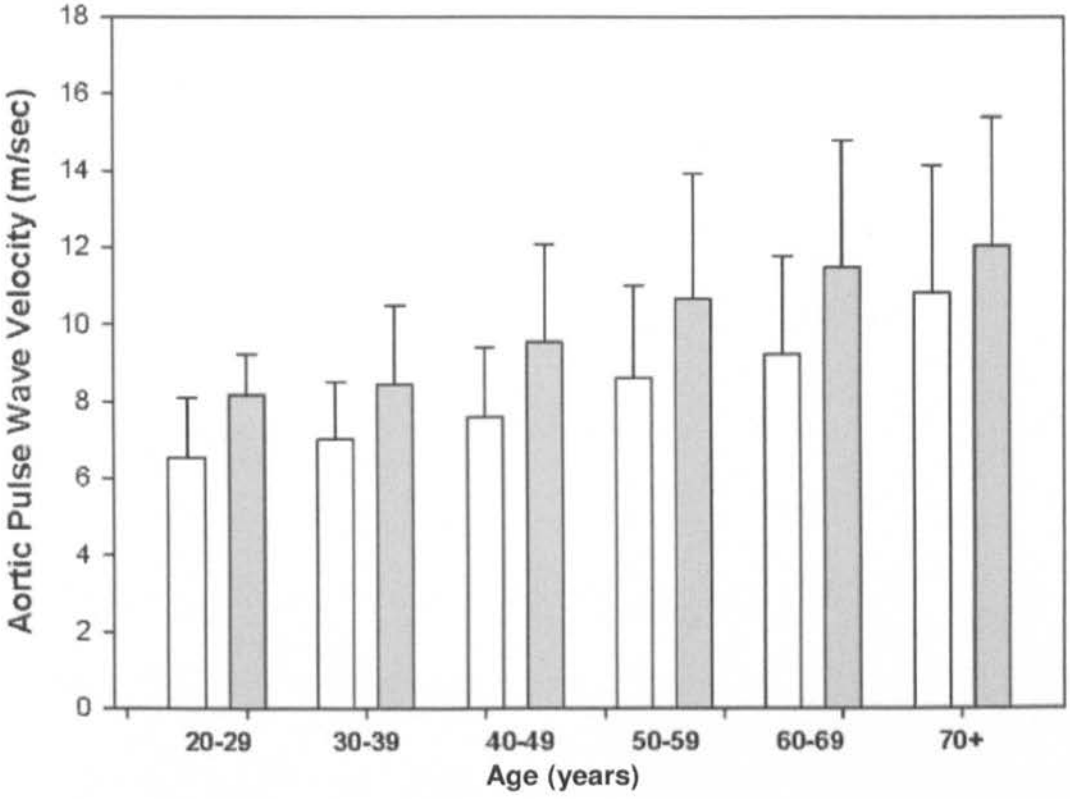



**Figure 1.3 - Bar chart of mean C-F PWV (m/s) plotted against 10 year increments of age (years) in patients with (shaded bars) and without (open bars) diabetes [21]**

There have been five published longitudinal studies of arterial stiffness, four of which have been published since the start of this study. Two of these studies are of pre-dialysis CKD patients. The results are summarised in table 1.1.

Whilst the association of systolic blood pressure (SBP) with arterial stiffness is a common theme, the results of other studies are mixed and the numbers of CKD patients studied are low.

| Study | No. of patients | CKD stage(s) | PWV | Rate of change (m/s/year) | Factors associated with change in PWV |
|---|---|---|---|---|---|
| Jung [26] | 67 | 5D on PD | H-F PWV | 0.44 ± 2.09 [§] | MBP, triglycerides |
| Fassett [27] | 34 | 3-4 | C-F PWV | 0.3 to 0.51 | Not stated |
| Chen [28] | 52 | 3-5 | B-A PWV | -0.06 | SBP |
| Benetos [29] | 483 | Not CKD [†] | C-F PWV | 0.14 ± 0.02 [††§] | Age, heart rate & serum creatinine |
| Tomiyama [30] | 2054 | Not CKD [†] | B-A PWV | 0.05 (-0.06; 0.18) [§§] | Age, BMI, MBP, ongoing smoking |

**Table 1.1 - Summary of studies of rate of change of arterial stiffness [†]General population, [††]Hypertensives only, [§]mean ± standard deviation (SD), [§§]median ($25^{th}$,$75^{th}$ centile) PD-peritoneal dialysis, BMI-Body Mass Index**

## Arterial Calcification and Arterial Stiffness

Whilst one might assume that calcification should be directly related to stiffness, the evidence in this field is unclear. Verbeke *et al* measured C-F PWV and assessed arterial calcification using a semi-quantitative Abdominal Arterial Calcification (AAC) score [31] in a large cohort of dialysis patients (n=1084) [32]. They found a significant univariate correlation between C-F PWV and AAC ($r=0.398$, $p<0.001$). However in multivariate analysis the incremental $R^2$ (inc. $R^2$) of C-F PWV attributable to AAC tertile was only 0.019 (total $R^2=0.393$). The remaining variation was explained by age (inc. $R^2=0.286$), diabetic status (inc. $R^2=0.052$) and MBP (inc. $R^2=0.036$). Prospective follow up of this incident cohort demonstrated significant interaction between C-F PWV and AAC score in determination of cardiovascular outcome [33] (see fig. 1.4).

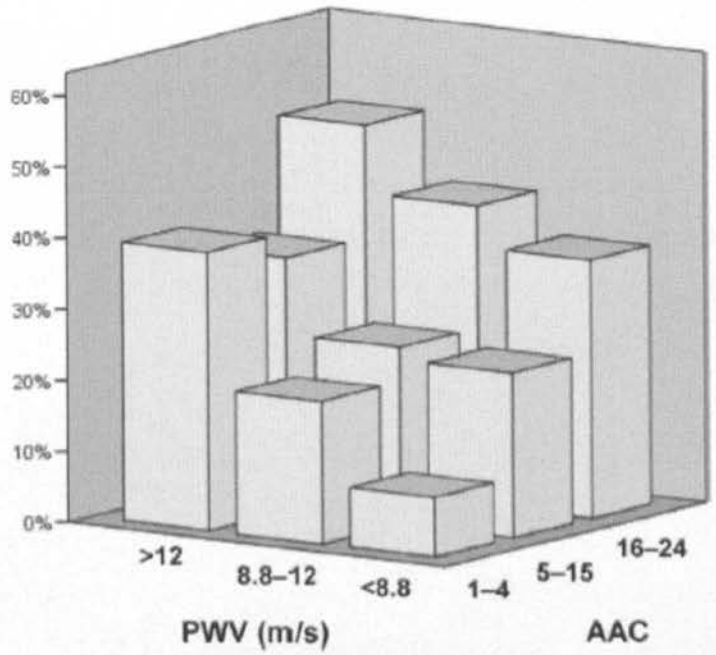



**Figure 1.4 - Association of C-F PWV (m/s) and AAC with two year risk of death or non-fatal CV event in 1034 prevalent European dialysis patients [33]**

Other groups have also found a relationship between aortic calcification and aortic stiffness. Raggi *et al* found a significant relationship between C-F PWV and abdominal calcification score in a study of 131 prevalent haemodialysis patients [34]. In addition, Temmar *et al* studied this relationship in a group of 150 CKD patients

(groups 2-5D) (see section 1.6). They found a significant difference in aortic calcification between patients with CKD stages 2 & 3 (n=52) and stage 5 on dialysis (5D) (n=47). In multivariate analysis of the factors associated with C-F PWV, aortic calcification was only significantly associated in the sub-group analyses of dialysis patients, but was not significant in the non-dialysis CKD patients (n=103) [35].

A further study of 193 healthy subjects from the Anglo-Cardiff Collaborative Trial (ACCT) found a strong correlation between C-F PWV and aortic abdominal calcification ($r=0.6$, $p<0.0001$). Aortic calcification, renal function, MBP, age and heart rate were independently associated with aortic stiffness in this group ($R^2=0.51$)[36].

It should be noted that the largest number of non-dialysis CKD patients in any of these studies was only 103. Of course, absence of an association in these small studies does not equate to an absence of an effect, so the relationship of aortic stiffness and aortic calcification in CKD stages 3 & 4 remains unknown. However, the finding of association in cohorts of both dialysis patients, where the effect size is likely to be greater, and larger cohorts of healthy subjects indicates that a relationship may be found if larger studies of non-dialysis CKD patients were to be performed. Notwithstanding this assumption, aortic stiffness is proposed as a measure of cardiovascular risk which integrates changes in the composition of the arterial wall due not only to calcification, but also to ageing and blood pressure related changes, as well as changes to the elastin and collagen content of the extracellular matrix and to the overall cellular composition. Outcome studies comparing these various measures directly are needed.

## 1.4 Arterial Changes in CKD

Arterial dysfunction may be due to changes in any of the arterial tunicae.

### Endothelium & Tunica Intima

In the healthy artery, nitric oxide (NO) is produced by the endothelium. NO diffuses through to the tunica media where it causes smooth muscle relaxation. Relaxation of the smooth muscle maintains arterial compliance. Dysfunction of the endothelium is common in CKD, and vascular muscle tone is implicated in arterial stiffness. Endothelial function is thought to be particularly important in the small and medium sized muscular arteries [10,37,38].

In the intima, CKD patients develop atherosclerotic disease earlier, more rapidly and to a greater extent and severity than the general population [39,40]. Atherosclerotic lesions often calcify producing intimal calcification.

In CKD additional abnormalities are present. These include increased ADMA, a naturally occurring inhibitor of nitric oxide synthase [41], and increased oxidative stress [42]. Lillitkarntakul *et al* recently reported a cohort study of CKD patients with minimal comorbidity, and found that blood pressure and age were the major determinant of arterial stiffness. Addition of biomarkers of endothelial function and oxidative stress produced a modest incremental $R^2$ of 0.07 to the model predicting arterial stiffness [25].

### Tunica Media

In CKD arteries dilate and undergo calcification in the tunica media, a process termed Monkeberg's arteriosclerosis (see fig. 1.5). Dilation is due to alteration of the elastic wall fibres, both via chemical degradation (calcification, lipid peroxidation and glycoxidation), alteration of the elastin:collagen ratio with relative reduction in elastin content, and thinning and fragmentation of the remaining fibres [43].

Vascular calcification is a complex active process. Whilst interest in, and understanding of, the vascular biology of CKD has increased dramatically in recent years, the mechanisms and factors determining this process remain incompletely understood. Within this thesis, papers mentioned as being 'recently' published indicate that they were published after October 2008. Numerous pathways and mediators have been implicated. These will now be explored.

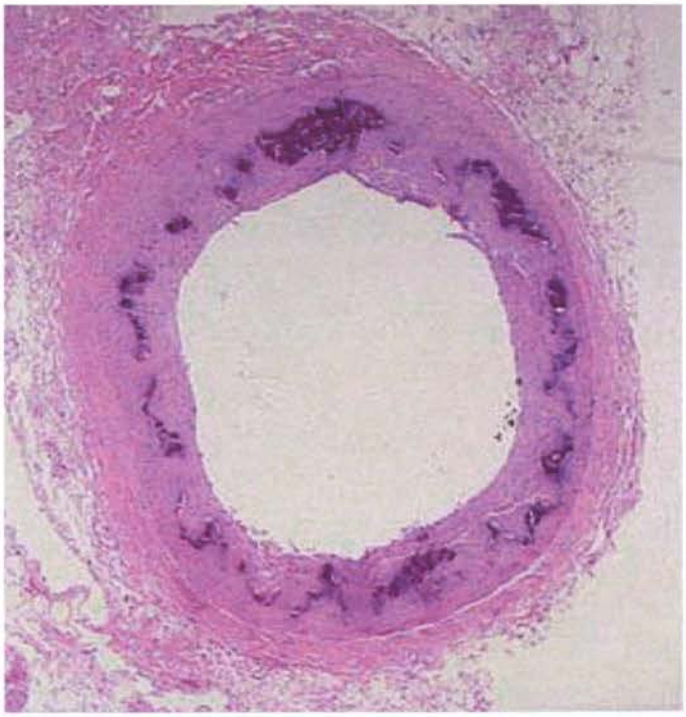


**Figure 1.5 - Histology of arterial cross section demonstrating deep purple staining of calcium deposits in tunica media with Von Kossa staining [44]**

Medial calcification occurs when VSMC undergo phenotypic transdifferentiation. This is associated with altered gene expression such that the VSMC assumes an osteoblastic phenotype. Shroff *et al* demonstrated an increase in total calcium load in medium sized muscular arteries taken from patients with pre-dialysis CKD. The total calcium load then further increased after the commencement of dialysis. This was associated with increased alkaline phosphatase activity, and *runx2* & *osterix* expression after the initiation of dialysis [45].

The same group subsequently demonstrated that aortic VSMCs in non-dialysed children with CKD stage 5 starting dialysis have an increased propensity to calcify when exposed to high calcium and phosphate milieu [46]. This finding further affirms the hypothesis that arterial physiology is disordered before the start of dialysis. It therefore acts as a basis from which to study the relationship of calcification inhibitor proteins on arterial stiffness in the pre-dialysis setting.

## Vascular Smooth Muscle Cells Transdifferentiation

Medial calcification is associated with changes within the VSMCs whereby the usually contractile myocyte transdifferentiates into a synthetic osteoblastic phenotype. This is made possible by the plasticity of the mesenchymal cell line. *In vitro* studies have shown that high levels of intracellular phosphate promote VSMC transdifferentiation [47]. A number of soluble proteins are thought to control this process. Bone morphogenic proteins (BMP) 2 & 7 act as potent competing osteogenic differentiating factors. BMP-2 acts via a master transcription factor *runx2* [48], which in conjunction with other downstream transcription factors *osterix* and *sox9,* act on the vascular mesenchymal cells pushing them towards an osteogenic phenotype. Further novel gene sets are activated causing the VSMC to express proteins including osteopontin, type I collagen, alkaline phosphatase and osteocalcin, in a phenotype similar to that seen in an osteoblast [49,50]. This metaplasia of the VSMC is also associated with changes to the extracellular matrix, the net effect of which is an increase in amorphous calcified matrix. BMP-7 opposes this effect by promoting the mesenchymal cells to develop towards VSMC with the expression of actin and myosin [51].

Transformation of the VSMC is associated with matrix vesicle release – a process seen in the arteries of CKD patients, but not in healthy subjects, which is thought to reflect excretion of calcium and phosphate from the cell [46].

## The Putative CaRP

There are many putative mediators of vascular calcification, with a diverse range of proposed mechanisms. In this study the role of fetuin-A, OPG & RANKL and FGF-23 are explored.

The reasons that these CaRP were chosen for study are varied. Fetuin-A was selected as data indicated a relationship between fetuin-A and outcome in dialysis cohorts. Preliminary analysis of the data from the ACADEMIC cohort indicated that this association may be mediated by arterial stiffening [2]. OPG and FGF-23 were selected for study as outcome data was again available in dialysis cohorts, whilst RANKL was measured as it is the physiological ligand of OPG. The data relating to the CaRP is reviewed in detail in this introduction.

Many other factors have been implicated in vascular calcification in CKD. Their effects are summarised in table 1.2.

| Variable | Effect |
|---|---|
| Phosphate [52,53] | Multiple. Increased extracellular phosphate transported via PIT1 causes increase in *runx2* expression |
| Calcium [52] | Multiple. Increases VSMC apoptosis & matrix vesicle release from VSMC – act as nidus for calcium phosphate deposition |
| Pyrophosphate (PPi) [54,55] | Physiological inhibitor of aortic calcification. Treatment with PPi reduces aortic calcium content in rats |
| Parathyroid hormone (PTH) [26,56] | High PTH associated with aortic calcification in presence of low calcium, normal phosphate & normal renal function. Associated with arterial stiffness |
| Vitamin D [57-59] | High doses of vitamin D reproducibly cause vascular calcification. Moderate doses of vitamin D analogues (+/- calcitriol) ameliorate atherosclerotic calcification |
| Phosphate binders [60,61] | Sevelamer attenuates progression of coronary and aortic calcification in dialysis and coronary artery calcification in pre-dialysis patients |
| Matrix Gla Protein (MGP) & warfarin [62,63] | Vitamin K dependent γ carboxylated MGP inhibits calcification via interaction with BMP 2 & 4. γ carboxylation is inhibited by warfarin. Warfarin is associated with arterial calcification in animal studies. Lack of prospective, controlled, non-confounded human studies |
| Osteocalcin [64] | Vitamin K dependent γ carboxylated protein. Independently associated with carotid artery calcification in humans |
| Osteopontin [65] | Expressed in calcified arteries. Thought to inhibit calcification via binding to mineralised crystal surface |

**Table 1.2 - Summary of proposed mediators of arterial calcification which are not the focus of the ACADEMIC study**

## 1.5 Cardiovascular Mortality and CKD

In addition to the arterial changes detailed previously, changes to the myocardium and the coronary arteries are also implicated in the increased cardiovascular mortality of CKD.

The increased cardiovascular mortality rate in CKD is partially explained by the shared causality of CKD and coronary artery disease. However, the pathogenesis of cardiovascular mortality in CKD differs from the normal population. A greater proportion of deaths are attributed to cardiac changes such as left ventricular hypertrophy (LVH) and related conditions including arrhythmias and left ventricular failure, and a lesser proportion to coronary artery atherosclerosis (see fig. 1.6) [66]. This variation in the causes of cardiovascular death in CKD explains the importance of non-traditional risk factors in this population and, in addition, explains the reduced efficacy of therapeutic interventions such as statin therapy in CKD when compared to the general population [67,68].

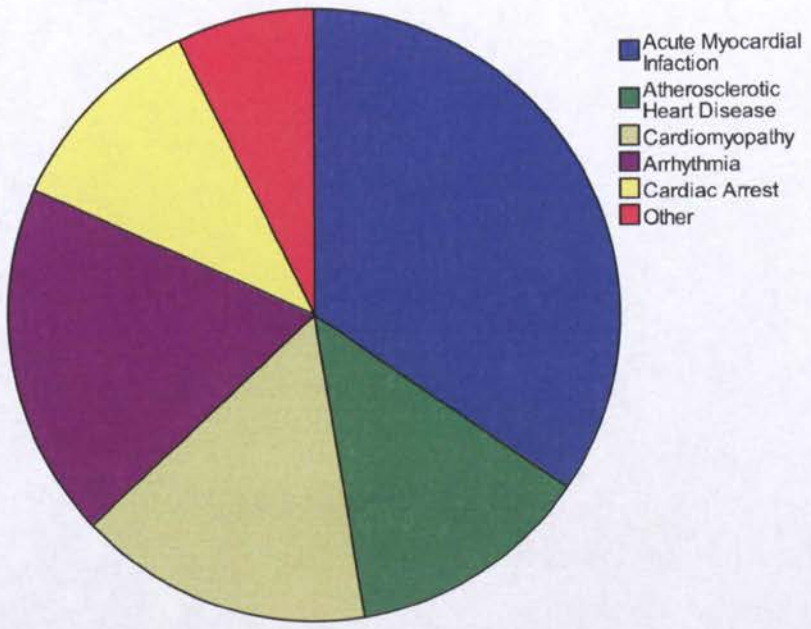



**Figure 1.6 - Pie chart of categories of cardiovascular mortality from United States Renal Data System (USRDS) database of dialysis patients 2006 [66]**

## Left Ventricular Hypertrophy

LVH is an increase in left ventricular mass and is widely accepted as a non-traditional cardiovascular risk factor. LVH is attributed to three main types of often interlinked pathology, i) excess pre-load, ii) excess afterload and iii) other biological variables. These pathological processes often occur simultaneously where they may have an additional or synergistic effect. Pathological pre-load is caused by chronic volume loading often due to salt and water excess, and is associated with lengthening of the cardiomyocyte. This results in eccentric LVH. Pathological afterload is the result of the integrated effects of systolic (SBP) and diastolic blood pressure, arterial resistance, aortic compliance and possibly calcification. Chronic afterload causes mechanical stretch of the ventricle, cardiomyocyte thickening and concentric LVH[69,70].

Other implicated biological mediators include activation of the renin-angiotensin-aldosterone system, phosphate and PTH [71,72]. In other studies OPG [73], and more recently FGF-23 [74,75] have also been associated with increased LV mass. There is also evidence that OPG and RANKL are expressed in the failing myocardium [76]. Recently in an animal model, LVH was induced by a direct action of a high concentration of FGF-23, potentially via the FGF-2 receptor [77].

In CKD all of these pathologies are often present. The combination of LVH with ischaemia triggers cardiomyocyte apoptosis and autophagy which finally results in an increase in the extracellular matrix and fibrosis. This fibrosis is thought to contribute to reduced contractility (i.e. systolic dysfunction), reduced ventricular compliance (i.e. diastolic dysfunction) and a disturbed electrophysiological state with a propensity to arrhythmogenesis. This phenotype is termed uraemic cardiomyopathy [69].

### Aortic Stiffness and Left Ventricular Hypertrophy

Aortic stiffening causes an increase in cardiac afterload and is important in the development of systolic hypertension and LVH (see fig. 1.7) [78]. LVH places a strain on myocardial perfusion via two mechanisms. Firstly, increase in the muscle mass of the left ventricle causes an increased myocardial oxygen demand. Secondly, coronary perfusion which occurs predominantly in diastole is impaired. The mechanism for this perfusion is controversial, with a classical view suggesting that it is dependent upon forward drive from diastolic pressure, which is maintained by the

arrival of the reflected pulse wave. Stiffening of the aorta causes earlier arrival of the reflected wave, augmenting the systolic pressure and relatively reducing diastolic perfusion [10]. However, Davies *et al* argue that coronary perfusion is driven by a dominant backwards travelling 'suction wave' which is generated via myocardial microcirculatory decompression [79]. Stiffening of the ventricular wall, such as that seen in LVH, is proposed to reduce suction and therefore perfusion.

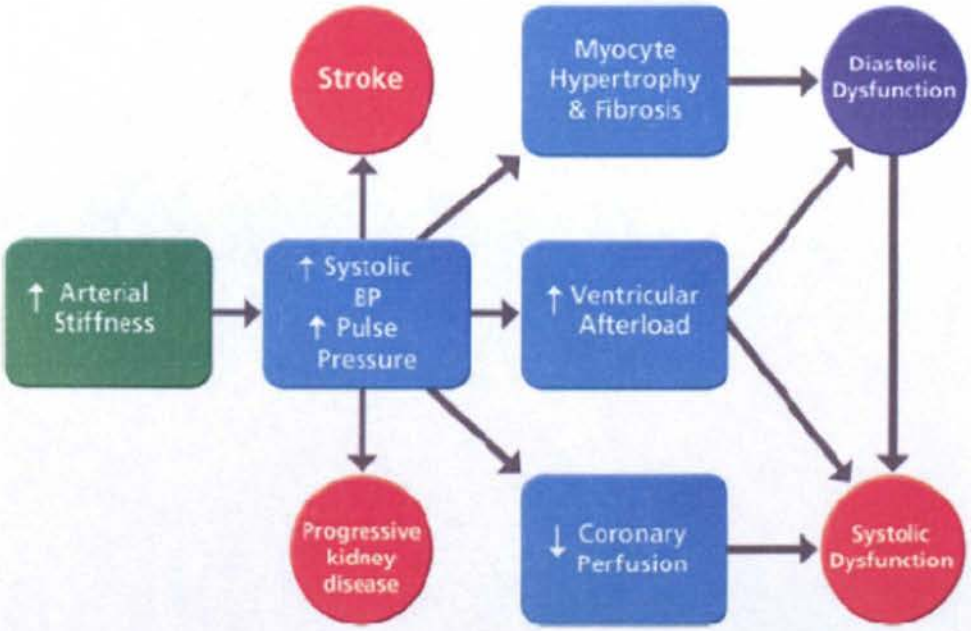



**Figure 1.7 - Schematic of effect of arterial stiffness on the cardiovascular and renal systems** [80]

Irrespective of mechanism, LVH is associated with sub-endocardial ischaemia and myocardial fibrosis. This triad of LVH, endomyocardial ischaemia and fibrosis linked to the increased incidence of traditional Framingham risk factors in the CKD population explains a significant proportion of the excess cardiovascular risk suffered by this population group [69].

## Aortic Stiffness and Survival

Aortic stiffness and arterial calcification are recognised independent predictors of all-cause and cardiovascular mortality in stage 5 CKD [81-83].

Increased C-F PWV has also been found to be an independent predictor of coronary heart disease and stroke within the apparently healthy general population [84,85]. There

is currently no published evidence that arterial stiffness is associated with mortality in any pure CKD stage 3 & 4 cohort.

## Other Cardiovascular Pathophysiology

There are three other major mechanisms which may contribute to the excess cardiovascular mortality of CKD.

### Coronary Artery Calcification

Calcification of the coronary arteries is associated with cardiovascular mortality [86]. The coronary arteries are however muscular arteries rather than elastic arteries (such as the aorta), and calcification here occurs predominantly within atherosclerotic plaque within the intima. However in patients with reduction in GFR, medial calcification is also seen [39,87]. Some of the processes implicated in large artery calcification, for example low fetuin-A, have also been associated with coronary artery disease [88]. Coronary artery calcification has been independently associated with C-F PWV after adjustment for age, sex, dialysis duration, inflammation and diastolic blood pressure in a mixed cohort of whom 48/55 were receiving renal replacement therapy (RRT) [89].

### Renal Osteodystrophy and Vascular Calcification

There is an inverse relationship between bone activity, arterial calcification and aortic stiffening in the dialysis population. Here histological low bone turnover, associated with low levels of PTH, is associated with increased calcification and stiffness of the aorta [90,91]. Absence of osseous buffering of calcium increases vascular calcium exposure and may exacerbate aortic calcification [92].

### Drug Effects

Patients with CKD are often treated with a variety of medications including diuretics, anticoagulants and erythropoiesis stimulating agents (ESAs). These have side effects which may contribute to the excess cardiovascular mortality. Diuretics cause electrolyte abnormalities which cause an increased risk of cardiac arrhythmia. A dose response relationship of diuretic with mortality has previously been found [93]. Warfarin treatment alters the carboxylation status of MGP with a resultant increased propensity for calcification [63]. ESA use has been associated with stroke in high risk CKD patients, though the mechanism for this continues to be debated [94].

## 1.6 Measurement of Renal Function

In 2002 the Kidney Disease Outcomes Quality Initiative (KDOQI) introduced a system for the classification of CKD [95] based on the abbreviated Modification of Diet in Renal Disease (MDRD) formula:-

186 x serum creatinine$^{-1.154}$ x age $^{-0.203}$ (x1.212 (if black)) (x 0.742 (if female))

This formula allows for the estimation of glomerular filtration rate (e(GFR)) at steady state (see table 1.3) [96]. This is validated in comparison to isotopic GFR measurement, accurate with GFR less than 60ml/min/1.73m$^2$ and has been widely adopted [97,98]. CKD is defined as either kidney damage or GFR ≤60ml/min/1.73m$^2$ for ≥3 months. Kidney damage is defined as pathological abnormalities or markers of damage, including blood or urine tests or imaging studies.

| Stage | Description | GFR (ml/min/1.73m$^2$) |
|---|---|---|
| 1 | Kidney damage with normal or ↑GFR | ≥90 |
| 2 | Kidney damage with mild ↓GFR | 60-89 |
| 3A | Moderate ↓GFR | 45-59 |
| 3B | Moderate ↓GFR | 30-44 |
| 4 | Severe ↓ GFR | 15-29 |
| 5(D) | Kidney failure | ≤15 (Dialysis) |

**Table 1.3 - KDOQI classification of CKD by GFR [99,100]**

Many factors determine the rate of change of renal function. GFR declines with age, and age is included in the MDRD, and the other commonly used equations for estimating eGFR [96,101,102]. This age related decline is accelerated by a diverse range of pathological processes including diabetes, the glomerulonephritides and inherited genetic disorders such as polycystic kidney disease. High blood pressure also causes a decline in renal function, though the GFR of many patients with high blood pressure is normal.

### Urinary Protein Loss

Proteinuria is common in CKD, and is a recognised cardiovascular risk factor in all patients, with and without reduction in GFR. Whilst previously proteinuria was quantified using a 24 hour urine collection, this has generally been replaced with measurement of total protein (or albumin) on a spot urine. The proportion of protein (or albumin) in this sample is expressed as a ratio to urinary creatinine (uPCR/uACR). A uPCR of 100mg/mmol is approximately equivalent to a urine protein loss of 1g in 24 hours [103]. A continuous association between proteinuria and cardiovascular risk is seen across the spectrum of urinary excretion [104].

The prevalence of proteinuria is dependent upon definition. Current National Institute for Health and Clinical Excellence (NICE) guidelines are based upon total urinary albumin excretion. They recommend that clinically significant proteinuria should be defined as uPCR>50mg/mmol, or uACR>30mg/mmol. CKD blood pressure treatment guidelines are currently based on a cut off uPCR of 100mg/mmol [105]. Other recent data suggest that uPCR may be a more sensitive measure of cardiovascular risk in CKD [106].

## Arterial Stiffening and Renal Function

Aortic stiffening has been proposed as a mechanism for acceleration of renal function decline. Pathophysiologically, stiffening of the vessel is thought to prevent dampening of the aortic pressure wave with resultant glomerular barotrauma and decline in filtration rate. [107]. This was first shown in a sub-group of the ACADEMIC study cohort (n=120) [108]. Recently three other groups have published their results looking at cohorts of patients with pre-dialysis CKD stages 3-5 (see table 1.4).

Three other groups have also found an association between AI (see appendix 2) and rate of renal function decline in cohorts of 111, 99 and 35 patients [109-111].

| Study | Arterial parameter(s) | Patient number and characteristics | Outcome measures | Adjustment | Significant results relating to outcome measure |
|---|---|---|---|---|---|
| ACADEMIC[108] | C-F PWV | 120 CKD stages 3 & 4 | i) reciprocal creatinine slope<br>ii) dialysis or >25% increase in Creatinine | ii) SBP and uPCR | i) C-F PWV indep. associated with outcome<br>ii) C-F PWV indep. associated with outcome |
| Nephrotest [112] | C-F PWV, CACWS and others | 180 mean eGFR 32 ± 16 ml/min/1.73m$^2$ | i) GFR slope<br>ii) start of dialysis | i) age, gender, BMI, smoking status, dyslipidaemia, uACR, pulse pressure<br>ii) as above + eGFR slope | i & ii) Association between CACWS and outcomes.<br>No sig. association between C-F PWV and outcomes |
| Chue et al [113] | C-F PWV and AI | 225 CKD stages 2, 3, 4 & 5<br>mean eGFR 43 ± 19ml/min/1.73m$^2$ | i) eGFR slope<br>ii) dialysis or >25% decline in eGFR | Age, gender, baseline eGFR, SBP, uACR, haemoglobin, calcium and phosphate | i) SBP and phosphate indep. associated with outcome (not C-F PWV)<br>ii) Phosphate indep. associated with >25% decline/ESRF<br>iii) No indep. association between AI and outcome |
| Chen et al [114] | B-A PWV | 145 CKD stages 3, 4 & 5 | i) eGFR slope<br>ii) dialysis or death | i) hypertension and baseline eGFR<br>ii) phosphate and baseline eGFR | i) B-A PWV associated with outcome |

**Table 1.4 - Studies of relationship of change of renal function with PWV and other arterial parameters in patients with CKD**
**CACWS-Carotid Artery Circumferential Wall Stress**

## 1.7 Fetuin-A

Fetuin-A is a 62 kDa, 349 amino acid protein which is synthesized in the liver. Fetuin-A is encoded on the *ahsg* gene on chromosome 3q27. A single nucleotide polymorphism in this gene is associated with significantly reduced fetuin-A irrespective of renal function [115-117]. Post translation, human fetuin-A is cleaved into a heterodimer, glycosylated and is phosphorylated at at least 2 sites [118]. Control of transcription, translation and post-translational modification and the effect of post-translational modification on protein activity are incompletely understood. Recent studies implicate malnutrition and interleukin 6 (IL-6) as downregulators of transcription [119,120].

**Total body and circulating forms of Fetuin-A**

The majority of the fetuin-A in the body is found complexed within bone [121]. Clinical studies have, in the main, measured circulating fetuin-A in the plasma.

Recent studies have characterised several forms of circulating fetuin-A within the serum of CKD patients. Ultra-centrifugation of serum separates a pellet consisting of fetuin-A, calcium, magnesium and inorganic phosphate. These calciprotein particles (CPP) or fetuin-mineral complexes (FMC) are formed particularly under conditions of increased calcium and phosphate [120]. The proportion of total fetuin-A present in CPP expressed as a reduction ratio (RR) is inversely related to eGFR. CPP were not detected in patients with normal renal function (see fig. 1.8) [122].

$$\text{Reduction Ratio} = \frac{\text{total serum fetuin-A - supernatant fetuin-A}}{\text{total serum fetuin-A}} \times 100$$

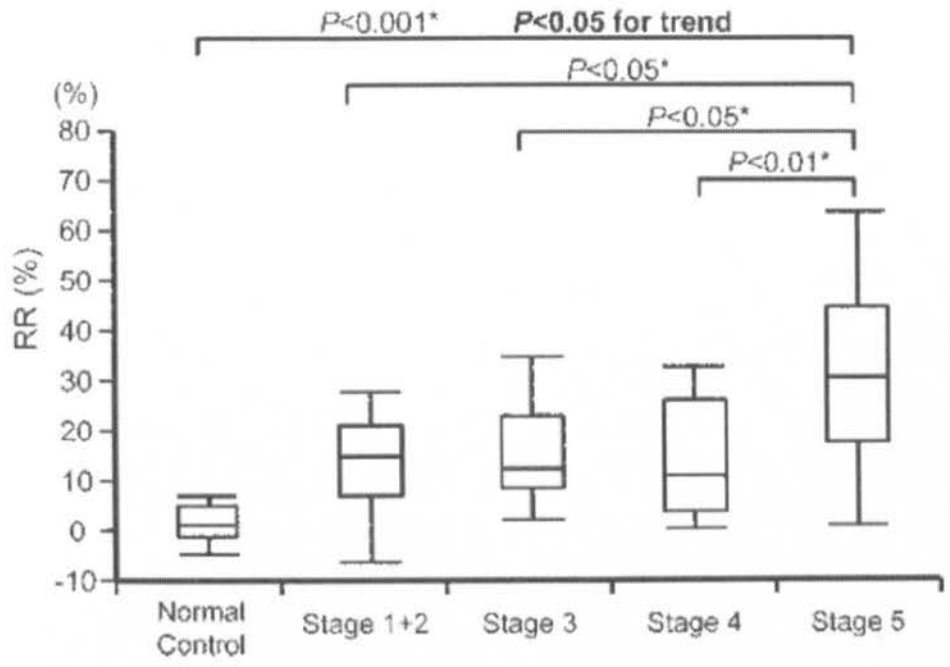



**Figure 1.8 - Reduction Ratio (RR) of CPP to total fetuin-A increases with severity of CKD in 84 patients (normal controls, n=11; stages 1-2, n=15; stage 3, n=28; stage 4, n=14; stage 5, n=15) Jonckheere-Terpstra test for trend** [122]

Fetuin-A has two seemingly distinct roles in the adult organism. Firstly in the control of calcification both in bone and at ectopic sites, and secondly in the modulation of insulin receptor sensitivity. These two roles will now be discussed.

## Calcification Inhibition

Fetuin-A is a mineral carrier protein which acts systemically both intra- and extracellularly to prevent spontaneous biomineralisation in the metastable environment. Fetuin-A acts to prevent extracellular calcification in several ways.

Fetuin-A binds calcium and phosphate to form CPP. These soluble colloidal spheres contain fetuin-A, matrix Gla protein, magnesium and basic calcium phosphate (the precursor of hydroxyapatite) in a mechanism analogous to the binding of apoproteins to cholesterol (see fig. 1.9) [120,123-125]. Stabilisation of the calcium and phosphate moeities prevent the formation of calcium phosphate nanocrystals which would otherwise precipitate from the supersaturated extracellular milieu. Nanocrystals upregulate expression of BMP-2 (a potent osteogenic factor) and osteogenic gene expression, and may also have the ability to regulate cell phenotype themselves [50].

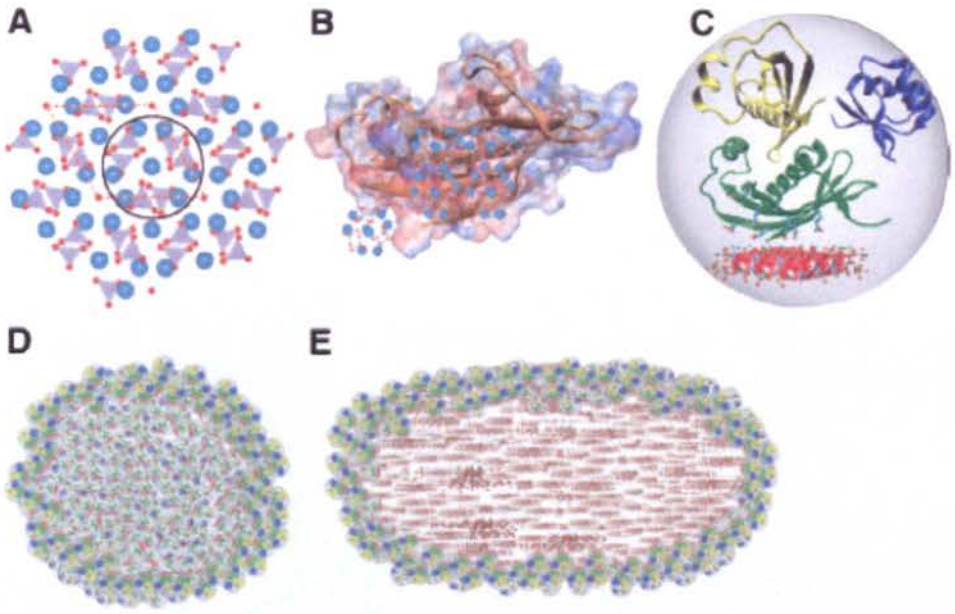



**Figure 1.9 - Schematic of formation of calciprotein particles from fetuin-A and calcium and phosphate A-Hydroxyapatite precursors (circle – $Ca_9(PO_4)_6$ Posner cluster); B-Interaction of hydroxyapatite precursors (Posner clusters) with fetuin-A; C-Interaction of hydroxyapatite precursors with three cystatin like domains of fetuin-A (yellow, red and blue); D & E-fetuin-A coating of unstructured (D) and structured (E) crystalline mineral core [126]**

Fetuin-A also acts to inhibit cell mediated mineralisation. In order for VSMC to maintain calcium and phosphate homeostasis and prevent apoptosis due to intracellular crystal formation, mineral excretion must take place. Fetuin-A is taken up into VSMCs, concentrated into excretory vesicles and subsequently excreted within matrix vesicles containing calcium released by healthy (and apoptotic) VSMCs. The fetuin-A maintains the minerals in solution and importantly abrogates the propensity of these vesicles to nucleate precipitation of calcium and phosphate [126,127]. Fetuin-A also enhances VSMC apoptotic body endocytosis to remove foci for potential calcification, and in addition has been postulated to inhibit intracellular apoptotic signalling in VSMCs [128].

### Excretion/Clearance

Fetuin-A is reduced by 19 to 29% during a standard four hour dialysis treatment [129,130]. The extent of fetuin-A clearance via renal degradation, peritoneal dialysis and urinary losses is currently unknown.

The pathway(s) by which the CPP are cleared from the circulation are not fully understood, although a role for reticuloendothelial system has been proposed (see fig. 1.10).

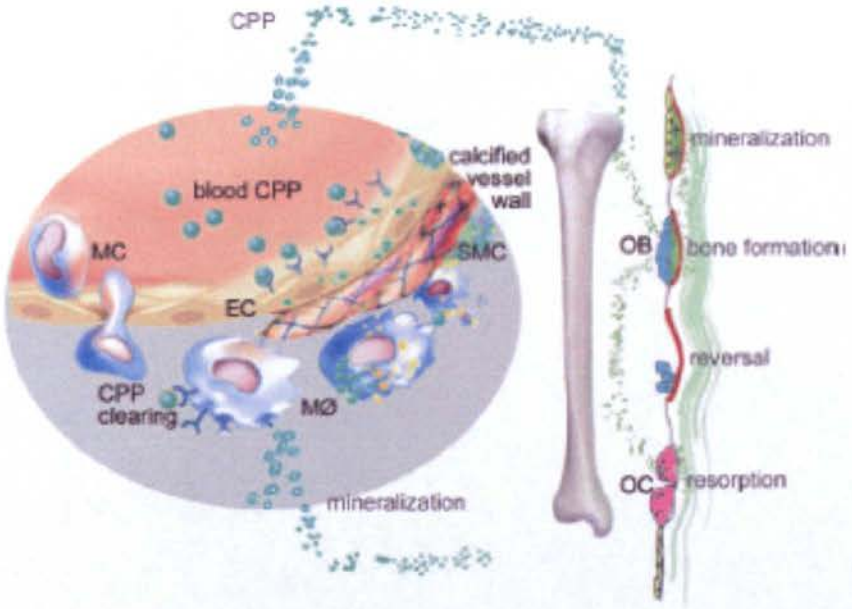



**Figure 1.10 - Hypothetical pathway of proposed CPP circulation and clearance by endothelium and tissue phagocytes - Osteoclasts (OC) generate CPP during bone resorption. These are taken up locally by osteoblasts (OB) and are stabilised by fetuin-A in the circulation. CPP in the interstitium are cleared by monocytes (MC) and macrophages (Mφ). Excess CPP generation may overwhelm reticuloendothelial clearance leading to smooth muscle cell (SMC) or endothelial cell (EC) apoptosis and deposition of calcified apoptotic cell remnants [50]**

## Fetuin Knockout Mice

Confirmation of the role of fetuin-A as an inhibitor of extraosseous calcification is provided by two *ahsg* -/- knockout mice models. When fed a mineral/vitamin D rich diet, *ahsg*-/- mice exhibit widespread soft tissue and small blood vessel calcification, despite normal serum calcium and phosphate concentrations (see fig. 1.11) [123]. The relative absence of large vessel calcification in these animals has been postulated to be due to local upregulation of counter-regulatory inhibitors such as MGP and osteopontin, or to the presence of an intact, non-atherosclerotic endothelium lacking the focus to trigger calcification [131,132].

The role of the endothelium in calcification is clarified by the *ahsg* -/- *apoE*-/- C57BL/6 mouse model which lacks intact endothelium. Unilateral nephrectomy with dietary phosphate supplementation (i.e. an artificial CKD environment) in this model increased aortic calcification compared to both the *ahsg* -/- *apoE* +/+ mouse and the

wild type. Crucially however, this calcification occurred in the intima as opposed to the media, and was associated with upregulation of MGP. The authors speculate that medial damage, lacking in this model, is required for medial calcification, and that MGP upregulation is an adaptive protective response [132]

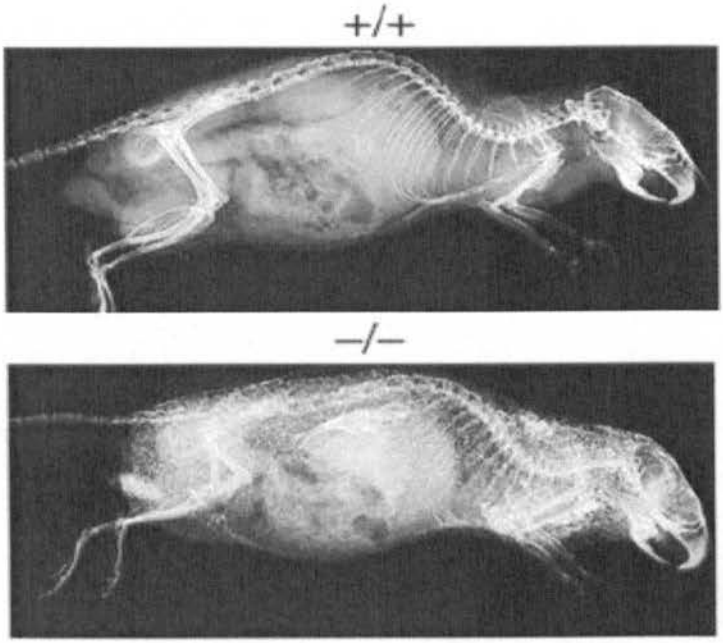



**Figure 1.11 - Extensive ectopic soft tissue calcification in the *ahsg*-/- mouse in comparison to the wild type *ahsg* +/+ [123]**

## Fetuin-A and Inflammation

Fetuin-A was recognised as a negative acute phase reactant in 1979 [133]. *In vitro* work has demonstrated that IL-1, IL-6, tumour necrosis factor (TNF) and interferon-γ (IFN-γ) are associated with reduction of fetuin-A post insult [134,135]. This work has been confirmed in observational human studies in which serum fetuin-A levels have been independently associated with IL-6 (β=-0.393) [136], and negatively correlated with IL-1β and TNF in CKD patients [137].

*In vivo*, it has recently been shown in animal models that increased fetuin-A is associated with reduced inflammatory responses. In addition, in animal models of severe sepsis, administration of exogenous fetuin-A is associated with improved outcome [126,134].

Fetuin-A has been identified as an inhibitor of meprin alpha, a metalloproteinase found in the wall of the intestine and in the kidney [138]. The clinical significance of this finding is not currently clear, although other meprin alpha inhibitors have been

demonstrated to be protective against renal injury in the ischaemic reperfusion mouse model [139]. Furthermore fetuin-A has been recognised to prevent neutrophil stimulation by hydroxyapatite [128].

This relationship further complicates any interaction between fetuin-A and vascular calcification in renal disease. CKD may be caused by inflammatory conditions whilst RRT, especially haemodialysis, is associated with frequent inflammatory stimuli. Soft tissue calcification is a common response to injury and inflammation. It is proposed that fetuin-A reduction secondary to inflammation may contribute to the calcification stress of CKD.

## Bone Turnover and Arterial Stiffness

Evidence linking increased calcium and phosphate with change in fetuin-A composition, and with altered VSMC matrix vesicle release and rates of VSMC apoptosis in the arterial wall indicates the importance of mineral metabolism in arterial disease [46,122].

Important recent advances indicate that the proportion of fetuin-A present as CPP increases with extraosseous calcification stress. Some data which suggests that CPP are reduced in the serum of patients using cinacalcet and in rats given alendronate is also supportive of this theory [120,122]. Bone is an important buffering mechanism for both calcium and phosphate. Cross-sectional studies in dialysis patients have shown that bone density is inversely associated with arterial stiffness [140] and also that adynamic bone disease is independently associated with both C-F PWV and aortic calcification [91].

## Fetuin-A and Renal Function

Studies examining the relationship between fetuin-A and renal function have also provided results that are difficult to interpret. All clinical studies except one [122] have studied total serum or plasma fetuin-A.

A study of the association of fetuin-A with renal function in 711 patients with relatively preserved renal function (mean creatinine clearance (CrCl) 80 ml/min/1.73m$^2$) and ischaemic heart disease found no independent relationship after adjustment for age, sex, ethnicity, diabetes, hypertension, serum albumin, haemoglobin and CRP. However, when the same adjusted comparison was performed using cystatin-C instead of CrCl, a significant relationship was found. An

increase in cystatin-C was associated with an increase in fetuin-A indicating an inverse association between renal function and fetuin-A concentration [141]. Unusually fetuin-A concentrations were also associated with serum corrected calcium and phosphorus in this study.

In contrast, Cottone *et al* examined the relationship of fetuin-A with $eGFR_{MDRD}$ in a population of patients with CKD stages 3-5. In multivariate analysis reduced fetuin-A levels were independently associated with IL-6, endothelin-1 and lower GFR [136]. Consistent with these findings, another small study of 32 CKD patients (mean CrCl 35 ± 11ml/min/1.73m$^2$) found that fetuin-A was inversely associated with serum creatinine [137].

However, these data are inconsistent and in further contrast, Stenvinkel *et al* studied a cohort of patients about to start dialysis (mean eGFR 6.8ml/min/1.73m$^2$) and found no direct correlation between eGFR and serum fetuin-A. However, potential confounders in this setting include inflammation, malnutrition and abnormalities of fluid and electrolyte homeostasis [115]. Weikert *et al* also failed to find any relationship between fetuin-A and creatinine in a population with normal renal function [142], and a further recent study of transplant recipients again found no relationship between total fetuin-A and eGFR [117].

As illustrated previously in the only published human study where renal function was related to CPP, the proportion of fetuin-A present as CPP (expressed as RR) increased significantly with stage of CKD (see fig. 1.8) [122].

## Fetuin-A and cardiovascular outcomes

### Normal renal function

Studies of fetuin-A in patients with normal renal function have shown contradictory results. Weikert *et al* showed a positive association between fetuin-A concentration and cardiovascular risk, however this study uncovered other unexpected correlations including a positive correlation between fetuin-A and hs-CRP. The authors of the paper postulate that the link between mortality and fetuin-A may be attributable to the link between fetuin-A and insulin resistance with the associated increased prevalence of atherosclerosis in that population [142].

Another study of healthy subjects found that fetuin-A was independently directly associated with carotid artery stiffness as measured by the stiffness parameter β (see appendix 2) [143]. Conversely sub-group analysis of a recently published study of subjects with normal renal function found that fetuin-A was inversely associated with PWV after adjustment for other variables [116].

**Fetuin-A in CKD-5D**

Fetuin-A concentrations are however convincingly lower in adult haemodialysis patients, in comparison both to healthy controls [144,145] and to patients receiving peritoneal dialysis [146]. Fetuin-A has been significantly inversely associated with cardiovascular and all-cause mortality in CKD-5D [115,144,146] (see fig. 1.12). Fetuin-A has been inversely independently associated with C-F PWV in children [147] and in Peritoneal Dialysys (PD) patients [26].

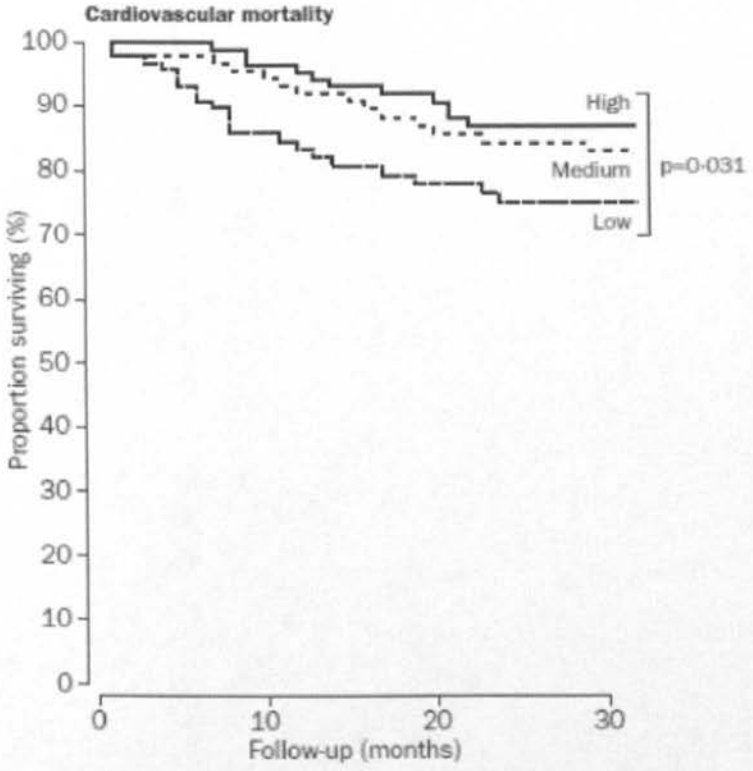



**Figure 1.12 - Kaplan-Meier survival plots of cardiovascular mortality within a propective cohort study of 312 prevalent European haemodialysis patients by tertile of fetuin-A Low 0.20-0.54g/l; Medium, 0.55-0.71g/l; High, 0.72-1.83g/l [144]**

However, the role of aortic stiffness as the mechanistic link between fetuin-A and cardiovascular mortality is questioned by the findings of Hermans *et al* who despite finding an independent relationship between fetuin-A and mortality (after

adjustment) found no association between fetuin-A concentration and C-F PWV in a cohort of 131 dialysis patients with low inflammatory status [148].

In other studies of dialysis populations, the significance of fetuin-A as a predictor of mortality has been confounded by inflammation [144,145,149]. Several studies have demonstrated a relationship between low fetuin-A and coronary artery calcification score (CACS). This relationship is seen in both dialysis and non-dialysis cohorts[88,149-152].

**Fetuin-A in pre-dialysis CKD**

Several recent studies have examined the association of fetuin-A with hard and surrogate outcomes in CKD stages 3 & 4. The first study investigating potential association between fetuin-A and cardiovascular and all-cause mortality in a large pre-dialysis CKD population (mean GFR 33 ± 11ml/min/1.73m$^2$) found no association [153]. In addition to quantification issues relating to Enzyme Linked ImmunoSorbent Assay (ELISA) type (discussed in section 3.3) [154], there are several possible clinical reasons for this. The 822 people followed up by this group were relatively young and healthy. The mean age was 52 years and only 9.7% of their study population had a history of coronary artery disease. Patients with polycystic kidney disease were heavily represented in this study (41% of patients) and these patients had higher concentrations of fetuin-A.

Sigrist *et al* examined the determinants of vascular calcification and arterial stiffness in a small heterogeneous group of patients with CKD stages 4, 5 & 5D. Whilst changes in vascular calcification was associated with change in R-DP PWV in this group, no association was found between fetuin-A and R-DP PWV or hs-CRP [155]. This cohort was somewhat limited by the inclusion of dialysis patients (88/134) with possible confounding due to the associated dialysis-mediated acceleration of stiffening and calcification [45] and the use of a PWV measure which incorporates predominantly muscular rather than elastic arteries. In addition there was significant variation in clinical parameters between comparator groups.

A recent study in transplant patients investigated the relationship of fetuin-A with coronary and aortic calcification. Whilst this study did not find an independent relationship between fetuin-A and CACS, an independent relationship between higher fetuin-A levels and reduced aortic calcification was found [117]. Survival

analysis in this cohort again found a significant interaction between inflammation and fetuin-A.

Manghat *et al* also studied the determinants of arterial stiffness in a CKD stage 1-4 population using Stiffness Index of the Digital Volume Pulse ($SI_{DVP}$) as the measure of arterial stiffness (see appendix 2). Only three parameters remained independently associated with this measure of arterial stiffness, bone specific alkaline phosphatase, tartrate resistant acid phosphatase and cholesterol ($R^2$=0.232). Fetuin-A, FGF-23, age and SBP were all excluded from the final model. This population had well controlled blood pressure, were relatively young and contained 29% CKD stages 1-2 [156]. Yilmaz *et al* found no independent relationship between fetuin-A and flow mediated dilatation in CKD stages 3 & 4 [157].

## Fetuin-A, Insulin Resistance, Adiponectin and the Metabolic Syndrome

During the course of this study significant advances have been made in the understanding of the role of fetuin-A in insulin resistance. These include a greater understanding of the interaction of fetuin-A with the insulin receptor and with adiponectin synthesis. Both these areas are relevant to this study.

Fetuin-A binds to the β subunit of the insulin receptor. Whilst fetuin-A undergoes complex post-translational modification including phosphorylation, the impact of these modifications on biological activity is not completely understood. However it is thought that only phosphorylated fetuin-A has the ability to cause downstream alteration to the activity of the insulin receptor tyrosine kinase, with resulting inhibition of receptor autophosphorylation and downstream tyrosine kinase activity [118,158]. Consistent with this, *in vivo*, *ahsg* knock-out mice have increased insulin sensitivity compared to the wild type [159].

Furthermore, in studies of healthy individuals, Stefan *et al* found fetuin-A levels were inversely associated with insulin sensitivity [160]. Further studies have also shown an association between fetuin-A levels, the metabolic syndrome and increased visceral adiposity (see fig. 1.13) [161-163], adding support to the theory that phosphofetuin has a role in the modulation of insulin resistance.

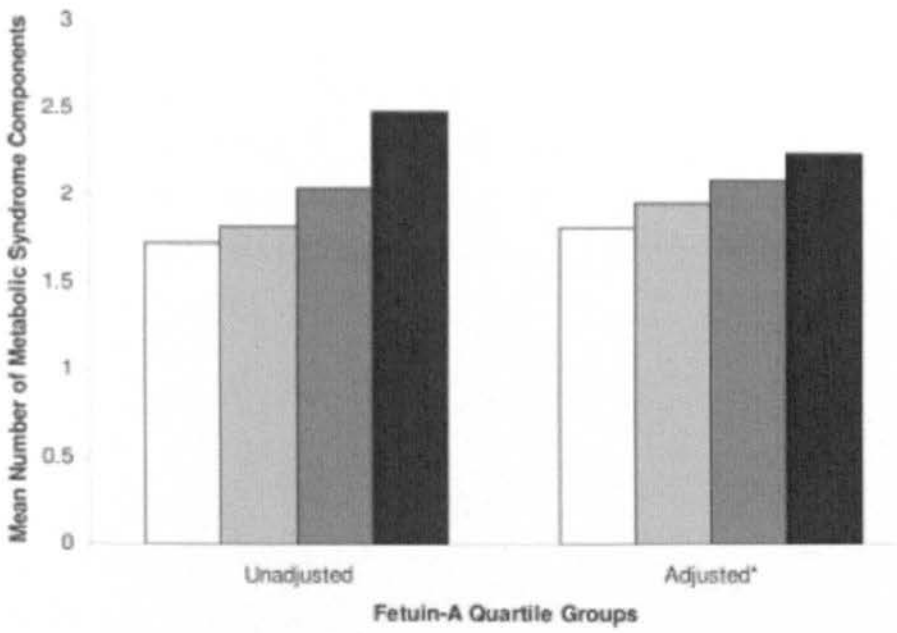



**Figure 1.13 - Relationship of fetuin-A quartile to mean number of components of metabolic syndrome in Heart and Soul Study of 711 non-diabetic patients from the USA - Quartile 1, <0.55g/l; quartile 2, 0.56-0.62g/l; quartile 3, 0.63-0.70g/l; quartile 4>0.71g/l - Adjusted for age, sex, race, alcohol, tobacco use, CrCl, BMI, hypertension, Low Density Lipoprotein (LDL) cholesterol, fibrinogen and CRP [162]**

Adiponectin is a cytokine, secreted from adipose tissue which increases body sensitivity to insulin. The genes *ahsg* and *adipoq* (encoding adiponectin) are located adjacently on chromosome 3q27 [164]. *In vitro* studies show fetuin-A treatment of adipocytes reduced transcription of *adipoq*. Consistent with this, administration of recombinant fetuin-A to mice is associated with reduction in circulating adiponectin, whilst cross-sectional analysis of a healthy human population showed a significant inverse association of fetuin-A with adiponectin [164].

Increased adiponectin, insulin resistance and the metabolic syndrome are associated with adverse outcomes in the general population [165-167]. It may therefore be expected that in the general population high fetuin-A would, in turn, also be expected to be associated with adverse outcomes. Indeed, this mechanism was proposed by Weikert *et al* in their study which demonstrated association between high fetuin-A and adverse cardiovascular (CV) outcomes [142]. This relationship has the potential to confound the inverse relationship between fetuin-A, arterial stiffness and cardiovascular disease which is seen in the CKD population.

## Fetuin-A and Pharmacological Intervention

There have been two intervention studies examining the response of serum fetuin-A to treatment with sevelamer.

Brandenburg *et al* studied 57 unselected haemodialysis patients in a cross-over study and found that sevelamer treatment increased plasma fetuin-A by 10% [5]. However, the significance of this finding is unclear since this was associated with an increase in CRP and a decrease in calcium during the treatment phase. Sevelamer is not thought to be absorbed from the gut lumen, and thus the mechanism involved in such an interaction is yet to be defined.

Caglar *et al* studied the effect of sevelamer in a small randomised controlled trial of stage 4 CKD patients [168]. They found similar increases in serum fetuin-A levels to Brandenburg *et al*, which were not seen in matched groups treated with calcium acetate.

There have been three other non-placebo controlled studies of the pharmaceutical manipulation of fetuin-A in man. A small study of 27 type II diabetic patients taking the Peroxisome Proliferator-Activator Receptor $\gamma$ (PPAR$\gamma$) inhibitor pioglitazone found significant reductions in total fetuin-A concentrations in the intervention arm [169]. A further study of 15 patients with metabolic syndrome taking sustained release niacin also found a significant reduction [170]. A third study of 12 haemodialysis patients observed a reduction in the CPP fraction after treatment with parathyroidectomy or cinacalcet for hyperparathyroidism [122].

## Summary

Fetuin-A is an abundant plasma protein whose primary function is to stabilise calcium and phosphate in solution. Fetuin-A is an integral part of the CPP found in CKD, which are proposed as a marker of extraosseous calcification stress. In addition to the role in calcification inhibition, fetuin-A is also thought to modulate insulin sensitivity and to be a negative acute phase reactant.

## 1.8 Osteoprotegerin and Receptor Activator of Nuclear Factor-κB Ligand

### Physiology

OPG and RANKL are cytokines which primarily control osteoclast activity and bone resorption. It has been proposed that this system may regulate differentiation of osteoclast-like cells at other sites, such as sites of vascular calcification [50].

RANKL is a 316 amino acid protein encoded on chromosome 13q12 [171]. The homotrimeric form of RANKL interacts with its' transmembrane receptor the Receptor Activator of Nuclear Factor-κB (RANK) which is expressed on pro-osteoclasts. Activation of the receptor causes osteoclast differentiation and functional activation causing bone resorption.

OPG is a 380 amino acid protein encoded on chromosome 8q24 which exists both in monomeric and dimeric forms. OPG has two active regions which correspond to the two recognised actions of OPG. Firstly OPG acts as a soluble decoy receptor for RANKL; effectively acting as a RANKL antagonist to inhibit osteoclastogenesis (see figure 1.14), and therefore inhibit bone resorption [172]. Secondly, OPG binds to TNF-Related Apoptosis Inducing Ligand (TRAIL) to inhibit apoptosis.

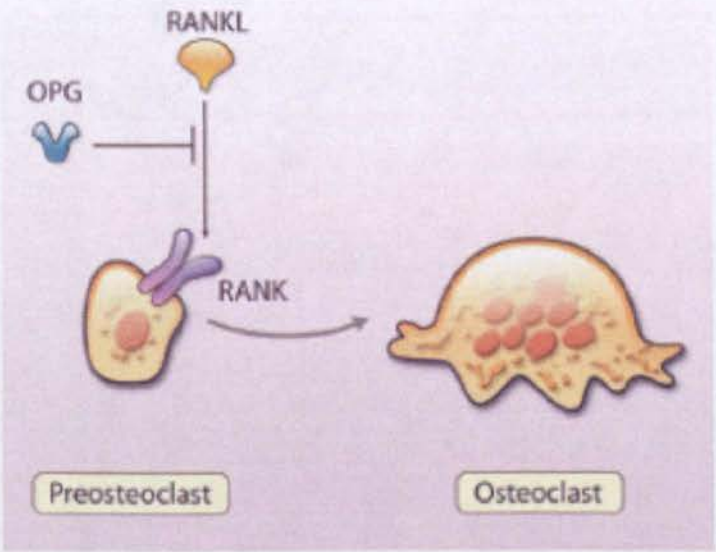



**Figure 1.14 – Binding of RANKL to RANK on the pro-osteoclast triggers osteoclast differentiation and activation. This interaction is inhibited by the binding of soluble decoy receptor OPG to RANKL [173].**

Bone marrow osteoblasts and stromal cells express both RANKL and OPG, thus osteoclast activity may be determined locally by the ratio of OPG:RANKL [174]. OPG is also expressed by endothelial and VSMCs. RANKL and RANK are usually undetectable in healthy vessels [175].

Soluble RANKL binds RANK in addition to OPG. Binding of RANKL to RANK is thought to stimulate chemokine release, monocyte chemotaxis and matrix metalloproteinase (MMP) activity thereby promoting vascular calcification [175-177]. Binding of RANKL to RANK on VSMC upregulates Bone Morphogenic Protein 4 (BMP-4) expression providing an autocrine stimulus for osteogenic transdifferentiation via the NF-κB pathway [177,178]. Recent VSMC cell culture studies have shown that this effect of RANKL is inhibited by OPG [177]. Furthermore studies in the rat 5/6 nephrectomy model have demonstrated that induction of CKD was associated with an increase of RANKL expression in the aorta. This co-localised with areas of calcification, but was not associated with an increase in OPG expression. Importantly however, these changes in expression were not mirrored by corresponding changes in the serum. In the serum, OPG levels were increased, whilst serum RANKL levels remained unchanged [177].

## Gene Disorders

The human phenotypes of the autosomal recessive OPG -/- and the gene disorders of RANK (which result in upregulation of RANK signalling) are of early onset Paget's disease with expansile skeletal hyperphosphatasia and familial expansile osteolysis respectively. These diseases are characterised by limb deformity, dental problems and deafness, but interestingly not by large artery calcification. However it should be noted that the oldest patient in the series of two reported with early onset Paget's disease was only 26 years old [179,180].

### Transgenic Animal Studies

Transgenic animal studies have yet to convincingly demonstrate a mechanistic link between OPG and vascular calcification. In fact, evidence from these studies indicates that OPG may actually have a protective role. OPG -/- mice develop large vessel medial calcification in response to inflammation [181]. OPG is expressed in wild type mouse aorta without RANKL or RANK expression. In the OPG -/- mouse, however, OPG is absent but RANKL and RANK are expressed, and a 4-fold increase in unbound RANKL is seen [182]. Shao *et al* proposed this may be due to

induction of RANKL expression by inflammation. Elevated OPG could therefore be proposed as an endogenous defence mechanism against increases in RANKL which acts under physiological conditions to prevent arterial calcification [178].

Consistent with this, OPG inhibited the development of vascular calcification in LDL receptor knockout mice fed an atherogenic diet. Exogenous OPG administration ***limited*** rather than exacerbated vascular calcification [183]. Furthermore knockout of the OPG gene in apolipoprotein deficient mice led to the acceleration of atherosclerosis and vascular calcification [182].

These findings suggest that the elevated OPG seen clinically may be reactive, rather than directly involved in the pathogenesis.

## Pathophysiology of OPG/RANKL

Control of OPG/RANKL expression is complex (see table 1.5). PTH upregulates RANKL messenger ribonucleic acid (mRNA) in bone marrow osteoblasts. There have been conflicting studies on the effect of PTH on OPG expression [175,184]. Moe *et al* investigated the differential effects of normal and uraemic serum on expression of OPG and RANKL in cultured bovine VSMCs and osteoblasts. Uraemic serum increased OPG and RANKL expression in VSMCs. In osteoblasts, RANKL expression was increased but OPG expression did not change [151]. This *in vitro* work provides supportive evidence of altered OPG/RANKL metabolism in the uraemic vasculature.

OPG and RANKL are both present in atherosclerotic lesions [185]. OPG is located at the margins of mineralizing lamellae, while RANKL is associated with the adjoining matrix [186].

Studies of the relationship between serum OPG and RANKL in the general population have found weak inverse correlations [187,188].

| OPG | | RANKL | |
|---|---|---|---|
| Increase | Decrease | Increase | Decrease |
| $1,25(OH)_2VitD_3$ | PTH | 1,25 $VitD_3$, PTH | TGF β |
| IL1α, TNF, IL6 IL-11, IL-17 | Glucocorticoids | Glucocorticoids | |
| Age, hypertension, renal impairment | $PGE_2$, IGF1 | $PGE_2$, IL-1α, TNF, IL-6, IL-11 & IL-17 | |
| Ang-II, FGF, PDGF | TGFβ, PPAR-ϒ | Immunosuppressants | |

**Table 1.5 - Factors influencing OPG and RANKL concentrations - $1,25(OH)_2VitD_3$-1,25 Dihydroxy cholecalciferol; PTH-parathyroid hormone; TGFβ-Transforming Growth Factor-β; IL-Interleukin; TNF-Tumour Necrosis Factor; $PGE_2$-Prostaglandin $E_2$; IGF1-Insulin-like Growth Factor 1; AngII-Angiotensin II; FGF-Fibroblast Growth Factor; PPAR-γ-Peroxisome Proliferator-Activated Receptor-γ; PDGF-Platelet Derived Growth Factor (after Vega & Collin-Osdoby) [175,179]**

## OPG

Clinical studies of OPG and cardiovascular parameters have yielded differing results. OPG concentration has been correlated with aortic calcification in haemodialysis [189-191] and transplant patients [192]. OPG has been associated with coronary artery calcification in dialysis and non-dialysis CKD patients and also in diabetic patients with normal renal function [151,193,194].

Increased OPG is also seen in patients with unstable angina [195], myocardial infarction [196] and significant coronary artery stenosis [197]. OPG has also recently been associated with mortality in both the GISSI-HF trial [198], a prospective cohort study of 1229 patients with heart failure and normal renal function, and a further study of a heterogeneous group of CKD stages 4, 5 and 5D [199].

Conversely, in a study of 135 patients with CKD stages 1-5 (but without traditional cardiovascular risk factors) no significant association between OPG, RANKL and cardiovascular events was found [200]. Jono *et al* also found no significant relationship with OPG and cardiovascular mortality in a study of 225 patients with normal renal function undergoing elective coronary angiography [201].

**OPG and Arterial Stiffening**

Several recent studies have reported an association between arterial stiffness and OPG. One study of children on dialysis using C-F PWV reported a significant direct correlation [147]. Sigrist *et al* also reported a direct correlation between OPG and C-R PWV in a mixed cohort of CKD stage 4, 5, & 5D [199]. Nakashima *et al* studied 151 haemodialysis patients and reported an independent association between B-A PWV and OPG [202]. Recently Zagura *et al* reported an independent association between OPG and C-F PWV in a study of patients with normal renal function [203].

**OPG Pathophysiology**

There are two mechanisms by which OPG is proposed to inhibit vascular calcification:-

**1. RANKL**

RANKL is expressed in the extracellular matrix of vessels with calcified plaque. RANKL is associated with osteogenic transdifferentation of VSMC via BMP-4. OPG is a direct inhibitor of RANKL and therefore would be expected to inhibit this process.

**2. Endothelial Dysfunction**

Endothelial dysfunction triggers inflammatory cell translocation into the vessel wall, where inflammatory cytokines are produced possibly promoting calcification. OPG may be upregulated as a negative feedback mechanism [204].

Conversely, there are several proposed mechanisms by which OPG may be associated with arterial stiffening and adverse outcomes in CKD. It is however noteworthy that some of the evidence relating OPG to outcomes comes from studies of non-CKD patients with non-stiffening related cardiovascular morbidity, i.e. coronary artery disease [194,196].

**1. Direct Effect**

OPG and RANKL expressed within the bone or the vasculature in the uraemic state may have either a distant endocrine or paracrine effect on the arterial system causing calcification, potentially via VSMC transdifferentiation.

OPG plays a key role in inhibiting osteoclastic bone resorption [205]. Patients with decreased renal function have an increased risk of osteoporosis [206]. Reduced bone

mineral density (BMD) has been associated with arterial stiffness and reduced survival in this setting [207,208]. Osteoporotic fracture with associated morbidity is however unlikely to be the main mechanism by which CKD-MBD is linked to mortality.

Whilst evidence of the role of OPG in the control of bone resorption is strong, pathophysiological evidence of the role played by OPG in vascular stiffening, calcification or VSMC transdifferentiation is less robust.

**2. Inflammation and Atherosclerosis**

Inflammation and atherosclerosis are associated with elevated OPG. The relationship of OPG and adverse outcomes may be direct, or may be an epiphenomenon.

OPG expression is increased by IL-1, TNF-α and other pro-inflammatory cytokines (see table 1.5) [179]. Inflammation is also associated with arterial stiffening. It is not clear if the elevated OPG of CKD is a marker of renal osteodystrophy or is related more closely to the process of vascular damage, perhaps through inflammation. In addition to OPG, inflammatory mediators increase the activity of alkaline phosphatase, a key osteogenic enzyme [172]. In CKD, inflammation and increased bone turnover co-exist. These processes may synergistically lead to accelerated vascular calcification.

**3. Apoptosis**

OPG acts as a decoy receptor for TRAIL [209,210]. TRAIL is known to induce apoptosis in cancer cells, however its role in non-cancer cells is not completely understood. TRAIL binds to four other non-OPG receptors. Binding of TRAIL to TRAIL death receptors 4 or 5 induces apoptosis in endothelial cells and VSMCs. The other two TRAIL receptors are thought to have anti-apoptotic effects.

Within the vasculature, OPG & TRAIL co-localise around areas of medial calcification and intimal atherosclerosis [211]. Clinically, serum TRAIL has been inversely associated with severity of coronary artery disease [212]. A study of uraemic *apoE-/-* mice found that uraemia enhanced TRAIL (& OPG) expression [213]. In addition TRAIL enhanced human VSMC calcification in cell culture. However in the same study, serum TRAIL was reduced in haemodialysis patients and was not correlated with vascular calcification.

The association of TRAIL with apoptosis and the associated increased concentrations of OPG seen in dialysis patients would be consistent with the observation that dialysis induces both apoptosis and vascular calcification [45]. Plasma OPG in this setting may therefore be a measure of vascular apoptosis, perhaps acting as a compensatory mechanism moderating TRAIL activity.

Moran *et al* demonstrated increased OPG staining in the tunica media of human abdominal aneurysm biopsy samples. In addition they demonstrated *in vitro* that incubation of healthy human VSMCs with OPG caused impaired cell proliferation, with associated apoptosis and production of MMPs, which are implicated in elastolysis and alteration of the extracellular matrix [214].

**4. Senescence Associated Secretory Pathway**

Ageing and uraemia are both associated with progressive DNA damage and exposure to oxidative stress [46,215]. These pathological triggers may cause the cell to leave the cell cycle. In addition to apoptosis or necrosis, the cell may enter a senescent phase [216,217]. This senescent phase has been defined as an irreversible growth arrest of previously mitotic cells, which is triggered by telomere shortening, oxidative stress or activated oncogenes [216,218,219]. This phenotype is associated with loss of function and loss of regenerative capacity. In addition, however, the senescent cell may further alter its gene expression to develop the senescence associated secretary pathway (SASP), which is cell type specific (CESP) [220,221]. Recent work on the VSMC has demonstrated that *in vitro* senescent VSMCs down regulate expression of genes including OPG and MGP, whilst upregulating expression of pro-calcific factors such as BMP-2 and MMPs [222].

Whilst this mechanism links the uraemic state and changes to the VSMC with the expression of cytokines which are mechanistically linked to vascular calcification, the change in expression of OPG and MGP is in the opposite direction to that which would be expected if VSMC senescence were to provide the mechanistic link between OPG and arterial stiffening and adverse outcome.

**5. Other**

Increased OPG expression has been demonstrated in the cardiomyocytes of patients with cardiomyopathy due to ischaemic or dilated cardiomyopathy [76]. Increased tissue

concentrations of OPG have been found within symptomatic carotid atherosclerotic plaque indicating a possible role in plaque destabilisation [185].

## RANKL

Three large epidemiological studies of patients with largely normal renal function have explored the association of RANKL with cardiovascular outcome.

RANKL and OPG were both independently associated with cardiovascular mortality risk in the Bruneck study of 909 subjects [187]. There was no significant correlation between RANKL and age, gender or serum creatinine in this study. There was also no correlation between RANKL and intimal media thickness or atherosclerosis score. OPG did not modify the strength of association of RANKL with outcome indicating an independent effect. The study authors proposed that the association of RANKL with CV mortality was due to its effect on monocyte migration and matrix degradation with subsequent destabilization of atherosclerotic plaque.

Conversely Lieb *et al* examined the relationship of OPG and RANKL with survival in the Framingham study. They found that serum OPG was independently associated with coronary calcification, cardiovascular disease and mortality, whilst RANKL was not [188]. The Framingham authors propose that the difference in results between their study and the Bruneck study could be explained by either the use of a mixed cardiovascular end point (rather than the acute vascular syndromes which would be expected to be related to a protein implicated in plaque rupture) or to the high analytical variability in their dataset.

Finally, in a sub-study of 2656 subjects from the European Prospective Investigation into Cancer (EPIC)-Norfolk study variation in the association of serum RANKL with regard to cardiovascular risk factors was reported according to gender. Whilst there was no difference between serum RANKL levels according to gender in males, RANKL was associated with age, lipid parameters, BMI and blood pressure, whilst in women it was associated with CRP. No association was found between RANKL and risk of coronary events after adjustment. Again in this study OPG was independently associated with coronary event risk, even after adjustment for Framingham risk factors. The authors suggested that in settings of high OPG concentration OPG may enhance the MMP inducing effects of RANKL [223].

### RANKL and Arterial Stiffening

There are only two published studies examining the relationship of RANKL with arterial stiffness. Othmane *et al* found no independent association between serum

RANKL and change in C-F PWV in an underpowered interventional study of the effect of sevelamer over 11 months in 26 haemodialysis patients [224]. Mangiafico *et al* also failed to find a relationship between RANKL and AI in a cross-sectional study of 182 cardiovascularly healthy post-menopausal osteoporotic women [225]. These results appear at odds with the *in vitro* and *in vivo* work in animal models.

**OPG & RANKL – A Summary**

There is mixed epidemiological evidence with regard to cardiovascular outcomes. However basic science studies have demonstrated that RANKL may be implicated in vascular calcification. Osteoporosis (a state of excess osteoclast activity) is associated with arterial stiffness. This association, when coupled with evidence of RANKL expression and arterial calcification in the OPG knockout mouse, and the work of Panizo *et al* who demonstrated RANKL expression co-localised with areas of aortic calcification in a rat CKD model, makes RANKL a reasonable candidate molecule for a mediator of arterial calcification, and therefore arterial stiffness. However, on balance, the clinical evidence indicates a stronger role for serum OPG than RANKL, both as a biomarker for arterial stiffness and more generally for cardiovascular risk.

## 1.9 Fibroblast Growth Factor-23 & Klotho

Hyperphosphataemia, elevated PTH and abnormal vitamin D levels are common in CKD and are associated with increased mortality. All these states are mediated, in part, by FGF-23 (see fig. 1.17). Recent studies have begun to investigate the contribution and mechanism(s) by which FGF-23 may be involved in this process. However, the relative importance of each of these potential mediators in the determining the final outcome is not clear.

### Physiology

FGF-23 is a 30kDa bone-derived protein with an important role in phosphate and vitamin D metabolism [226,227]. FGF-23 is expressed in osteoblasts in response to hyperphosphataemia and elevated $1,25(OH)_2$ Vitamin D[228].

FGF-23 binds to the ubiquitous FGF-Receptor (FGF-R) in the presence of the obligate cofactor Klotho [229]. Klotho expression is limited to the renal tubule, parathyroid, reproductive organs and pituitary gland determining the tissue selectivity of FGF-23 under physiological conditions [230]. Klotho has not been detected in vascular or cardiac tissue. FGF receptor binding in the kidney leads to suppression of the epithelial Na/Pi -2a and -2c transporters, with reduced tubular phosphate re-absorption and a subsequent increased in urinary phosphate excretion [231,232].

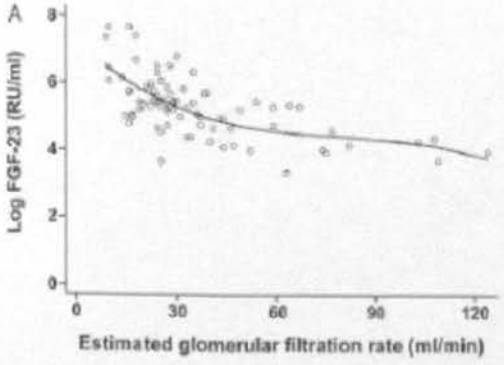



**Figure 1.15 – Relationship of eGFR (ml/min) with log cFGF-23 (RU/ml) in 80 adult CKD outpatients from USA [237]**

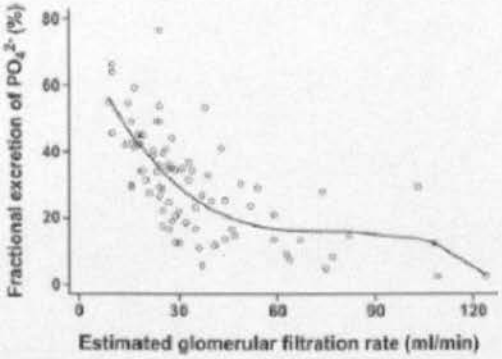



**Figure 1.16 – Relationship of eGFR (ml/min) with fractional excretion of phosphate (%) in 80 adult CKD outpatients from USA [237]**

Measurement of FGF-23 is not straightforward and studies variously report concentrations of intact FGF-23 (iFGF-23) or the c-terminal fragment (cFGF-23)[233]. However FGF-23 increases as GFR drops below 90ml/min/1.73m$^2$ [234,235] preceding changes in serum calcium and phosphate (see figs 1.15 & 1.16). The associated increased fractional excretion of phosphate represents an appropriate osseous response to the reduction in phosphate excretion due to GFR reduction. In advanced CKD however, serum phosphate rises as the fractional excretion of phosphate is no longer able to compensate for reduction in glomerular filtration despite further exponential increases in FGF-23.

FGF-23 influences vitamin D metabolism by suppressing 1α-hydroxylase whilst increasing expression of 24-hydroxylase, resulting in a reduction in active 1,25 $(OH)_2$ vitamin $D_3$ [236-238].

The actions of FGF-23 are demonstrated by animal models and hereditary syndromes in which there is excess or absent FGF-23. Klotho and FGF-23 knockout mice demonstrate widespread vascular calcification [239,240]. Familial hypophosphataemic rickets is a syndrome of pathological excess FGF-23 and is characterised biochemically by hyperphosphaturia and hypophosphataemia [241,242]. In contrast, inactivation of the FGF-23 gene causes tumoral calcinosis with hyperphosphataemia, excess 1,25$(OH)_2$ vitamin $D_3$ and widespread soft tissue and vascular calcification [243].

Klotho is also expressed in the parathyroid gland. FGF-23 reduces PTH secretion, which also causes reduction in tubular phosphate re-absorption. Klotho expression is significantly reduced in CKD [244,245], however it remains unclear if the reduction in klotho is a result, or trigger, of increased FGF-23. Whilst a beneficial activity of klotho has been recently demonstrated in mice [244], the clinical evidence associating the FGF-23 klotho axis to outcomes has been strongly weighted towards FGF-23. This evidence will now be reviewed.

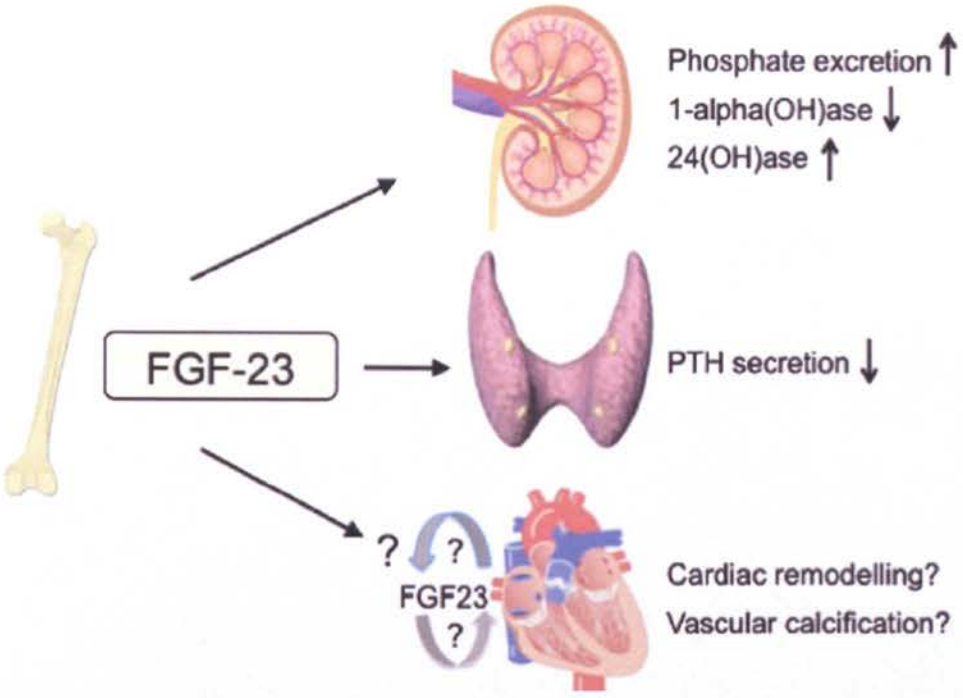



**Figure 1.17 - Physiological and proposed pathophysiological actions of FGF-23 on the kidney, parathyroid and cardiovascular system leading to increased phosphate excretion, altered vitamin D metabolism, reduced PTH secretion and possible cardiac remodelling and vascular calcification** [246]

## Pathophysiology of FGF-23

### Vascular Calcification

Nasrallah *et al* found FGF-23 was independently associated with aortic calcification score, in addition to age and systolic blood pressure (SBP), in a small haemodialysis cohort [247]. Jean *et al* also found an association of FGF-23 with aortic calcification in a larger prevalent cohort of patients [248]. FGF-23 has also been associated with calcification of small calibre arteries in 56 non-diabetic haemodialysis patients, although no association was found between FGF-23 and aortic calcification in this study [249].

Conversely in a cohort with normal renal function, Roos *et al* found no relationship between FGF-23 and CACS [250].

In mouse models of CKD, FGF-23 has been associated with aortic calcium content [251]. However in an apparently contradictory model, FGF-23 knockout mice and mice with non-functional klotho alleles both demonstrated widespread arterial calcification. However, as expected, these phenotypes have severe

hyperphosphataemia and elevated levels of $1,25(OH)_2$ vitamin D, which have the potential to mask any causal relationship between FGF-23 and vascular changes [252].

Thus phenotypes with both low and high FGF-23 have both been associated with ectopic calcification. The role of phosphate must be considered a significant confounder in the absent FGF-23 model, where the associated hyperphosphataemia is likely to trigger arterial calcification irrespective of FGF-23 status. In the CKD model however, increases in phosphate are generally limited until ESRF is reached, whereas FGF-23 undergoes substantial increases below a GFR of 60ml/min/1.73m$^2$ (see fig 1.15).

**Klotho non-specific effects of FGF-23**

FGF receptors are present on cardiomyocytes and vascular endothelium [253], but klotho is not expressed within the cardiovascular system, and thus any effects of FGF-23 within the vasculature are thought to occur via a klotho-independent mechanisms. It has been suggested that low affinity binding of FGF-23 to FGF receptors may mimic binding of FGF-2 [254], which has previously been implicated in myocardial hypertrophy [255,256]. This theory has recently been broadened by the *in vitro* demonstration that FGF-23 causes cardiomyocyte hypertrophy via a FGF-R dependent pathway [77]. Furthermore this effect was also seen *in vivo*, in both wild type and klotho deficient mice, confirming the mechanism to be klotho independent, and providing a clear proof of concept link between phosphate excess and LVH.

## FGF-23 and Outcomes - Cardiovascular Risk

Gutierrez *et al* investigated the relationship of FGF-23 with mortality in dialysis and non-dialysis CKD. In a haemodialysis cohort, a dose response relationship between FGF23 level and mortality risk was found in an analysis which controlled for the potential impact of phosphate and adjusted for PTH. There was no significant difference in $1,25(OH)_2$ vitamin $D_3$ levels between groups (see fig. 1.18) [257].

**Non-dialysis Cohorts**

In a study of 162 pre-dialysis CKD patients, the same group also found a strong independent relationship between FGF-23 and LVH. However, no relationship was found between FGF-23 and coronary artery calcification in this study [74]. Seiler *et al* studied a group of 149 CKD patients with mean eGFR 36 ± 23ml/min/1.73m$^2$ and found that cFGF-23 was independently associated with an increased risk of

cardiovascular events and death (Hazard Ratio (HR) 2.49 (95% Confidence Interval (CI) (1.40-4.39)). This analysis adjusted for eGFR, diabetic status, phosphate and cardiovascular co-morbidity, but failed to adjust for vitamin D status or proteinuria – both important confounders. This relationship lost significance in the subgroup with eGFR≤30ml/min/1.73m$^2$ [258]. The Chronic Renal Insufficiency Cohort (CRIC) study of 3879 patients with eGFR 20-70ml/min/1.73m$^2$ has also recently reported that elevated FGF-23 is associated with risk of death [263]. Other studies of patient groups with normal renal function have found association between FGF-23 and left ventricular mass [259], and cardiovascular events and all cause mortality rates [260].

There is also emerging evidence that FGF-23 may be associated with coronary artery disease. A recent study of patients with eGFR 30-90ml/min/1.73m$^2$ found that FGF-23 was independently associated with a score of extent of coronary artery occlusion [88]. However, this association was not replicated by Gutierrez *et al* in their study of predialysis CKD patients [74].

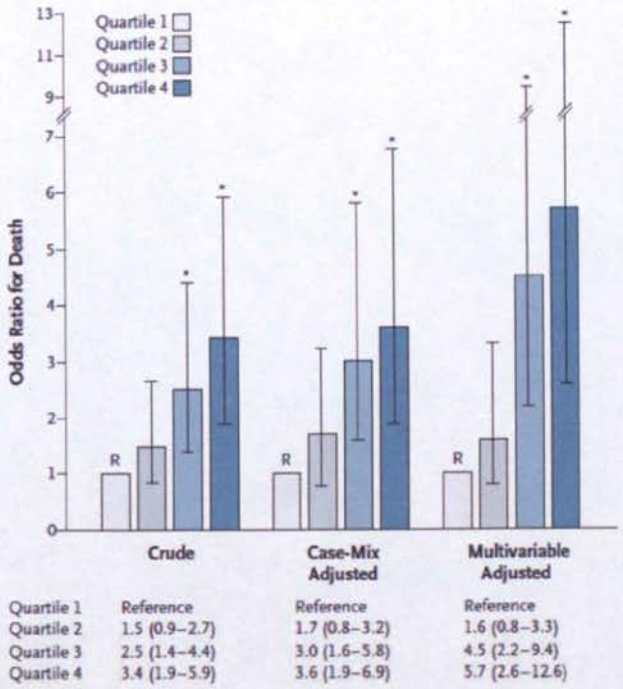



**Figure 1.18 - Increased Odds Ratios (OR) (+95%CI) for death across quartiles of c-FGF23 in nested case control study of 400 incident US haemodialysis patients - Quartiles of FGF-23: 1, <1090RU/ml; 2, 1090-1750RU/ml; 3, 1751-4010RU/ml; 4, >4010RU/ml - Case mix adjustment -age, sex, ethnicitiy, BP, BMI, dialysis centre, adequacy & access, cause of CKD and comorbidity; multivariate adjustment - case mix plus phosphate, calcium, albumin, PTH, creatinine and ferritin [257]**

### FGF-23 and the Artery

The associations of FGF-23 with several surrogates of vascular dysfunction have been explored in the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) cohort, a Swedish community study in which approximately 22% of patients have eGFR<60ml/min/1.73m$^2$ [261]. In this study arterial stiffness was measured using a change in reflection index (ΔRI) (see appendix 2) and patients were dichotomised by estimated GFR calculated from a cystatin based formula. Notwithstanding these unusual methodologies, in crude and fully adjusted cross-sectional models the authors found that increased FGF-23 was independently associated with increased 'stiffness'. However ΔRI is primarily a measure of peripheral resistance and may reflect predominantly endothelium related NO production rather than large vessel compliance. This relationship was only present in the subgroup of 150 patients with cardiovascular co-morbidity. The relationship was particularly strong in the subgroup of 42 patients with reduced renal function ($eGFR_{Cys}$ <50ml/min/1.73m$^2$). A further PIVUS cohort sub study with mean eGFR 83.7 ± 18ml/min/1.73m$^2$ found an association between FGF-23 and atherosclerotic load [262] and concentric LVH [259].

The relationship of FGF-23 with flow-mediated dilatation (FMD) of the artery (a test of endothelial function) was also measured in another study of a clean CKD population with limited CV comorbidity examined. In an adjusted analysis increased FGF-23 was associated with greater flow mediated dilatation [157]. Arterial stiffness was not measured in this study.

### FGF-23 and Rate of Change of Renal Function

The CRIC study also examined the relationship of FGF-23 with probability of reaching ESRF. FGF-23 was not associated with risk of ESRF in the fully adjusted population analysis, although a significant relationship was found in a subgroup analysis of the patients with eGFR>30/min/1.73m$^2$ [263]. Fliser *et al* had previously found association between FGF-23 and rate of renal function decline in the Mild Moderate Kidney Disease (MMKD) study comprising predominantly patients with CKD stages 1 & 2 [264].

## Summary – FGF-23

FGF-23 is a bone derived phosphatonin which in the healthy state and early stage CKD controls serum phosphate via mediation of renal phosphate re-absorption. In

later stage CKD, FGF-23 levels rise exponentially and appear to be associated with LVH and cardiovascular mortality. FGF-23 has been associated with rate of decline of renal function. Limited data on the relationship with arterial stiffness is available.

## 1.10 Outcome Measures

The outcome measures used in this study will now be reviewed.

### Arterial Stiffness – Baseline and Rate of Change

As previously discussed, there is limited published data examining the relationship of any of the CaRP with the gold standard measure of aortic stiffness in CKD stages 3 and 4. Whilst several studies have addressed the determinants of rate of change of PWV (see table 1.1), only one small study has measured interval C-F PWV in pre-dialysis CKD patients [27].

### Rate of Decline of Renal Function

Whilst a point estimate of reduced GFR is a recognised cardiovascular risk factor in the general 'healthy' population [265], other studies have suggested that rate of decline of renal function may also predict cardiovascular events in both healthy populations [266,267] and in CKD [9].

Given the evidence that CaRP are associated with arterial stiffening and with adverse outcome, we hypothesised that these proteins would be independently associated with rate of renal function decline. No studies have been published relating fetuin-A, OPG or RANKL to rate of renal functional decline.

### Troponin

Troponin (cTn) is a macromolecular complex of three tightly interlinked proteins (cTn Tropomysin (cTnT), cTnI & cTnC) which are involved in the regulation of cardiac contraction. cTnT is a 37kDa, 228 amino acid polypeptide subunit. 92% of cTnT is bound to the sarcolemmal membrane whilst the remaining 8% is free in the cytosol [268]. Routine serological investigation of ischaemic myocardial injury is based upon quantification of circulating cTnT. Diagnosis of myocardial ischaemia requires a troponin above the 99$^{th}$ centile of a healthy population using a test with an acceptable precision (a coefficient of variation (CVr) of $\leq$10% at that concentration) [269]. Historically this level of accuracy at low concentrations has been hard to achieve in commercial assays which have lacked precision towards the lower limit of detection with an associated reduction in sensitivity.

**Troponin a biomarker for adverse outcome**

The significance of an elevated troponin level outwith the setting of symptomatic acute cardiac ischaemia has been explored by several groups. A consistent association with adverse outcomes has been found.

A meta-analysis of the risk of cardiovascular mortality in asymptomatic dialysis patients associated with abnormally elevated cTnT found a relative risk of 2.55 (CI 1.93-3.37) [270]. Consistent results were found in patients with CKD stages 3-5 [271]. However standard assays were used in these studies and the majority of patients had undetectable cTnT.

A new generation of high sensitivity cTnT (hs-cTnT) assays were introduced in 2009. These assays are able to detect significantly lower levels of circulating troponin T with greater precision. General population studies using the new 5$^{th}$ generation assays have demonstrated that hs-cTnT was detectable in a significant proportion of 'healthy' subjects. Koerbin *et al* selected a 'cardio- and renal healthy' population using stress echocardiography and serum creatinine and measured hs-cTnT. All patients had a cTnT below the standard concentration (0.01mcg/l), but 74% of patients had a hs-cTnT titre >3ng/l [272].

Recent data from three large US community cohort studies of individuals with normal renal function has shown that hs-cTnT was detectable in 22-66% of individuals (see fig. 1.19) [273-275]. In all these studies hs-cTnT was associated with mortality risk. In addition, in the Dallas Heart Study (mean Cr~70μmol/l) hs-cTnT was associated with a variety of measures of LVH [274]. In the Cardiovascular Health Study (CHS), which excluded patients with known heart failure, hs-cTnT was associated with incident heart failure [275]. Finally in the Atherosclerosis Risk In Communities (ARIC) study, which excluded patients with known coronary heart disease and stroke, hs-cTnT was associated with incident CHD and stroke [273]. Studies of patients with coronary artery disease or heart failure have found hs-cTnT in nearly all participants [276,277].

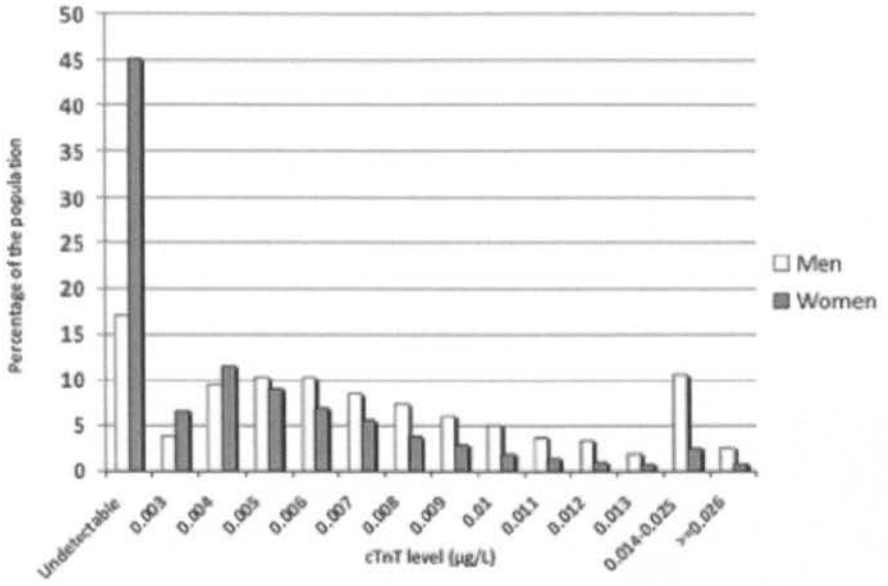



**Figure 1.19 - Histogram showing gender-specific distribution of hs-cTnT in 9698 general US population subjects in ARIC study (mean age 63.9 years)** [273]

Within CKD cohorts, hs-cTnT assay use increases the sensitivity of long term cardiovascular risk determination over standard troponin in asymptomatic haemodialysis patients [278]. The significance of detectable hs-cTnT in asymptomatic pre-dialysis CKD remains incompletely understood. Few CKD stage 3 & 4 cohorts have examined the relationship of troponin with outcome.

In the Chronic Renal Impairment in Birmingham (CRIB) cohort of 382 non-dialysed patients with CKD stages 3 to 5 (of whom 88 had CKD stage 3, 178 stage 4 & 116 stage 5) troponin was detected in 22% of patients using a standard assay. This group incorporated troponin in their final model for the prediction of mortality, in conjunction with age, current smoking and n-terminal pro Brain Natriuretic Peptide (NT-pro-BNP) and calculated a relative risk of death due to elevated troponin (>0.01µg/L) of 1.83 (95% CI 1.26-2.66) in a mean follow up period of six years [279]. Testing of this model in another United Kingdom (UK) cohort, the East Kent cohort showed a consistent result (C-statistic 0.82 (95%CI 0.75-0.89) [279].

Finally, Abbas *et al* used a standard troponin assay in their UK based study of 222 CKD stages 3-5 patients, of whom 95 had detectable troponin. Whilst they found evidence of an independent relationship between eGFR and cTnT, they did not find a significant independent relationship between cTnT and Left Ventricular Mass Index (LVMI). However cTnT was predictive of survival in this cohort [271].

Despite the use of troponin to diagnose myocardial infarction, the elevation of troponin is not solely dependent upon obstructive epicardial coronary disease. It is therefore instructive to consider the other reasons why troponin may be elevated.

Teleologically, one might expect that detection of a low concentration of a circulating human protein, albeit one which is expressed only within the myocardium, may reflect normal physiology rather than a pathological process. Development of an exquisitely sensitive assay would therefore be able to detect tiny amounts of protein released during cell turnover. Troponins have been studied in athletes. Elevated levels are found, particularly after prolonged intense activity, but have been correlated with reduced ventricular contractility in this setting [280]. Proposed mechanisms leading to troponin release in athletes include an increase in myocardial sarcolemmal membrane permeability permitting leakage of cytosolic troponin. Other mechanisms proposed to lead to this increase in permeability in athletes include oxidative stress and altered acid-base balance [281] - both processes present in patients with CKD.

Alternatively troponin may be released from the cell cytosol during myocyte turnover [282,283]. These are physiological processes and differ from troponin release secondary to cardiomyocyte necrosis as seen in myocardial infarction. The physiological release of troponin is however not consistent with the association of troponin with adverse outcomes in either asymptomatic or symptomatic patients.

Alternatively, and perhaps more relevantly, myocardial stretch with stimulation of integrins has been implicated in elevated troponin levels. Integrins are bi-directional signalling molecules involved in cardiac remodelling after myocardial infarction or with pressure overload. Stimulation of these integrins mediates export of troponin out of viable cardiomyocytes [284,285]. Whilst it is likely that there is significant overlap, it is proposed that in high risk populations hs-cTnT is predominantly a biomarker of ongoing cardiac damage or dysfunction [286].The mechanism associating the presence of low concentrations of circulating cTnT with vascular pathology remains to be established.

**Elevated Troponin in Renal Impairment**

In order to inform an interpretation of serum troponin titres in CKD it is useful to review the impact of reduced GFR upon clearance of this molecule. Consensus on

the role of renal excretion of troponin is lacking. Whilst an in depth review of the clearance kinetics of troponin in CKD is beyond the scope of this study, and indeed to the best of my knowledge no such studies have been published in the era of high sensitivity troponin assays, it is useful to note some of the published data.

Early theories that positive troponin results in CKD were the result of foetal expression of cTnT in skeletal muscle or cross reactivity of skeletal TnT with early assays have been discounted [287,288].

There are four current potential explanations for the increased circulating concentrations of cTnT in CKD patients:-

1. cTn-T is normally renally excreted and elevated cTnT is a reflection of reduced filtration
2. In CKD, metabolism of troponin is altered leading to the generation of fragments which cross react with the epitope utilized in the assay.
3. Elevated cTnT in CKD is a manifestation of increased cardiac remodelling or increased cardiac strain
4. Elevated cTnT in this population is a manifestation of cardiac ischaemia

Evidence that elevated troponin titres measured in haemodialysis patients are due to circulating immunoreactive fragments of TnT have again generally been discounted [289]. Troponin fragments present are not thought to cross react with hs-cTnT assay [286].

In healthy individuals, it has been suggested that troponin has a 2-5% renal clearance [290]. Wiessner *et al* studied circulating troponin concentrations post coronary artery bypass grafting in a two groups, one with CKD (4/15 on RRT) and a control. The CKD group were found to have cTnT levels that were significantly higher for longer with a greater area under the curve compared to the control group [291]. cTnT has also been detected in the urine in pre-dialysis CKD patients. The cTnT:creatinine ratio increased with severity of renal failure but was thought likely to be dependent upon the proportion of tubular secretion of troponin [292].

We postulated that the improved ability of the hs assay to detect cTnT at low concentrations may allow us to detect a relationship between either aortic stiffness or the CaRPs and cardiac risk. The mechanism for this could be either a direct relationship between these proteins and TnT release – i.e. a direct expression of the

protein in the ischaemic, failing or hypertrophic myocardium, or indirectly due to sub-endocardial ischaemia due to impaired diastolic cardiac perfusion due to earlier arrival of the reflected wave in the patients with the stiffer arteries.

## 4. Survival

The results of any study which examines the predictors of survival are dependent upon the recruited population. It is therefore instructive to consider cohorts with similar characteristics to this study.

In addition to the CRIB study[279], a much larger, but retrospective cohort study of the referred CKD stage 4 population of north west Canada found that age, lower DBP, high phosphate, low haemoglobin and high calcium were predictive of mortality whilst vitamin D use was associated with survival [293].

Both traditional and non-traditional risk factors are therefore associated with mortality risk in CKD. All of the calcification regulatory proteins, except CPP, studied in this thesis have been associated with mortality in CKD populations. We will explore the relationship between C-F PWV and the CaRP in a cohort of CKD stage 3 and 4 patients.

## 1.11 Aims in the Wider Context

### KDIGO & EURECA-m

The area of study of this thesis has been highlighted for study by the Kidney Disease: Improving Global Outcomes (KDIGO) foundation in 2009. Their recommendations to the research community include:

1. To develop a risk-stratification tool based on CKD-MBD components and evaluate its predictive accuracy for clinical outcomes inpatients with CKD stages 3-5, 5D, and 3-5T [100].

2. To prospectively study circulating biochemical markers (including bone specific alkaline phosphatase, procollagen type 1 n- and c-terminal propeptides, and n- and c-telopeptide cross links, tartrate-resistant acid phosphatase, and osteoprotegerin) to determine if they can predict fractures or other clinical outcomes in CKD stages 3-5, 5D, and transplanted patients [100].

In addition, the European Renal Association – European Dialysis and Transplant Association (ERA-EDTA) working group, European Renal Cardiovascular Medicine (EURECA-M) which focuses on the interaction between pathologies of the cardiovascular and renal systems have included the following priorities:

1. Clarification of the relative contribution of renal factors for cardiovascular disease versus renal disease progression in patients with CKD [294].

2. Identification of novel risk factors and markers of cardiovascular disease and for progression in patients with CKD [294].

3. Longitudinal assessment of the relationship between typical biochemical alterations found in CKD and ESRF (oxidative stress, inflammation, endothelial dysfunction, and bone and mineral disorders) in relationship to arterial changes (PWV) and clinical outcomes [295].

4. Determine the role of arterial stiffening in left ventricular hypertrophy and left ventricular failure dysfunction (sic) in patients with CKD and/or CV diseases [295].

Consistent with these bodies, the specific aims of this study, as previously stated, were:

1. To explore the relationship between CaRP and rates of aortic stiffening and change in renal function.
2. To explore the relationship of the CaRP with myocardial dysfunction.
3. To explore the relationship of plasma fetuin concentration with inflammatory markers and urinary fetuin-A loss.
4. To explore the relationship of the CaRP with hard outcomes, i.e. mortality and end stage renal failure

# Chapter 2

# Patients and Methods

## 2.1 The ACADEMIC Study

This doctoral study is a sub-study of a larger project entitled the ACADEMIC study. The ACADEMIC study is a prospective observational cohort study of patients with CKD, which set out to investigate the relationship between stiffening of the blood vessels and oxidative stress. The pre-specified end points of the study were (i) rate of decline of renal function/ renal replacement therapy, (ii) cardiovascular events and (iii) death.

200 patients were recruited in total. The last patient entered the study in September 2010.

**Previous Investigator**

The first patient was recruited into this study by LT in March 2006. I took over full time involvement in the study from 1st October 2008. Prior to 1st October, I had been involved in data analysis relating to comparison of different methods of assessment of arterial stiffness using data derived from 24 hour ambulatory blood pressure measurement [296].

LT performed analysis on the results available from the first 133 patients (i.e. those she recruited to the study), censoring her follow up on 1st September 2008. In her thesis, LT reported her analysis of the relationship of total fetuin-A to C-F PWV. LT also reported preliminary results regarding the rate of change of renal function [2]. We have progressed these analyses during the course of this study and the results have now been published [108].

**Consistency of Method**

In order to ensure methodological consistency throughout the study period, I learned the measurement techniques from LT and a dedicated research nurse (AL) over a period of one month prior to taking over responsibility of the day to day running of the study. AL was present at over 95% of enrolment visits during the study. A repeatability study was performed. The results of the repeatability study are presented in appendix 3.

## 2.2 Recruitment and Selection Criteria

The source of referral of the study patients is listed in table 2.1 below.

| Source of referral | Number |
|---|---|
| Renal outpatients | 182 |
| Diabetic outpatients | 7 |
| Care of the Elderly | 5 |
| Worthing and Southlands NHS Trust | 2 |
| Word of mouth | 3 |
| General practice | 1 |

**Table 2.1 - Source of patient referrals - NHS-National Health Service**

The inclusion and exclusion criteria for the study are listed in table 2.2.

| Inclusion | Exclusion |
|---|---|
| Age 40-90 years | Left ventricular ejection fraction <35% † |
| CKD stages 3 & 4 | Aortic stenosis with gradient >30mmHg † |
| | Atrial fibrillation with apical rate >100bpm |
| | Parathyroidectomy |
| | Renal transplantation |

**Table 2.2 - Inclusion and exclusion criteria for ACADEMIC study - †from previous echocardiograph only if performed**

## 2.3 Study Protocol

### First Visit

On recruitment to the study patients underwent baseline assessment including a full medical history exploring potential comorbidities with particular regard to cardiovascular disease, diabetic status, and cause and duration of CKD. Drug history, tobacco exposure and alcohol use were recorded. Blood pressure was measured. The patient was examined, and anthropometry and electrocardiogram (ECG) were performed. Data was entered onto a standardised case report form. The case notes of the patients and any relevant imaging were reviewed to verify information where possible. Blood and urine were sampled, analysed and stored as detailed below.

**Blood Pressure Measurement**

Subjects reclined on an examination couch for five minutes in a temperature controlled room at 21°C. Blood pressure and heart rate recordings were then measured using a validated oscillometric device (Omron HEN-705CP, Tokyo, Japan) at the right brachial artery using an appropriately sized cuff. The mean of two BP recordings was taken. Mean blood pressure (MBP) was calculated as:

$$MBP = \frac{(SBP - DBP)}{3} + DBP$$

**Anthropometry**

Height and clothed weight were measured. BMI was calculated.

$$BMI\ (kg/m^2) = \frac{weight\ (kg)}{height^2\ (m)}$$

A tape measure was used to measure carotid-femoral and carotid-radial distances were measured as illustrated in fig. 2.1.

Examination included auscultation of the carotid artery to identify bruits. If a significant bruit was detected, or if previous imaging had found significant stenosis of the right carotid artery, then carotid measurements were not performed.

## Follow Up Assessments

Patients were seen at six monthly intervals. These visits consisted of a review of health and medications since the previous study visit, measurement of weight and clinic blood pressure. Measurement of C-F and C-R PWV was performed at annual intervals

Blood and urine sampling was performed at each visit. Routine tests were performed as listed below. Samples of urine, serum and plasma were frozen at -80° C for subsequent batch testing for non-routine tests.

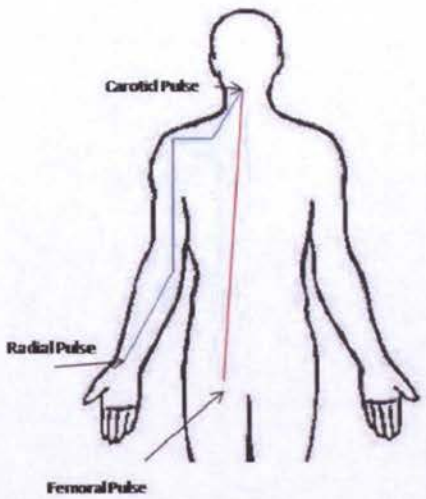



**Figure 2.1 - Diagram of distance measurement for carotid-radial (blue) and carotid-femoral (red) distances** [297] (adapted)

## Measurement of Arterial Stiffness

PWV was measured in the carotid-femoral (aortic) and carotid-radial segment of the arterial tree using Complior™ (Colson, Les Lilas, France), a validated automated system [298]. The patient was placed in a semi-recumbent position on an examination couch. Piezo-electric mechanotransducers were manually applied to the skin over the right femoral or right radial artery and base of the right carotid artery (see fig. 2.2).

The transducers were adjusted to obtain optimum transduction of the waveforms. The Complior™ software analysed the traces for fidelity and calculated the time interval between the point of maximal upstroke of the pressure wave in the carotid and the radial or femoral arteries (see fig. 2.3).

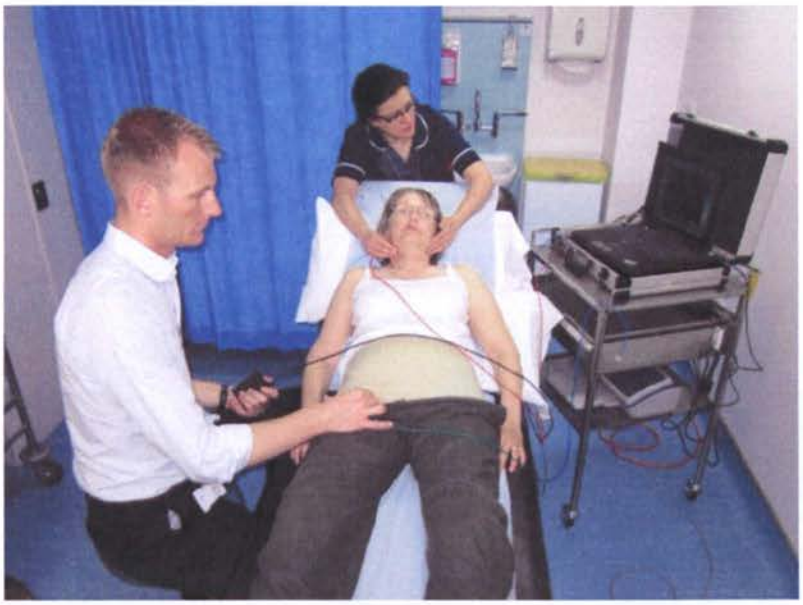


**Figure 2.2 - Measurement of C-F PWV with piezo-electrical mechanotransducers applied simultaneously to the femoral and carotid pulses (with kind permission of patient (appendix 4))**

PWV recordings were measured twice on each occasion. The recordings were visually inspected to determine the number of waveforms designated valid by the software. The recording with the greatest number of valid waveforms was used. If the recordings were of similar quality, a mean value was calculated. The vast majority of C-F PWV measurements were performed by two of three operators (MF, LT and AL).

PWV was calculated using the equation:-

$$\text{velocity}(\text{m/s}) = \frac{\text{distance (m)}}{\text{time (s)}}$$

Due to logistic constraints, limitations were placed on the standardisation of conditions between patients, such that patients had C-F PWV measured, and blood and urine sampled at different time points throughout the day.

AI was also measured in this study. The C-F PWV results alone are reported and analysed in this thesis.

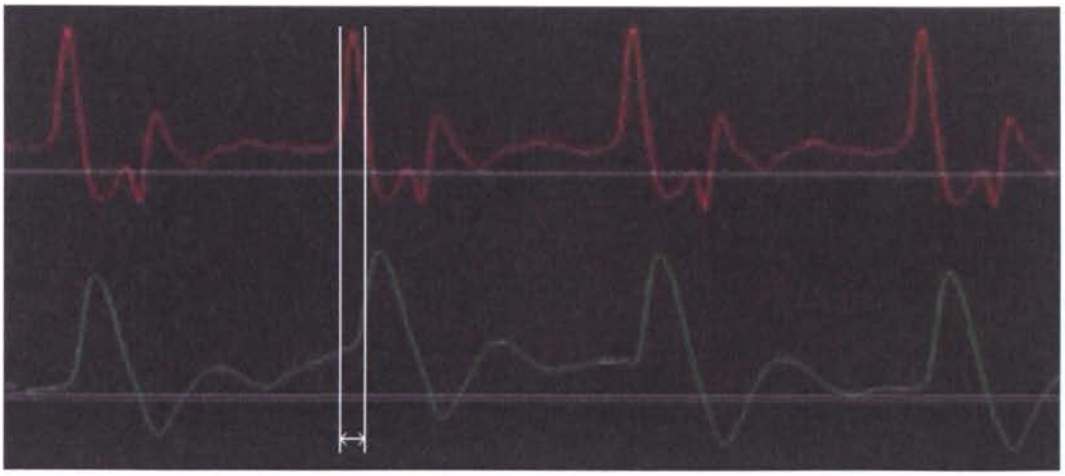


**Figure 2.3 - Carotid (top) and femoral (bottom) pulse pressure profiles as generated by Complior™ - Time interval between points of maximal upstroke indicated by white arrow**

### Categorisation of Cause of Renal Failure

Whilst the KDIGO classification of CKD into stages provides a useful framework with which to study the severity of renal impairment, the processes underlying the development of CKD should also be appreciated. The cause of CKD of the patients was classified after review of the notes and relevant histology, imaging and biochemistry. Patients were classified into three categories by two observers (MF and LT):

**1. Renal Pathology**

These patients had clear evidence of a renal pathology based upon serology, histology, urinalysis, biochemistry, imaging and/or history. This category included diabetic nephropathy, the glomerulonephritides, polycystic kidney disease and interstitial nephropathy.

**2. Hypertensive/Glomerulosclerosis**

These patients had no clear evidence of active renal disease and no overt proteinuria (uPCR<350mg/ml) or history of other active urinary sediment. They had either overt cardiovascular disease or several cardiovascular risk factors.

**3. Other**

This group comprised patients with obstructive renal failure, stone disease and reflux. Patients with small kidneys on ultrasound were also included, as were patients for whom no other diagnosis was available and no cardiovascular risk factors other than hypertension were present.

## Cardiovascular Comorbidity

Cardiovascular co-morbidity was assessed using patient history, review of case record and ECG. LVH and hypertension were not classified as cardiovascular co-morbidities. Patients were classified into three categories:

**1. Overt cardiovascular comorbidity**

This group had either a history of transient ischaemic attack (TIA), stroke, myocardial infarction or angina, or had undergone treatment for cardiovascular disease such as coronary artery bypass grafting or angioplasty of peripheral artery disease.

**2. Subclinical cardiovascular comorbidity**

This group had neither history of overt disease or treatment thereof. This group consisted of patients who had undergone imaging which demonstrated significant arterial disease which was either asymptomatic or had not been deemed to require treatment; for example unilateral renal artery stenosis.

**3. No cardiovascular comorbidity**

None of the above

## ECG and LVH

A standard 12 lead ECG was recorded for each individual upon entry to the study. The ECG was assessed using Sokolow-Lyon (S-L) [299] and Cornell [300] voltage criteria for presence of LVH. Patients were classed as having LVH if either criterion was met.

**Sokolow-Lyon Criteria**

The sum of the height of the S wave in $V_1$ + R wave in $V_5$ or $V_6$ (whichever is larger) $\geq$ 35 mm or R wave in aVL $\geq$ 11 mm.

**Cornell voltage Criteria**

The sum of the height of the S wave in $V_3$ + R wave in aVL> 28 mm (men) or 20 mm (women).

## Medication Classification

A drug history was taken from the patient on entry to the study. This was corroborated using the computerised medical report, patient notes and prescription information supplied by primary care to the patient where available.

Loop diuretics were not classified as antihypertensive agents. Calcium based phosphate binder use was classified as intake of additional elemental calcium >1g/day.

## Ethics & Consent

Ethical approval was originally obtained from the West Sussex Research Ethics Committee on 21st January 2006. I wrote an ethical amendment which was submitted and approved in order to perform the assays for OPG and RANKL in January 2009 (see appendix 4). The original protocol included approval for transthoracic echocardiography to be undertaken at six monthly intervals. This was subsequently not performed due to budgetary constraints.

Patients when approached to enter the study were given a copy of the patient information sheet. They were then called at home several days later in order to determine if they understood the proposed study and if they wanted to participate. All patients signed a written consent form on enrolment to the study. The study was conducted in accordance with 'Good Clinical Practice' [301] and the Declaration of Helsinki [302].

## 2.4 Laboratory Assays

### Routine

Haemoglobin; creatinine; albumin; corrected calcium; phosphate; alkaline phosphatase; parathyroid hormone; total cholesterol; CRP; glycated haemoglobin (diabetics only) and urine total protein:creatinine ratio were measured using routine automated analysers at the Departments of Clinical Biochemistry (Roche Modular, Haywards Heath, UK) and Haematology (Sysmex UK Ltd, Milton Keynes, UK) at the Royal Sussex County Hospital (RSCH).

### Non-Routine Laboratory Assays

Samples of serum, plasma and random urine were taken at each visit. Serum and plasma samples were spun at 3500r.p.m. (eqv. 2000g) for ten minutes. Aliquots of serum and plasma were taken for frozen storage at -80°C. These were used for batched analysis of non-routine assays.

Non-standard biochemical analysis was performed by ES in the Department of Clinical Biochemistry at the Royal Sussex County Hospital (RSCH). Assays were performed in duplicate and the mean values used.

During the course of this study, ES undertook work on aspects of biochemistry which are directly relevant to this thesis:-

1. The variability between the two different types of commercially available fetuin-A assays [154].
2. The instability of intact FGF-23 post venepuncture and the impact of CKD upon this instability [233]
3. The classification of total fetuin-A into free fetuin-A, phosphorylated fetuin-A and CPP [303].

I participated in collection of the samples, discussion of the relevance of such results to both this cohort and the wider area of study, and contributed to the write up of these results prior to publication or submission/presentation in abstract form. The relevance of these studies is discussed in chapter 3.

### Fetuin-A

Total fetuin-A was measured in plasma samples using an ELISA (Biovendor, Brno, Czech Republic (CZ)). This kit uses a polyclonal goat anti-human fetuin-A antibody.

Measurement of total fetuin-A was performed using thawed stored plasma samples. Inadequate volume of stored plasma necessitated the use of thawed stored serum for analysis of the various fractions of fetuin-A. There was a close correlation between titres measured using different sample types (see fig. 2.4).

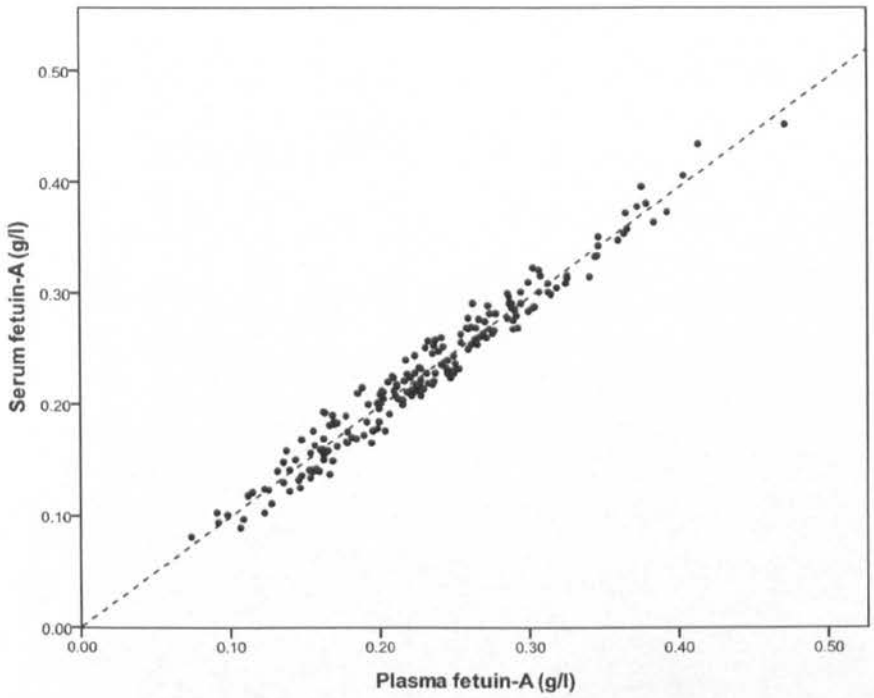



**Figure 2.4 - Scatter plot of fetuin-A titres (g/l) measured in matched serum and plasma samples (r=0.982, p<0.001)**

### Fetuin-A Sub fraction Analysis

Fetuin-A sub-fractions were measured using an adaption of the method described by Hamano *et al* [122].

### CPP

In order to quantify the various forms of fetuin-A in serum, total serum fetuin-A was first quantified. Total serum fetuin-A was measured using the ELISA technique described above for plasma. 500µl of serum was then ultra-centrifuged at 24,000g for 2 hours. This produced a small translucent pellet containing CPP. The supernatant fetuin-A was then re-quantified using the same assay. CPP levels were then expressed as a reduction ration to total fetuin-A as previously described.

**Phospho-isoforms**

Phosphorylated and non-phosphorylated isoforms were separated using phos-tag electrophoresis (NARD Institute, Hyogo, Japan). This method retards proteins depending upon the number and position of sites of phosphorylation. Post electrophoresis, the gels were blotted, incubated with anti-fetuin-A antibody (Biovendor) and quantitated using chemiluminescence to produce a measurement of phosphofetuin for each study participant [303].

There was no significant difference in ELISA-based measurements of native phosphorylated serum fetuin-A and dephosphorylated samples (paired t-test, n=39, $p<0.05$). Data regarding assay specificity for the different phospho-isoforms and CPP is not currently available.

**OPG, RANKL & FGF-23**

OPG and RANKL were measured in plasma with sandwich ELISAs (Biovendor, Brno, CZ). The current OPG ELISA is reported to detect total OPG, both mono- and dimeric and OPG bound to ligands (i.e. RANKL) in serum [204].

Serum intact FGF-23 was measured using a sandwich ELISA kit (Immutopics, San Clemente, USA). This assay employs two affinity purified goat polyclonal antibodies that detect epitopes in the N- and C-terminal portions of the molecule. A sub-study on the stability of FGF-23 post venesection was performed by ES. This demonstrated that FGF-23 was unstable when collected into specimen tubes containing ethylenediaminetetraacetic acid (EDTA) [233]. This instability was greater in CKD patients than controls, but could be minimised by stabilisation with a protease inhibitor cocktail (2mM aminoethylbenzenesulfonyl fluoride hydrocholoride, 1.6mM aprotinin, 100μM bestatin,30μM E-64, 40μM leupeptin, 20μM pepstatin A (Sigma, Gillingham, UK)).

Serum samples for FGF-23 analysis were therefore treated with this protease inhibitor cocktail prior to analysis in order to prevent degradation.

**High Sensitivity Cardiac Troponin-T**

Serum cTnT was measured in batches using the Elecsys Troponin T hs (high sensitivity) assay on the Modular Analytics E170 analyser (Roche, Haywards Heath, UK). The assay employs two monoclonal antibodies specifically directed against

human cTnT. The antibodies recognize two epitopes (amino acid position 125-131 and 136-147) located in the central part of the cTnT protein.

The detection antibody in the hs-cTnT assay has been genetically re-engineered to reduce interference from heterophilic antibodies. In addition, the sample volume is greater, and ruthenium based signalling from the detector antibody has been optimised, to give a limit of detection of 0.005µg/l [304].

**Inflammatory Proteins**

High sensitivity C-reactive protein (hs-CRP) was measured by high sensitivity particle-enhanced immunonephelometry on the Dade Behring ProSpec analyser (Siemens, Camberley, UK). Serum IL-1β, IL-6 and TNF-α were measured by high sensitivity ELISA (RnD, Abingdon, UK). All the ELISA assays were adapted for use on an automated platform (Triturus, Grifols, UK). Limits of detection, and coefficients of variation (CVr) are listed in table 2.3 below.

**Urinary Protein Loss**

Proteinuria was measured on a random spot urine, and expressed as a ratio to urine creatinine, urine protein:creatinine ratio (uPCR) (mg total protein per mmol creatinine). In patients for whom urinary protein excretion was below the level of routine laboratory detection, a uPCR was calculated using the lower limit of the total protein assay.

Urinary fetuin-A was measured using an adaption of the Biovendor (Brno, CZ) ELISA kits. Fetuin-A concentration in the urine was expressed as ratio to urine creatinine concentration (uFCR) (µg fetuin-A per mmol creatinine).

| Assay | Limit of Detection | Within assay variation (%) | Between assay variation (%) | Concentration at which CVr measured |
|---|---|---|---|---|
| Fetuin-A [†§] | 3.5ng/ml | 5.2 | 7.5 | 30ng/ml |
| Fetuin-A [†§§] | 0.4ng/ml | 3.8 | 6.1 | 30ng/ml |
| Fetuin-A [††§§] | 1.0ng/ml | 5.9 | 7.4 | 100µg/l |
| OPG | 0.12pmol/l | 5.2 | 6.0 | 3.5pmol/l |
| RANKL | 0.25pmol/l | 5.9 | 6.6 | 450pmol/l |
| iFGF-23 | 0.7pg/ml | 5.3 | 7.2 | 0.7pg/ml |
| hs-CRP | 0.10mg/l | 3.6 | 5.2 | 0.45mg/l |
| hs-cTnT | 0.003µg/l | ND | 10[§§§] | 0.011µg/l |
| IL-1β | 0.06pg/ml | 5.1 | 6.8 | 1.75pg/ml |
| IL-6 | 0.04pg/ml | 6.2 | 7.0 | 2.55pg/ml |
| TNF-α | 0.10pg/ml | 4.2 | 5.7 | 10.55pg/ml |

**Table 2.3 - Assay characteristics - [†]plasma, [††] urine, [§]Epitope, [§§] Biovendor. [§§§]total imprecision CVr=mean/standard deviation; Total imprecision =$\sqrt{(SD_{within\ run}^2 + SD_{between\ run}^2)}$; ND-not determined**

## Blood Pressure Treatment Targets

All participants remained under the care of their primary care physician, and most remained under review of the Sussex Kidney Unit. Choice of blood pressure medication remained at the discretion of the primary care provider, but generally followed current guidelines for CKD, such as United Kingdom Renal Association targets for BP in CKD in 2006 – target BP<130/80 for patients with uPCR <100mg/mmol and target BP <125/75 if uPCR>100mg/mmol [305]. Blood pressure guidelines changed over the course of the study with a loosening of the targets but an increased incentivisation to reach the targets in primary care [105].

## 2.5 End Points & Statistics

### Outcomes

Analysis of results was performed by comparison of clinical, biochemical and demographic data against both baseline measurements and longitudinal changes of outcome variables.

### Change in Renal Function

Rate of change of renal function was assessed using all the measurements of serum creatinine available for each participant at Brighton and Sussex University Hospitals NHS Trust (BSUH) over the five years prior to analysis. Creatinine values were converted to eGFR using the MDRD formula. Data was censored for follow up on 16th June 2011. Details of dates of start of dialysis were determined using review of the electronic patient record and biochemistry data. Follow up data was censored at start of RRT or death.

Decline in renal function was considered in three ways:

1. Analysis of the dichotomous combined end-point of commencement of dialysis or ≥25% decline in $eGFR_{MDRD}$ (sustained for over 30 days) since study entry was used.

2. Analysis of gradient of GFR plotted against time.

3. Analysis of gradient of 1/creatinine plotted against time.

### Gradient Analyses

All available GFR estimations for all patients in the study were retrieved from hospital biochemistry databases dating back six months prior to recruitment into the study. Data was censored at the beginning of dialysis. eGFR values were plotted against time and the gradient of the line of best fit calculated. This was then used as a measure of rate of change of renal function. The process was repeated using 1/creatinine measurements to give a second gradient coefficient.

The raw data were then reviewed. eGFR estimations and 1/creatinine values which related to episodes of acute kidney injury (AKI) and other obviously outlying values were removed to generate a further 'cleaned' slope coefficient.

### Change in Arterial Stiffness

At the time of analysis, not all patients had had three years of C-F PWV measurement performed. Assessment of rate of change at one, two and three years was calculated by subtraction of baseline C-F PWV from C-F PWV at the time of measurement.

### Survival

Survival data was gathered prospectively through the course of the study. Survival follow up was censored on 1st August 2011. At this time the CV5 database in the Department of Renal Medicine and the BSUH OASIS patient tracking database were used to check patients were not registered to have died. If a patient was not registered to have died, the Electronic Patient Record (laboratory results system) was reviewed. Patients were classified to be alive if they had been seen in a BSUH outpatient clinic or had any BSUH laboratory investigation after 1st June 2011. The sub-group of patients who did not fulfil this criterion had follow up censored two months after the last recorded laboratory investigation or clinic letter.

## Statistics

C-F PWV is blood pressure dependent [10]. Values were therefore adjusted for MBP at the time of measurement. A linear regression of the two variables was performed. The residual values were then added to the mean C-F PWV. Values relate to adjusted C-F PWV measurements unless stated to be unadjusted.

Data was examined for distribution. Normality was assessed using the Kolmogorov-Smirnov or Shapiro-Wilk test depending on sample size. Data was log transformed to a normal distribution where appropriate. Correlation was assessed using Pearson's correlation for parametric data, or Spearman's rank correlation for non-parametric data. Comparisons between means of continuous variables were performed using Student's t test and Mann-Whitney U test as appropriate, and between groups of dichotomous variables using Chi-square test, or Fisher's exact test where appropriate due to small sample size. Throughout the study, continuous parametric data is presented as mean ± standard deviation (SD); non-parametric data is presented as median ($25^{th}$, $75^{th}$ centiles). The statistical probability considered significant was 0.05 in a 2 tailed test.

Throughout the thesis significance will be denoted such that * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$. Results obtained using log transformed data will be denoted with $^{\$}$.

**Linear Regression Analysis**

Linear regression analysis was performed to investigate independent association with continuously distributed outcome variables. The method for selection of parameters in the final model was determined by the type of analysis performed. A forward stepwise model was employed for exploratory analysis, whilst a forced entry model was used to adjust for confounders in analyses where a specific hypothesis was being tested. Confounders were selected from review of the literature or the finding of a significant relationship in univariate analysis. Unstandardised B values with 95% CI are reported.

Validity of models was tested by review of outliers and plots of residuals. Assessment of collinearity was performed upon centred data. Variance Inflation Factors (VIF) and Eigenvalues were calculated and reviewed for size and stability.

**Logistic Regression**

Logistic regression analysis was used to calculate adjusted and unadjusted OR for categorical outcome variables. OR with 95% CI are reported. Multinomial logistic regression was used to calculate OR for an analysis with three discrete outcome variables.

**Receiver Operator Characteristic Curves**

The relationship between ratios of true and false positive results of the various models generated was performed using receiver operating characteristic (ROC) curves. The area under the curve (AUC) of the ROC curves was used to compare the predictive ability of the various models.

**Survival Analysis**

Kaplan-Meier survival plots were drawn for study parameters in order to investigate the associated risk of the dichotomous outcomes of ESRF and death. Continuously distributed parameters were dichotomised using the median value. The log rank test was used to determine significance of difference between plots.

Parameters under investigation in the study hypothesis and those with univariate significance were tested to see if they fulfilled the requirement for proportional

hazards using a log minus log survival plot. If this requirement was fulfilled they were entered into a Cox Proportional Hazards Regression Model (CPHRM). If the proposed hazard parameters did not fulfil this requirement either a logistic regression model was built, or alternatively, the variable in question was entered into the proportional hazards model along with an interaction term of that variable multiplied by time. Hazard ratios (HR) with 95% CI are reported.

Statistical analysis was carried out using SPSS version 18 (SPSS, Chicago, IL, USA).

# Chapter 3

# Baseline Results

## 3.1 Baseline Characteristics

The baseline demographic and clinical characteristics of the cohort are given in table 3.1. The ethnic distribution was Caucasian 96.5%, Arab 2%, South Asian 1% and Black African 0.5%. The age distribution of the cohort is shown in fig. 3.1. The male patients were significantly younger than the female patients (mean age 68 ±12 years vs. 72 ± 9 years, p=0.011).

| Parameter | Value |
|---|---|
| Gender (male: female) | 144:56 (72%:28%) |
| Age (yrs) | 69 ± 11 |
| Smoking history (%) | 63 |
| Tobacco exposure† (pack years) | 29 ± 28 |
| Alcohol use (units/week) | 8 ± 10 |
| BMI ($kg/m^2$) | 29.8 ± 7.5 |
| Diabetes (%) | 26 |
| Cardiovascular comorbidity (%) | 44 |
| Mean SBP (mmHg) | 151 ± 22 |
| Mean DBP (mmHg) | 81 ± 11 |
| Mean heart rate (bpm) | 70 ± 12 |

**Table 3.1 - Baseline demographic and clinical characteristics - †smokers only**

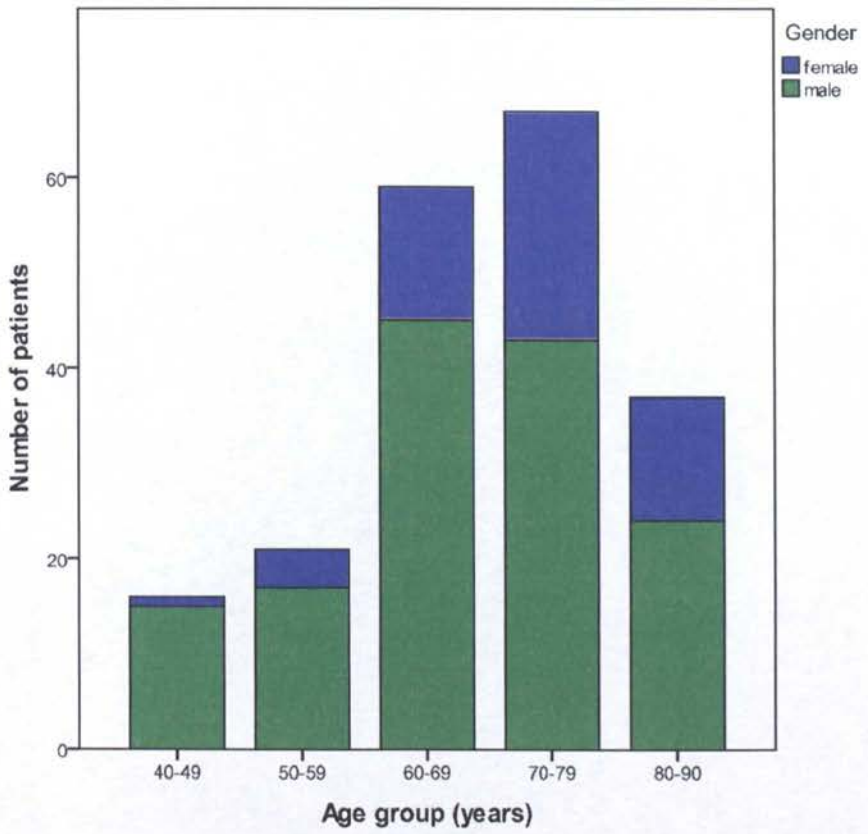



**Figure 3.1 - Bar chart of gender and age distribution of ACADEMIC patients (n=200) - Green-male; blue female**

The mean clinic blood pressures were above current treatment guidelines for CKD patients [105] despite the use of a mean 2.1 ± 1.3 antihypertensive medications, as listed in table 3.2.

Antihypertensive medication was taken by 82% of patients in the study. 60 patients (30%) in the study had a SBP less than 140mmHg, of whom six (3%) were not taking antihypertensive medication. 32 patients (16%) in the study had SBP less than 130, of whom three (2%) were not taking antihypertensive medications. There were 93 patients (46%) in the study who had a DBP less than 80mmHg and 28 patients (14%) had a DBP less than 70mmHg. Of these 28 patients, only one was not taking blood pressure medication.

| Medication | %/Mean |
|---|---|
| Number of antihypertensive agents | 2.1 ± 1.3 |
| ACEi/ARB | 65% |
| Beta blocker | 33.5% |
| Calcium channel blocker | 46% |
| Thiazide diuretic | 22% |
| Alpha blocker | 32.5% |
| Statin | 60% |
| Erythropoesis Stimulating Agent (ESA) | 6.5% |
| 1(OH) vitamin $D_3$ | 8% |
| 25(OH) vitamin $D_3$ | 4% |
| Calcium based phosphate binder | 5% |
| Non-calcium based phosphate binder | 1.5% |
| Bisphosphonate | 8% |

**Table 3.2 - Medication use at entry to study - ACEi/ARB, Angiotensin Converting Enzyme inhibitor, Angiotensin Receptor Blocker**

Routine biochemical and haematological parameters of the cohort are shown in table 3.3. 30 patients had CKD stage 3A, 82 stage 3B and 88 stage 4.

Most patients (93%) had a six month mean corrected calcium within the normal range, 85% of patients had a six month mean phosphate within the normal range despite a low prevalence of phosphate binder use. Three patients in the study were taking sevelamer, no patients were taking lanthanum carbonate. Only five patients in the study had a six month averaged phosphate above the local reference range.

Vitamin $D_3$ usage was low. 8% of patients were receiving 1(OH) vitamin $D_3$, and 4% were receiving 25(OH) vitamin $D_3$ (cholecalciferol). The mean haemoglobin was 12.7 ± 1.6g/l despite a low prevalence of ESA use.

| Parameter | Value |
|---|---|
| eGFR (ml/min/1.73m$^2$) | 33 ± 11 |
| Phosphate$^\dagger$ (mmol/l) | 1.07 ± 0.18 |
| C. Calcium$^\dagger$ (mmol/l) | 2.30 ± 0.11 |
| Albumin$^\dagger$ (g/l) | 42.3 ± 3.2 |
| iPTH (ng/l) | 75 (50, 116) |
| uPCR (mg/mmol) | 27.7 (13.3, 69.0) |
| uPCR>100mg/mmol (no pts) | 32 |
| uPCR>350mg/mmol (no pts) | 7 |
| Cholesterol (mmol/l) | 4.4 ± 0.9 |
| Haemoglobin$^\dagger$ (g/dl) | 12.7 ± 1.6 |
| Haemoglobin A1c (%) | 7.6 ± 1.6 |
| Cardiovascular comorbidity | 44% |
| LVH | 20.5% |

**Table 3.3 - Routine biochemical and haematological parameters of cohort - $^\dagger$Mean of all measured values at BSUH in preceding 6 months including baseline visit - reference range PTH 15-65ng/ml; corr. calcium 2.15-2.55mmol/l; phosphate 0.87-1.45mmol/l**

The age distribution of the cohort across stages of CKD is shown in fig. 3.2. The subgroups of patients with CKD stages 3B and 4 were significantly older than those with stage 3A.

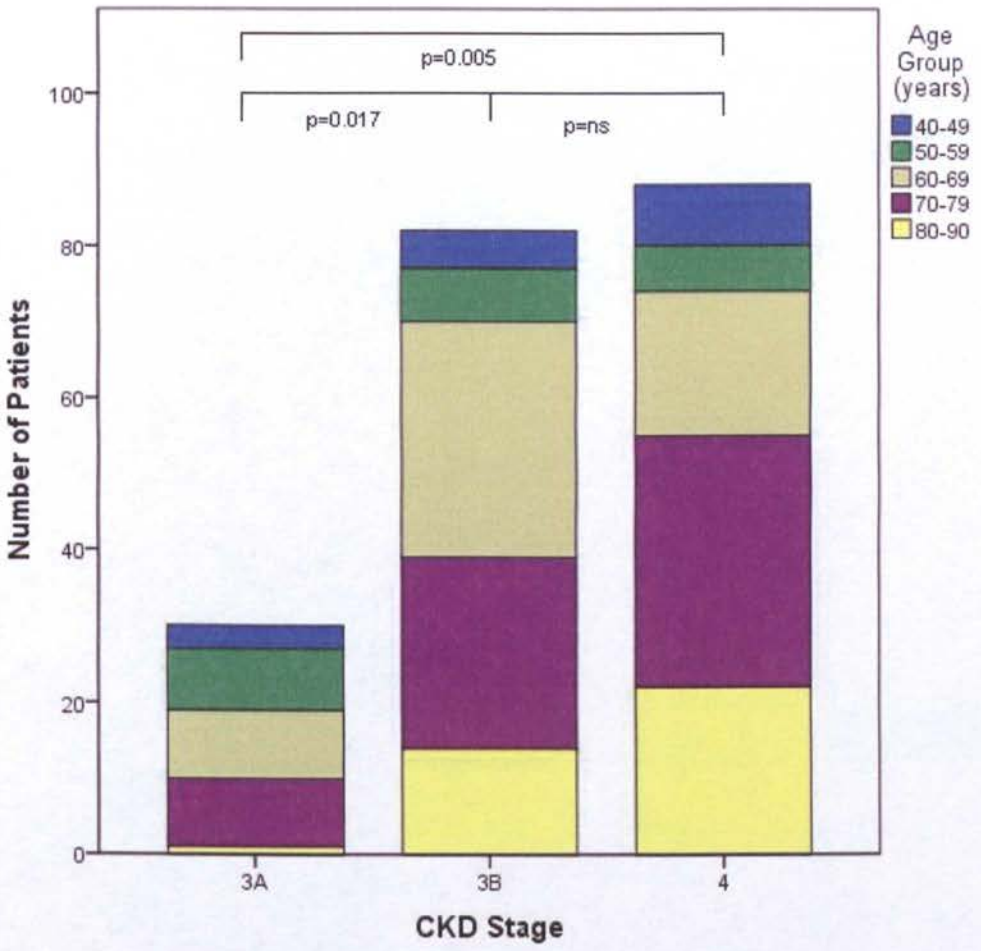



**Figure 3.2 - Histogram of age distribution by CKD stage. p values relate to comparison of mean age between groups - Mean age: 3A-64 ±11years (n=30); 3B-69±11 years (n=81); 4-70±12 years (n=89)**

## Cause of CKD

The cause of CKD was determined at entry to the study using review of the case notes, biochemistry and histology. The results are listed in table 3.4.

| Diagnosis | Number of patients |
|---|---|
| Hypertension /glomerulosclerosis | 68 |
| Non specified glomerulonephritis | 27 |
| Diabetic nephropathy | 12 |
| Interstitial nephritis | 12 |
| Obstructive uropathy | 11 |
| ANCA-associated vasculitis | 10 |
| Reflux nephropathy or chronic pyelonephritis | 9 |
| Polycystic Kidney Disease | 9 |
| Nephrectomy | 5 |
| Congenital | 3 |
| Non resolving loss of GFR post acute insult | 3 |
| Multicystic kidney disease | 2 |
| Nephrolithiasis | 2 |
| Unknown | 27 |

**Table 3.4 - Cause of CKD in ACADEMIC patients (n=200) - ANCA-Anti Neutrophil Cytoplasmic Antibody**

These diagnoses were subsequently split into three pre-specified categories (see chapter 2) in order to permit meaningful comparison of distribution of diagnoses by age and CKD stage. 34% were classified with hypertensive CKD, 42% with intrinsic renal CKD and 24% as CKD of other aetiology. There was no significant difference in the distribution of diagnostic categories by stage of CKD.

There was a significant variation in age and blood pressure across diagnostic categories of CKD. The youngest tertile had a majority of patients with intrinsic renal disease, and also the lowest SBP and highest DBP. The oldest tertile had a predominance of hypertension/glomerulosclerosis mediated CKD, but still had the highest SBP. There was no difference in DBP between the two eldest tertiles (see fig. 3.4). There was no significant difference between the mean numbers of antihypertensive medications used between tertiles. There was however a significant decrease of uPCR towards the older tertiles (p=0.035). There was no significant variation in the distribution of diagnostic categories when the cohort was examined by stage of CKD (p=0.221).

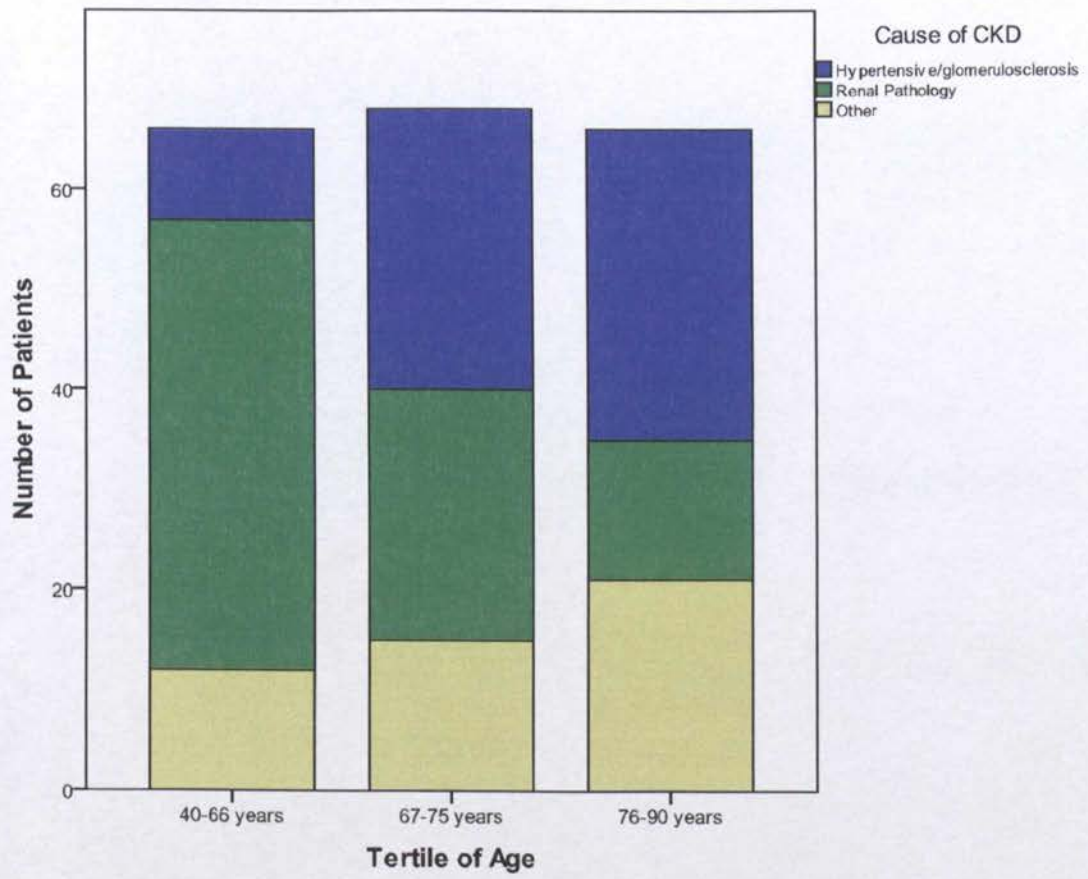
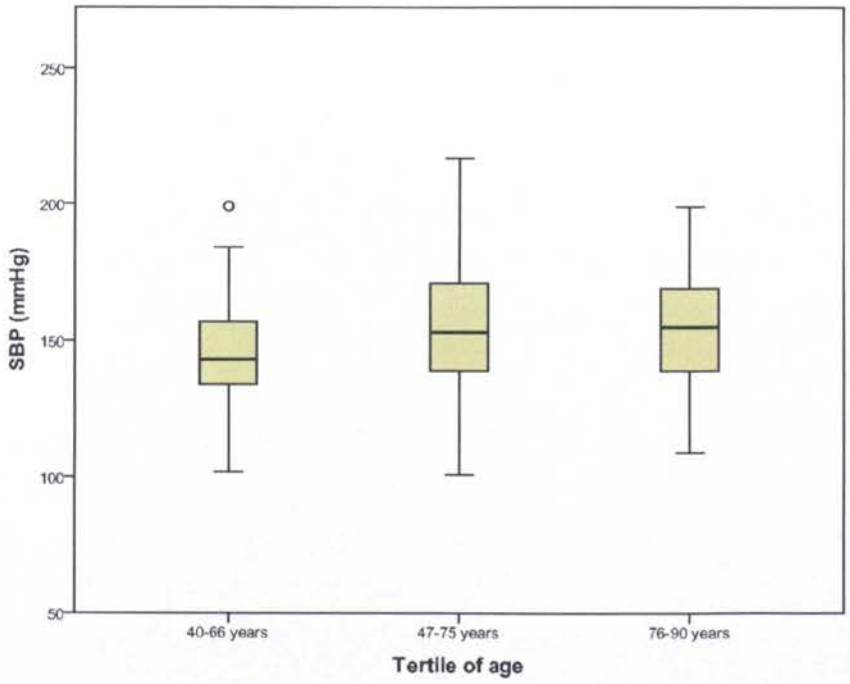



**Figure 3.3 - Bar chart of cause of CKD by tertile of age (n=200) (Chi sq =33.070, p<0.001$^{***}$)**

A




B


**Figure 3.4 - Boxplots of (A) SBP (mmHg) and (B) DBP (mmHg) by tertile of age in ACADEMIC cohort (n=200) - ANOVA for trend (A) p=0.003$^{**}$; (B) p<0.001$^{***}$**

## Discussion

The entry criteria for the ACADEMIC study were relatively wide. The study therefore contains patients with a range of ages, causes of CKD and a variety of co-morbidities. This was a predominantly elderly, Caucasian cohort. The majority of patients were hypertensive despite receiving antihypertensive medication, 26% of patients had diabetes and there was a significant incidence of cardiovascular comorbidity. A large proportion of the population had smoked and the tobacco use of those who had smoked was substantial. 44% of the population had had a previous cardiovascular event. Cholesterol control was reasonable within the population. This was, therefore, a population with significant exposure to traditional cardiovascular risk factors. However the population were generally well, with maintained haemoglobin and little evidence of malnourishment as evidenced by the serum albumin.

With regard to non-traditional risk factors, many of the patients had significant proteinuria. The mean BMI was above both the recommended range of 18.5 to 25, and the UK mean [306]. As expected for a cohort of patients with CKD stages 3 & 4, the serum phosphate was within or below the normal range for 97.5% of patients. This cohort therefore was a cohort at high cardiovascular risk due to both traditional and non-traditional risk factors.

Within the cohort there were two heterogeneous sub-populations; a younger group of patients with predominantly intrinsic renal disease and less severe impairment of GFR, and an older group of patients with lower GFR who were more likely to have hypertensive/glomerulosclerotic CKD. These patients generally reflect the population of the nephrology outpatient clinics at BSUH from where the large majority of the patients were recruited.

The period of recruitment spanned a significant increase in the understanding and awareness of the importance of CKD within primary care, with an associated significant change in specialist referral patterns within the UK [307]. This was due, in part, to the widespread adoption of the MDRD formula which allowed reporting of estimated GFR, and also, in part, to the inclusion of CKD within the primary care Quality and Outcomes Framework (QOF) in 2006 [308]. This was updated in 2009 with an increased focus on blood pressure control.

Guidelines for the diagnosis and treatment of hypertension also changed over the period of the study [105,309,310]. The 2008 NICE CKD guidelines recommend keeping blood pressure less than 140/90mmHg, with a target for treatment of below 130/80mmHg, whilst the current Renal Association CKD guidelines recommend keeping blood pressure below 140/90 in CKD, and less than 130/80 in diabetic patients and those with uPCR>100mg/mmol [310]. The applicability of the results of this cohort to other UK cohorts recruited before and after these changes may therefore be limited.

## 3.2 Arterial Stiffness

C-F PWV was successfully measured in 185 of 200 study patients. Measurement was unsuccessful in 15 patients. The software was unable to detect the pulse waveform in eight patients, four patients had a carotid bruit, or impalpable carotid or femoral pulse and three had previously undergone either abdominal aortic aneurysm repair or femoral popliteal bypass surgery. Comparison was made between the groups who did, and did not have successful measurement of C-F PWV. There was no significant difference between groups, with regard to demographics, blood pressure, mineral parameters or CaRP.

Blood pressure is widely recognised as a determinant of arterial stiffness [14,311]. For this reason, in order to permit meaningful comparison without reliance upon regression analysis, C-F PWV values were adjusted for MBP as previously outlined. The distribution of the C-F PWV measurements within the cohort is shown in fig. 3.5. The mean adjusted C-F PWV was 13.0 ± 2.6 m/s.

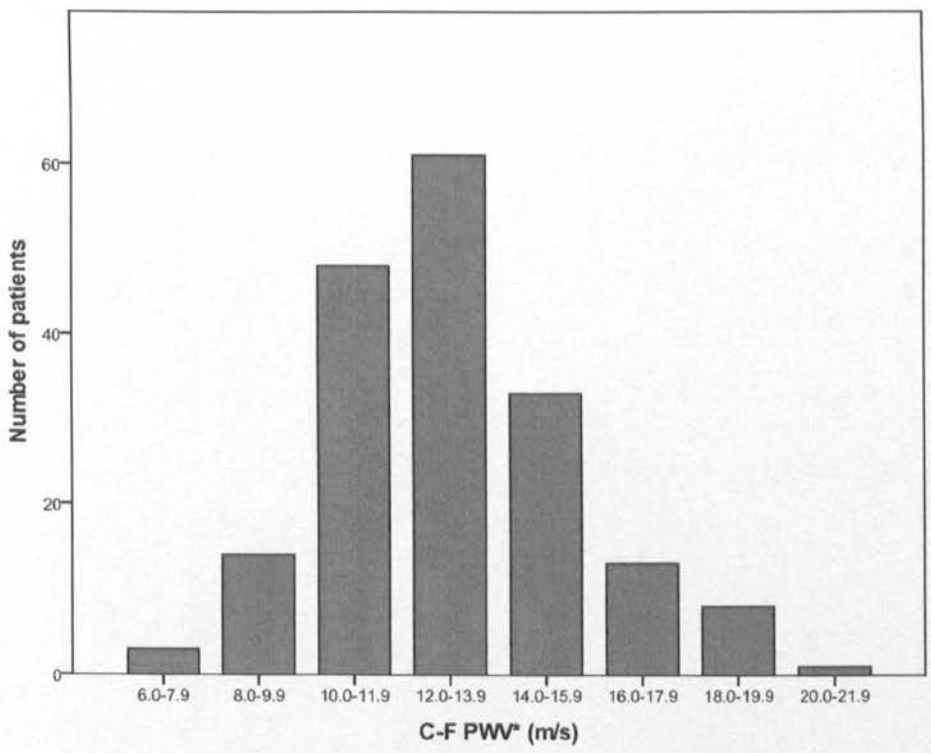



**Figure 3.5 - Bar chart of distribution of C-F PWV (m/s) (*adjusted for mean blood pressure) within the 185 study patients with C-F PWV measurement**

The univariate correlation of C-F PWV with continuously distributed variables is shown in table 3.5. Increased C-F PWV was significantly correlated with increased age, SBP and hs-CRP, and with decreased eGFR, haemoglobin and DBP. The significant positive and negative correlations with SBP and DBP respectively are a reflection of the manifestation of arterial stiffness as an increased pulse pressure. As expected, C-F PWV was significantly correlated with pulse pressure.

| Variable | r | p value |
|---|---|---|
| eGFR | -0.156 | 0.034* |
| Age | 0.535 | <0.001*** |
| SBP | 0.220 | 0.003** |
| DBP | -0.248 | 0.001*** |
| Pulse pressure | 0.390 | <0.001*** |
| iPTH$ | 0.110 | 0.139 |
| hs-CRP$ | 0.188 | 0.011* |
| Haemoglobin | -0.150 | 0.047* |
| uPCR$ | 0.043 | 0.565 |
| C. Calcium | 0.065 | 0.390 |
| Phosphate | 0.062 | 0.416 |

**Table 3.5 - Univariate correlation of C-F PWV with demographic and biochemical parameters**
**$ln transformed**

In order to understand the association of medication and comorbidity with C-F PWV within this cohort, comparison was made between mean values in sub-groups according to anti-hypertensive and statin use, smoking history, gender, diabetic status and cardiovascular comorbidity (see table 3.6).

| Medication/Comorbidity (no taking medication/with condition) | Mean (± SD) C-F PWV (m/s) Without | With | p value |
|---|---|---|---|
| ACEi/ARB (121) | 13.8 ± 2.3 | 12.8 ± 2.6 | 0.001** |
| Beta Blocker (62) | 12.9 ± 2.5 | 13.5 ± 2.7 | 0.369 |
| Calcium Channel Blocker (82) | 12.6 ± 2.4 | 13.5 ± 2.7 | 0.027* |
| Diuretic† (102) | 12.6 ± 2.4 | 13.5 ± 2.7 | 0.609 |
| Alpha Blocker (60) | 12.8 ± 2.6 | 13.3 ± 2.6 | 0.260 |
| Statin Use (110) | 12.8 ± 2.3 | 13.1 ± 2.8 | 0.442 |
| Diabetes (41) | 12.7 ± 2.5 | 13.7 ± 2.6 | 0.032* |
| CV comorbidity (78) | 12.3 ± 2.3 | 14.0 ± 2.6 | <0.001*** |
| Gender (M:F-135:50) | 13.1 ±2.6 | 13.0 ± 2.6 | 0.806 |
| Smoking history (111) | 12.7 ± 2.6 | 13.2 ± 2.5 | 0.292 |

**Table 3.6 - Comparison of C-F PWV by medication use, diabetic status and gender (n=185)**
**†Any diuretic, but no significant difference if examined by loop or thiazide only**

Patients with diabetes and cardiovascular comorbidity had significantly increased C-F PWV. There was no significant difference between C-F PWV according to gender, smoking history and statin usage. In addition, patients taking calcium channel blockers also had a significantly higher C-F PWV, whilst patients taking ACEi/ARB had a significantly lower C-F PWV. Multiple regression analysis of the factors independently associated with C-F PWV is included in section 3.4.

## Discussion

Ninety three percent of study participants had successful measurement of aortic stiffness using C-F PWV. The reasons for unsuccessful measurement (bruits, absent pulses or previous vascular surgery) are likely to disproportionately affect arteriopathic patients. However comparison of demographic, clinical and biochemical characteristics of the groups did not demonstrate any statistically significant differences. This is in contrast to the CRIC study which reported that C-F PWV measurement was not successful in 22% of patients (using the Sphygmacor™ system). These patients were more likely to be diabetic, female, have worse renal impairment, increased BMI, higher phosphate and urinary protein excretion [21]. Notwithstanding any undetected significant difference, which may be due to the small number of patients in whom measurement was not possible, exclusion of patients with high C-F PWV would be expected to bias the results to the null hypothesis.

The mean adjusted C-F PWV was 13.0 ± 2.6m/s. Unfortunately C-F PWV reference data is not available for patients with CKD. However, this value falls within the upper part of the reference range from the recently reported European reference range collaborative study [311]. In this study of non-diabetic patients without cardiovascular comorbidity or antihypertensive treatment, a mean value of 12.2m/s with reference range of 5.7-18.6m/s was given for patients aged between 60-69 years and BP between 140/90 and 160/100mmHg. However the absence of the influence of CKD and diabetes in this reference population limits the validity of this comparison.

Many factors have been associated with arterial stiffness in cross-sectional studies. As expected, and consistent with the findings of both the systematic review of cohorts with normal renal function [312] and the 2009 CRIC study of over 2500 US CKD patients [21], blood pressure and age were strongly correlated with C-F PWV in the ACADEMIC study. Patients with diabetes also had a significantly higher C-F PWV. As previously discussed the mechanisms of age and blood pressure related stiffening include deterioration of the elastin fibres in the tunica media, and an increased collagen content. Diabetes is also recognised to cause stiffening of the arteries via alteration of the extracellular matrix [313].

In the study cohort, patients with cardiovascular comorbidity had an increased C-F PWV. The aetiology of this relationship is less clear. Hypertension, diabetes, ageing and CKD are all associated with both arterial stiffening and cardiovascular comorbidity. It is possible that any of these factors could independently cause arterial stiffening which would, in turn, increase left ventricular afterload leading to LVH with an associated increased risk of cardiovascular events. The independent association of these factors will be explored using multivariate analysis later in the study.

ACEi/ARB and calcium channel blocker use was associated with significant differences in C-F PWV. The lower PWV in the group of patients not taking ACEi/ARB may be explained by the significantly lower age of patients in this group (ACEi/ARB mean age 67 ± 11 years vs. no ACEi/ARB mean age 72 ± 11 years, p=0.02). This difference is consistent with guidelines for choice of hypertensive in the elderly [309,314] and also with the variation in distribution of CKD category. In addition, treatment of diseases included within the intrinsic renal pathology category disproportionately indicates the use of ACEi/ARB to target proteinuria. There was no significant difference in age between those taking and not taking calcium channel blockers.

Despite some evidence suggesting that calcium channel blockers may slow arterial calcification [52], a recent randomised study of the effect of different classes of antihypertensives on C-F PWV in patients with essential hypertension found that whilst all the drugs studied (atenolol, perindopril, lercanidipine or bendroflumethiazide) gave a similar reduction in blood pressure, none had any effect on C-F PWV [315]. A recent metanalysis again studied the four major antihypertensive classes. Whilst differences were seen in the short term, no significant difference between major antihypertensive classes was seen beyond four weeks [316]. It therefore remains likely that the difference in C-F PWV seen in this observational study between calcium channel blocker usage categories can be explained by indication and demographics, in that calcium channel blockers are recommended as first line treatment in patients over 55 years of age [314].

The significant association of haemoglobin with C-F PWV in this cohort may be explained in two ways. Firstly, as previously detailed, PWV is inversely related to density of blood. Alternatively, eGFR is itself associated with haemoglobin

(r=0.313, p<0.001). Partial correlation of C-F PWV with haemoglobin after adjustment of eGFR is no longer significant ($r_p$=-0.095, p=0.209), indicating that the association can be explained by the association of eGFR with haemoglobin.

Methodological consideration is also important. Whilst C-F PWV is regarded as the gold standard method of non-invasive arterial stiffness measurement, it is not without drawbacks. Firstly, the time interval calculated does not correspond directly to the time taken for the pulse to pass from the carotid to femoral artery. Indeed anatomically the pulse does not pass from the carotid to the femoral artery. The right carotid artery is a branch of the brachiocephalic trunk which is itself a branch of the aortic arch. This problem is, in essence, related to nomenclature of the measurement rather than anything more significant.

Secondly, estimation of the carotid femoral distance is flawed. The distance routinely used in calculations (and that used in this study) is the distance from the carotid pulse to the femoral pulse measured using a tape measure across the anterior aspect of the body. This overestimates the carotid femoral distance, particularly in people with large BMI, and contains no assessment of aortic tortuosity [317]. The overestimation of the carotid femoral distance in overweight subjects has the potential to bias cross-sectional results.

Thirdly, whilst exhaustive comparison of the different methods of C-F PWV measurement is beyond the scope of this thesis, there are differences between the two most widely used methods of measuring C-F PWV. Complior™, the method employed in this study, uses a mathematical algorithm to calculate the point of maximal upstroke during systole. It has previously been demonstrated that this algorithm underestimates the transit time of the pulse and therefore underestimates C-F PWV in comparison to Sphygmacor™ measurement [318].

These methodological considerations limit direct comparison of data from this study to other studies using different PWV methodologies. However, this measure of arterial stiffness has been used extensively and has strong associated outcome data. Importantly the majority of comparisons made with other studies are of statistical relationships, and not of crude values. Comparison with other studies is therefore valid.

## 3.3 Calcification Regulatory Proteins

The measured concentrations and distribution of the CaRP are listed in table 3.7.

| Parameter | Titre |
|---|---|
| Total fetuin-A$^{\dagger}$ (g/l) | 0.231 ± 0.071 |
| Total fetuin-A$^{\dagger\dagger}$ (g/l) | 0.228 ± 0.072 |
| Phosphofetuin-A$^{\dagger}$ (mg/l) | 22.5 (16.0, 29.5) |
| CPP$^{\dagger}$ (%) | 10.5 (5.3, 17.2) |
| OPG$^{\dagger\dagger}$ (pmol/l) | 9.6 (7.3, 12.3) |
| RANKL$^{\dagger\dagger}$(pmol/l) | 601 ± 258 |
| iFGF-23$^{\dagger\dagger}$ (pg/ml) | 60.8 (45.7, 78.8) |

**Table 3.7 - Titres of CaRP including sub-fractions (n=200) - $^{\dagger}$measured in plasma or $^{\dagger\dagger}$serum**

The correlations of titres of the CaRP with eGFR, age and standard mineral parameters at baseline were calculated (see table 3.8). Stepwise multiple linear regression models of the parameters independently associated with CaRP were then constructed.

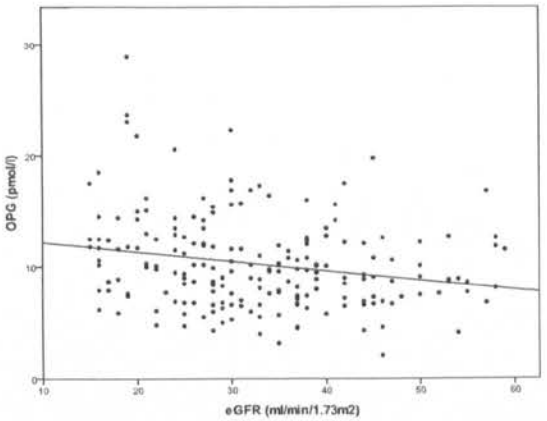
CPP, OPG and iFGF-23 were all significantly associated with age. Within this cohort, eGFR will be a significant potential confounder in many relationships. There was also a significant relationship between eGFR and both age ($r=-0.182$, $p=0.010$) and iPTH ($r=-0.432$, $p<0.001$).

CPP, OPG and iFGF-23 were significantly correlated with eGFR (see fig. 3.6). No significant correlation was seen between eGFR and RANKL or total fetuin-A. iFGF-23 and CPP were significantly associated with phosphate. Notably there was no significant correlation between any of the other CaRP and either calcium or phosphate which were, as discussed, within the normal range for the majority of study subjects. There was no significant correlation between any of the CaRP and either BMI or haemoglobin

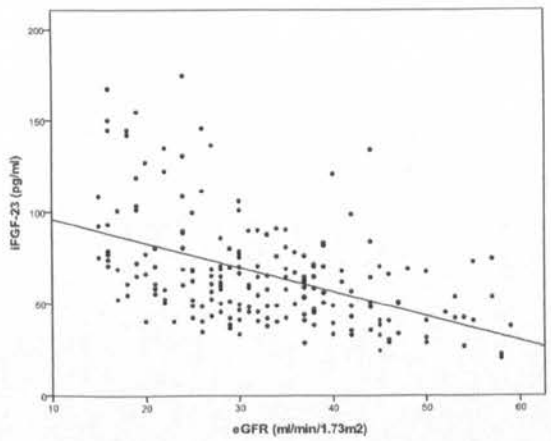
| CaRP | eGFR | Age | C. Calcium | Phosphate | iPTH[$] |
|---|---|---|---|---|---|
| Total fetuin-A | 0.031 | 0.085 | 0.059 | -0.020 | -0.054 |
| Phosphofetuin-A[$] | -0.089 | 0.017 | 0.059 | 0.020 | -0.026 |
| CPP | -0.427*** | 0.260*** | -0.046 | 0.209** | 0.252*** |
| OPG[$] | -0.200** | 0.361*** | -0.013 | -0.001 | 0.164* |
| RANKL | 0.070 | 0.059 | 0.036 | 0.043 | -0.058 |
| iFGF-23[$] | -0.529*** | 0.164* | -0.057 | 0.235** | 0.271*** |

**Table 3.8 - Univariate correlation coefficients of CaRPs with eGFR, age and routine bone parameters (n=200)**

A




B




C

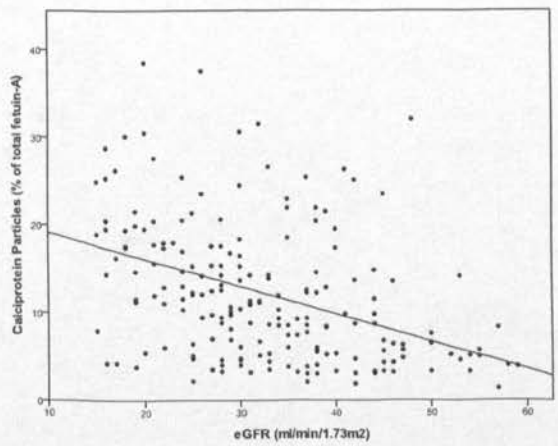



**Figure 3.6 - Scatterplots of signficant associations of eGFR (ml/min/1.73m$^2$) with (A) OPG (pmol/l) (r=-0.200, p=0.004$^{**}$); (B) iFGF-23 (pg/ml) (r=-0.529, p<0.001$^{***}$); and (C) CPP (% of total fetuin) (p=-0.427, p<0.001$^{***}$) within the ACADEMIC cohort (n=200)**

Comparison was made of the CaRP titres according to medication use (see table 3.9).

| Medication | CaRP | Without medication | With medication | p value |
|---|---|---|---|---|
| Phosphate binder | No. pts | 187 | 13 | |
| | Fetuin-A | 0.23 ± 0.07 | 0.25 ± 0.08 | 0.350† |
| | CPP | 10.6 (5.3, 16.8) | 10.3 (7.0, 19.4) | 0.496 |
| | OPG | 9.6 (7.3, 12.4) | 9.9 (7.6, 12.1) | 0.614 |
| | RANKL | 742 ± 222 | 593 ± 258 | 0.062 |
| | FGF-23 | 60.8 (45.5, 75.6) | 68.7 (50.6, 77.1) | 0.368 |
| Bisphosphonate | No. pts | 184 | 16 | |
| | Fetuin-A | 0.23 ± 0.07 | 0.19 ± 0.05 | 0.018* |
| | CPP | 10.2 (5.5, 17.3) | 11.2 (5.1, 15.3) | 0.804 |
| | OPG | 9.8 (7.4, 12.3) | 8.1 (5.8, 13.5) | 0.284 |
| | RANKL | 601 ± 258 | 606 ± 268 | 0.939 |
| | FGF-23 | 60.8 (46.7, 75.6) | 65.0 (39.3, 77.2) | 0.752 |
| Vitamin $D_3$ | No. pts | 176 | 24 | |
| | Fetuin-A | 0.23 ± 0.07 | 0.23 ± 0.07 | 0.724 |
| | CPP | 10.1 (5.2, 16.3) | 12.2 (6.9, 19.5) | 0.121 |
| | OPG | 9.6 (7.3, 12.2) | 9.9 (7.0, 13.5) | 0.875 |
| | RANKL | 598 ± 262 | 621 ± 228 | 0.694 |
| | FGF-23 | 61.4 (45.3, 76.5) | 60.1 (50.6, 74.6) | 0.693 |

**Table 3.9 - Comparison of distribution of CaRP by category of medication †p=0.915 if comparison made with non-calcium based phosphate binder**

The only significant difference found was the reduced total fetuin-A in those patients taking bisphosphonates. Of note, there was no significant difference in CPP or other parameters in relation to bisphosphonate use. There was no significant difference between fetuin-A or CPP titres in the groups of patients taking vitamin $D_3$ or

phosphate binders. Sensitivity analysis examining only those taking non-calcium based binders again showed no significant difference.

Total fetuin-A, phosphofetuin-A and CPP titres were compared within the diabetic and non-diabetic populations in this study. The results are shown in table 3.10.

| Fetuin-A fraction | Non-Diabetic (n=148) | Diabetic (n=52) | p value |
|---|---|---|---|
| Total fetuin-A (g/l)[†] | 0.22 ± 0.06 | 0.27 ± 0.08 | <0.001*** |
| Phosphofetuin-A (mg/l) | 22.3 (15.4, 28.2) | 23.6 (17.7, 34.0) | 0.033* |
| CPP (%) | 10.4 (5.3, 16.6) | 10.9 (5.5, 17.5) | 0.811 |

**Table 3.10 - Titres of fetuin-A and subfractions according to diabetic status in ACADEMIC cohort [†]plasma**

Mean total fetuin-A and phosphofetuin-A were significantly greater in the diabetics. There was no significant difference in CPP between groups.

The correlations of the various CaRP with each other were also examined (see table 3.11 below).

| | CPP | | OPG[$] | | RANKL | | iFGF-23[$] | |
|---|---|---|---|---|---|---|---|---|
| | r | p | r | p | r | p | r | p |
| Total Fet-A | 0.022 | 0.974 | -0.033 | 0.640 | -0.009 | 0.900 | 0.016 | 0.826 |
| CPP | | | 0.198 | 0.005** | -0.059 | -0.408 | 0.308 | <0.001*** |
| OPG[$] | | | | | -0.100 | 0.160 | 0.311 | <0.001*** |
| RANKL | | | | | | | -0.115 | 0.105 |

**Table 3.11 - Correlation matrix of the CaRP in ACADEMIC cohort (n=200)**

### Fetuin-A – Inflammation and Urinary Loss

Fetuin-A has been characterised as a negative acute phase reactant [133]. This pre-dialysis cohort has relatively low levels of inflammation. 74% of patients had a CRP≤5g/l at baseline. Standard laboratory CRP measurement however lacks accuracy at low concentration, preventing meaningful examination of the relationship of fetuin-A with inflammation in this setting.

In order to investigate the relationship of inflammation with CaRP, titres of inflammatory cytokines (hsCRP, IL-1β, IL-6 and TNF-α) were measured. The results are shown in table 3.12.

| Parameter | Median titre |
|---|---|
| hsCRP (mg/l) | 2.30 (0.95, 5.76) |
| IL-1β (pg/ml) | 3.01 (2.3, 3.82) |
| IL-6 (pg/ml) | 6.51 (4.47, 9.49) |
| TNF-α (pg/ml) | 16.70 (12.60, 21.91) |

**Table 3.12 - Baseline titres of inflammatory mediators (n=200)**

The correlation of the CaRP with the inflammatory markers was calculated (see table 3.13). Again, no significant relationship between inflammatory markers and total fetuin-A was seen. Analysis of the various sub-fractions of fetuin-A allowed further study of the relationship of fetuin-A with inflammation. A statistically significant correlation was seen between both phosphofetuin-A and CPP and hs-CRP , and also between CPP and IL-6. No significant relationship was found between any of the fetuin-A fractions with IL-1β or TNF (data not shown).

| CaRP | hsCRP[$] | | IL-1β[$] | | IL-6[$] | |
|---|---|---|---|---|---|---|
| | r | p | r | p | r | p |
| Total fetuin-A | 0.073 | 0.302 | 0.092 | 0.197 | 0.018 | 0.803 |
| Phosphofetuin- A[$] | 0.231 | 0.001** | 0.121 | 0.088 | 0.109 | 0.125 |
| CPP | 0.429 | <0.001*** | 0.096 | 0.174 | 0.193 | 0.006** |
| OPG[$] | 0.289 | <0.001*** | 0.109 | 0.125 | 0.161 | 0.023* |
| RANKL | -0.147 | 0.038* | 0.027 | 0.707 | -0.008 | 0.910 |
| iFGF-23[$] | 0.160 | 0.024* | 0.068 | 0.341 | 0.250 | <0.001*** |

**Table 3.13 - Univariate correlation of CaRP with inflammatory mediators (n=200)**

### Urinary Fetuin Loss

Uncomplexed fetuin-A has a molecular mass of 62kDa. Albumin has a molecular mass of 67kDa. We investigated the extent of urinary fetuin-A loss in our cohort. Urine total protein and fetuin-A concentrations were measured and expressed as a ratio to urinary creatinine (uFCR). The relationship of uFCR to uPCR was examined in the 197 patients in whom sufficient urine was available.

There was a strong correlation between uFCR and both uPCR ($r=0.582$, $p<0.001$) and uACR ($r=0.570$, $p<0.001$). This correlation persisted after exclusion of the subgroup with uPCR<100mg/ml ($r=0.431$, $p<0.001$).

There was no significant correlation between total fetuin-A and uFCR in the full cohort ($r=-0.038$, $p=0.548$). A correlation with borderline significance was seen in the non-diabetic patients ($r=-0.147$, $p=0.076$) (see fig 3.7). However, there was also a significant relationship between eGFR and both uPCR ($r=-0.249$, $p<0.001$) and uFCR ($r=-0.145$, $p=0.042$). Within this study, as discussed no significant correlation was seen between total fetuin-A and eGFR. The relationship of urinary fetuin-A loss with arterial stiffness will be considered alongside other relevant parameters in the subsequent section.

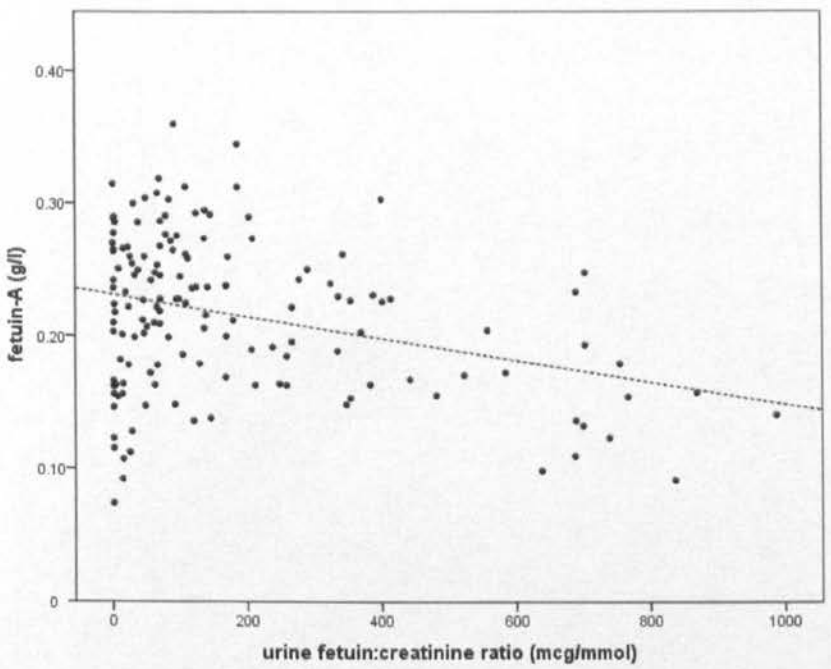



**Figure 3.7 - Scatterplot of the relationship of uFCR (mcg/mmol) with total plasma fetuin-A (g/l) in non-diabetic patients (n=147) (r=-0.147, p=0.076)**

## Multivariate Analysis

A stepwise multiple linear regression model was constructed to investigate the factors independently associated with total fetuin-A (see table 3.14). Diabetic status and uFCR were the factors independently associated with total fetuin-A, in a model which explained 18.9% of the variation in total fetuin-A.

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| Diabetes | 0.063 | 0.144 | 0.042 | 0.084 | <0.001*** |
| uFCR† | -0.074 | 0.189 | -0.117 | -0.031 | 0.001** |

**Table 3.14 - Stepwise multiple linear regression of factors independently associated with total fetuin-A (n=197) - Excluded variables: phosphate, c. calcium, iPTH, eGFR, hsCRP, IL-1β, IL-6, TNF-α, other CaRP & bisphosphonate use - Model $R^2$=0.189 - † pg/mmol**

### Calciprotein Particles

The proportion of total fetuin-A present as CPP was not normally distributed and not amenable to log transformation. The population was therefore split into tertiles by CPP. The relationship of CPP to study variables is shown in table 3.15 below.

| Variable | Low | Intermediate | High | p value |
|---|---|---|---|---|
| CPP (%) | 4.4 ± 1.2 | 10.5 ± 2.2 | 21.1 ± 5.6 | |
| Gender (% male) | 76 | 67 | 73 | 0.527 |
| Age (yr) | 65 ± 11 | 69 ± 12 | 72 ± 10 | 0.002** |
| CV comorbidity (%) | 36 | 43 | 52 | 0.181 |
| Diabetes (%) | 27 | 21 | 28 | 0.564 |
| eGFR (ml/min/1.73m$^2$) | 39 ± 11 | 33 ± 8 | 27 ± 9 | <0.001*** |
| uPCR (mg/mmol) | 19 (10, 44) | 28 (13, 59) | 34 (19, 102) | 0.004** |
| uFCR (mcg/mmol) | 81 (25,152) | 84 (15, 217) | 137 (59, 348) | 0.015* |
| Albumin (g/l) | 43 ± 3 | 42 ± 3 | 43 ± 3 | 0.413 |
| C. Calcium (mmol/l) | 2.29 ± 0.11 | 2.29 ± 0.12 | 2.29 ± 0.12 | 0.993 |
| Phosphate (mmol/l) | 1.04 ± 0.19 | 1.07 ± 0.18 | 1.13 ± 0.22 | 0.043* |
| iPTH (ng/l) | 65 (46, 99) | 74 (50, 95) | 108 (62, 162) | <0.001*** |
| hs-CRP (mg/l) | 1.12 (0.60, 2.92) | 2.40 (1.20, 4.68) | 5.10 (1.80, 9.90) | <0.001*** |
| IL-1β (pg/ml) | 2.73 (2.35, 3.58) | 3.06 (2.30, 4.00) | 3.10 (2.42, 3.85) | 0.389 |
| IL-6 (pg/ml) | 6.0 (3.8, 8.0) | 6.4 (4.5, 9.5) | 7.9 (5.3, 10.3) | 0.021* |
| TNF-α (pg/ml) | 15.6 (12.7, 18.1) | 17.9 (12.5, 22.9) | 16.1 (12.3, 22.2) | 0.165 |

**Table 3.15 - Comparison of study population characteristics by tertile of CPP (n=200) - (Low <6.7%, Medium 6.8-14.2%, High >14.2%)**

A multinomial logistic regression was performed to demonstrate the factors independently associated with risk of categorisation by tertile. The results are shown below in table 3.16.

| Parameter | B | OR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| Low to middle tertile | | | | | |
| eGFR | -0.059 | 0.943 | 0.909 | 0.978 | 0.001** |
| Age | 0.030 | 1.031 | 0.998 | 1.064 | 0.062 |
| Low to high tertile | | | | | |
| eGFR | -0.140 | 0.869 | 0.829 | 0.911 | <0.001*** |
| Age | 0.052 | 1.053 | 1.016 | 1.092 | 0.005** |

**Table 3.16 - Multinomial logistic regression model of factors independently associated with tertile of CPP (n=197) - Excluded variables: hsCRP, IL-6, phosphate, uFCR & iPTH**

Patients with a lower eGFR and increased age had a significantly increased likelihood of a CPP in the highest tertile compared to the lowest tertile. There was a similar relationship between the middle and lowest tertile, although age did not reach significance in this comparison.

**OPG and RANKL**

OPG was inversely correlated with eGFR (see fig. 3.6), but directly correlated with age, hsCRP, iPTH & iFGF-23. No significant relationship was found between OPG and phosphate, or corrected calcium (see table 3.8). OPG was significantly associated with hsCRP and IL-6, but not with TNF or IL-1β (see table 3.13).

A stepwise multiple linear regression analysis of the factors independently associated with OPG was performed (see table 3.17). Age and iFGF-23 were the only variables independently associated with ln OPG.

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| Age | 0.010 | 0.110 | 0.006 | 0.014 | <0.001*** |
| iFGF-23$ | 0.263 | 0.182 | 0.138 | 0.388 | <0.001*** |

**Table 3.17 - Stepwise multiple linear regression of factors independently associated with OPG$ (n=200) - Excluded variables: phosphate, c. calcium, iPTH, eGFR, hsCRP, IL-1β, IL-6, TNF, other CaRP & bisphosphonate use - Model $R^2$=0.182**

RANKL was not significantly correlated with eGFR. However, RANKL was significantly correlated with hsCRP and with uPCR. RANKL was not significantly correlated with OPG (r=-0.010, p=0.160).

If a stepwise regression analysis of the factors independently associated with RANKL was performed, no parameters were retained with p<0.05. If the model criteria were relaxed to include parameters with a significance of p<0.10 then hsCRP and TNF were retained (see table 3.18). However, the model produced is weak with wide confidence intervals, and explained only 3.7% of variation in RANKL. Interestingly, neither OPG nor bisphosphonate use were retained in this model.

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| hsCRP$ | -27.912 | 0.019 | -54.985 | -0.840 | 0.043* |
| TNF | 5.130 | 0.037 | -0.205 | 10.465 | 0.059 |

**Table 3.18 - Stepwise multiple linear regression of factors independently associated with RANKL (n=200) - Excluded variables: Phosphate, c. calcium, iPTH, eGFR, diabetic status, IL-1β, IL-6, other CaRP & bisphosphonate use - Model $R^2$=0.037**

### FGF-23

iFGF-23 was significantly correlated with serum phosphate in addition to eGFR, iPTH and age (see table 3.8 & fig. 3.6). There was also a significant correlation between iFGF-23 and both uPCR (r=0.309, p<0.001) and hsCRP (r=0.160, p=0.024). There was a significant association between iFGF-23 and both OPG and CPP. A stepwise regression model of the parameters independently associated with iFGF-23 was performed (see table 3.19).

In this model, eGFR, OPG and uPCR were independently associated with iFGF-23 in a model which explained 34.9% of the variation in ln FGF-23. Phosphate, iPTH and the other CaRP were excluded from the final model.

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | -0.016 | 0.267 | -0.021 | -0.012 | <0.001*** |
| OPG$ | 0.214 | 0.313 | 0.095 | 0.333 | <0.001*** |
| uPCR$ | 0.072 | 0.349 | 0.028 | 0.116 | 0.001** |

**Table 3.19 - Stepwise multiple linear regression of factors independently associated with iFGF-23$ (n=200) - Excluded variables: age, phosphate, c. calcium, iPTH, diabetic status, IL-1β, IL-6, other CaRP & bisphosphonate use - Model $R^2$=0.349**

## Discussion

### Fetuin-A

The results of the multivariate analysis demonstrate that, in this population, the factors independently associated with total fetuin-A are diabetic status and uFCR. There was no independent relationship between the inflammatory mediators, calcium, phosphate, eGFR or any of the other CaRP.

This study was not designed to study insulin resistance or the metabolic syndrome, and so fasting glucose, cholesterol subfractions and triglycerides were not measured. Nonetheless, the differences between the diabetic and non-diabetic groups described are in line with the association of fetuin-A with increased risk of type 2 diabetes previously reported in the general population [161].

The finding that total serum fetuin-A is inversely and independently related to uFCR is interesting, and has not been previously demonstrated in either the CKD or general populations. The majority of patients with ESRF are oligo/anuric, it is therefore difficult to envisage a significant role for urinary fetuin-A loss in this population where the evidence relating low total fetuin-A with adverse outcome is strongest. However, our findings suggest that urinary fetuin-A loss may have a role in determining total fetuin-A in non-dialysis patients, in particular in those with heavier proteinuria.

There was no significant relationship between eGFR and total fetuin-A. This is consistent with the large population studies of patients with relatively preserved renal function [141,142], though not with the smaller studies of Cottone *et al* [136] or Dervisoglu *et al* [137]. There was also no significant relationship between fetuin-A and either corrected calcium or phosphate. Whilst fetuin-A is integral to maintenance of

calcium and phosphate within solution and to control of mineral solubility at sites of bone turnover, there is no previously published evidence that total serum calcium and phosphate titres are related to circulating titres of fetuin-A within CKD stages 3 & 4.

However care must be taken in the comparison of fetuin-A titres between studies. Using data and samples from the first 178 patients recruited to this cohort study, ES undertook laboratory work to compare the analytical performance of the two commercially available assays, from Biovendor (Brno, CZ) and Epitope Diagnostics (CA, USA). The results of this work demonstrated significant differences between the performance of the two assays with regard to precision, limit of detection and comparability. The Epitope Diagnostics assay had a lower precision, higher limit of detection and positive bias. During the course of this work, a comparison of the total fetuin-A in the ACADEMIC cohort was made with total fetuin-A in a healthy control group. Both kits demonstrated that the total fetuin-A in the control serum was higher than that in the CKD serum. However, again no correlation was found between total fetuin-A and eGFR if the Epitope Diagnostics kit was used [154].

Commercially available recombinant fetuin-A is asialylated, and often used as a standard. Native human fetuin-A has a number of glycosylation sites. ES repeated the analysis after deglycosylation of the samples. We reported that the titres of the samples analysed using the Biovendor kit were markedly reduced, and concluded that the level of glycosylation is important in antibody specificity between assays. This may explain some of the differences between assays, suggesting that the specificity of the Biovendor kit to native fetuin-A was greater than the other commercially available kit [154]. We therefore restricted our subsequent analysis to the results obtained using the Biovendor kit as it seemed more physiologically relevant.

If the previously published cohort data is reviewed with this knowledge, then it is noted that one of the larger studies, which showed no relationship between eGFR and total fetuin-A using the Epitope diagnostics assay [141], whilst the other study by Weikart *et al* used an immunoturbidometric method [142]. Of the two smaller studies which reported an association between eGFR and fetuin-A, one used the Biovendor kit [137] and the other the Epitope Diagnostic kit [136]. Therefore, whilst performance of the assays does differ, choice of assay does not seem to be a significant factor in determination of any potential relationship between eGFR and total fetuin-A

concentration. In this thesis, the results obtained using the Biovendor assay are reported.

These experiments were performed and reported prior to the appreciation of the data of the Matsui/Hamano group which highlighted the importance of the CPP sub-fractions of fetuin-A [120,122]. The influence of glycosylation on the proportion of fetuin-A present as CPP, or vice verse, is not known. The influence of any variation in the proportion of fetuin-A subfraction upon assay performance is also not known.

**Fetuin CPP**

In the ACADEMIC study, age and eGFR were independently associated with the likelihood of a high CPP. The significant relationship between CPP and eGFR is consistent with the stepwise relationship described by Hamano *et al* in their study of 78 CKD patients in which they propose CPP as a biomarker of extraosseous calcification stress [122]. The ACADEMIC cohort is however significantly larger than the Hamano cohort in which the mean CPP in CKD stages 3 and 4 were not significantly different (see fig. 1.8). This relationship is consistent with the proposed theory of CPP as a marker of extraosseous calcification, as seen in patients with more severe renal impairment.

**Inflammation**

In this study, no significant independent association was found between total fetuin-A and any of the inflammatory mediators measured.

Fetuin-A was first characterised as a negative acute phase reactant in a cohort of 23 patients suffering from acute infectious diseases [133]. This is a markedly different setting to the stable CKD outpatient scenario in this study. The lack of significant association between total fetuin-A and hs-CRP in our cohort is consistent with the findings of Ix *et al* in his analysis of the MDRD CKD study [153], but in contrast to studies of dialysis patients where an inverse relationship has been reported between fetuin-A and markers of inflammation [144,145].

This study is not sufficiently large for robust subgroup analysis, and subgroup analysis of any variation of the relationship of fetuin-A with inflammatory mediators dependent upon renal function was not specified *a priori*. However, in order to investigate whether the relationship of fetuin-A with inflammation was dependent upon renal function within the confines of the ACADEMIC study, the correlation of

total fetuin-A with the inflammatory mediators was tested in the subgroup of patients with CKD stage 4. No significant relationship was found (data not shown).

The relationship of fetuin-A with inflammation has also been examined within secondary cardiovascular prevention studies. These cohorts have generally normal renal function. In this setting, a positive relationship between total fetuin-A and hsCRP has been found [142,162,164]. There are several explanations for the lack of association found between fetuin-A and the cytokines measured.

Firstly, it is possible that the changes in bone and vascular milieux may confound the relationship of total fetuin-A with inflammation. The studies in which a positive relationship between total fetuin-A and inflammatory markers was found are general population studies. As mentioned previously, in the general population there is emerging evidence that fetuin-A is associated with risk of type II diabetes. Consistent with this theory, mean total fetuin-A and phosphofetuin-A were both significantly higher in the diabetic patients in this study.

Secondly, in our non-inflamed cohort the effect size of the association of total fetuin-A with inflammation may not be sufficiently strong to be detected over background variation due to other factors.

Finally, interpretation of the relationship between IL-1β, IL-6 and TNF-α and other factors in this study must be tempered with appreciation of the sites of action of these cytokines. The inflammatory cytokines studied exert a predominantly paracrine effect [319], however these substances were measured using a general serum sample which is unlikely to accurately represent the microenvironment of either the bone or the liver [164].

It therefore appears likely that the association of total serum fetuin-A with inflammation is dependent upon other metabolic factors. In patients with ESRF this association may be confounded by other effects such as extraosseous calcification stress or the pro-inflammatory dialysis milieu whereas in the general population the association of fetuin-A with inflammation may be linked to its association with the metabolic syndrome.

The direct association between CPP and hsCRP and IL-6 must be reconciled with the current knowledge base. Whilst hsCRP and IL-6 were not retained in this regression

analysis, it could be argued that if total fetuin-A is reduced in inflammation, but the CPP (expressed as a proportion of total fetuin-A) remained constant then this would provide a direct association between inflammation and CPP. However, in this study, no independent association was found between inflammation and total fetuin-A to support this theory.

Alternatively the association between CPP and IL-6 and hsCRP may reflect the pathway of inflammation mediated vascular calcification [178,303].

The subgroup of patients taking bisphosphonates had a lower total plasma fetuin-A concentration, but there was no significant difference between CPP using either group comparison or multivariate analysis. The group of patients using bisphosphonates did not have a significantly different age or eGFR, and bisphosphonate use was not retained in the stepwise multivariate model of factors independently associated with fetuin-A titre. Notwithstanding the observational nature of these results, they are at odds with those of Hamano *et al* who have demonstrated that administration of alendronate led to the loss of CPP within the serum, but no change in total serum fetuin-A in the adenine CKD rat model. Whilst studies in rats have shown that bisphosphonates protect against vascular calcification, these studies used much larger relative doses than those used in humans. Results in human studies have been mixed, and larger studies are underway.

It has been proposed that patients under significant pro-calcific stress (such as a high serum calcium or calciphylaxis) may undergo depletion of circulating fetuin-A. The low circulating titres of fetuin-A may be a reflection of the binding of free fetuin-A to calcium, to form complexes which are subsequently either deposited locally or cleared by the reticulo-endothelial system [126], i.e. that fetuin-A is low as it has been consumed.

Hamano *et al* also studied the effect of parathyroidectomy and cinacalcet on CPP titre. However parathyroidectomy was an exclusion criterion for the ACADEMIC study and no patients were taking cinacalcet. It was therefore impossible to investigate if the effect is also seen in CKD stages 3 & 4 within the confines of this study. There were no significant differences in titre of CPP according to diabetic status, or uFCR.

Other factors which are thought to influence fetuin-A titres include *ahsg* genotype [320]. *ahsg* genotyping was not performed in this study.

**OPG/RANKL**

In the ACADEMIC study, increased OPG was associated with increased titres of hs-CRP and IL-6. Whilst consistent with previously published data (summarised in table 1.5) this relationship did not persist in the multivariate model. Instead OPG was independently associated with age and iFGF-23 in a model explaining 18.2% of the variation in OPG.

FGF-23 is produced by osteoblasts in response to hyperphosphataemia and increased vitamin D. OPG is produced by a variety of cell types, including osteoblasts. Both OPG and iFGF-23 are increased in patients with CKD, however, to the best of my knowledge, no evidence demonstrating a direct link between OPG and the transcription or translation of FGF-23 has been published.

A similar study of OPG in 351 US patients with CKD stages 3 & 4 has been published very recently [321]. In this study age, eGFR, serum albumin, SBP and female gender were all independently associated with OPG. A sensitivity analysis was performed on our data whereby these variables were entered into the stepwise multivariate model. None of the additional parameters were retained. These studies appear therefore to be in conflict. The reasons for this are not clear, however demographic factors may play a role as the 351 US patients were significantly younger, with a greater proportion of black ethnicity and with diabetes.

In the ACADEMIC study, RANKL was only weakly associated with inflammatory mediators in the stepwise multivariate analysis. This is consistent with the previously published evidence.

In addition to the biological factors influencing titres of OPG & RANKL such as the overriding influence *in vivo* of uraemia, consideration should also be given to methodological factors such as the site of cytokine sampling (i.e. plasma rather than the bone or vascular wall microenvironment) or to the overriding influence *in vivo* of uraemia on the titres of cytokines.

Titres of RANKL are approximately 60 times greater than those of OPG. The lack of significant correlation between OPG and RANKL in the ACADEMIC study (r=-

0.010, p=0.160) is consistent with the study of Semb *et al* who also found no significant relationship [223]. Conversely, Lieb *et al* found a weak, but significant inverse relationship in their study of the Framingham population [188].

**FGF-23**

The factors independently associated with iFGF-23 were eGFR, OPG and uPCR. eGFR explained 26.7% of the variation in iFGF-23. Notably, despite the well documented causal relationship between phosphate and FGF-23, phosphate was not independently associated with iFGF-23.

As previously discussed, serum phosphate was within, or below the normal range for 97.5% of study patients. Within the range of eGFR studied, phosphate homeostasis is abnormal, but homeostatic mechanisms including iFGF-23 remained effective at maintaining phosphate within the normal physiological range. The significant correlation of iFGF-23 with phosphate in this cohort of patients with CKD stages 3 & 4 is consistent with other studies which demonstrate that iFGF23 is increased with only minor reduction in GFR and whilst phosphate is normal [235]. The correlation between iFGF-23 and phosphate was stronger and more significant for baseline phosphate (r=0.223, p=0.002) rather than the six month averaged value (r=0.194, p=0.007). Despite the stronger correlation between iFGF-23 and baseline phosphate, time averaged values were used for calcium, phosphate, albumin and haemoglobin throughout the study, as these reflect the milieu to which the blood vessels and heart were chronically exposed during the period preceding baseline PWV measurement.

Vitamin D and its analogues were not measured in the ACADEMIC study, primarily due to cost considerations. The association of circulating vitamin D with any of the CaRP cannot therefore be assessed. Comparison of CaRP titres between groups using vitamin $D_3$ was also limited by indication bias, and low numbers. Few patients were taking vitamin $D_3$ supplementation; 4% used 1(OH) vitamin $D_3$ and 8% used 25(OH) vitamin $D_3$. 1,25 $(OH)_2$ vitamin $D_3$ administration would be expected to be associated with increased iFGF-23, OPG and RANKL. However, there was no significant difference between FGF-23 titres in those using vitamin $D_3$ compared to the rest of the study cohort.

## 3.4 CaRP and Arterial Stiffness

The correlations of C-F PWV with standard demographic and clinical variables are shown in table 3.5, and the correlations of C-F PWV with CaRP are shown in table 3.20 below.

| Variable | r | p value |
|---|---|---|
| Total fetuin-A | -0.069 | 0.351 |
| CPP | 0.338 | <0.001*** |
| OPG$ | 0.395 | <0.001*** |
| RANKL | -0.008 | 0.917 |
| iFGF-23$ | 0.262 | <0.001*** |

**Table 3.20 - Univariate correlation of CaRP with C-F PWV (n=185)**

Significant univariate correlation was seen between C-F PWV and OPG, iFGF-23 and CPP. C-F PWV was not significantly correlated with total fetuin-A or RANKL.

In order to further understand the factors independently associated with C-F PWV various multiple linear regression models were constructed. Whilst previous studies have reported associations between fetuin-A, OPG, iFGF-23 and a variety of arterial parameters, the pathophysiological mechanisms for the relationships of the CaRPs remain incompletely understood. For this reason, initial exploratory analysis was performed using stepwise multivariate analysis. Variables were selected for entry to the model if there was published evidence of independent association, significant univariate correlation or the variable was one of the CaRP under investigation.

Age, OPG, CPP and diabetic status were independently associated with C-F PWV, whilst the other parameters listed, including FGF-23 and RANKL, were excluded from the stepwise model (see table 3.21). If total fetuin-A was substituted for CPP then total fetuin-A was not retained in the final model, and in addition diabetes was lost from the final model. If cardiovascular comorbidity was entered in this model, then it replaced diabetes in the final model with a similar power ($R^2$=0.374).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95%CI | p value |
|---|---|---|---|---|---|
| Age | 0.119 | 0.281 | 0.092 | 0.1478 | <0.001*** |
| OPG$ | 1.489 | 0.327 | 0.632 | 2.345 | 0.002** |
| CPP | 0.061 | 0.357 | 0.019 | 0.102 | 0.004** |
| Diabetes | 0.702 | 0.371 | 0.003 | 1.401 | 0.049* |

**Table 3.21 - Stepwise multiple linear regression model of factors independently associated with C-F PWV (n=185) - Excluded variables: gender, eGFR, SBP, DBP, RANKL, iFGF-23$, ACEi/ ARB use & CCB use - Model $R^2$=0.371**

### Forced Entry Models

Stepwise multivariate analysis is based on purely mathematical criteria. In order to adjust for the effect of factors previously recognised to influence arterial stiffness a series of forced entry models were constructed. The results of this analysis are shown in table 3.22.

OPG, CPP, age and diabetic status remained significantly independently associated with adjusted C-F PWV whilst the other parameters forced into the model did not reach statistical significance. After construction of a fully adjusted model (model 2), iFGF-23 was forced into this model. However, the significance of the univariate correlation of iFGF-23 was lost after adjustment for OPG, age and other standard haemodynamic parameters in this model. Whilst there was a very small increase in the proportion of variation of C-F PWV explained in the model ($R^2$ increased from 0.381 to 0.387), the F test for the final model in comparison to the previous was not significant (p=0.425), indicating that there was no significant improvement in model fit from model 2. Model 2 can therefore be considered to be the final fully adjusted model.

| Parameter | Unadjusted[a] | | Model 1[b] | | Model 2[c] | | Model 3[d] | |
|---|---|---|---|---|---|---|---|---|
| | B | p value | B | p value | B | p value | B | p value |
| Age | 0.108 | <0.001*** | 0.094 | <0.001*** | 0.088 | <0.001*** | 0.089 | <0.001*** |
| eGFR | -0.017 | 0.261 | -0.010 | 0.507 | 0.007 | 0.663 | 0.017 | 0.336 |
| SBP | 0.015 | 0.064 | 0.012 | 0.113 | 0.012 | 0.130 | 0.011 | 0.158 |
| HR | 0.015 | 0.254 | 0.014 | 0.270 | 0.010 | 0.411 | 0.010 | 0.416 |
| DM status | 0.856 | 0.024* | 0.756 | 0.042* | 0.803 | 0.028* | 0.771 | 0.035* |
| OPG[$] | | | 1.298 | 0.004** | 1.230 | 0.005** | 1.075 | 0.018* |
| CPP | | | | | 0.062 | 0.006** | 0.060 | 0.009** |
| iFGF-23[$] | | | | | | | 0.602 | 0.192 |

**Table 3.22 - Adjusted multiple linear regression model of factors independently associated with C-F PWV (Adjusted for MBP) (n=185)**
**a-Model $R^2$=0.322; b-$R^2$=0.353; c-$R^2$=0.381; d-$R^2$=0.387**

## Discussion

This is the first published study to examine the relationship between all the putative CaRP and aortic stiffness in a cohort of patients with CKD stage 3 & 4 patients using the gold standard measurement of C-F PWV. As discussed in the introduction, various groups have studied the associations of fetuin-A, OPG and FGF-23 with a variety of arterial parameters.

Assessing the multivariate models constructed, there are four major findings. Firstly OPG is independently associated with C-F PWV. Secondly CPP is independently associated with C-F PWV, whilst total plasma fetuin-A is not. Thirdly, no significant independent association was found between C-F PWV and either FGF-23 or RANKL. Finally C-F PWV was not independently associated with eGFR after adjustment for other factors.

The data findings from this study will now be reviewed in relation to other published information relating to arterial stiffness and the CaRP in question.

### OPG

We found an independent association between OPG and C-F PWV. Two studies of OPG and arterial stiffness in adult pre-dialysis CKD have previously been published. A recent study demonstrated an independent association between OPG and C-F PWV which persisted after adjustment for both traditional and non-traditional risk factors [321].

A further study measured C-R PWV in both dialysis and non-dialysis CKD patients from the UK, [199]. A univariate correlation between OPG and C-R PWV was found but regression analysis was not reported. C-R and C-F PWV cannot be used interchangeably. However the finding of an independent association between OPG and C-F PWV in the ACADEMIC study is also consistent with two further studies, both also published since the beginning of this study.

Nakashima *et al* studied 151 prevalent haemodialysis patients. They found that OPG and age were the only factors independently associated with C-F PWV. Furthermore, they demonstrated that OPG was independently associated with hazard of both all cause and cardiovascular death in this group [202].

Zagura *et al* also measured C-F PWV in two groups of patients with preserved renal function; one group with peripheral vascular disease and a control group. OPG and MBP were significantly independently associated with C-F PWV in both groups, along with age in the healthy controls and Ankle Brachial Pressure Index (ABPI) in those with peripheral vascular disease (model $R^2$ =0.44 and 0.47 respectively) [203].

**RANKL**

No evidence was found of any association between RANKL and C-F PWV in either univariate or multivariate analysis. Interestingly only two studies have been published relating RANKL to arterial stiffness parameters, neither of which found any significant association [224,225]. No other studies have been published relating RANKL to PWV in CKD. Whilst it is possible that this is because these studies have not been performed, the evidence of both vascular calcification in the OPG -/- mouse, the association of OPG with adverse cardiovascular outcome and the association of reduced bone density with cardiovascular mortality, all point towards RANKL as a major player. It is therefore possible that this reflects positive publication bias.

Indeed, recent work by Panizo *et al* has demonstrated that *in vitro* incubation of human VSMC with RANKL causes a dose dependent increase in VSMC calcification [177]. This process is abolished by co-incubation with OPG. The authors then demonstrated that this process was mediated through RANK via a BMP-4 dependent pathway. Furthermore the authors studied OPG, RANKL and BMP-4 expression in a 5/6 nephrectomy with excess calcitriol rat model of arterial calcification of CKD. Within this model, the serum RANKL:OPG ratio was decreased in the intervention (CKD) arm. This was however associated with an **increased** ratio of RANKL RNA:OPG RNA in the aortic wall, and an increased BMP-4 RNA level. Particularly relevant for interpretation of the ACADEMIC study however, is the disconnect between RNA staining at the aortic wall and the serum cytokine concentrations in this study, in which serum RANKL concentrations did not reflect the increased RNA staining seen in the wall of the aorta. This disconnect is complicated by the strong correlation between OPG and eGFR which was seen in the rat model, mirroring the findings of the ACADEMIC study. As serum OPG concentrations increase in CKD, expression of RANKL (which was not significantly correlated with eGFR) as a ratio to OPG is liable to misinterpretation due to

alteration in OPG rather than in the potentially pathophysiologically implicated RANKL.

Moreover, it appears increasingly as if OPG may play a protective role against vascular calcification. Di Bartolo *et al* have recently shown *in vitro* that OPG can protect against calcium induced VSMC calcification via the Insulin-like Growth Factor 1 receptor (IGF1R) [322]. Translation of this work to the *in vivo* setting is awaited.

In summary, whilst the findings of this study are consistent with others in the literature, they are not wholly consistent with the understanding of the mechanisms of OPG or RANKL at a cellular level.

**Total Fetuin-A**

No correlation was found between total plasma fetuin-A and baseline C-F PWV. The published evidence in non-dialysis patients is contradictory. Roos *et al,* studying patients with normal renal function, found that fetuin-A was associated with C-F PWV after adjustment for age and blood pressure – however this relationship was only present in the sub-group of male subjects [116]. This small study did not have predetermined subgroup analyses and to my knowledge no studies have suggested a possible sex-linked element to fetuin-A metabolism. Fetuin-A has also been independently associated with C-F PWV in three studies of dialysis patients – two in adults [26,148] and one in children [147]. Two other studies have not found a significant relationship [148,199].

Fetuin-A was not forced into our first multivariate model as the evidence that total fetuin-A is physiologically implicated in arterial stiffening is not strong. Indeed there was no significant correlation between total fetuin-A and C-F PWV in our cohort.

*In vivo* work on the arteries of paediatric CKD patients, in whom other confounding arterial pathologies are minimised, has shown that whilst the arteries of both pre-dialysis and dialysis patients are more sensitive to changes in calcium and phosphate (which are thought to be partially mediated by fetuin-A), those on dialysis are significantly more sensitive that those with pre-dialysis CKD [46]. The lack of association of fetuin-A with C-F PWV in the ACADEMIC study may therefore be due to a reduced effect size in the pre-dialysis setting which is either obscured by unadjusted confounding or not detectable in a sample of this size.

**Calciprotein Particles**

However, the proportion of fetuin-A present as CPP was significantly independently associated with C-F PWV, in the fully adjusted model. This is the first time that CPP proportion has been related to arterial stiffness. Strikingly we have demonstrated this relationship in a cohort of patients with CKD stages 3 & 4 and multiple comorbidities rather than in a dialysis cohort where this relationship would be expected to be stronger. Whilst Hamano *et al* did not report any investigation of the relationship between CPP and arterial stiffness, these results are compatible with, and indeed compliment, their findings of an association between CPP and CACS in 73 pre-dialysis CKD patients [122].

This is a dynamic field and no mechanisms have yet been proven. However these results are consistent with the current theoretical pathways that suggest that excess CPP are formed during periods of 'mineral stress', i.e. when calcium and phosphate concentrations rise, and that they may then be endocytosed by VSMCs [323]. Under physiological conditions, CPP may be processed within the cell, and the excess calcium excreted in matrix vesicles in which the calcium is stabilised by fetuin-A. When an excess of CPP is present in the circulation, the capacity of this system may be overwhelmed leading to either mineral formation directly within the matrix vesicles which lack adequate fetuin-A, or alternatively, the excess calcium may damage the cell. Any cellular damage may lead to apoptotic (or senescent) change, with the generation of apoptotic bodies which themselves act as nidi for calcification [126].

**FGF-23**

iFGF-23 was significantly correlated with C-F PWV, but, as discussed above, the association was lost after adjustment for other factors. These results are consistent with other studies which have not demonstrated any association of FGF-23 with arterial stiffness.

One study examining the relationship of FGF-23 with arterial stiffness in pre-dialysis CKD patients has been published recently. This study employed an unusual method of digital pulse wave analysis ($SI_{DVP}$) to measure arterial stiffness (see appendix 2). No independent association was found between FGF-23 (or fetuin-A) and this stiffness measurement [156]. A further study has explored the relationship between iFGF-23 with arterial parameters using change in Reflective Index ($\Delta$RI) derived

using pulse wave analysis to measure stiffness. While FGF-23 was associated with stiffness in this study, ΔRI is primarily a measure of endothelial function [261].

However, FGF-23 has been independently associated with aortic calcification in an analysis of patients with CKD on dialysis, after adjustment for age, dialysis vintage, phosphate and lipid variables. CPP were not measured in this study [247]. This association has not been shown in pre-dialysis CKD patients.

**Sensitivity Analysis**

In our model, age and diabetic status were significantly independently associated with C-F PWV in addition to OPG and CPP. In our published model diabetic status and CPP were not included as covariates [324]. However, diabetes is a recognised determinant of arterial stiffness [23]. A sensitivity analysis in this paper was performed showing an increased model power in the non-diabetic patients alone. However, the most robust method of testing variables is to use the entire study population. In this cohort, the diabetic patients have a statistically significantly increased mean C-F PWV. Diabetic status was therefore included in the final, fully adjusted model.

CPP was not included in our first published model [324], as the work of Hamano *et al* and our subsequent analyses were not available at the time of manuscript preparation.

The relationships in the final model remained significant after sensitivity analyses for calcium & vitamin $D_3$ supplementation and bisphosphonate use. Finally, if crude C-F PWV was used as the dependent variable, SBP joined the other four parameters previously significantly associated with adjusted C-F PWV in a model with a similar total $R^2$.

It is interesting to contrast our findings with those of two other cohorts. Firstly, the CRIC cohort study of 2564 patients from the USA with mean eGFR of 39.7 ± 16.0ml/min/1.73m$^2$. In that study, the parameters found to be independently associated with C-F PWV were age, eGFR, fasting glucose, black ethnicity, waist circumference, MBP, male gender and diabetes [21]. Secondly, the Edinburgh cohort reported by Lilitkarntakul *et al* which contained 113 patients with CKD stages 1 to 5 with few comorbidities. In that cohort, the factors independently associated with C-F PWV were age, MBP, hsCRP and ADMA. The association of fasting glucose and cholesterol were lost after adjustment [25].

In neither of these studies were CaRP titres measured, and therefore any potential relationships with CaRP within these cohorts cannot be identified. Fasting glucose was not measured in the ACADEMIC study, and therefore we have been unable to validate this association in this study. ADMA titres were only available for the first 133 patients in the study. Multivariate analyses including ADMA (performed by, and included in the thesis of LT [2]) demonstrated that C-F PWV was not independently associated with ADMA in these 133 patients [325].

The epidemiological nature of this study limits the potential to draw definitive conclusions on the mechanisms of aortic stiffening. However, the finding of an independent association of particularly CPP, but also OPG (which were themselves significantly correlated with eGFR) with C-F PWV, rather than eGFR itself as measured in the CRIC study and others [19,21], indicates that the strength of association of these CaRP is stronger than that of eGFR. This provides support, albeit epidemiological and cross-sectional, to the concept that these proteins are either involved in, or directly associated with the process of arterial stiffening.

However, our findings and those of the CRIC study and others, should be contrasted with the Edinburgh cohort in which eGFR was not independently associated with C-F PWV [25]. The authors of that study suggest that the association of C-F PWV with eGFR in other studies may be driven by cardiovascular comorbidity. Our findings are in partial agreement with this, as if cardiovascular comorbidity is entered into our stepwise model then it is retained. However, importantly, OPG and CPP are also retained in the model. Thus it could be suggested that these proteins are more likely to be implicated in the process of stiffening than eGFR *per se*, as eGFR is, above all, an estimation of the rate of glomerular filtration rather than more directly of extraosseous stress.

In summary, in this analysis, we have found an independent association between both OPG and CPP and baseline C-F PWV within the ACADEMIC cohort. Total fetuin-A, iFGF-23 and plasma RANKL were not independently associated with baseline C-F PWV. This data and analysis has subsequently been published [303,324].

## 3.5 Troponin, Arterial Stiffness and the CaRP

Within the study, limited cardiac data is available; a medical history was taken and an ECG performed at entry to the study. The introduction of the highly sensitive cardiac troponin T (hs-cTnT) assay during the course of the study provided a novel method of biomarker based assessment of medium and long term cardiovascular risk.

### Results

Forty one patients were found to have LVH using ECG criteria. No significant differences were found between age, renal function and titres of CaRP within these two groups.

#### Troponin T

The distribution of hs-cTnT within the study population is shown on fig. 3.8.

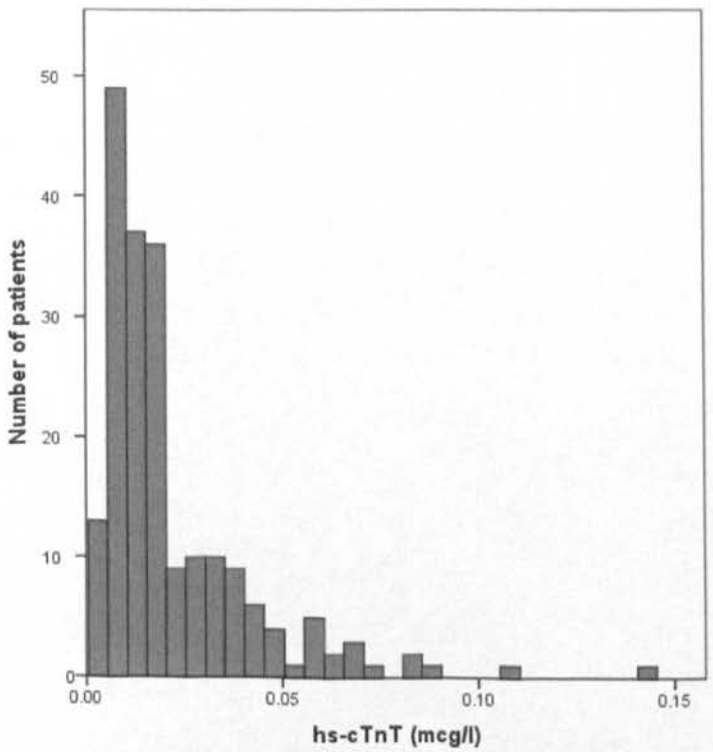



**Figure 3.8 - Histogram of distribution of hs-cTnT(mcg/l) in ACADEMIC cohort (n=200)**

The median hs-cTnT was 0.016mcg/l (0.009, 0.028). This distribution is right skewed, and so was log transformed prior to further analysis.

The relationship of hs-cTnT to LVH as measured by ECG criteria and overt and sub-clinical cardiovascular comorbidity are shown in table 3.23.

| CV Parameter (no. with characteristic) | hs-cTnT median ($25^{th}$, $75^{th}$ centile) Yes | No | p value |
|---|---|---|---|
| $LVH_{Cornell}$ (20) | 0.013 ( 0.007, 0.253) | 0.017 (0.009, 0.029) | 0.121 |
| $LVH_{S-L}$ (28) | 0.012 (0.006, 0.308) | 0.016 (0.010, 0.028) | 0.182 |
| $LVH_{Cornell\ and/or\ S-L}$ (41) | 0.012 (0.007, 0.027) | 0.017 (0.010, 0.028) | 0.097 |
| CV comorb. (overt) (88) | 0.019 (0.012, 0.031) | 0.013 (0.008, 0.023) | 0.005** |
| CV comorb.(subclin) (101) | 0.018 (0.012, 0.031) | 0.013 (0.008, 0.023) | 0.004** |
| Diabetes (149) | 0.019 (0.012, 0.033) | 0.014 (0.008, 0.025) | 0.013* |

**Table 3.23 - Comparison of hs-cTnT titre by ECG LVH criteria and comorbidity**

There was no significant difference in hs-cTnT measurements between groups with and without LVH by ECG criteria. However there was a significantly higher concentration of hs-cTnT in patients with previous cardiovascular disease or diabetes.

The correlation of hs-cTnT with biochemical and demographic variables are shown in table 3.24.

hs-cTnT was significantly correlated with eGFR, age, OPG, RANKL and iFGF-23. hs-cTnT was not significantly correlated with total fetuin-A but was significantly correlated with CPP. There was also significant correlation between hs-cTnT and C-F PWV, in addition to uPCR, hs-CRP and phosphate – other recognised non-traditional cardiovascular risk factors.

| Variable | r | p value |
|---|---|---|
| eGFR | -0.434 | <0.001*** |
| Age | 0.456 | <0.001*** |
| SBP | 0.130 | 0.066 |
| DBP | -0.129 | 0.068 |
| C-F PWV | 0.399 | <0.001*** |
| Fetuin-A | 0.012 | 0.866 |
| CPP | 0.382 | <0.001*** |
| OPG$ | 0.554 | <0.001*** |
| RANKL | -0.270 | <0.001*** |
| iFGF-23$ | 0.468 | <0.001*** |
| iPTH$ | 0.247 | 0.001** |
| hs-CRP$ | 0.325 | <0.001*** |
| Haemoglobin | -0.141 | 0.053 |
| uPCR$ | 0.255 | <0.001*** |
| Corrected calcium | 0.051 | 0.482 |
| Phosphate | 0.203 | 0.005** |
| Cholesterol | -0.123 | 0.082 |

**Table 3.24 - Univariate correlations of hs-cTnT$ with demographic and biochemical parameters within ACADEMIC cohort (n=200) (n=185 for C-F PWV)**

A series of multivariate models of the parameters independently associated with hs-cTnT were constructed for the whole population. Variables were initially selected for entry to the models if there was published evidence of independent association, univariate correlation with $p<0.05$ or the variable was the focus of investigation. Initially a model containing traditional and non-traditional cardiovascular risk factors

excluding C-F PWV and the CaRP was constructed. In this model, eGFR, age, uPCR and hs-CRP were independently associated with ln hs-cTnT. These parameters remained independently associated with ln hs-cTnT after full adjustment (see table 3.25).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | -0.014 | 0.171 | -0.024 | -0.005 | 0.003** |
| Age | 0.032 | 0.332 | 0.024 | 0.040 | <0.001*** |
| uPCR$ | 0.183 | 0.400 | 0.099 | 0.026 | <0.001*** |
| hsCRP$ | 0.094 | 0.427 | 0.028 | 0.160 | 0.005** |
| CV comorbidity | -0.013 | 0.428 | -0.194 | 0.169 | 0.892 |
| Diabetes | 0.146 | 0.434 | -0.053 | 0.345 | 0.149 |
| Phosphate | 0.463 | 0.445 | -0.020 | 0.946 | 0.060 |
| iPTH$ | 0.054 | 0.446 | -0.092 | 0.200 | 0.892 |

**Table 3.25 - Adjusted multiple linear regression model of factors associated with hs-cTnT$ (n=200) - Model $R^2$=0.446**

A further set of models were then constructed incorporating the CaRP and C-F PWV (see table 3.26). The total $R^2$ of the fully adjusted model incorporating the CaRP was 0.572, an increase of 0.126 from the model without the CaRP.

### Sensitivity Analysis

If diabetic status and cardiovascular comorbidity were forced into the final model the $R^2$ was reduced from 0.572 to 0.563 (data not shown). Forced inclusion of these comorbidity parameters did not alter the parameters found to be significantly independently associated in the final model, and the coefficients for the other parameters remained stable. C-F PWV was not significant in either model.

Exclusion of the diabetic patients made no difference to the significance of the final parameters in the model, whilst exclusion of the patients with cardiovascular comorbidity led to the loss of significance of phosphate. In the final model, the other parameters remained unchanged.

CPP and iPTH were also not included in the final fully adjusted model as forced inclusion neither increased the overall power of the final model, nor altered the stability of selection of other significant parameters.

| Parameter | Unadjusted[a] | | Model 1[b] | | Model 2[c] | | Model 3[d] | | Final Model[e] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B | p value | B | p value | B | p value | B | p value | B | p value |
| FGF23[$] | 0.977 | <0.001*** | 0.727 | <0.001*** | 0.713 | <0.001*** | 0.427 | 0.001** | 0.176 | 0.001** |
| OPG[$] | | | 0.822 | <0.001*** | 0.699 | <0.001*** | 0.568 | <0.001*** | 0.248 | <0.001*** |
| C-F PWV[†] | | | | | 0.049 | 0.008** | -0.002 | 0.902 | - | - |
| Age | | | | | | | 0.024 | <0.001*** | 0.010 | <0.001*** |
| eGFR | | | | | | | -0.010 | 0.035* | -0.004 | 0.069 |
| SBP | | | | | | | -0.003 | 0.148 | -0.001 | 0.411 |
| uPCR[$] | | | | | | | 0.087 | 0.036* | 0.045 | 0.015* |
| hsCRP[$] | | | | | | | 0.041 | 0.183 | 0.016 | 0.220 |
| Phosphate | | | | | | | 0.471 | 0.030* | 0.202 | 0.031* |
| RANKL | | | | | | | 0.001 | 0.030* | 0.001 | 0.003** |

**Table 3.26 - Adjusted linear regression models of the factors associated with hs-cTnT[$] (n=185) [†]adjusted for MAP**
**a-Model $R^2$=0.259; b-$R^2$=0.416; c-$R^2$=0.468; d-$R^2$= 0.596; e-$R^2$=0.572**

## Discussion

The emergence of hs-cTnT as a biomarker of adverse cardiovascular outcome within asymptomatic outpatient populations has been very recent. To the best of my knowledge no other study has examined hs-cTnT within a population of pre-dialysis CKD and nor have any other published studies explored the relationship of the CaRP with this biomarker. Several of the studies that have examined the relationship between the cardiovascular system and the CaRP have used patients with overt cardiovascular pathology including acute coronary syndrome and heart failure, and various measures of outcome and/or cardiovascular risk. For these reasons validation of the ACADEMIC study findings against these cohorts is challenging.

There was no difference in the frequency of LVH by ECG criteria in patients with cardiovascular or diabetic comorbidity. There was no difference in CaRP titres by ECG LVH classification. We were unable to perform echocardiography or other cardiac imaging with measurement of LV mass and therefore could not explore this relationship further. Whilst hs-cTnT is yet to be validated as an independent predictor of medium to long term cardiovascular risk within the setting of CKD stages 3 & 4, evidence of the predictive power of this biomarker in other clinical settings, both with and without CKD, indicates that it is a promising tool for risk stratification [273-275,278]. On this basis, hs-cTnT was examined as a surrogate outcome measure. An interim analysis of the predictive power of hs-cTnT in the setting of CKD stage 3 and 4 is presented in chapter 4.

The model has several noteworthy features. Firstly iFGF-23, OPG and RANKL were independently associated with hs-cTnT in this cohort. Secondly, C-F PWV was not independently associated with hs-cTnT and thirdly, uPCR and serum phosphate (other non-traditional risk factors) were independently associated with hs-cTnT in this relatively strong model.

There are several potential mechanisms by which FGF-23, OPG and RANKL may be associated with a myocardial biomarker.

The original hypothesis that CaRP are associated with cardiovascular morbidity and mortality via increased afterload due to arterial stiffening was *not* supported by the models previously discussed. RANKL and iFGF-23 were not independently associated with C-F PWV. Moreover, after adjustment, C-F PWV was no longer

independently associated with hs-cTnT suggesting that the association of arterial stiffness with hs-cTnT may simply be an epiphenomenon. Alternatively, the effect of OPG upon hs-cTnT (or *vice versa*) may be greater than the effect of C-F PWV to such an extent that C-F PWV loses significance, or again it is possible that the study population may be too heterogeneous or simply too small to detect any effect size.

The lack of association of C-F PWV with hs-cTnT in the ACADEMIC study is, however, in contrast to the recently published findings of a large study of 1479 community dwelling Chinese people which found that hs-cTnT was independently associated with C-F PWV even after extensive appropriate adjustment for traditional risk factors [326]. CaRP were not measured in this study.

**FGF-23**

This is the first study to demonstrate an independent association between iFGF-23 and hs-cTnT. This association was independent of eGFR and phosphate. The findings of our multivariate model add to other published evidence in CKD stages 3 & 4 which link FGF-23 to adverse cardiovascular outcomes, i.e. the independent association between FGF-23 and both LVMI and concentric LVH [74,259].

These results are also consistent with the proposed theory of non-specific binding of FGF-23 to the FGF-2 receptor with subsequent myocardial fibrosis [327] or indeed the recently established evidence of direct specific interaction between FGF-23 and myocardium [77]. However, this non-interventional cohort study can never be more than supportive of this theory.

**OPG/RANKL**

This is the first published study to demonstrate that OPG is independently associated with hs-cTnT. Within the general population, OPG has been independently associated with LVH [73]. OPG is expressed within both the endothelium and VSMC of normal arteries, and increased expression has been demonstrated in cardiomyocytes of patients with dilated and ischaemic cardiomyopathy [328]. The pathophysiological role of OPG in this setting is not completely understood. If cTnT were to be released in patients with LVH or heart failure then a direct association between OPG and cTnT may be seen. OPG could therefore simply be a biomarker of cardiac dysfunction.

Alternatively since inflammation upregulates OPG expression, OPG could therefore be acting as marker of inflammation. However hs-CRP was not significantly independently associated in our fully adjusted model. Removal of OPG from the model does change the outcome, resulting in a significant independent association between hs-CRP and hs-cTnT (data not shown). This provides evidence, albeit weak, that the association of OPG to hs-cTnT may be influenced by inflammation. Removal of OPG from the model however, does not result in a significant independent association between C-F PWV and hs-cTnT.

RANKL was also significantly independently associated with hs-cTnT in the final fully adjusted model. As mentioned, RANKL is upregulated in the unstable atherosclerotic plaque and also within the failing myocardium, but again the study was not designed to investigate the mechanistic aspects of this relationship.

RANKL has previously been linked with cardiovascular death. In the Bruneck study, a prospective study in the general population, RANKL was independently associated with cardiovascular death in a model incorporating OPG, hsCRP and a variety of other traditional risk factors [187]. The authors concluded that the ability of RANKL to enhance monocyte/macrophage migration and stimulate osteogenic transdifferentiation of VSMC may lead to deposition of focal calcium deposits which could then alter atherosclerotic plaque stability. Interestingly, the authors also commented that the association of RANKL with cardiovascular disease was strongest for acute vascular events such as myocardial infarction and ischaemic stroke, but less strong for stable arterial occlusive disease such as femoral or carotid artery occlusion.

Our cross-sectional analysis, and the incomplete understanding of the significance of hs-cTnT in the stable but high risk outpatient setting, limits our interpretation. However, the ACADEMIC cohort differs significantly from the Bruneck cohort, particularly in regard to renal function. Whilst occlusive coronary artery disease still plays a role in the cardiac mortality burden in our population, medial calcification is a relatively greater problem with differing pathophysiology.

Conversely, in the Framingham study no significant association between RANKL and incident cardiovascular disease was found [188]. The authors suggested that this may be because they included stable coronary heart disease and heart failure in their

end point. Furthermore, the EPIC-Norfolk study also failed to find an independent association between RANKL and coronary events [223]. These differing results, and the issue posed by the evidence indicating a lack of association between the concentration of RANKL in the blood and the level of staining of RANKL RNA within the aortic wall [177] remain unreconciled.

**Fetuin-A**

Total fetuin-A and CPP were not significantly correlated with hs-cTnT. Fetuin-A has been associated with adverse cardiovascular outcome, and there is evidence that fetuin-A and CPP are associated with coronary artery calcification in CKD [88,122] However the proposed mechanistic link between low fetuin-A and cardiovascular outcome is thought to be mediated via arterial calcification rather than by direct interaction with the myocardium. Therefore whilst CPP was correlated with hs-cTnT, CPP was not forced into the final model.

**Non CaRP Risk Factors**

In the final model age, uPCR and phosphate were also significantly associated with hs-cTnT. These variables have been previously associated with adverse cardiovascular outcome and inclusion of these parameters within the final model is further supportive evidence of the validity of hs-cTnT as a biomarker of cardiovascular risk.

Microalbuminuria is proposed as a measure of endothelial function [329], however in the study population the presence of patients with heavy proteinuria necessitates caution in this interpretation. In the ACADEMIC study 140 patients had a uPCR less than 50mg/mmol. Urinary protein loss and albumin excretion have been demonstrated to be a durable predictor of cardiac risk in a variety of settings, and have been associated with left ventricular hypertrophy and cardiovascular mortality [330,331]. Serum phosphate was also significantly independently associated with hs-cTnT in this study. Phosphate has been identified as a risk factor for cardiovascular mortality in other studies of patients with CKD stages 3 & 4 [332,333].

**C-F PWV**

Aortic stiffness was not independently associated with hs-cTnT in this model. This finding is unexpected and noteworthy as aortic stiffness is proposed as an integrated measure of cardiac afterload. Potential explanations for the lack of association

include that the other parameters incorporated in the model may more closely reflect the process studied, the outcome measure chosen may not reflect the process studied (i.e. cardiac afterload) or that this may be a chance finding.

In the ACADEMIC study, hs-cTnT has been interpreted as a biomarker of cardiovascular risk. However, the factors leading to a raised troponin should be considered further. Importantly, outwith the setting of diagnosis of acute cardiac ischaemia, troponin is elevated in heart failure with particularly marked increases in advanced and decompensated heart failure where it has been associated with sudden cardiac death [334]. This may explain the association of hs-cTnT particularly with OPG. OPG has been associated with parameters of left ventricular function in cohorts of patients with both preserved LV function and overt heart failure [73,76,328]

The ACADEMIC study population contained a large proportion of elderly patients and the burden of comorbidity was large. It is possible that the absence of structural echocardiography or other tests of cardiac function at entry to the study may have led to a significant number of patients entering the study with sub-clinical heart failure – both systolic and diastolic. The statistical relationship of OPG with hs-cTnT may be a reflection of this process as opposed to any relationship with arterial stiffness, or indeed arterial calcification.

hs-cTnT was significantly correlated with eGFR in this cohort, and therefore univariate correlations of troponin with the CaRP may be explained by confounding. eGFR is forced into the final model in order to adjust for this confounding effect.

The study of the traditional and non-traditional risk factors in this section with construction of a model of factors independently associated with hs-cTnT provides a novel perspective of cardiovascular risk in this population. This model requires validation in two ways. Firstly, evidence of the prognostic ability of hs-cTnT in this population must be established. Given the validation in dialysis and general populations, this seems plausible. Secondly the variables independently associated with the surrogate outcome must be independently associated with actual hard outcomes, i.e. cardiovascular events and mortality. Repetition of this study in other populations and comparison with other cardiovascular risk prediction models should therefore be performed. For this model to have clinical utility, it must be

demonstrated that this model provides additional relevant, accessible, reliable, non-cost prohibitive information which would inform clinical management.

Notwithstanding these concerns, the finding of an independent statistically significant association between OPG, iFGF-23 and other cardiovascular risk factors with hs-cTnT is a novel finding, and this data has been published [324].

## Limitations of Study

In order to interpret the findings of any study, the design of the study and the context within which it was performed must be appreciated. This chapter of the thesis reports statistical associations based upon a cross-sectional analysis of the baseline characteristics of a prospective, non-interventional cohort study.

The main limitations of this investigation are the design of the study and the large number of confounders present. The cross-sectional nature of the observations in this chapter simply represent statistical association. They do not, and cannot, represent evidence of a pathophysiological mechanism. However the finding of statistical association can be interpreted, with care, as evidence that a process previously identified at a cellular level may have a role within a population.

Many of the patients in the study have a large number of comorbidities, which have the potential to confound any potential relationship. Comorbidity status was determined by classification based on history and the patient case record, but formal comorbidity scoring was not performed. Cardiovascular disease and diabetes are significant confounders and C-F PWV values differed in some treatment groups.

Patients in this study were recruited over a period of four years – itself a source of bias as treatment guidelines and referral patterns changed over this period. Patients in the study were required to be able to consent, and to attend the hospital every six months. The patients for whom this was most difficult were the eldest and most immobile, and also those in full time employment. Thus the ability to generalise the results of the study are limited by this selection bias.

Interest in the CaRP, and their relationship with cardiovascular outcomes, arterial stiffness and arterial calcification has increased dramatically during the three years of this study. This has been associated with a large increase in publications and knowledge in this topic area. Some of these publications have been directly relevant, and sometimes have overlapped with the findings in this study; for example, the introduction of the hs-cTnT assay and the advances in fetuin-A biology especially with regard to the link between CPP and extraosseous stress. For this reason, some of the analyses in this study were not specified *a priori*. However, data from patients in this study has been used to advance biochemical and laboratory methods and these advances have been applied to this study as appropriate.

Laboratory data must also be interpreted with an understanding of its limitations. As discussed quantification of the CPP was based upon an adaption of the method of Hamano *et al*, and we are, to our knowledge, the first group to report outcome findings related to this sub-fraction analysis. The potential for error in this setting is therefore increased.

A further potential source of error in this study is the use of estimated $GFR_{MDRD}$ rather than isotopic GFR measurement. eGFR is however widely used in clinical practice and is more appropriate than other measurements such as Cockcroft-Gault at this range of GFR.

Measurement of C-F PWV was performed on 185/200 study participants. Notwithstanding the lack of statistical difference between those measured and the total population, anecdotally, those not measured were less healthy that those measured. The limited number of operators involved in measurement and the use of the Complior™ PWV system reduced the potential for inter operator error. Similarly review of notes and ECGs were all performed by one of two operators.

Finally, in this study, aortic stiffness has been used as an end point against which titres proteins proposed to be involved in arterial calcification have been correlated. However it should again be noted that aortic calcification and aortic stiffness are not interchangeable parameters.

In order to validate our results and to understand the importance of the C-F PWV and CaRP as risk factors for adverse cardiovascular outcome, longitudinal follow up of sufficiently large cohorts for a sufficiently long period is required. Only in this setting can a comparison of the predictive power of the various biomarkers be adequately performed.

## Summary

Within this chapter, the demographic, clinical and biochemical details of the cohort of patients in the ACADEMIC study have been summarised, identifying two sub-populations: the elderly with elevated blood pressure and hypertensive CKD and a younger group with intrinsic renal disease and generally less severe renal impairment. The relationship of the four CaRP under study has been presented, including novel work on the relationship in CPP within CKD stages 3 & 4.

The distribution of C-F PWV has been explored, and an independent association between OPG, age and CPP with C-F PWV was been found.

hs-cTnT is proposed as a biomarker of cardiovascular risk. No relationship was found between hs-cTnT and ECG LVH, or C-F PWV. However the novel independent association between OPG, FGF-23 and RANKL and hs-cTnT was noted in a model which explained 57% of the variation of hs-cTnT within this population. In order to understand the significance of these cross-sectional reports, longitudinal analysis is required.

# Chapter 4

# Rate of Change of Renal Function and Survival

## 4.1 C-F PWV, CaRP and Rate of Change of Renal Function

### eGFR slope

There are cogent theories of how arterial stiffening might be linked with renal dysfunction and a number of groups have reported such a cross-sectional association[19,20], while some others have not substantiated this link [25]. Additional studies have found relationships with other markers of renal dysfunction [335]. The direction of causality, if any, of this relationship remains unclear.

Longitudinal analysis of data from 120 of the first 133 patients in this study with a mean follow up of 551 days (presented in the thesis of LT [2]), formed the basis of the first publication to demonstrate an independent association between C-F PWV and rate of decline of renal function [108]. As summarised earlier, four other longitudinal analyses of the relationship between arterial parameters and the change in renal function have been published during the last year, of which two tested the relationship between C-F PWV and the rate of renal function decline (see table 1.4). To date, no other study has confirmed our previous observation. However, relationships between other vascular parameters and decline in renal function have been demonstrated. Viewed together, these results suggest that vascular changes which result in stiffening of some arterial territories may be an independent risk factor for progression of renal disease, but this remains unproven, largely as none of these studies has been sufficiently powered to definitively address the issue. In addition, the evidence associating FGF-23 with change in renal function remains incomplete, especially in patients with advanced CKD.

Given the apparently incongruous results in these studies, we re-evaluated the change in renal function data of the initial 133 patients in ACADEMIC cohort after the addition of the data from the final 67 patients. This analysis differs from the previously published analysis as there are more patients in the study, the follow up period is longer and rate of decline was calculated using slope of an eGFR against time plot rather than a plot of reciprocal creatinine against time.

### Results

No patients were transplanted prior to starting dialysis. One patient was excluded from both analyses. This patient had a single functioning kidney with renal artery stenosis in the single patent renal artery. The patient's renal function was stable on

entry to the study, but then abruptly deteriorated (presumably due to arterial occlusion), giving an apparent eGFR slope of -45ml/min/1.73m$^2$/year.

**eGFR slope**

The median period of follow up was 1149 days (range 176 to 2058 days). The distribution of the length of follow up period demonstrated two peaks consistent with a period of reduced recruitment at the time of transfer of the day to day running of the study from LT to myself in 2008 (see fig. 4.1). The median time period from first eGFR plot to baseline visit was 102 (range 0 to 218 days). The median period of follow up from baseline visit to the final eGFR plot was 1147 days (range 37-1892 days).

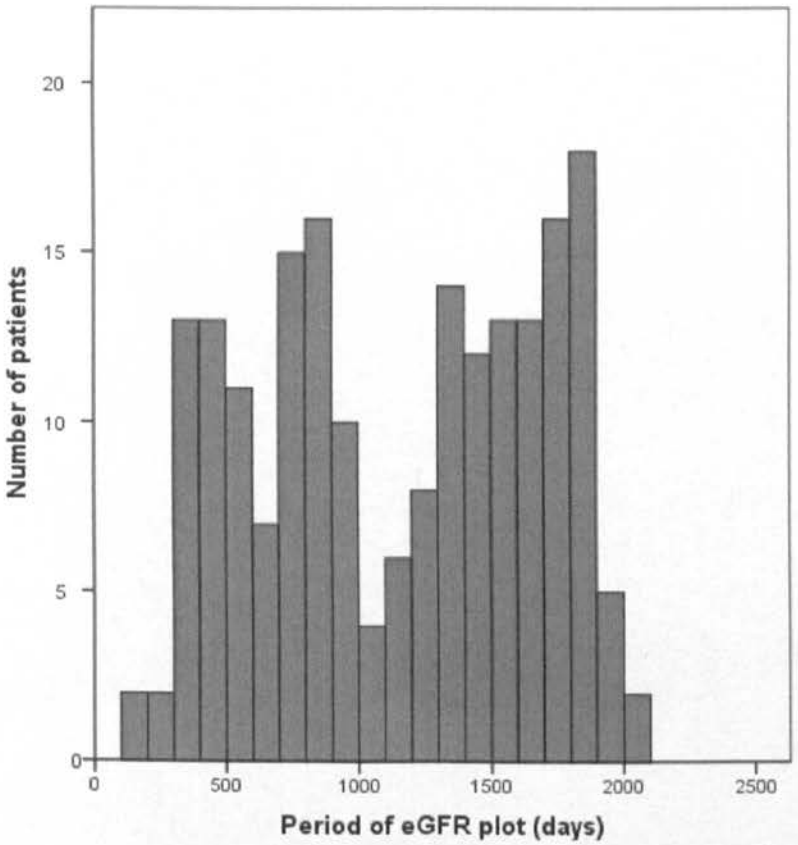



**Figure 4.1 - Bar chart of period of eGFR plots (days) in ACADEMIC cohort (n=199)**

## Uncleaned Data

The distribution of the eGFR slope coefficients is demonstrated in fig. 4.2. The median eGFR slope was -0.61ml/min/1.73m$^2$/year (-2.64, 1.41) indicating that the majority of patients experienced a decline in renal function over the period of the study. As illustrated, there was an approximately normally distributed central core of patients. However in a small but significant number of patients, a large change in renal function was seen over the period of study. Excluding the patient with the abrupt renal decline discussed above, a further four patients had a decline in renal function of >10ml/min/1.73m$^2$/year whilst five experienced an increase in GFR of >10 ml/min/1.73m$^2$/ year.

In order to explore the relationship between change in eGFR and other demographic and clinical variables including C-F PWV, univariate correlations were calculated (see table 4.1).

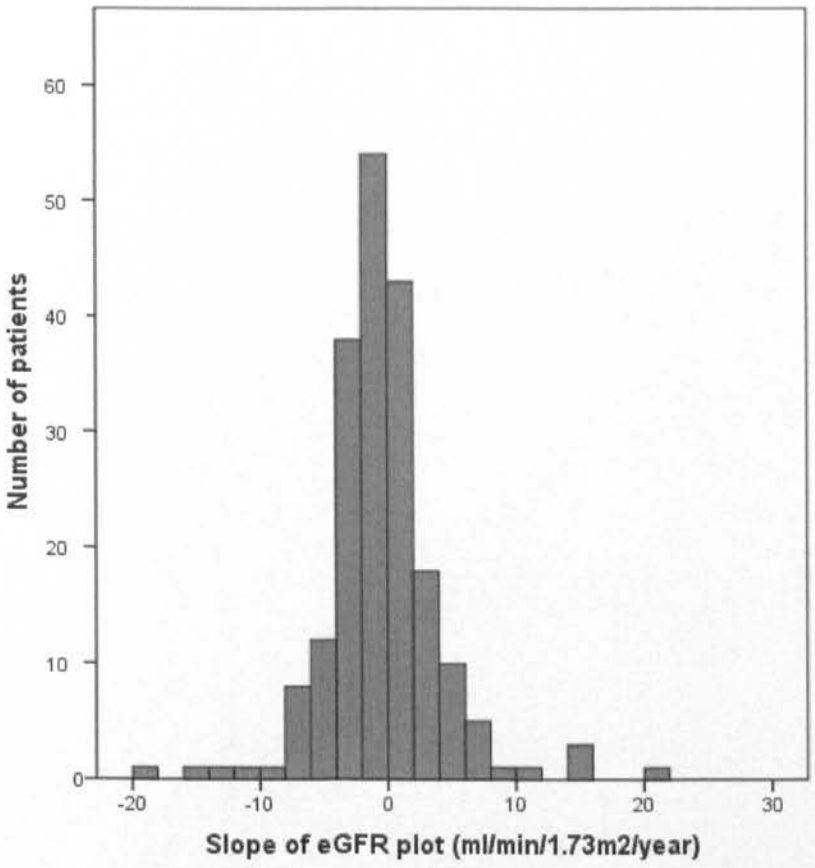



**Figure 4.2 - Bar chart of distribution of eGFR slope (ml/min/1.73m$^2$/year) in ACADEMIC cohort (n=199)**

| Parameter | r | p value |
|---|---|---|
| SBP | -0.081 | 0.258 |
| eGFR | 0.269 | <0.001*** |
| uPCR | -0.274 | <0.001*** |
| Age | 0.163 | 0.022* |
| Phosphate | -0.103 | 0.148 |
| C-F PWV | 0.081 | 0.272 |
| Tobacco exposure† | 0.042 | 0.558 |

**Table 4.1 - Correlation of eGFR slope with demographic and clinical variables (n=199) - †pack years**

There was a highly significant correlation between eGFR slope and both baseline eGFR and uPCR. There was also a significant correlation between age and eGFR slope indicating that in this population the younger patients were those with the greatest deterioration in eGFR. Interestingly there was no significant correlation between eGFR slope and SBP, C-F PWV, phosphate or tobacco exposure in this analysis of the entire cohort.

The correlations between eGFR slope and the CaRP were calculated. The results are shown in table 4.2.

| CaRP | r | p value |
|---|---|---|
| Total Fetuin-A | -0.084 | 0.236 |
| CPP | -0.132 | 0.063 |
| OPG | -0.130 | 0.067 |
| RANKL | 0.069 | 0.333 |
| FGF-23 | -0.182 | 0.010* |

**Table 4.2 - Correlation of eGFR slope with CaRP (n=199)**

There was a significant relationship with eGFR slope and iFGF-23, but not with total fetuin-A, CPP, OPG or RANKL. Comparison was also made of eGFR slope by diabetic status and gender (see table 4.3).

| Parameter (no. with characteristic) | Median eGFR slope (ml/min/1.73m$^2$/year) Yes | No | p value |
|---|---|---|---|
| Diabetes (51) | -1.50 (-4.46, 1.37) | -0.45 (-2.12,1.43) | 0.048* |
| Gender (M) (143) | -0.61 (-2.43,1.41) | -0.60 (-2.86, 1.61) | 0.711 |
| ACEi/ARB use (130) | -0.78 (-2.96, 1.42) | -0.37 (-2.19, 1.42) | 0.164 |
| Statin use (119) | -0.53 (-2.29, 1.22) | -0.69 (-3.01, 1.59) | 0.747 |

**Table 4.3 - Comparison of median eGFR slope with dichotomous variables (n=199)**

There was a significantly greater rate of decrease in eGFR in the diabetic patients compared to the non-diabetic patients, but no significant difference according to gender or ACEi/ARB or statin usage.

A stepwise multivariate analysis of the factors independently associated with slope of eGFR across the whole population was performed. Parameters were chosen for entry to the model if there was significant univariate correlation or they had previously been recognised to be associated with rate of change of renal function. Baseline eGFR, age and SBP were all independently associated with eGFR slope in a model explaining 15.6% of the variation in eGFR slope (see table 4.4).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | 0.118 | 0.065 | 0.064 | 0.172 | <0.001*** |
| Age | 0.102 | 0.110 | 0.049 | 0.154 | <0.001*** |
| SBP | -0.045 | 0.156 | -0.073 | -0.017 | 0.020* |

**Table 4.4 - Stepwise multiple linear regression of parameters independently associated with eGFR slope (n=184). Excluded variables: uPCR, diabetic status, C-F PWV and iFGF-23$^{\$}$ Model $R^2$=0.156**

In order to adjust for the effect of diabetes, a forced entry model was constructed. However this model was not significantly different from the model shown. Forced entry of OPG, iFGF-23 or phosphate to the adjusted model did not significantly increase the power of the model and none of the CaRP were significant in the final model. They were therefore not included (data not shown).

## Cleaned Data

Incorporation of all creatinine measurements in the plot from which the coefficient of rate of change was calculated has several problems. Firstly, episodes of AKI are disproportionately represented due to the increased frequency of measurement of renal function during these periods, and secondly the MDRD formula for estimation of GFR is not validated for use during periods of AKI. The crude data was reviewed and episodes of AKI and other obviously outlying values were censored. After this process, the median eGFR slope for the 199 patients was -0.52 ml/min/1.73m$^2$/year (-2.33, 1.30).

However there were nine outlying values with a slope coefficient greater than two standard deviations of the mean. These outlaying values posed two problems. Firstly unstable patients with superimposed pathologies may mask any effects of aortic stiffness, and secondly outlying points may unduly influence any constructed models. The influence of these values upon the analysis was investigated with the use of Cook's distances. This demonstrated that several of the patients with high value slope coefficients had large Cook's distances indicating that they may have been leverage points in the analysis.

These outlying patients were therefore excluded from further eGFR slope analysis. Of the remaining patients, 178 had C-F PWV measurement performed at baseline. The analysis was re-run in this group. The mean eGFR slope was -0.53 ± 2.83 ml/min/1.73m$^2$/year. The correlations of eGFR slope with standard biochemical and clinical parameters, C-F PWV and the CaRP were re-examined (see table 4.5).

| Parameters | r | p value |
|---|---|---|
| SBP | -0.104 | 0.165 |
| eGFR | 0.262 | 0.001** |
| uPCR | -0.259 | <0.001*** |
| Age | 0.079 | 0.297 |
| Phosphate | 0.035 | 0.645 |
| C-F PWV | 0.094 | 0.212 |
| Tobacco exposure† | 0.032 | 0.676 |
| Total Fetuin-A | -0.028 | 0.715 |
| CPP | -0.107 | 0.157 |
| OPG$ | -0.153 | 0.042* |
| RANKL | -0.010 | 0.892 |
| FGF-23$ | -0.152 | 0.042* |

**Table 4.5 - Correlation of eGFR slope with demographic and clinical variables in the cleaned, stable renal function sub-group with C-F PWV measurement (n=178) - †pack years**

eGFR, uPCR, OPG and iFGF-23 remained significantly correlated with eGFR slope in the stable patients.

Male patients had a significantly greater decline in eGFR compared to females. There was no significant difference between eGFR slope according to diabetic status, ACEi/ARB or statin use in this sub-group analysis (see table 4.6).

| Parameter (no. with characteristic) | Mean eGFR slope (ml/min/1.73m$^2$/year) Yes | No | p value |
|---|---|---|---|
| Diabetes (43) | -0.59 (3.44) | -0.50 (2.67) | 0.875 |
| Gender (M) (130) | -0.67 (2.79) | 0.13 (3.04) | 0.028* |
| ACEi/ARB use (116) | -0.75 (2.89) | -0.09 (2.77) | 0.136 |
| Statin use (106) | -0.39 (2.75) | -0.71 (3.03) | 0.478 |

**Table 4.6 - Comparison of eGFR slope with dichotomous variables in the cleaned, stable cohort with C-F PWV measurement (n=178)**

Initially, a stepwise multivariate analysis of the factors independently associated with eGFR slope was re-run. uPCR and baseline eGFR were independently associated with slope of eGFR within in this model ($R^2$=0.106) (see table 4.7).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| uPCR$ | -0.598 | 0.077 | -0.975 | -0.222 | 0.002** |
| eGFR | 0.047 | 0.106 | 0.008 | 0.087 | 0.018* |

**Table 4.7 - Stepwise multiple linear regression of the parameters independently associated with eGFR slope in the cleaned, stable cohort with C-F PWV measurement (n=178) - Excluded variables: age, diabetic status, SBP, C-F PWV & gender - Model $R^2$=0.106**

A further series of models was constructed to allow for adjustment for the effect of diabetes, age and SBP. This power of this adjusted model was not significantly greater than the unadjusted model. C-F PWV was then forced into both the adjusted and unadjusted models. Again, there was no significant increase in the model power.

In order to explore the association of the CaRP, OPG and iFGF-23 were forced into both unadjusted and adjusted models. Again there was no significant increase in the proportion of variation in the coefficients explained by the models. They were therefore not included in the final model.

### Sensitivity Analysis

A sensitivity analysis was performed in which serum phosphate and ACEi/ARB use were entered into the model. ACEi/ARB use was retained in the model (see table 4.8). In this model, ACEi/ARB use was associated with an increased rate of decline

in eGFR. Serum phosphate was not retained in the model. Addition of diabetes, age and SBP to this model was not associated with an increase in model power.

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| uPCR[$] | -0.673 | 0.077 | -1.051 | -0.294 | 0.001** |
| eGFR | 0.045 | 0.106 | 0.006 | 0.084 | 0.024* |
| ACEi/ARB use | -0.919 | 0.128 | -1.768 | -0.069 | 0.034* |

**Table 4.8 - Multiple linear regression analysis of the parameters independently associated with eGFR slope in the cleaned, stable cohort with C-F PWV measurement (n=178) - Model $R^2$=0.128**

In summary, in the final model uPCR, eGFR, and ACEi/ARB use were independently associated with cleaned eGFR slope in a model which adjusted for the effect of SBP and explained 12.8% of the variation in eGFR slope.

## Discussion

Within this analysis, we have not replicated the findings from the analysis of the first 120 patients. Instead, the results of the entire cohort were used to construct a model in which baseline eGFR, age and SBP were independently associated with eGFR slope.

We hypothesised that the pathophysiological processes by which arterial stiffness may interact with the glomerulus to cause reduction of GFR may play a significant role only in patients with relatively stable renal function. In patients in whom relatively large changes in GFR are seen, other pathological or regenerative processes may occur which could mask any effects of arterial stiffness on the glomerulus. Therefore in order to further investigate the association of C-F PWV with rate of change of renal function within the ACADEMIC cohort we undertook a further analysis using cleaned data in the patients with stable renal function.

In this model baseline eGFR, uPCR and ACEi/ARB use were independently associated with eGFR slope. Neither C-F PWV nor the CaRP were significantly associated with eGFR slope in the final model.

The relationships of the various CaRP with rate of change of renal function are discussed together in section 4.2.

The results of the analyses presented above do not provide more evidence to support the hypothesis that arterial stiffening causes decline in renal function. These results are however consistent with the two other recently published studies which also failed to demonstrate any independent association between C-F PWV and rate of change of renal function (see table 1.4). Instead, those studies proposed that serum phosphate [113] and CACWS [112] were associated with rate of change of renal function.

Briet *et al* calculated the rate of change of isotopically measured GFR against time for a group of 180 patients with a similar distribution of GFR as the ACADEMIC cohort. 126 patients remained under follow up and not on dialysis at three years. This 'Nephrotest' cohort differed from the ACADEMIC cohort as the patients were younger (mean age 60 ± 14 years v 69 ± 11 years), with lower mean blood pressures (134/74 v 151/81mmHg), a smaller proportion were diabetic and there was a greater use of ACEi/ARB and 1(OH) vitamin $D_3$. The mean slope in the 'Nephrotest' group was -1.7 ± 3.7ml/min/1.73m$^2$/year. Whilst there was no mean significant change in C-F PWV over the three years in this group, the authors did detect significant changes in the carotid artery. The carotid artery diameter and circumferential wall stress (CACWS) increased, whilst the wall to lumen ratio decreased. After multivariate analysis adjusting for a variety of traditional risk factors including pulse pressure (but not including SBP or MBP), CACWS was associated with rate of change of measured GFR in addition to age, previous CV event and uACR, whilst C-F PWV was not.

One possible explanation for the lack of independent association between C-F PWV and change in GFR in the Nephrotest study may be that the regression model was adjusted for pulse pressure, a parameter directly influenced by stiffness of the aorta. The authors proposed that the absence of the usual thickening of the carotid artery (an increased wall:lumen ratio expected in response to dilatation of the artery seen in non-CKD ageing) may be due to excessive matrix turnover or increased VSMC apoptosis. Mechanistically, the authors also proposed that the alterations to the remodelled carotid artery phenotype that they found may have impacted upon the transmission of systemic pulse pressure more peripherally, leading to capillary rarefaction and increased small arterial stiffness with a resulting decrease in GFR. However, as in the ACADEMIC study, no histological or haemodynamic studies of renal blood flow were available to support or refute this theory.

The evidence relating change in CACWS to outcome in either this, or other clinical scenarios, is lacking. It is also unclear why localised measures of arterial strain, compliance and distensibility should have greater relevance than C-F PWV, a measure which integrates arterial stiffness over a possibly more relevant anatomical section of the arterial tree.

Phosphate has also been proposed as a risk factor for decline in renal function [113,336]. In 2011, Chue *et al* published the findings of their study of 225 CKD patients [113]. Again this cohort was younger and had lower blood pressure and adjusted C-F PWV than the ACADEMIC cohort. Their cohort had a higher mean eGFR with a wider distribution (43 ± 19 ml/min/1.73m$^2$ v 33 ± 11 ml/min/1.73m$^2$), a higher serum phosphate and lower levels of urinary protein excretion. Notably, this cohort also had significantly more stable renal function than the ACADEMIC cohort with a mean change in eGFR of -0.11 ± 0.54 ml/min/1.73m$^2$/year [113]. There was no significant association between C-F PWV and rate of change of eGFR in this cohort in either univariate analysis or after adjustment for age, gender, baseline eGFR, SBP, uACR, haemoglobin, calcium and phosphate. Adjustment for gender, haemoglobin and calcium were not performed in the analysis above of the ACADEMIC study as the evidence that these parameters have a role in the determination of rate of change of renal function is weak. However, if the analysis was re-run adjusting for these variables then the model produced did not change significantly. Phosphate and C-F PWV remained insignificant in the final model, with no significant increase in model power.

In the 'Nephrotest' and Chue cohorts, diabetes was not independently associated with rate of change of renal function. This mirrored our findings, despite the inclusion of patients with diabetic nephropathy in the ACADEMIC cohort. Adjustment was performed for diabetic status in both other cohorts. The non-significance of diabetes in the final models may be explained by the inclusion of measurements of proteinuria in both models. Whilst proteinuria is not a specific marker for diabetic nephropathy, it is a marker of the glomerular disease process typically caused by diabetes. In addition, a significant proportion of diabetic patients in the ACADEMIC cohort have CKD which was not thought to be due solely to diabetic nephropathy.

**Why do these findings differ from those previously published by this group?**

The most striking result within this analysis is the absence of a significant independent association between C-F PWV and eGFR slope. This larger study with longer follow up is less influenced by short term changes in the renal function on the overall gradient of eGFR (or reciprocal creatinine) plots, but the potential exposure to episodes of AKI is increased. This necessitated review of the data.

The results from the analysis of the cleaned data demonstrated that baseline eGFR, uPCR and ACEi/ARB use were independently associated with rate of change of renal function. ACEi/ARB use was associated with an increased rate of decline of renal function.

Methodologically wider interpretation of this data is limited by the observational study design and heterogeneous study population. In addition, review of the data was not pre-specified and was performed in a subjective manner. However we hypothesised that the factors associated with rate of decline of renal function would differ across diagnostic categories. We therefore undertook studies of the stable ACADEMIC patients using the diagnostic category classification previously described.

## Rate of Change of Renal Function in Diagnostic Categories

The sub-group categorised with intrinsic renal disease (n=74) had a significantly greater rate of change of eGFR when compared to the relatively more stable sub-groups classified with hypertensive renal disease (n=60) or other (n=44) (see fig. 4.3).

Separate multiple linear regression models were constructed for the different categories. Stepwise analysis was first performed and then a model adjusting for eGFR, SBP, uPCR and age was constructed. Finally C-F PWV and OPG were forced into the finally adjusted model and the overall fit of the model tested.

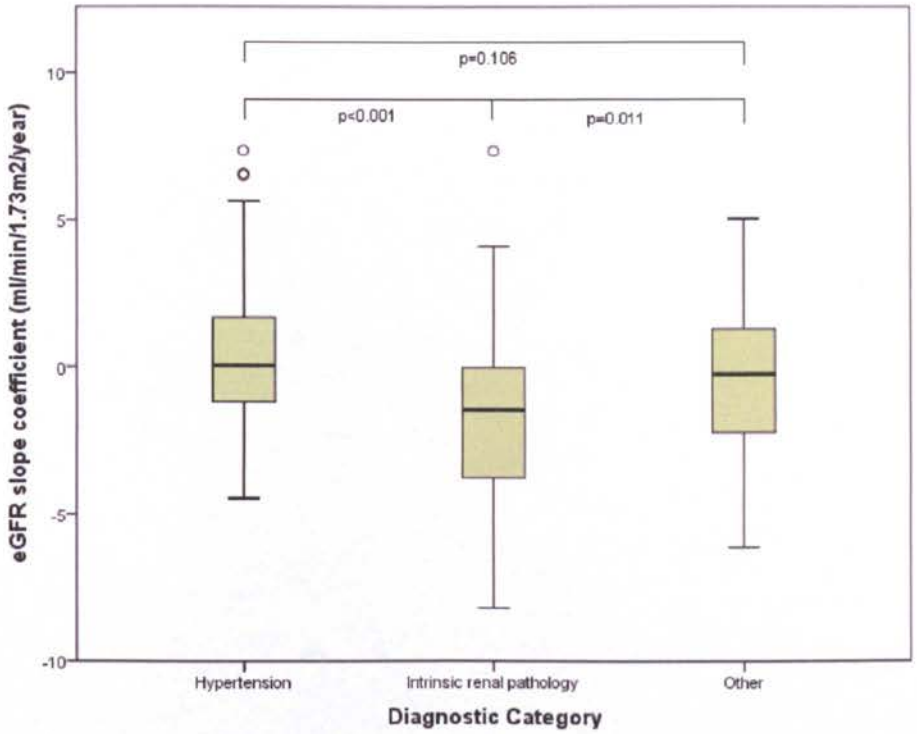



**Figure 4.3 - Boxplots of slope of patients with stable eGFR by diagnostic category Hypertension (n=60); intrinsic renal disease (n=74); other, (n=44)**

## Hypertensive Renal Disease Category

eGFR and ACEi/ARB use were independently associated with eGFR slope in the stepwise model for the 60 patients classified with hypertensive CKD (see table 4.9).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | 0.109 | 0.136 | 0.037 | 0.182 | 0.004** |
| ACEi/ARB use | -1.325 | 0.199 | -2.602 | -0.048 | 0.042* |

**Table 4.9 - Stepwise multiple linear regression of factors independently associated with eGFR slope in stable hypertensive subgroup (n=60) - Excluded variables: SBP, age, uPCR, DM & C-F PWV - Model $R^2$=0.199**

Adjustment of the model in this subgroup for uPCR did not improve the fit of the model, or the relationship between eGFR and eGFR slope. Adjustment for the effect of SBP, uPCR and age did not significantly increase the fit of this model (F test p=0.274). Forced inclusion of C-F PWV into the finally fully adjusted model did not improve the fit. The most parsimonious model for this subgroup is therefore the model containing only baseline eGFR and ACEi/ARB use.

### Intrinsic Renal Disease Category

uPCR was the only parameter retained in the stepwise model for the 73 patients classified with intrinsic renal pathology (see table 4.10).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| uPCR[$] | -0.813 | 0.116 | -1.342 | -0.285 | 0.003[**] |

**Table 4.10 - Stepwise multiple linear regression of factors independently associated with eGFR slope in stable 'renal disease' subgroup (n=74) - Excluded variables: SBP, age, eGFR, DM & C-F PWV - Model $R^2$=0.116**

After adjustment, uPCR remained a significant independent predictor of slope of eGFR, but the other parameters were not significant (data not shown). If C-F PWV was forced into the adjusted model, then C-F PWV was significantly independently associated with eGFR slope (B=0.379 (CI 0.038-0.721), p=0.030).

### Other CKD Category

Stepwise multivariate analysis of the parameters independently associated with eGFR slope in the 'other' CKD category demonstrated that SBP was independently associated with eGFR slope (see table 4.11).

| Parameter | B | Inc. $R^2$ | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| SBP | -0.050 | 0.205 | -0.081 | -0.020 | 0.002[**] |

**Table 4.11 - Stepwise multiple linear regression of factors independently associated with eGFR slope in stable 'other' subgroup (n=44) - Excluded variables uPCR, age, eGFR, DM & C-F PWV - Model $R^2$=0.205**

Adjustment for the effect of age, eGFR and uPCR did not significantly increase the amount of variation explained by the model. Addition of C-F PWV to either unadjusted or adjusted model did not improve the fit of the model.

OPG and FGF-23 were not retained in the models of any of the diagnostic categories.

In order to further investigate the cause for the variation in results between this analysis and the previous analysis of LT, I repeated the analysis using the coefficients of reciprocal creatinine plots as used in the previous analysis. The results obtained using both raw and cleaned data were similar between the eGFR and

reciprocal creatinine slope analyses. C-F PWV was not significantly independently associated with reciprocal creatinine plot slope.

## Discussion

The analysis of subgroups within the cohort by diagnostic category provides interesting results, which further highlights the importance of the composition of the study cohort in the determination of the final results.

In the group categorised with hypertensive CKD, baseline eGFR and ACEi/ARB use were the factors independently associated with eGFR slope, indicating that in this cohort the patients with the lowest eGFR, and those taking ACEi/ARBs were those most likely to have progressive renal failure. In contrast, in the group categorised as having intrinsic renal disease, proteinuria was the only risk factor significantly independently associated with eGFR slope, retaining significance after adjustment. In the final adjusted model, C-F PWV was significantly independently associated with eGFR slope, although the direction of this relationship was the opposite of that expected. Finally, in the smallest subgroup, classified as CKD due to other factors, SBP was independently associated with eGFR slope.

This analysis should be interpreted with caution due to the relatively small size of the study and the risks of repeated analysis of such a dataset. In addition, whilst the system of classification of CKD into three diagnostic categories used is based upon general principles of clinical nephrology, this system has not been validated in the wider population. The classification was based upon clinical history, laboratory measurements and histological and radiological data where available. As such, whilst the system is clinically relevant in so far as it reflects what happens in clinical practice, there is significant potential for misclassification. However, the results are relatively consistent with the current knowledge base, and are compatible with current widely accepted therapeutic strategies.

In summary, the factors independently associated with rate of renal function decline varied by diagnostic category. Evidence of a weak association between C-F PWV and rate of change of renal function was found in the intrinsic renal pathology group alone. No evidence was found that any of the CaRP were independently associated with rate of eGFR slope in any diagnostic category.

## 4.2 Rate of Change of Renal Function

## Combined Dichotomous End Point

Change in renal function was also assessed using the fixed end point of start of RRT or a drop in eGFR of ≥25%. Whilst some other studies have used dichotomous end points including doubling of serum creatinine [264] or ≥ 25% decline in eGFR [113], these studies do not state the duration of the decline in renal function required for the end point to be reached. It is therefore possible that patients with reversible AKI have been included in these groups.

In order to minimise this potential confounding, the ACADEMIC cohort was dichotomised using the combined renal end point of commencement of RRT or decline in eGFR ≥25% from baseline which lasted over 30 days.

Logistic regression models were initially constructed using stepwise, and then forced entry models, to produce an adjusted analysis. Initially recognised risk factors and those with univariate significance were entered into the stepwise analysis. C-F PWV, the CaRP and hs-cTnT were then entered into the adjusted model. A comparison of the various models was performed using measurement of the area under the curve of ROC curves.

### Results

Twenty patients started RRT after entering the study. The mean duration of follow up of those starting dialysis was 908 days (range 42-1620). 60 patients experienced a one month or greater ≥25% drop in eGFR. 63 patients experienced either of these end points comprising the progressor group. Comparison of the characteristics of the progressor and non-progressor groups is displayed in table 4.12.

The progressor group had a significantly lower eGFR, were more likely to be male and had higher levels of proteinuria. This group also had higher CPP, and higher titres of iFGF-23 and hs-cTnT.

| Parameter | Non-progressor | Progressor | p value |
|---|---|---|---|
| Age (years) | 70 ± 11 | 67 ± 12 | 0.065 |
| eGFR (ml/min/1.73m$^2$) | 35 ± 10 | 28 ± 10 | <0.001*** |
| Diabetes (%) | 24% | 29% | 0.499 |
| Gender (% male) | 68% | 81% | 0.039* |
| SBP (mmHg) | 150 ± 22 | 154 ± 21 | 0.255 |
| DBP (mmHg) | 80 ± 11 | 84 ± 11 | 0.031* |
| uPCR (mg/mmol) | 21 (11,45) | 43 (20,138) | <0.001*** |
| C-F PWV (m/s) † | 13.1 ± 2.5 | 12.7 ± 2.7 | 0.439 |
| Phosphate (mmol/l) | 1.06 ± 0.19 | 1.09 ± 0.17 | 0.242 |
| Tobacco exposure†† | 4 (0, 30) | 5 (0,38) | 0.785 |
| Total fetuin-A (g/l) | 0.23 ± 0.07 | 0.24 ± 0.07 | 0.134 |
| CPP (%) | 8.8 (4.9, 15.2) | 12.8 (8.5, 19.4) | 0.044* |
| OPG (pmol/l) | 9.3 (7.3, 12.1) | 10.2 (7.4, 12.5) | 0.321 |
| RANKL (pmol/l) | 609 ± 261 | 582 ± 263 | 0.502 |
| iFGF-23 (pg/ml) | 58(42, 74) | 66 (53, 78) | 0.013* |
| hs-cTnT (mcg/l) | 0.014 (0.008, 0.024) | 0.019 (0.012, 0.033) | 0.021* |

**Table 4.12 - Comparison of patient characteristics between progressor (eGFR decline ≥25% or RRT) (n=63) and non-progressor (eGFR decline <25% and no RRT) (n=137) groups †progressor:non-progressor (57:128); ††pack years**

Medication use was compared between progressor and non-progressor groups. There was no significant difference in the proportion of patients using any of the major classes of anti-hypertensive between groups. There was a significantly lower proportion of patients using statin therapy in the progressor group (25% v 41% (p=0.020)).

Several of the biological variables are directly linked to GFR. A stepwise logistic regression analysis was performed to explore the independent association of these variables to the risk of renal disease progression using the dichotomous variable.

Initially the variables with a significant difference between the two groups were entered into a stepwise forward logistic regression model. The results are shown in table 4.13.

| Parameter | B | OR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | -0.059 | 0.943 | 0.911 | 0.977 | 0.001** |
| uPCR | 0.006 | 1.006 | 1.002 | 1.011 | 0.006** |
| Statin use | -0.844 | 0.430 | 0.221 | 0.835 | 0.013* |

**Table 4.13 - Logistic regression model of parameters independently associated with risk of reaching combined renal end point (n=200) - Excluded variables: Gender, DBP, CPP, iFGF-23 & hs-cTnT**

In order to adjust for recognised determinants of renal disease progression, an adjusted model was constructed containing SBP and diabetes. This did not alter the significance of the variables selected in the stepwise model.

A further adjusted model was constructed including age (see table 4.14). Age was significantly associated with risk of progression in this model, however uPCR lost significance.

| Parameter | B | OR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | -0.073 | 0.930 | 0.895 | 0.966 | <0.001*** |
| uPCR | 0.004 | 1.004 | 1.000 | 1.010 | 0.060 |
| Statin use | -0.920 | 0.398 | 0.201 | 0.791 | 0.009** |
| SBP | 0.016 | 1.017 | 0.999 | 1.034 | 0.062 |
| Diabetes | 0.252 | 1.287 | 0.592 | 2.795 | 0.524 |
| Age | -0.038 | 0.963 | 0.931 | 0.995 | 0.025* |

**Table 4.14 - Adjusted logistic regression model of parameters independently associated with risk of reaching combined renal end point (n=200)**

Further adjusted models were then constructed containing C-F PWV, phosphate, CPP, iFGF-23 and hs-cTnT. There was no significant independent association of the additional variable with risk of attainment of the combined end point in any of the models. Forced inclusion of the other CaRP (RANKL, OPG & total fetuin-A) also

failed to either improve the fit of the models or show significance of the CaRP tested (models not shown).

In order to investigate any potential interaction between statin therapy and proteinuria, an interaction term was included in the analysis. This was not significant indicating no significant interaction was seen in this model in this population.

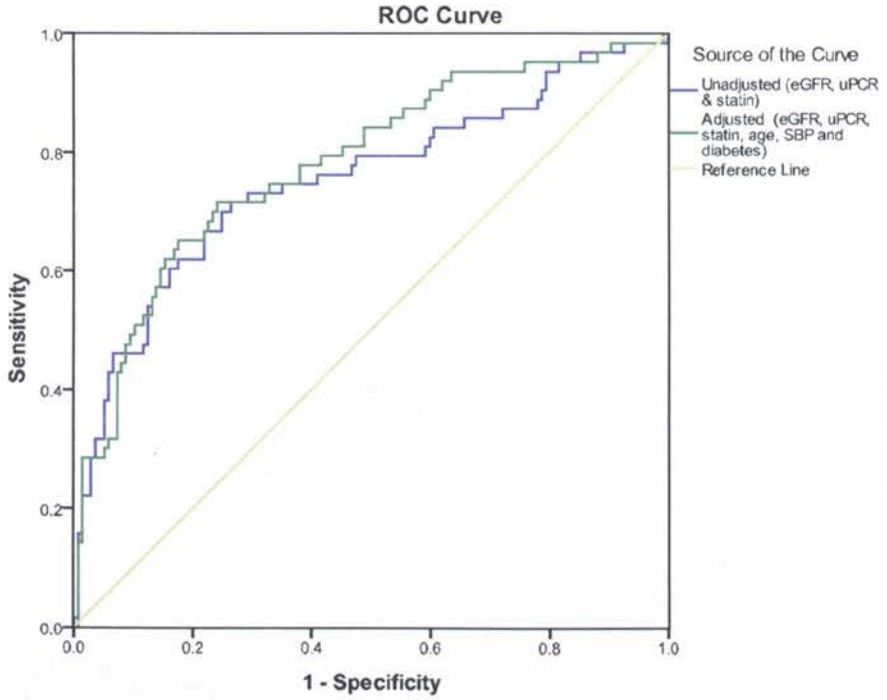
ROC curves were drawn for the stepwise logistic regression model and for the adjusted model incorporating SBP and diabetes (see fig 4.4A). ROC curves were then drawn for the models which incorporated C-F PWV, phosphate, CPP and iFGF-23 and hs-cTnT (see fig 4.4B). Curves were compared using area under the curve (AUC) (see table 4.15).

| Model | AUC | 95% lower CI | 95% upper CI | p value |
|---|---|---|---|---|
| Unadjusted | 0.736 | 0.659 | 0.814 | <0.001*** |
| Adjusted | 0.802 | 0.731 | 0.874 | <0.001*** |
| Adjusted + C-F PWV† | 0.802 | 0.732 | 0.872 | <0.001*** |
| Adjusted + CPP | 0.802 | 0.730 | 0.874 | <0.001*** |
| Adjusted + PO4 | 0.804 | 0.733 | 0.876 | <0.001*** |
| Adjusted + iFGF-23 | 0.802 | 0.730 | 0.873 | <0.001*** |
| Adjusted + hs-cTnT | 0.802 | 0.731 | 0.873 | <0.001*** |

**Table 4.15 - AUC of ROC curves predicting the combined renal end point (n=200) †n=185**

The AUC for the various models varied from 0.736 to 0.804, indicating a good discriminant ability for all models, which was greater in the adjusted models. However the adjusted curve and the other curves from models incorporating the CaRP, phosphate, C-F PWV and hs-cTnT appear very similar, and superimposed in places. Whilst formal testing of the difference between the AUC of the various models was not been performed, the widely overlapping 95% CI for the AUC of the various curves indicates that there was not likely to be any significant difference between the models.

A




B

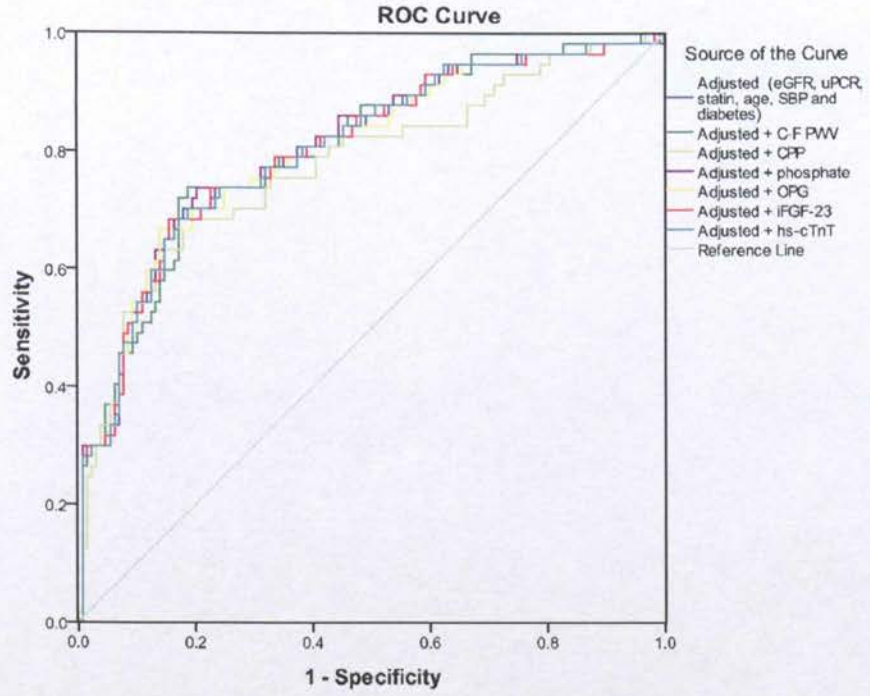



**Figure 4.4 - ROC curves of (A) unadjusted and adjusted (for age, SBP & DM) models and (B) adjusted models including other proposed variables (C-F PWV, CPP, phosphate, OPG, iFGF-23 & hs-cTnT) as predictors of the combined end point of dialysis or ≥25% eGFR drop in ACADEMIC cohort (n=200) (n=185 for model incorporating C-F PWV)**

## Discussion

The results of this analysis of the factors associated with starting dialysis or having a significant sustained drop in eGFR are slightly different from those presented previously in section 4.1. The results of the stepwise unadjusted analysis demonstrate the independent association of three variables with the risk of attainment of the combined dichotomous end point of starting dialysis or a sustained drop in eGFR of ≥25%. Two of these parameters, baseline eGFR and uPCR, are widely recognised as determinants of progression of renal disease.

Patients with a lower baseline GFR are more likely to reach the combined end point for several reasons. Firstly, they have a reduced reserve prior to the need of RRT, and secondly the absolute drop in GFR required for a loss of 25% is less. In addition however, patients with greater severity of renal impairment are more likely to be having a destabilised GFR in the case of an acute event [337,338]. Proteinuria is a parameter which is both recognised to be associated with progressive renal impairment, and indeed is used as a therapeutic target to reduce progression of CKD [339].

Interestingly, neither SBP nor diabetic status reached statistical significance in the final analysis. They were, therefore, not independently associated with probability of reaching the combined renal end point despite the relatively high blood pressure values in our cohort. The absence of SBP from the stepwise analysis is unexpected. As previously stated only 25/200 patients in the study had blood pressure controlled below Renal Association targets [310]. In a sensitivity analysis, substitution of SBP for either a categorical variable of attainment of the Renal Association guidelines, or SBP greater or less than median SBP (150mmHg), did not affect selection of significant variables for the stepwise model. The borderline significance of SBP in the adjusted model is likely to be a reflection of the power of the study.

SBP was significantly associated with the risk of renal disease progression in the model which was adjusted for C-F PWV (data not shown). However, given the absence of wider published evidence that C-F PWV is independently associated with rate of change of renal function, there is little justification for adjustment of the model for C-F PWV, and this evidence should be regarded as statistically very weak.

The absence of diabetes from the stepwise model may be explained by the composition of the cohort. Whilst 26% of patients in the cohort had diabetes, diabetic nephropathy was classified as the cause of CKD in only 6%. In this study, diabetes is generally a comorbid condition rather than the aetiological factor of CKD in that subpopulation.

Forced inclusion of diabetes and SBP into the logistic regression model did not significantly alter the AUC of the ROC curve. Addition of other subsequent parameters again did not alter the selection of significant parameters in the final model. Interpretation of the series of models must therefore be undertaken with care, as the predictive power of the models did not differ greatly. In particular, despite the finding that the adjusted models incorporating phosphate had the greatest AUC, the confidence intervals for the AUC of this model incorporated all the other models' AUC. The equivalence in the AUC of the various models therefore indicated that within this dichotomous analysis there was, again, no evidence that C-F PWV is associated with risk of decline in renal function within this cohort.

Statin therapy was the third variable associated with a reduced likelihood of progression in this analysis. The association of statin use with a reduced progression of CKD in the dichotomous analysis was not seen in the linear regression analysis in the previous section. If statins are forced into the linear regression model in section 4.1, then no improvement is seen in the fit of the model. The published evidence regarding any effect of statins on kidney disease progression is conflicting. The findings in relation to statins may therefore be a type 1 error.

Interpretation of the statistical relationship of interventional parameters such as medication in this (and other) observational analyses should be performed with caution as there is a significant likelihood of indication bias. Indication bias is particularly likely to be present in the case of ACEi/ARB which are widely used for progressive renal disease, particularly when accompanied by proteinuria. Interestingly the recently reported Study of Heart and Renal Protection (SHARP) trial, in which data was collected prospectively on 6029 patients with CKD stages 3-5 with a mean eGFR of 27 ± 13ml/min/1.73m$^2$ reported no significant reduction in risk of ESRF or doubling of serum creatinine with lipid lowering treatment [68].

Further contrast with the results of those dichotomous studies which have shown an association between arterial parameters and renal end points is therefore required. Notwithstanding the differences between AI and PWV, Weber *et al* recently published data suggesting an association between AI and risk of progression to ESRF or doubling of serum creatinine in 111 patients with CKD [111]. Sub-group analysis revealed that the association was only present in those with eGFR <32 ml/min/1.73m$^2$. However this was a particularly heterogeneous cohort, 43% of which had received a kidney transplant. These patients should be presumed to be taking immunosuppression and are likely also to have reached ESRF and received dialysis prior to transplantation. These factors limit comparison with other cohorts.

However, interestingly the population of Taal *et al* in which AI and B-A PWV were associated with renal disease progression also had relatively more severe CKD than either the ACADEMIC study, or the studies of Breit *et al* or Chue *et al* discussed earlier [112,113] (see table 1.4). In order to establish if this relationship was present in the patients with more severe CKD, a sensitivity analysis in the ACADEMIC cohort using only the patients with CKD stage 4 was performed. Again however, this did not show a significant independent association between C-F PWV and risk of progression using either the dichotomous end point or eGFR slope (data not shown).

hs-cTnT was increased in the progressor group in univariate analysis. This finding is likely to be explained by the lower eGFR in this group. There was no association of hs-cTnT with risk of reaching the combined dichotomous end point independent of eGFR. This finding is in contrast to the recently published findings of a sub-analysis of 1000 patients from the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) study - a study of the effect of darbopoetin alfa in type II diabetic patients with CKD stages 3 and 4 [340]. In analysis of the factors associated with reaching ESRF in that study, addition of cTnT and BNP to their logistic regression model (which incorporated baseline eGFR and uPCR) increased the AUC of their model significantly. Proposed mechanisms for this association include the loss of cardiac output due to incident ischaemic heart disease leading to reduction in GFR and an increased propensity of the physician to start RRT in those with incident ischaemic heart disease. Alternatively, the authors suggested that cTnT titres may be a reflection of the uraemic state such as increased neurohormonal activation,

inflammation or oxidative stress which could impact upon disease progression within the kidney and the heart.

In the ACADEMIC study high sensitivity cTnT measurement was performed. However the number of patients under follow up was only 20% of that in the TREAT sub-study. Therefore whilst the ACADEMIC study used the more sensitive hs-cTnT assay, it remains relatively underpowered compared to this larger study.

Methodologically, the combined dichotomous end point in this analysis was employed as it increased the proportion of patients who reached the end point and therefore increased the power of the statistical analysis. The main problem with this analysis however is the potential for inclusion of patients in the progression group with AKI as opposed to progressive CKD. Despite the stipulation that the duration of eGFR decline be greater than 30 days, it is still possible that some patients with prolonged episodes of AKI may have been included in the progression group.

In summary, in this analysis of the factors associated with reaching the combined dichotomous end point, only baseline eGFR and uPCR and statin therapy should be regarded as being robustly independently associated with risk of renal disease progression in this population.

**CaRP and rate of change of renal function**

Despite the previous findings of an independent association between FGF-23 and rate of decline of renal function in two studies discussed earlier [263,264], within the ACADEMIC study no significant independent association between FGF-23 and rate of renal function decline was found. In addition, no evidence was found of any significant independent association between total fetuin-A, CPP, OPG or RANKL and change in renal function, using either slope of eGFR or the dichotomous end point.

## 4.3 Rate of Change of Renal Function – Dialysis

In order to remove the potential effect of reversible AKI on the combined dichotomous end point of starting dialysis or ≥25% decline in eGFR, the dichotomous analysis was repeated including only those patients who started dialysis in the progressor group. Comparison of the patients who started dialysis was made with those who experienced a ≥25% decline in eGFR but did not start dialysis.

Twenty patients started RRT, of whom 17 had C-F PWV measurement at baseline. Comparison of clinical characteristics of the two progression groups are displayed in table 4.16.

| Parameter | Dialysis | ≥25% eGFR decline | p value |
|---|---|---|---|
| Age (years) | 66 ± 12 | 67 ± 12 | 0.723 |
| eGFR (ml/min/1.73m$^2$) | 24 ± 9 | 30 ± 10 | 0.028* |
| Diabetes (%) | 35 | 26 | 0.411 |
| SBP (mmHg) | 157 ± 21 | 152 ± 21 | 0.344 |
| DBP (mmHg) | 88 ±11 | 73 ± 11 | 0.079 |
| uPCR (mg/mmol) | 107 (32, 280) | 34 (16, 98) | 0.005** |
| C-F PWV† (m/s) | 13.1 ± 3.0 | 12.6 ± 2.5 | 0.537 |
| Statin use (%) | 45 | 49 | 0.777 |
| ACEi/ARB use (%) | 60 | 72 | 0.337 |

**Table 4.16 - Comparison of characteristics between dialysis (n=20) and ≥25% decline in eGFR (n=43) groups - †dialysis:≥25% decline (17:35)**

The only significant differences were that the group who started dialysis had a lower baseline eGFR and greater protein excretion.

A stepwise logistic regression model of the factors independently associated with risk of starting RRT within the ACADEMIC cohort was built (see table 4.17).

| Parameter | B | OR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| eGFR | -0.096 | 0.909 | 0.849 | 0.972 | 0.005** |
| uPCR | 0.006 | 1.006 | 1.002 | 1.010 | 0.004** |

**Table 4.17 - Logistic regression model of parameters independently associated with starting dialysis (n=185)**

Baseline eGFR and uPCR were independently associated with risk of progression to dialysis. Two adjusted models were then constructed. Firstly the initial model was adjusted for recognised risk factors for progression. Diabetes, SBP, age and statin therapy were not significantly associated with risk of progression to dialysis. A further model was then constructed incorporating C-F PWV (see table 4.18). In this model, statin use was associated with a significantly reduced risk of dialysis in addition to eGFR and uPCR.

| Parameter | B | OR | Lower 95% CI | Upper 95%CI | P value |
|---|---|---|---|---|---|
| eGFR | -0.160 | 0.852 | 0.773 | 0.939 | 0.001** |
| uPCR | 0.004 | 1.004 | 1.000 | 1.009 | 0.044* |
| Statin use | -1.568 | 0.208 | 0.053 | 0.823 | 0.025* |
| SBP | 0.032 | 1.032 | 0.996 | 1.069 | 0.080 |
| Diabetes | 0.839 | 2.314 | 0.570 | 9.402 | 0.241 |
| Age | -0.067 | 0.935 | 0.868 | 1.007 | 0.075 |
| C-F PWV | 0.022 | 1.022 | 0.734 | 1.423 | 0.897 |

**Table 4.18 - Adjusted logistic regression model of parameters (incorporating C-F PWV) independently associated with starting dialysis (n=185)**

Comparison of these models was performed using ROC curve analysis, with measurement of the AUC (see fig 4.5 and table 4.19).

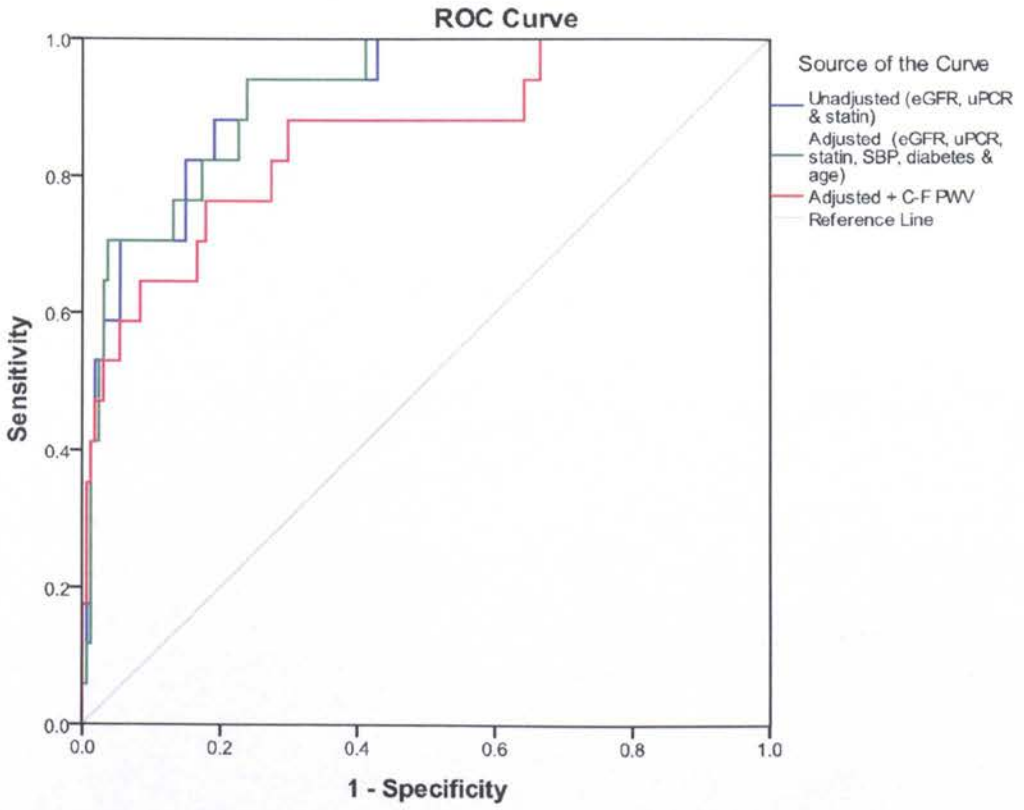



**Figure 4.5 - ROC curves of unadjusted model, and adjusted (for age, SBP and DM), and adjusted with C-F PWV models as predictors of progression to dialysis within ACADEMIC cohort**

| Model | AUC | 95% lower CI | 95% upper CI | p value |
|---|---|---|---|---|
| Unadjusted | 0.918 | 0.860 | 0.977 | <0.001*** |
| Adjusted | 0.918 | 0.860 | 0.977 | <0.001*** |
| Adjusted + C-F PWV† | 0.856 | 0.754 | 0.959 | <0.001*** |

**Table 4.19 - AUC of unadjusted and adjusted ROC curves predicting dialysis (n=200) - †(n=185)**

This analysis demonstrated that the unadjusted model and the adjusted model had the same predictive capacity in this population, and were significantly better than the adjusted model which incorporated C-F PWV. The most parsimonious model therefore is the first unadjusted model generated using the stepwise analysis.

## Discussion

The strength of this analysis is that start of dialysis is a relatively hard end point. However this end point also has weaknesses. Firstly initiation of dialysis is a joint decision made between physician and patient whereas measurement of eGFR has no additional operator driven component. Secondly, patients who entered the maximum conservative care programme and would otherwise have started dialysis would not be recorded as reaching the end point.

These models must be interpreted with caution due to the relatively low number of events. However, the results of the logistic regression models are consistent with the regression models constructed using both the combined dichotomous end points and the eGFR slope coefficients. Baseline eGFR and uPCR were independently associated with the risk of progression to dialysis within this cohort. This relationship is robust, persisting after adjustment for a variety of other parameters previously recognised as risk factors for disease progression. The AUC of the ROC curves of the models are very similar to those of the combined end point models. There was no evidence that C-F PWV predicted progression to dialysis in this cohort.

In summary, the factors independently associated with risk of progression to dialysis in the ACADEMIC cohort are baseline eGFR and uPCR.

## 4.4 Patient Survival Analysis

Twenty six study patients died during the follow up period. Twenty two of these patients were from the group of 185 patients with C-F PWV measurement at baseline. The median period of follow up of all patients was 1346 days (range 101-1956 days). The median period of follow up from baseline visit to death was 797 days (range 101-1663 days). Comparison was made between the surviving and non-surviving patients (see table 4.20).

A greater proportion of the patients who died had cardiovascular comorbidity and diabetes. The group of patients who died also had a significantly higher mean C-F PWV, significantly higher levels of hsCRP, OPG and hs-cTnT, but lower levels of RANKL at baseline (see fig 4.6). The difference in eGFR between groups reached only borderline significance. Comparison of medication usage between groups was performed. The patients who died were less likely to be using an ACEi/ARB (46% v 68%, p=0.022), but there were no other statistically significant relationships.

| Parameter | Survivors | Non-survivors | p value |
|---|---|---|---|
| Age (years) | 68 ± 12 | 73 ± 7 | 0.219 |
| Tobacco exposure†† | 2 (0, 26) | 35 (1,44) | 0.146 |
| BMI (kg/m$^2$) | 29.1 ± 7.6 | 30.3 ± 6.3 | 0.815 |
| CV comorbidity (%) | 5% | 23% | <0.001*** |
| LVH (%) | 10 | 14 | 0.928 |
| Diabetes (%) | 20 | 11 | 0.038* |
| SBP (mmHg) | 150 ± 21 | 161 ± 20 | 0.205 |
| DBP (mmHg) | 81 ± 11 | 81 ± 11 | 0.446 |
| C-F PWV† (m/s) | 12.9 ± 2.7 | 14.0 ± 1.6 | 0.004** |
| eGFR (ml/min/1.73m$^2$) | 33 ± 11 | 28 ± 8 | 0.060 |
| Phosphate (mmol/l) | 1.07 ± 0.18 | 1.06 ± 0.17 | 0.639 |
| C. Calcium(mmol/l) | 2.22 ± 0.31 | 2.31 ± 0.11 | 0.152 |
| Albumin (g/l) | 42.5 ± 3.1 | 42 ± 3.6 | 0.758 |
| uPCR (mg/mmol) | 25 (13, 65) | 40 (20, 81) | 0.232 |
| hsCRP (mg/l) | 2.1 (0.9, 5.2) | 5.3 (2.9, 13.6) | 0.001** |
| Total fetuin-A (g/l) | 0.23 ± 0.07 | 0.23 ± 0.09 | 0.591 |
| CPP (%) | 10 (5,16) | 15 (9, 23) | 0.104 |
| OPG (pmol/l) | 9.1 (7.0, 12.1) | 11.6 (9.9, 14.2) | 0.007** |
| RANKL (pmol/l) | 623 ± 253 | 453 ± 243 | <0.001*** |
| iFGF-23 (pg/ml) | 59.8 (45.4, 72.3) | 73.3 (55.3, 95.1) | 0.113 |
| hs-cTnT (mcg/l) | 0.021 ± 0.019 | 0.033 ± 0.023 | 0.015* |

**Table 4.20 - Comparison of surviving (n=174) and non-surviving (n=26) groups - † (163:22); ††pack years**

RANKL, CPP and total fetuin-A did not fulfil the proportional hazards assumption required for Cox proportional hazards modelling. We noted that increased OPG and

lower RANKL were associated with an increased risk of death. Given their physiological interaction within bone, these parameters were expressed as a ratio. This ratio met the proportional hazards requirement, had univariate significance and was therefore entered into the Cox model. CPP and total fetuin-A were not entered into the Cox model.

A stepwise Cox proportional hazards analysis (without hs-cTnT) was performed. The results are shown in table 4.21 below. Patients with cardiovascular co-morbidity or with an increased OPG:RANKL ratio were at an increased risk of death. Patients taking ACEi/ARB medication were at a borderline reduced risk of death.

| Parameter | B | HR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| CV disease | 1.678 | 5.335 | 1.932 | 14.844 | 0.001** |
| OPG:RANKL† | 0.084 | 1.088 | 1.024 | 1.156 | 0.007** |
| ACEi/ARB use | -0.858 | 0.424 | 0.178 | 1.011 | 0.053 |

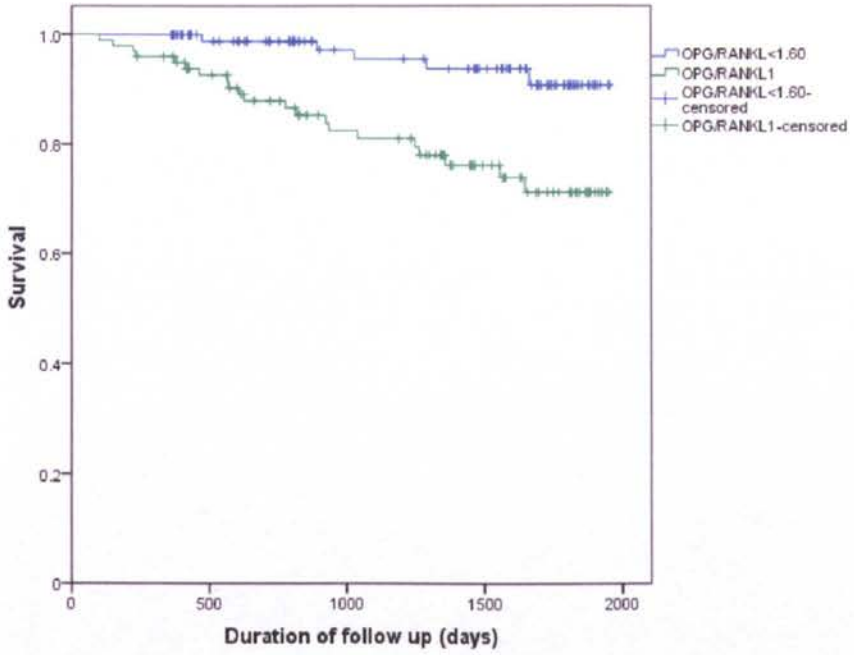
**Table 4.21 - Stepwise Cox proportional hazard regression model of parameters independently associated with mortality (n=185) - Excluded variables: C-F PWV, hsCRP, DM & eGFR - †x10²**

A further stepwise model was then constructed incorporating hs-cTnT. ACEi/ARB use was replaced by hs-cTnT in this model (see table 4.22).

| Parameter | B | HR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| CV disease | 1.696 | 5.542 | 1.937 | 15.436 | <0.001*** |
| OPG:RANKL† | 0.080 | 1.084 | 1.019 | 1.153 | 0.011* |
| hs-cTnT†† | 0.213 | 1.237 | 1.048 | 1.460 | 0.012* |

**Table 4.22 - Stepwise Cox proportional hazard regression model for parameters independently associated with mortality (n=185) - Excluded variables: C-F PWV, hsCRP, DM, eGFR & ACEi/ARB - †-x10²; ††-x10⁻¹**

A




B

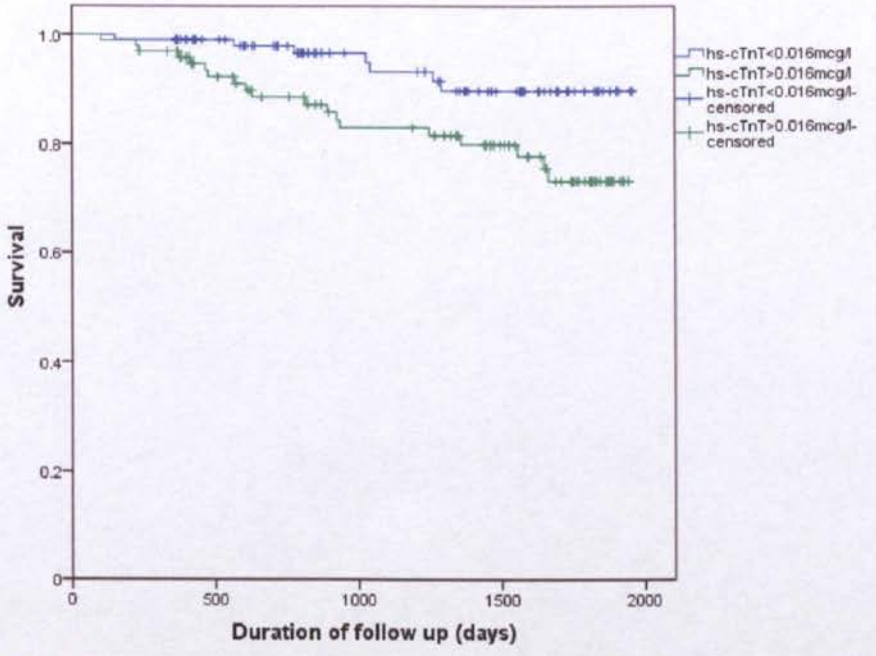



**Figure 4.6 - Kaplan-Meier plots of patient survival in ACADEMIC cohort by median (A) OPG:RANKL (1.60) ($p=0.001^{**}$); and (B) hs-cTnT (0.016mcg/l) ($p=0.015^{*}$) (n=200 at $t_0$)**

A sensitivity analysis was run in which the models were adjusted for eGFR. The significant parameters in the first model (without hs-cTnT) remained unchanged. In the second model, the significance of hs-cTnT became borderline (HR1.182 (CI 0.977 – 1.431) p=0.09), whilst the significance of the other parameters remained essentially unchanged. If iFGF-23 or phosphate were entered into the stepwise model, they were not retained. In addition, if a model was run containing both OPG and OPG:RANKL then the ratio was retained in the final model in preference to OPG alone.

## Discussion

There are two striking novel results from this analysis. Firstly we have demonstrated that hs-cTnT is associated with increased hazard of mortality in a cohort of patients with CKD stages 3 & 4. As previously discussed, this relationship has been demonstrated in the general population and in ESRF cohorts, but not in the pre-dialysis CKD setting. The findings of this study are a logical extension of the results of these other studies, and confirm that high circulating levels of troponin T are associated with adverse outcome in the pre-dialysis CKD population. Furthermore, our findings demonstrate that whilst hs-cTnT is independently associated with eGFR, after adjustment for eGFR the relationship between hs-cTnT and mortality risk is preserved, albeit with borderline significance. Given the significant findings in other larger studies of populations at lower risk this borderline risk is likely to be explained by the relatively low power of the ACADEMIC study in this regard.

Secondly, we have demonstrated that the OPG:RANKL ratio is also associated with an increased hazard of death in this setting.

Again, models based upon a relatively small number of events in a relatively small and heterogeneous CKD population must be interpreted with caution. There have been three previously published larger studies examining the association of RANKL with cardiovascular events. All these studies were performed in the general population. In the Bruneck study, increased RANKL was associated with risk of cardiovascular events [187]. In the EPIC-Norfolk study, RANKL showed no association with cardiovascular event rate [223], whilst conversely, in the Framingham study, RANKL was inversely associated with multiple cardiovascular risk factors including smoking, diabetes and antihypertensive treatment [188]. Clearly it is difficult to reconcile these findings, however it could be proposed that in the ACADEMIC

study RANKL may be acting as a surrogate for the multiple cardiovascular risk factors as identified in the Framingham study. Several other studies have identified the association of OPG with adverse cardiovascular outcome as previously discussed.

**Proportional Hazards**

This analysis is limited by the necessity of potential independent parameters to fulfil the proportional hazards assumption. This assumption indicates that the hazard attributed to the parameter at baseline is stable over the period of follow up. As would be expected, parameters such as diabetes and cardiovascular comorbidity meet this requirement. A patient at high cardiovascular risk due to a previous myocardial infarction (an expression of accumulated cardiovascular risk) will remain at high cardiovascular risk, notwithstanding any attempts to mitigate this. Equally, it could be theorised that biochemical parameters which reflect the steady state would continue to be markers of cardiovascular risk over time. This theory however fails to explain why other CaRP such as RANKL, fetuin-A or CPP do not fulfil the requirement.

In circumstances where the proportional hazards requirement is not met, several other options are available. The period of follow up may be shortened, the analysis stratified by the parameter which fails to fulfil the proportional hazard assumption, a logistic regression analysis may be performed, or alternatively a survival analysis with a time dependent covariate may be performed.

Shortening the period of follow up was not useful approach as the number of events in the analysis was already low. Equally stratification of the cohort was not useful in this setting, both due to the low number of events and to the fact that this would prevent examination of the influence of the stratification parameter. Instead an interaction term of time and RANKL was calculated and entered into the proportional hazard model. This allowed for the change in hazard over time. In this situation, if the model was rerun with RANKL and RANKL*time interaction term, then RANKL, CV comorbidity and hs-cTnT were retained in the final model (data not shown).

## Logistic Regression Analysis

In order to further investigate the factors independently associated with risk of death in the cohort, a logistic regression analysis was therefore performed. Comparison was made between the surviving and non-surviving patients, using student's t test for parametric data or appropriate test for non-parametric data.

In addition to the parameters which were significantly different using the log rank test, there was also a significant difference between groups in tobacco exposure (p=0.001), eGFR (p=0.011), CPP (p=0.003) and iFGF-23 (p=0.012).

A stepwise logistic regression model was constructed incorporating all variables with univariate significance. The results are shown in table 4.23.

| Parameter | B | OR | Lower 95% CI | Upper 95% CI | p value |
|---|---|---|---|---|---|
| Tobacco exposure[†] | 0.017 | 1.007 | 1.001 | 1.013 | 0.036* |
| CV disease | 1.571 | 4.811 | 1.531 | 15.128 | 0.007** |
| eGFR | -0.081 | 0.922 | 0.863 | 0.986 | 0.017* |
| RANKL | -0.003 | 0.997 | 0.996 | 0.999 | 0.012* |

**Table 4.23 - Stepwise logistic regression model of factors independently associated with mortality (n=185) - Excluded variables: age, SBP, C-F PWV, CPP, OPG, iFGF-23, ACEi/ARB & hs-cTnT - AUC 0.827 (0.756-0.902) - [†]pack years**

Patients with cardiovascular comorbidity and previous smoking exposure had an increased risk of mortality, as did those with a lower baseline eGFR and RANKL. The retention of tobacco exposure (classified as a continuous variable) indicates a dose response relationship with mortality risk. This relationship was not explored in the Cox proportional hazard model as this variable was not statistically significantly associated with mortality risk in the Kaplan-Meier analysis. However, if the Cox model was re-run then tobacco exposure was not significant in the final model. A further difference is that hs-cTnT was not retained within the logistic regression model, whereas it was retained within the Cox model. In a final sensitivity analysis, if RANKL was replaced with OPG:RANKL in the logistic regression model then this parameter was not retained within the final stepwise model.

In summary, the logistic regression model produced results which were complementary, but not identical, to the Cox proportional hazards model.

These models identified that both traditional 'Framingham' risk factors such as tobacco exposure and cardiovascular comorbidity. Additionally other non-traditional risk factors including RANKL or OPG:RANKL and hs-cTnT were associated with an increased mortality risk.

## 4.5 Survival Analysis – Death or Dialysis

In order to increase the power of the previous analysis we investigated the factors associated with a combined hard end point of start of dialysis or death. Survival analysis was censored when patients started RRT.

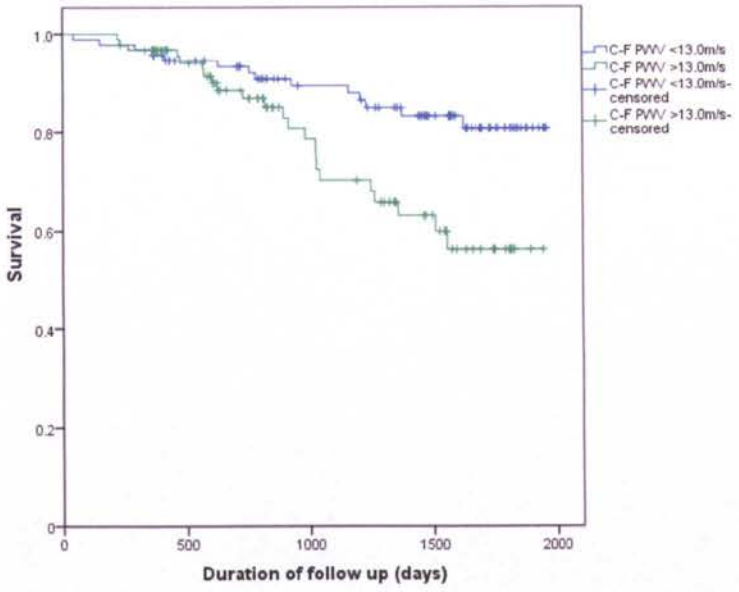
Forty four patients reached the combined end point of death or dialysis in the study. Twenty patients started dialysis of whom two subsequently died within the follow up period. A further twenty four patients died without starting dialysis. A comparison of the demographic, clinical and biochemical parameters in the death/dialysis group and the non-death/dialysis group was performed using log rank analysis. The results are shown in table 4.24. Kaplan-Meier plots are shown for eGFR and C-F PWV dichotomised using median values (see fig. 4.7).

A comparison of the proportion of patients taking various anti-hypertensive medications and statins between the two groups was also performed. The results are shown in table 4.25.

| Parameter | Death or dialysis | Non death/dialysis | p value |
|---|---|---|---|
| Age (years) | 70 ± 11 | 68 ± 11 | 0.488 |
| Tobacco exposure† | 30 (1, 44) | 2 (0,23) | 0.027* |
| CV comorbidity (%) | 59 | 40 | 0.010* |
| LVH (%) | 18 | 21 | 0.486 |
| Diabetes (%) | 36 | 22 | 0.010* |
| SBP (mmHg) | 159 ± 21 | 149 ± 21 | 0.247 |
| DBP (mmHg) | 84 ± 11 | 81 ± 11 | 0.386 |
| C-F PWV†† (m/s) | 13.7 ± 2.3 | 12.8 ± 2.6 | 0.008** |
| eGFR (ml/min/1.73m$^2$) | 26 ± 8 | 35 ± 10 | <0.001*** |
| Phosphate (mmol/l) | 1.10 ± 0.20 | 1.06 ± 0.18 | 0.284 |
| C. Calcium (mmol/) | 2.27 ± 0.19 | 2.22 ± 0.32 | 0.258 |
| Albumin (g/l) | 41 ± 1 | 43 ± 3 | 0.057 |
| uPCR (mg/mmol) | 50 (23, 143) | 23 (12, 50) | <0.001*** |
| hsCRP (mg/l) | 4.6 (1.4, 10.0) | 2.0(0.9, 4.7) | 0.002** |
| Total fetuin-A (g/l) | 0.23 ± 0.08 | 0.23 ± 0.07 | 0.231 |
| CPP (%) | 17 (9, 22) | 9 (5,14) | <0.001*** |
| OPG (pmol/l) | 12 (10,14) | 9 (7,12) | 0.002** |
| RANKL (pmol/l) | 515 ± 254 | 625 ± 254 | 0.006** |
| iFGF-23 (pg/ml) | 79 (65, 106) | 58 (43, 70) | <0.001*** |
| hs-cTnT (mcg/l) | 0.024 (0.015, 0.042) | 0.014 (0.008, 0.023) | 0.008** |

**Table 4.24 - Comparison of demographic and clinical parameters of surviving (n=156) and death/dialysis (n=44) groups - †pack years; †† surviving:non-surviving (141:44)**

A

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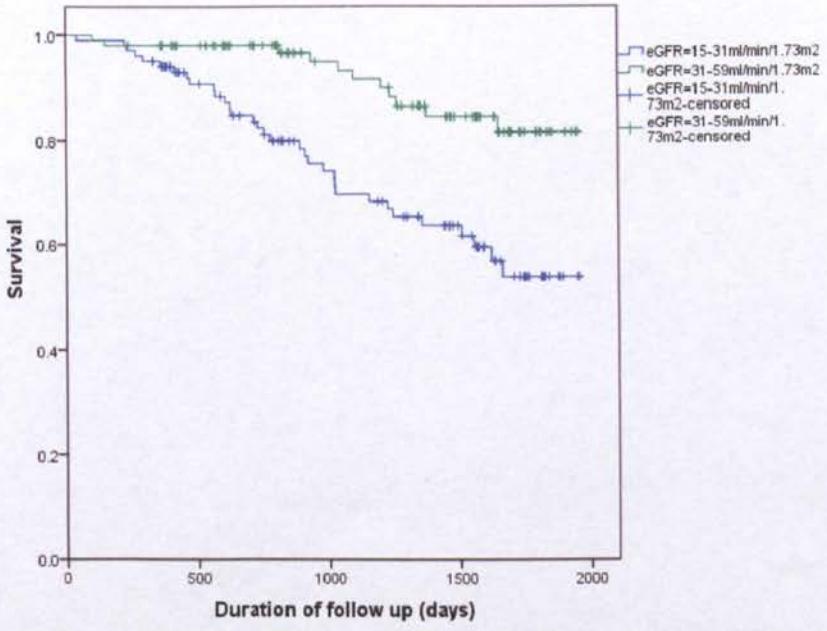


**Figure 4.7 - Kaplan-Meier plot of combined patient and renal survival in ACADEMIC cohort by (A) median C-F PWV (13.0m/s - adj. for MBP) ($p=0.008^{**}$) (n=185) and (B) eGFR ($31ml/min/1.73m^2$) ($p<0.001^{***}$) (n=200)**

| Medication | Death or dialysis | Non death or dialysis | p value |
|---|---|---|---|
| ACEi/ARB (%) | 52 | 69 | 0.018* |
| Beta Blocker (%) | 32 | 34 | 0.865 |
| CCB (%) | 57 | 43 | 0.332 |
| Diuretic (%) | 64 | 53 | 0.686 |
| Statin (%) | 48 | 63 | 0.068 |

**Table 4.25 - Comparison of medication use of surviving (n=156) and death/dialysis (n=44) groups**

All parameters with univariate significance fulfilled the requirement for proportionality of hazard except for CPP and C-F PWV. A Cox proportional hazards analysis was run without these parameters (see table 4.26).

| Parameter | B | HR | Lower 95% CI | Upper 95%CI | p value |
|---|---|---|---|---|---|
| Tobacco exposure† | 0.015 | 1.015 | 1.007 | 1.023 | <0.001*** |
| Diabetes | 0.838 | 2.312 | 1.156 | 4.622 | 0.018* |
| uPCR | 0.003 | 1.003 | 1.001 | 1.004 | 0.003** |
| eGFR | -0.112 | 0.894 | 0.853 | 0.938 | <0.001*** |

**Table 4.26 - Stepwise Cox proportional hazard regression model of parameters independently associated with progression to combined end point of death or dialysis (n=200) - Excluded variables: CV comorbidity, hsCRP, OPG, RANKL, iFGF-23, hs-cTnT & ACEi/ARB use - †pack years**

However, if C-F PWV and CPP are entered into the stepwise model with an interaction term with time for each parameter (i.e. CPP*time and C-F PWV*time) then the model changed significantly (see table 4.27)

| Parameter | B | HR | Lower 95% CI | Upper 95%CI | p value |
|---|---|---|---|---|---|
| Tobacco exposure[†] | 0.014 | 1.013 | 1.002 | 1.024 | 0.017* |
| eGFR | -0.163 | 0.850 | 0.796 | 0.907 | <0.001*** |
| C-F PWV | 1.044 | 2.841 | 2.008 | 4.018 | <0.001*** |
| C-F PWV*time | -0.002 | 0.998 | 0.998 | 0.999 | <0.001*** |
| CV comorbidity | 1.169 | 3.217 | 1.199 | 8.631 | 0.020* |
| ACEi/ARB use | -1.338 | 0.262 | 0.108 | 0.636 | 0.003** |

**Table 4.27 - Stepwise Cox proportional hazard regression model of parameters independently associated with progression to death or dialysis (combined renal end point) (n=185) - Excluded variables: hsCRP, OPG, RANKL, iFGF-23, CPP, CPP*time & hs-cTnT - [†]pack years**

In this final model tobacco exposure, baseline eGFR, C-F PWV, CV comorbidity and ACEi/ARB use were all independently associated with risk of reaching the combined end point of death and dialysis. The inclusion of the interaction term in the stepwise model indicated that as time from study enrolment increased, then the risk attributable to increased C-F PWV was diminished. Diabetes and uPCR were no longer retained in this final stepwise model.

If a sensitivity analysis was performed in which OPG:RANKL was substituted for OPG and/or RANKL then this variable was still not retained within the final stepwise model.

## Discussion

Death and progression onto dialysis are hard, clinically relevant end points. The most striking result from this study is the finding of a statistically significant independent association between C-F PWV and the combined end point of death and dialysis. Whilst this falls short of the holy grail of arterial stiffness in pre-dialysis CKD, i.e. an independent association between C-F PWV and death in this cohort, this is, to the best of my knowledge, the first time that C-F PWV has been associated with such an outcome in this population.

As previously discussed, baseline renal function is a recognised risk factors for progression of renal disease to dialysis. Similarly tobacco exposure and previous cardiovascular disease are classical traditional risk factors for cardiovascular events in the general population.

However, interpretation of these results must be tempered by appreciation of the heterogeneity of the cohort, and the inclusion of ACEi/ARB use in the final model must again be interpreted with regard to indication bias.

A greater proportion of younger patients with proteinuric renal disease would be expected to progress to dialysis whilst conversely, older patients with non-proteinuric renal disease would be expected to have a significantly greater risk of death than of progression to dialysis. These proportions are borne out in the results of this study. If the study population is split into tertiles by age, the number starting RRT was greatest in the youngest tertile, compared to the middle and oldest tertile (9, 6 and 5 patients), whilst the number of patients who died increased from 6 in the youngest tertile to 9 in the middle and 11 in the eldest. Whilst these trends are not significant due to the low total number of events, this would explain the absence of age as a significant predictor of the combined endpoint.

In summary, in this final combined renal and patient survival analysis, tobacco exposure and low eGFR were consistently associated with adverse outcome. Diabetes, proteinuria, cardiovascular comorbidity and absence of ACEi/ARB use were also associated with death and dialysis, whilst the risk associated with C-F PWV declined in a time dependent manner.

## Limitations of Study

The cohort studied is a sub-population of the CKD population seen within the nephrology service of the Sussex Kidney Unit. The extent to which the cohort is reflective of the general clinic population has not been studied. Referral management guidelines used within the NHS and BSUH are likely to further limit the applicability of this cohort study to the wider community of patients with CKD stages 3 & 4, both within Sussex and beyond.

As discussed, the population studied is heterogeneous, including patients with differing ages, and CKD of varying aetiologies across a relatively wide range of eGFR. We have attempted to explore the varying responses in the differing categories of CKD where possible. However these sub-group analyses are exploratory and underpowered. The lack of finding of significant relationships within these sub-group analyses should not therefore be interpreted as the lack of a significant relationship in the wider CKD population. Equally, statistically, the problems of multiple comparisons within a relatively small dataset are acknowledged. A recognised hazard of stepwise modelling is that parameters may be selected into a model due to mathematical chance rather than pathophysiological relevance. This probability is especially high in analysis of cohorts with a relatively small number of events.

Within this chapter, problems have arisen with interpretation of renal function data in patients in whom relatively large changes of renal function were demonstrated to have a significant leverage on overall analysis. Whilst the number of patients with unstable renal function could have been minimised by more stringent entry criteria to the study, this would not have precluded unpredictable changes.

In order to model survival data, it is assumed that the behaviour of the patients for whom follow up is censored is similar to the behaviour of those who remain under follow up. However, whilst this assumption was not formally tested as it is not easy to study the behaviour of people who have been lost to follow up, it is likely that this assumption was violated for a variety of reasons. Patients who have been lost to follow up may have moved from the area. The ability to relocate is not uniform and is likely to be a marker of health status. Alternatively, patients who have ceased to attend clinic appointments may have done so because they are either moribund at

home, or alternatively may have been admitted to a residential care facility. Both of these scenarios are associated with additional mortality risk.

In addition, the violation of the proportional hazards assumption by certain variables in the survival analysis necessitated manipulation of the variables or use of a sub-optimal logistic regression analysis rather than the preferred Cox proportional hazards regression modelling.

## Summary

Within this chapter we have investigated the association of recognised and novel risk factors with rate of change of renal function across the study cohort. We have demonstrated the importance of traditional factors (baseline eGFR and uPCR) for renal disease progression within our cohort. In addition, we found that ACEi/ARB use was associated with rate of change of eGFR. Using the dichotomous end points for renal disease progression, baseline eGFR and uPCR were again independently associated with progression, in conjunction with statin use.

In the survival analysis, we extended the observation of the association of hs-cTnT with mortality from large general population studies to a population composed exclusively of CKD stages 3 & 4. RANKL was associated with reduced mortality risk in this analysis, whilst OPG was associated with an increased mortality risk in combination with other risk factors. In addition, increased C-F PWV was independently associated with risk of death or dialysis in the combined end point Cox analysis in a time dependent manner, in conjunction with other traditional and non-traditional risk factors.

# Chapter 5

# Rate of Change of C-F PWV

## 5.1 Rate of Change of PWV

185 patients had C-F PWV measurement performed at baseline, 141 patients were measured at one year, 90 patients at two years and 69 patients at three years. The mean of the PWV at annual intervals are shown in table 5.1 and fig. 5.1.

| Visit | SBP (mmHg) | DBP (mmHg) | C-F PWV (m/s) | No. antihypertensives |
|---|---|---|---|---|
| Baseline | 151 ± 21 | 82 ± 11 | 13.0 ± 2.6 | 2.1 ± 1.3 |
| 12 months | 142 ± 21 | 79 ± 11 | 13.1 ± 2.9 | 2.2 ± 1.3 |
| 24 months | 136 ± 18 | 75 ± 11 | 13.1 ± 2.9 | 2.1 ± 1.3 |
| 36 months | 136 ± 18 | 74 ± 10 | 13.1 ± 2.8 | 2.2 ± 1.3 |

**Table 5.1 - Mean (±SD) SBP, DBP and adjusted C-F PWV across study period - Baseline (n=185), 12 months (n=141), 24 months (n=90) & 36 months (n=69)**

There was no significant change in the group mean C-F PWV over time (p=0.975 ANOVA for trend). There was a significant reduction in SBP and DBP over the study period (SBP, p=0.003; DBP p<0.001). There was no significant change in the number of antihypertensives taken throughout the study period.

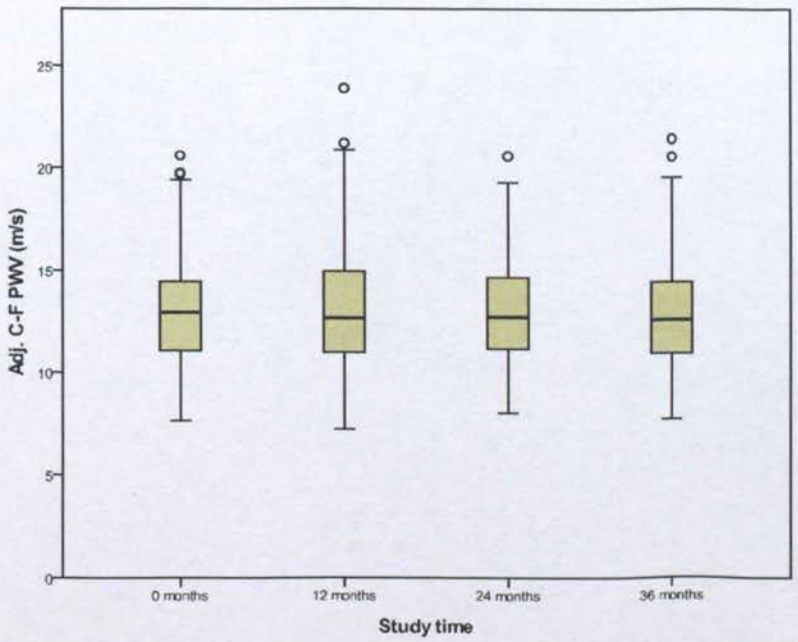


**Figure 5.1 - Boxplots of mean C-F PWV (m/s) (adjusted for MBP) across study period 0 months, n=185; 12 months, n=141; 24 months, n=90; 36 months, n=69 (p=0.975 ANOVA for trend)**

Despite the absence of change in mean C-F PWV over time within the cohort, C-F PWV changed in individual patients. In order to investigate this, changes in C-F PWV at 12, 24 and 36 months were calculated. There was a highly significant correlation between paired results over all three comparator periods ($p<0.001$ for all groups). However, if paired t-tests were used a sequential significant increase was seen in C-F PWV over time (see table 5.2).

| Period of comparison | C-F PWV at baseline (m/s) | C-F PWV at end point (m/s) | Mean change from baseline (m/s) | p value |
|---|---|---|---|---|
| 12 months | 12.8 ± 2.4 | 13.1 ± 2.9 | 0.3 ± 1.9 | 0.035* |
| 24 months | 12.4 ± 2.5 | 13.1 ± 2.9 | 0.7 ± 1.9 | 0.001** |
| 36 months | 12.1 ± 2.5 | 13.1 ± 2.8 | 0.9 ± 1.9 | <0.001*** |

**Table 5.2 - Sequential mean C-F PWV (m/s) of groups remaining under follow up across study periods - 12months (n=141), 24 months (n=90), 36 months (n=69)**

This is explained by the preferential dropout rate amongst those with high C-F PWV at baseline, such that the baseline C-F PWV in those who remained under follow up at 36 months was significantly lower than that of the whole cohort.

### Characteristics of patients not measured at 12 months

There are many patients in whom sequential annual measurement of C-F PWV was not performed. Patients ceased to have C-F PWV measurements for a variety of reasons. At 12 months, four patients had died, three had started RRT, one had been lost to follow up, three missed appointment, five had unrecordable pulses, and a further 28 had left the study or were too unwell to attend further appointments.

In order to investigate if this subgroup of patients differed from the study cohort who remained under follow up at 12 months, a comparison was made of the two groups. (see table 5.3).

| Parameter | Measured at 12 months (n=141) | Not measured at 12 months (n=44) | p value |
|---|---|---|---|
| Age (years) | 68 ± 11 | 71 ± 13 | 0.266 |
| Diabetes (%) | 39 | 51 | 0.181 |
| CV comorbidity (%) | 23 | 33 | 0.163 |
| Gender (% male) | 76 | 62 | 0.087 |
| SBP (mmHg) | 151 ± 21 | 151 ± 23 | 0.955 |
| DBP (mmHg) | 82 ± 11 | 81 ± 11 | 0.553 |
| Heart rate (bpm) | 71 ± 12 | 69 ± 14 | 0.495 |
| Tobacco exposure[†] | 2 (0, 30) | 8 (0, 35) | 0.232 |
| Baseline C-F PWV (m/s) | 12.8 ± 2.4 | 13.7 ± 2.9 | 0.048* |
| eGFR (ml/min/1.73m$^2$) | 34 ± 11 | 29 ± 9 | 0.014* |
| uPCR (mg/mmol) | 28 (13, 68) | 19 (14, 51) | 0.308 |
| C. Calcium (mmol/l) | 2.30 ± 0.12 | 2.29 ± 0.10 | 0.720 |
| Phosphate (mmol/l) | 1.06 ± 0.19 | 1.06 ± 0.17 | 0.931 |
| Total fetuin-A (g/l) | 0.23 ± 0.06 | 0.22 ± 0.08 | 0.270 |
| CPP (%) | 9.3 (5.0, 16.5) | 11.5 (7.7, 18.3) | 0.088 |
| OPG (pmol/l) | 9.5 (7.0, 12.5) | 10.1 (7.6, 12.1) | 0.464 |
| RANKL (pmol/l) | 623 ± 241 | 578 ± 297 | 0.328 |
| iFGF-23 (pg/ml) | 58.6 (45.6, 73.1) | 65.0 (45.5, 90.1) | 0.190 |
| hsCRP (mg/l) | 2.3 (0.9, 5.8) | 2.2 (1.1, 5.4) | 0.840 |
| hs-cTnT (mcg/l) | 0.017 (0.012, 0.033) | 0.014 (0.008, 0.024) | 0.115 |

**Table 5.3 - Comparison of patients who underwent baseline C-F PWV measurement by 12 month follow up status (n=185) - [†]pack years**

Patients who were lost to follow up within the first year had a significantly higher mean C-F PWV and a lower eGFR. No other significant differences were seen.

### Change at 12 months

141 patients had C-F PWV measurement performed at 0 and 12 months. The distribution of the change in C-F PWV from month 0 to month 12 was not parametric. The population was therefore dichotomised into two groups; those in whom C-F PWV increased and those in whom it decreased or remained stable. 80 patients experienced an increase in C-F PWV (mean increase 1.4 ± 1.6m/s), whilst 61 experienced either a reduction in C-F PWV or no change (mean 1.1 ± 1.1m/s). The characteristics of the groups were then compared (see tables 5.4 & 5.5).

| Medication | Decrease/stable | Increase | p value |
|---|---|---|---|
| ACEi/ARB (%) | 64 | 76 | 0.111 |
| Beta Blocker (%) | 30 | 33 | 0.704 |
| Calcium Channel Blocker (%) | 46 | 45 | 0.915 |
| Diuretic (%) | 66 | 53 | 0.119 |
| No. antihypertensives | 2.2 ± 1.4 | 2.1 ± 1.2 | 0.600 |
| Statin (%) | 53 | 65 | 0.133 |
| Bisphosphonate (no. pts) | 7 | 4 | 0.466[§] |
| Calcium and/or vitamin D (%) | 13 | 13 | 0.914 |

**Table 5.4 - Comparison of medication use between patients in whom C-F PWV increased (n=80) or decreased/remained stable (n=61) at 12 months - [§]Fisher's exact test**

| Parameter | C-F PWV decrease/stable | C-F PWV increase | p value |
|---|---|---|---|
| Age (years) | 68 ± 11 | 69 ± 11 | 0.569 |
| Diabetes (%) | 20 | 24 | 0.562 |
| Gender (% male) | 75 | 78 | 0.771 |
| CV comorbidity (%) | 33 | 44 | 0.186 |
| SBP (mmHg) | 149 ± 18 | 152 ± 22 | 0.415 |
| DBP (mmHg) | 81 ± 11 | 83 ± 11 | 0.394 |
| Heart rate (bpm) | 70 ± 11 | 71 ± 13 | 0.395 |
| Tobacco exposure† | 7 (0, 30) | 1 (0, 20) | 0.308 |
| BMI ($kg/m^2$) | 29.6 ± 5.6 | 28.6 ± 5.2 | 0.297 |
| eGFR ($ml/min/1.73m^2$) | 34 ± 12 | 33 ± 10 | 0.610 |
| uPCR (mg/mmol) | 32 (12, 99) | 23 (13, 44) | 0.221 |
| C. Calcium (mmol/l) | 2.30 ± 0.11 | 2.29 ± 0.12 | 0.954 |
| Phosphate (mmol/l) | 1.06 ± 0.19 | 1.06 ± 0.19 | 0.980 |
| Total fetuin-A (g/l) | 0.24 ± 0.06 | 0.23 ± 0.06 | 0.191 |
| CPP (%) | 8.7 (5.0, 14.4) | 9.3 (5.0, 17.7) | 0.349 |
| OPG (pmol/l) | 7.7 (6.6, 11.8) | 10.0 (8.1, 12.7) | 0.009** |
| RANKL (pmol/l) | 583 ± 257 | 647 ± 226 | 0.117 |
| iFGF-23 (pg/ml) | 55.4 (45.0, 68.8) | 64.6 (47.3, 81.4) | 0.020* |
| hsCRP (mg/l) | 2.3 (0.9, 4.8) | 2.2 (0.9, 5.9) | 0.868 |

**Table 5.5 - Comparison of patients in whom C-F PWV increased (n=80) or decreased/remained stable (n=61) at 12 months - †pack years**

The only significant differences were increased mean titres of OPG and iFGF-23 in the group with increased C-F PWV.

A stepwise logistic regression model was constructed, in order to identify the factors independently associated with risk of increase in C-F PWV (see table 5.6).

| Parameter | B | OR | Lower 95% CI | Upper 95%CI | p value |
|---|---|---|---|---|---|
| iFGF-23 | 0.015 | 1.015 | 1.001 | 1.028 | 0.033* |

**Table 5.6 - Stepwise logistic regression model of factor(s) independently associated with risk of increased of C-F PWV at 12 months (n=141) - Excluded variable: OPG**

The sole factor independently associated with the risk of increase in C-F PWV was iFGF-23. Patients with an increased iFGF-23 were more likely to have an increase in C-F PWV over one year.

### Change at 24 months

Within the cohort of 90 patients for whom C-F PWV measurement was performed at 0 and 24 months, 27 experienced a reduction in or stable C-F PWV from the baseline value (mean change 1.3 ± 1.8m/s) and 63 experienced an increase in C-F PWV (mean change 1.5 ± 1.2m/s). Again, the distribution of the change in C-F PWV at 24 months was not parametrically distributed and so the population was dichotomised into those for whom C-F PWV had increased and those for whom it had decreased/remained stable. Comparison of the parameters listed in tables 5.4 and 5.5 was then made. In order to avoid excessive duplication of non-significant results only the parameters where the distributions differed with a $p<0.10$ are listed (see table 5.7).

| Parameter | C-F PWV decrease/stable | C-F PWV increase | p value |
|---|---|---|---|
| Diabetes (no. patients) | 2 | 15 | 0.083§ |
| Heart rate (bpm) | 67 ± 11 | 72 ± 12 | 0.078 |
| iFGF-23 (pg/ml) | 49.7 (33.4, 68.9) | 62.4 (50.5, 74.6) | 0.049* |
| Beta blocker use (%) | 48 | 22 | 0.014* |

**Table 5.7 - Comparison of factors varying between patients in whom C-F PWV increased (n=63) and decreased/remained (n=27) stable at 24 months - §Fisher's exact test**

### Change at 36 months

Within the cohort of 69 patients for whom C-F PWV measurement was performed at 0 and 36 months, 14 experienced a reduction in, or stable C-F PWV from the baseline value (mean change 1.4 ± 2.2 m/s) and 55 experienced an increase in C-F PWV (mean change 1.5 ± 1.4m/s). Again, the distribution of the change in C-F PWV

at 36 months was not parametrically distributed and so the population was dichotomised. Comparison of the parameters listed in tables 5.4 & 5.5 above were then made. In order to avoid excessive duplication of non-significant results only the parameters with $p<0.10$ are listed (see table 5.8).

| Parameter | C-F PWV decrease/stable | C-F PWV increase | p value |
|---|---|---|---|
| Diabetes (no. pts) | 0 | 13 | 0.056[§] |
| SBP (mmHg) | 161 ± 17 | 150 ± 19 | 0.070 |
| uPCR (mg/mmol) | 15.5 (11.5, 28.1) | 27.2 (12.5, 64.7) | 0.065 |
| CPP (%) | 12.4 (6.2, 20.7) | 7.4 (5.0, 13.6) | 0.076 |
| Diuretic use (no. pts) | 5 | 37 | 0.063[§] |

**Table 5.8 - Comparison of factors varying between patients in whom C-F PWV increased (n=55) and decreased/remained stable (n=14) at 36 months - [§]Fisher's exact test**

Logistic regression models were not calculated for rate of change over 24 and 36 months due to the interim nature of the analysis.

**Non-Diabetics Only**

In order to verify the previously published analysis in which SBP and total fetuin-A were independently associated with rate of change of eGFR over 12 months year in the 73 non-diabetics patients recruited by LT [341], this analysis was repeated in the subgroup of non-diabetic patients from the entire ACADEMIC cohort of 200 patients.

There was a significantly greater increase in C-F PWV over 12 months in the 31 diabetic patients compared to the 110 non-diabetic patients (1.1 ± 2.5 m/s/year vs. 0.1 ± 1.6m/s/year ($p=0.011$)) for whom data was available. The distribution of the 12 month change in C-F PWV in the non-diabetic patients was non-parametrically distributed. The parameters listed above in tables 5.4 & 5.5 were compared between the 61 non-diabetic patients in whom C-F PWV increased and 49 in whom it decreased or remained stable. The significant results are shown in table 5.9.

| Parameter | C-F PWV decrease/stable | C-F PWV increase | p value |
|---|---|---|---|
| Total fetuin-A (g/l) | 0.24 ± 0.06 | 0.22 ± 0.05 | 0.019* |
| OPG (pmol/l) | 7.5 (6.5, 11.8) | 9.5 (8.0, 12.5) | 0.023* |
| Diuretic use (%) | 65 | 46 | 0.042* |

**Table 5.9 - Comparison of factors varying between non-diabetic patients in whom C-F PWV increased (n=61) and decreased/remained stable (n=49) at 12 months**

A stepwise logistic regression model was then constructed (see table 5.10).

| Parameter | B | OR | Lower 95% CI | Upper 95%CI | P value |
|---|---|---|---|---|---|
| Total fetuin-A | -0.845 | 0.429 | 0.208 | 0.886 | 0.022* |

**Table 5.10 - Stepwise logistic regression model of factor(s) independently associated with risk of increased of C-F PWV at 12 months in non-diabetic patients (n=110) - Excluded variables: OPG, diuretic use (+/- SBP and CPP)**

The results of the logistic regression model are consistent with the previously published results of the first tranche of patients from the study. Reduced total fetuin-A was independently associated with an increased risk of an increase in C-F PWV. OPG was excluded from the model. Whilst there was no difference in CPP or SBP between groups, they were entered into the stepwise model as SBP was independently associated with rate of change of C-F PWV in the multivariate analysis of the first tranche of patients. CPP was entered into the model as it was independently associated with C-F PWV at baseline. Neither variable was retained in the final stepwise model.

## Discussion

There are several noteworthy results within this analysis. Firstly, in patients who remained under active follow up within the study, there was a sequential increase in the C-F PWV. Secondly, in this group of patients iFGF-23 was independently associated with the risk of increase in C-F PWV. Finally, in the subgroup analysis of the non-diabetic patients who remained under follow up total fetuin-A was independently associated with risk of increase in C-F PWV.

The progressive increase in C-F PWV in the cohort is, in itself, a factor of interest. Whilst caution must be taken in extending the interpretation of the repeatability data (see appendix 3), if the same change seen in the healthy volunteers had been present in the ACADEMIC patients, then a decrease in C-F PWV in those remaining under follow up would have been expected. Instead a progressive increase in C-F PWV of approximately the same magnitude is seen at 12 monthly intervals. Whilst the ACADEMIC cohort and the repeatability cohort have not been formally compared, there are many differences between these groups. In particular the repeatability cohort was much younger, was not known to have CKD and were not using any regular medications. All three of these factors have the potential to explain the differences. A further alternative explanation is that the CKD cohort, already familiar with the patient role, may have had less of a stress response at their first visit, thereby reducing the potential for blood pressure changes to subsequent visits. Notably, however, the BP in the cohort who remained under follow up dropped progressively and significantly from the 0, 12 and 24 month visits, despite no significant change in the number of antihypertensives taken throughout the study.

Total fetuin-A and iFGF-23 were independently associated with rate of change of C-F PWV in this study. Non pre-specified subgroup analysis in a cohort is liable to criticism as it may be used to selectively report significant results. However, in this situation, where there is significant evidence that diabetic patients undergo an aggressive pattern of vascular change which is thought to have a partially different mechanism to that of CKD, we would argue that this analysis is valid. This is particularly true when, as in the case of diabetes in this study and others, there was a significant difference in titre of total fetuin-A in non-diabetic and diabetic patients[161]

Our results can be contrasted with those of five other studies, four of which have been published since the beginning of 2010 (see table 1.1). Three of these studies

have followed patients with CKD, however only one of these studies used C-F PWV. All three of the CKD studies are smaller than the ACADEMIC study. Jung *et al* studied 67 South Korean PD patients using H-F PWV [26]. Whilst this measure of arterial stiffness is not identical to C-F PWV, as H-F incorporates the ascending aorta, it is similar to C-F PWV and should be considered to be comparable. In this group, 36% of whom were diabetic, a mean increase in H-F PWV of 0.44 ± 2.09m/s/year was seen. The rate of change of H-F PWV was independently associated with change in MBP and baseline serum triglycerides. Fetuin-A was quantified in this cohort, but was not independently associated with rate of change of H-F PWV. No diabetic subgroup analysis was performed in this small study. This may explain the lack of any significant association between fetuin-A and H-F PWV.

Fassett *et al* studied 34 CKD patients with a mean eGFR of 36ml/min/1.73m$^2$ and mean age of 64 years [27]. This cohort had a mean increase in C-F PWV of 0.4m/s/year. This rate of change of PWV is consistent with that found in the ACADEMIC study. Chen *et al* studied a group of 52 Taiwanese patients with CKD stages 3-5 using B-A PWV over a two year time interval [28]. The mean B-A PWV decreased by 0.064m/s/year over two years. Change in SBP was the only factor independently associated with rate of change of B-A PWV, but no CaRP were measured in this study.

Two other much larger studies have examined the factors associated with rate of change of arterial stiffness in non-CKD populations. Benetos *et al* studied 483 Parisians using C-F PWV over a period of 6 years [29]. 61% of these patients were hypertensive. The hypertensive subgroup had a greater rate of increase of C-F PWV when compared to the normotensive group (0.15m/s/year vs. 0.08m/s/year). Increased heart rate and increased creatinine at baseline were independently associated with the rate of increase of C-F PWV in the hypertensive subgroup.

Finally, Tomiyama *et al* studied 2054 Japanese of working age using B-A PWV over a period of five to six years [30,342]. The group found a mean increase in B-A PWV of 0.05m/s/year which was independently associated with age, BMI, baseline MBP and ongoing high tobacco exposure.

No other studies have examined the association between iFGF-23 and rate of change of arterial stiffness. The association between these two parameters found in the

ACADEMIC study is not however robust. iFGF-23 was not independently associated with baseline C-F PWV after adjustment. Logistic regression analysis of the factors associated with increase or decrease in C-F PWV at 24 and 30 months has not been reported due to the interim nature of the analysis.

However, if a logistic regression analysis was performed using the 24 month data then iFGF-23 was not retained in the stepwise model (data not shown). Furthermore, interestingly there was no significant difference in iFGF-23 titres in the 36 month comparison. Importantly, there is little evidence of a direct effect of FGF-23 within the arterial wall. For these reasons I think that this result, whilst the end point of a valid analysis, requires repetition both in further later analyses of this study and in other study cohorts to avoid the attachment of any undue significance.

There are several features of this analysis which require comment. Firstly, there appears to be a large drop-out rate in this study, especially with regard to the 24 and 36 month analyses. There are still patients in the study who have yet to reach this stage in the follow up. A complete analysis is planned at the end of the PWV measurement period. However, as evidenced by the difference in eGFR and baseline C-F PWV in the comparison between those who completed follow up at one year and those who did not, this longitudinal analysis is likely to be affected by survivor bias. In addition to having better renal function and less stiff arteries, the patients who remained in the study were more likely to be healthy, more mobile and more engaged with health services.

Secondly, in this analysis, all measures of C-F PWV were adjusted for MBP at the time of measurement. A progressive decline in BP is seen in the cohort over time. The cause of this is unclear. It may represent survivor bias, therapeutic success or alternatively increased familiarity with the clinic and measurement. In this analysis therefore, we are unable to comment further on the role of blood pressure in the determination of change of arterial stiffness.

Thirdly, the effect of change of various medication subclasses on arterial stiffness over time has not been fully explored. Whilst this is a possible confounder, the impact is likely to be limited as the number of anti-hypertensive medications did not change significantly over the 12, 24 or 36 month intervals. Notwithstanding this caveat, interpretation of the data with regard to the protective, or otherwise, effects

of various classes of medication should again be tempered by appreciation of both the observational nature of this study (with likely indication bias) and the low numbers of patients using particular drug classes, in particular phosphate binders.

## Summary

In summary, in this analysis, in patients who remained under follow up, arterial stiffness increased progressively over the study period. At one year, increased iFGF-23 was independently associated with the risk of increase in arterial stiffness over twelve months. In the sub-group of patients without diabetes, total fetuin-A was associated with an increased risk of progressive arterial stiffening.

# Chapter 6

# Discussion & Conclusion

## Study Strengths & Limitations

This part of the ACADEMIC project studied four proteins important in the disordered mineral metabolism of CKD and their relationship with arterial stiffness, rate of stiffening, change in renal function and outcomes of death or dialysis.

The study was relatively small and single centred. This had several advantages. A limited number of operators were involved in the measurement of arterial stiffness. One of three operators (MF, AL or LT) was present at every measurement and indeed the vast majority of measurements were performed by two of these operators. This limited the potential for inter-operator error and the development of divergent methodology. The relatively stable population of Brighton and Sussex studied, with little overlap between nephrology services, helped ensure that only relatively few patients were lost to follow up. End points such as death and progression onto dialysis can therefore be calculated with relative confidence and completeness. The prospective nature of the study removed some of the potential selection bias which can be present in retrospective studies, and thereby increased the wider applicability of the results of the study to other cohorts.

However, inevitably such a study also has drawbacks, the largest and most important of which is that multiple statistical analyses have been performed on a relatively small number of study participants. Scientific convention suggests that statistical significance is determined by a $p<0.05$. This convention has been followed in the vast majority of analyses within the study. However, by virtue of this definition, statistical examination of a set of random data would be expected to generate a 1 in 20 rate of type 1 error. The chance of this type 1 error is increased by the multiple comparisons within a dataset. In order to minimise this potential for error, the risk of over-interpretation of results has been limited by use of *a priori* analyses where possible, and attempt has been made to set the findings in a relevant clinical context.

Equally one must not over interpret the negative findings of this study. The study did not recruit to the target of 300 patients and the results of the survival analysis and the analyses of rate of change of arterial stiffness and renal function presented in this thesis form interim analyses. Several novel scientific findings have been reported in this study. However the lack of a significant result in an underpowered study does not equate to an absence of effect. It is hoped that contribution of these results to the

UREKA collaboration will permit a more powerful examination of the role of arterial stiffness in the cardiovascular mortality of CKD in the UK.

Many of the results contained in this thesis are from cross-sectional analysis. It is important therefore to further caution against over interpretation. The finding of an independent statistical association between two variables is exactly that. It is not evidence of a mechanistic link, nor is it evidence that change in one parameter would lead to change in another. This limitation is particularly exemplified in the incomplete appreciation of the association between OPG and C-F PWV where the findings from *in vitro* and animal studies would indicate that the inverse relationship would have been expected.

The single centre nature of the study also limits the applicability of the findings for several reasons. Firstly, the population characteristics of the local area impact on the characteristics of the study cohort. For example, the ACADEMIC study comprises a predominantly white population with 26% of diabetic patients. This differs significantly from the large USA based CRIC study in which 57% of patients are black and 52% have diabetes [21,321]. It would not be feasible to recruit a study containing 114 black patients with CKD stages 3 & 4 from Brighton and Sussex.

Secondly, the interpretation of association between medication use and outcome in this study should be tempered by the appreciation of indication bias. For example, patients who are younger with heavier proteinuria have a stronger indication for ACEi/ARB use than the elderly hypertensive patients. The younger group are more likely to suffer progressive loss of GFR, but less likely to die than the elderly group. This does not necessarily mean that ACEi/ARB use causes loss of renal function. The potential for significant indication and other biases is increased in single centre studies by both local prescribing practices and other factors such as referral guidelines.

Appreciation of the likely multiple concurrent pathologies is also relevant in choice of end points within the study. The cohort studied has a clinically relevant heterogeneity of risk. The 85 year old patient with a stable but reduced eGFR with minimal proteinuria is more likely to die than to progress to ESRF, whereas the younger patient with progressive proteinuric CKD, not responsive to therapy, is

more likely to progress to ESRF. The processes driving these processes are likely to differ and the choice of end points in the study reflects this.

The entry criteria for the study permitted recruitment of a wide variety of patients ranging from young people with progressive proteinuric glomerulonephritis to older patients with stable mild impairment of renal function. Prevalent CKD stage 3 & 4 patients with a range of co-morbidities were recruited from the outpatient clinics at BSUH over a five year period.

The wide entry criteria of the study led to recruitment of a very heterogeneous cohort which contained many potential confounders. Whilst judicious adjustment has been performed where possible, it is likely that residual confounding remains. Nevertheless, the entry criteria are likely to reflect the patient population seen in outpatient nephrology clinics throughout the UK during this period and do not, therefore, unduly limit the wider applicability of the results beyond the centre of study. However the profile of the outpatient population is changing, in part due to referral guidelines, in part due to demographic factors and in part due to the increasing expertise of primary care in the management of patients with CKD stage 3.

Fitting of data to a model is dependent upon the selection of parameters. The robustness of the constructed models has been tested using study of residuals and tests of multi-collinearity. The ultimate test of such models is however transferability to other similar cohorts. Whilst this is beyond the scope of this thesis, critical appraisal of the models with regard to the currently available literature has been performed where appropriate.

The power of this study was limited by extent and scope of observation. Not every biochemical, haemodynamic or physiological parameter could be measured. As such, surrogate markers have been used, for example ECG diagnosis of LVH was used over more complex, expensive and invasive measurements of left ventricular function. This is likely to have resulted in the gathering of less reliable data, eroding the ability of the study to detect any association which may have been present, for example the association between OPG and left ventricular function.

In addition, the study could be criticised for measuring arterial stiffness rather than arterial calcification. This decision was based upon the availability of local expertise,

the lower cost of arterial stiffness measurement and the difficulty obtaining permission and funding for radiation exposure. However, simple measures such as arterial stiffness are not necessarily less satisfactory than other more complex measures.

## What are we measuring?

Within the study there was no use of imaging, either to quantify calcification in the aorta, peripheral or coronary vessels, or indeed to quantify bone mineral density. Whilst these parameters would have added interesting relevant information, it is useful to revisit the information provided by C-F PWV. C-F PWV is an integrated measure which reflects the load placed on the left ventricle by the elasticity of the aorta. C-F PWV measures stiffening throughout the wall of the aorta. This is caused not only by medial calcification, but also by changes to the extracellular matrix and also probably by calcification of the tunica intima. Whilst the mechanisms behind these processes differ, they share a common end point; a less compliant aorta. The ability of non-endovascular imaging to differentiate between calcification in the tunica media and the tunica intima has not yet been proven. In elderly patients with CKD stages 3 & 4, changes to the extracellular matrix and calcification of atherosclerotic plaque may play a significant role in arterial stiffening, which may confound any effect of the CaRP on medial calcification.

C-F PWV, whilst the gold standard method of non-invasive measurement of aortic stiffness, is not a perfect measure. C-F PWV does not incorporate the time interval corresponding to the pulse wave traversing the ascending aorta, the part of the aorta with the greatest compliance. Secondly, interpretation of the relationship of C-F PWV with compliance is reliant upon the assumption that other variables in the Moens–Korteweg equation remain constant. This assumption, whilst convenient, is unlikely to completely reflect physiological behaviour of the artery [343].

Similarly, only a certain number of CaRP could be measured. At the onset of my study a decision of which parameters to measure was taken based upon the current literature. Were the decision to be revisited with the benefit of hindsight, the choices made may differ. Indeed, the decisions made in the planning of the study should be reviewed cognaissent of the literature at the time of planning. This is a fast moving field and there have been numerous advances in the understanding of the process of arterial pathology, both in CKD patients and beyond, during the course of this study.

Whilst it may appear that some of the findings in this thesis replicate published data, many relevant papers have been published in the three years since October 2008.

Appreciation of this literature may either address the initial hypothesis, or may suggest other hypotheses which could have been addressed to give a deeper insight. Indeed, there have been major advances in all of the areas of study during the three year period of my doctoral study, of which perhaps the most relevant are the understanding of the role of the fetuin sub-fractions in the control of mineralisation, the introduction of the hs-cTnT assay potentially allowing serological quantification of cardiovascular risk in the general population, and finally the emerging field of the senescent VSMC phenotype which is associated with altered OPG secretion.

## Summary of Findings

During the period of this doctoral study, results from this study and others have demonstrated significantly differing roles for the CaRP studied. In chapter 3, the heterogeneous nature of the study cohort is demonstrated using both recognised variables such as age, proteinuria, and stage of CKD, but also using a categorisation of CKD by cause. Extending the work of other groups in humans and other animals, we have shown that in addition to recognised risk factors, OPG was independently associated with C-F PWV, whilst eGFR was not. CPP, a fetuin-A subfraction thought to be a marker of extraosseous mineral stress was also studied and found to be associated with C-F PWV and reduced eGFR. We also demonstrated that total plasma fetuin-A was independently associated the extent of fetuin loss in the urine, and with diabetic status.

RANKL was independently associated with inflammatory markers, whilst OPG and iFGF-23 were independently associated with each other. iFGF-23 was also associated with eGFR and uPCR, whilst OPG was additionally independently associated with age.

hs-cTnT was proposed as a marker of medium and long term cardiovascular risk in this study. This approach was validated in the Cox proportional hazard modelling in chapter 4 and in other studies [273-275,278]. At baseline, hs-cTnT was independently associated with age, eGFR, hsCRP and proteinuria. The adjusted model associating hs-cTNT with these variables was significantly strengthened by the inclusion of OPG, iFGF-23 and RANKL and other non-traditional risk factors.

In chapter 4, baseline eGFR, proteinuria, age and SBP were associated with rate of change of renal function. Further subgroup analysis of the stable cohort by diagnostic category of CKD indicated that the inclusion of age within this model was likely to be a reflection of the different behaviour of these categories. A dichotomous end point analysis again found that eGFR and age were associated with risk of CKD progression. Statin use was protective in this analysis.

Analysis of the factors independently associated with risk of mortality in the study was limited by the non-proportionality of some of the proposed hazard parameters. However, cardiovascular comorbidity, hs-cTnT and OPG:RANKL were independently associated with risk of death. In a final end point analysis, a combined model incorporating renal and patient survival was constructed in which C-F PWV was independently associated with outcome in a time dependent manner, along with tobacco exposure, eGFR and cardiovascular comorbidity. ACEi/ARB use was protective.

Finally, the factors associated with change in aortic stiffness were explored. iFGF-23 was associated with increased stiffening in the entire cohort, whilst total fetuin-A was associated with aortic stiffening in the non-diabetic population.

## Are the CaRP calcification regulatory proteins?

Many of the study findings are compatible with other published work in the field. However, in order to fully comprehend the excess cardiovascular risk in this population, the processes underway at a cellular and sub-cellular level must be clearly understood. As such the advances in the understanding of fetuin-A biology, especially in relation to the scavenger role of the fetuin-A, perhaps within the CPP, but also in matrix vesicles and apoptotic bodies are particularly exciting. Epidemiological evidence supporting the importance of fetuin-A and CPP at a population level is provided by this study.

Conversely, evidence directly implicating some of the other CaRP in the process of arterial stiffening remains lacking or incompletely understood. This is particularly the case for OPG and RANKL. For whilst evidence that OPG has a direct physiological role in arterial stiffening remains notable by its absence, epidemiological studies, including this one, have repeatedly demonstrated that OPG is associated with arterial stiffness and cardiovascular morbidity and mortality.

Moreover, *in vitro* and animal work both indicate that OPG may be protective against vascular calcification.

Recently reported advances in the understanding of the VSMC cell cycle have reinforced this paradox. In order to appreciate this new research, OPG should perhaps be regarded from a different perspective. Instead of viewing OPG solely as a soluble decoy receptor for RANKL, perhaps OPG could be viewed as a cytokine secreted by the failing myocardium or the senescent VSMC.

The relevance of the senescence pathway to the findings of the ACADEMIC study is illustrated by the findings of a recent study. Here microgene array analysis of human aortic VSMC, grown *in vitro* under extended conditions until senescence, demonstrated up regulation of a variety of genes including those encoding BMP-2, MMP and the inflammatory cytokines IL-1β and IL-8 [222]. BMP-2 is a potent inducer of osteoblastic differentiation, as it leads to initiation of *runx-2* expression, a core transcription factor for osteoblastic gene expression [344]. Transcription of OPG was reduced under these conditions.

A reasonable hypothesis to explain these findings would be that a component of the uraemic state, perhaps oxidative stress, leads to progressive damage of the VSMC. This theory is based upon *in vitro* work, but is consistent with work done by other groups demonstrating an increased rate of DNA damage in cells in the aortic wall in patients with CKD [46]. This progressive DNA damage will eventually trigger the cell to leave the cell cycle and enter a senescent phase whereby the VSMC adopts a CESP secreting a variety of factors including BMP-2 – i.e. the VSMC undergoes osteoblastic transdifferentiation.

Were OPG expression to be increased this could neatly explain the association of OPG with arterial stiffness. However OPG expression appears to be **decreased** [222]. In order to accommodate the reduced expression of OPG within any theory of CKD senescence mediated arterial stiffening, the *in vitro* work in which exogenous OPG administration attenuated vascular calcification must be considered [183,345]. It is possible that the role of OPG is to protect against RANKL mediated calcification, and the reduced expression of OPG is therefore consistent with increased expression of pro-calcific BMP-2, with the overall phenotypic aim to increase calcification. However, this theory fails to explain the association of OPG with arterial stiffness.

Studies demonstrating altered OPG expression in the failing myocardium do however provide a potential pathophysiological link. It could be proposed that increased arterial stiffness (which increases left ventricular afterload leading to the development of heart failure) would lead to the increased expression of OPG, thereby associating OPG with aortic stiffness. Other studies have previously demonstrated increased OPG expression in the failing myocardium.

It is unclear if this theory could encompass the association of OPG with cTnT and adverse survival seen in this and other studies. The mechanisms leading to OPG expression within the cardiomyocyte are currently not well understood. Is this related to senescence within the myocardium? If so, is this associated with an oxidative stress mediated SASP, and does this lead to the secretion of OPG? Or alternatively, is this related more directly to increased afterload, hypertrophy, fibrosis and/or ischaemia? Ultimately many questions regarding OPG remain unanswered. What is the source of the increased OPG seen in CKD, the bone, the myocardium or the wider vasculature? What pathological role, if any, does the OPG play within the vasculature?

RANKL was chosen for investigation in this study as it is the circulating ligand to which OPG binds under physiological conditions in the control of bone resorption. As illustrated the epidemiological evidence linking RANKL to arterial stiffening and to cardiovascular outcomes is much less strong than that for OPG. However, notwithstanding this, in the ACADEMIC study RANKL was independently associated with hs-cTnT, and OPG:RANKL was associated with risk of death within the study. RANKL was not associated with arterial stiffness.

Finally, the findings of the study in relation to iFGF-23 must be reconciled. The association of iFGF-23 with hs-cTnT is entirely compatible with other studies in which iFGF-23 has been associated with LVH and mortality. Previously FGF-23 has been implicated in LVH via reports of non-specific binding to FGF receptors, which were theorised to mimic binding of FGF-2, which has previously been implicated in myocardial hypertrophy [346]. Excitingly however, a recent study has demonstrated a klotho independent FGF-23 mediated mechanism of LVH [77,346].

This study and others have not, however, demonstrated an association between FGF-23 and arterial stiffness. Indeed, evidence linking FGF-23 to the VSMC, or alteration

in the extracellular matrix is notable by its' absence. How, therefore, is iFGF-23 associated with progressive arterial stiffening over the first 12 months of the study? This may reflect an unrecognised pathological process or is this association may be either an epiphenomenon or a chance finding

## Implications for Intervention

It is not appropriate to make recommendations on safety or efficacy of patient treatment based upon the results of this observational study of a cohort with a heterogeneous risk profile.

Whilst there is some evidence that sevelamer can increase serum fetuin-A, this association was not tested in the ACADEMIC cohort due to the study design and low number of patients taking this medication. Phosphate was associated with hs-cTnT in the multivariate analysis in this study. However, before taking the leap to suggest that this implies that sevelamer or other phosphate binders should be used in patients with CKD stage 3 & 4 to prevent arterial stiffening and mitigate cardiovascular risk, it would first be important to validate the association of arterial stiffness with adverse outcome in CKD stage 3 & 4 cohorts. Then a repeat demonstration of the association of fetuin-A (or perhaps more relevantly CPP) with progressive arterial stiffening would be required. Other mechanisms must also be explored. Is the increase in fetuin-A associated with an altered glycaemic risk profile? Do phosphate binders reduce FGF-23? Does this have any effect on myocardial fibrosis? Is this effect dependent or independent of vitamin D? What is the role of other medications, for example aldosterone antagonists, on this process?

However, in the clinical domain, medications which directly impact the area of this study are already being used. Denosumab is a fully human monoclonal antibody which binds specifically to RANKL, mimicking the effect of OPG. Denosumab is an effective drug in the treatment of osteoporosis and malignant bone metastases, which has completed phase 3 trials and was licensed in Europe and the USA in 2010 [347-349]. No difference in cardiovascular mortality was seen in the large randomised controlled trials which preceded licensing [348,349]. Denosumab is not identical to OPG. However, whilst care must be taken not to overinterpret the findings of these studies, the results cast doubt upon the theory that particularly OPG, but also RANKL, are causatively linked to cardiovascular outcomes. Instead, they add weight to the theory that OPG may be a molecular bystander.

## Non-studied Areas

There are several aspects of the uraemic cardiovascular phenotype which have not been addressed in this thesis which have the potential to confound the results of the study. Notably, endothelial function was only assessed in the patients recruited prior to my involvement in the study. The impact of oxidative stress is also not addressed in this thesis. Isoprostanes were measured, however it is beyond the scope of this thesis to discuss this aspect of cardiovascular risk. However the role of oxidant stress may come to the fore if the SASP mechanism of vascular calcification proves physiologically relevant.

Importantly, there was also no measure of vitamin D status. Assessment of vitamin D status is complex and expensive with significant variation between assays, physiological variation in titres across the seasons and little consensus on definitions of insufficiency or interpretation of titres after supplementation [350]. Notwithstanding these challenges, vitamin D data would have been of interest, given the general low vitamin D levels associated with CKD and the potential interactions with FGF-23. In addition, there are many other parameters which could have been measured in the study, including BMP 2 and 7, elastin degradation products and pyrophosphate.

Other future areas of study relevant to this project include the development of a greater understanding of the metabolism of the CaRP, particularly fetuin-A. Currently the mechanism of clearance of fetuin-A, and the various sub-fractions of fetuin-A (incorporating scavenged calcium and phosphate) are not well understood and yet appear directly relevant to the process of arterial calcification.

Within the bounds of epidemiological study, however, there are also outstanding areas of interest. Whilst arterial stiffness has been associated with adverse cardiovascular outcomes in the general population and in dialysis cohorts, there is limited published evidence of this relationship in patients with CKD stage 3 & 4. This may be partially explained by the clinically significant heterogeneity of risk in this and other study populations, and the lack of power in relatively small studies.

Completion of repeated measurements of C-F PWV in the full ACADEMIC cohort to three years will provide unique data. In addition, it is hoped that survival data from this and other studies in the UREKA collaboration will allow the hypothesis

that arterial stiffness is associated with mortality in pre-dialysis CKD to be adequately addressed.

## Conclusion

In summary, in this thesis I am reporting aspects of the ACADEMIC study cohort. These results include the study findings relating to the metabolism of total fetuin-A, but also to the CPP fraction. The relationships of the CaRP with arterial stiffness and stiffening at 12 months have been reported. The interim analysis of the association of the CaRP with arterial stiffening at 24 and 36 months are also described. hs-cTnT is proposed as a biomarker of cardiovascular risk within CKD stages 3 & 4, and the factors associated with hs-cTnT are demonstrated. Finally, the relationship of the CaRP with risk of dialysis, death and decline in renal function are detailed.

## Glossary

| | |
|---|---|
| AAC | Abdominal Arterial Calcification |
| ABPI | Ankle Brachial Pressure Index |
| ACADEMIC | Arterial Compliance And oxiDant strEss as predictors of rate of loss of renal function, morbidity and Mortality In CKD |
| ACCT | Anglo-Cardiff Collaborative Trial |
| ACEi | Angiotensin Converting Enzyme Inhibitor |
| ARB | Angiotensin Receptor Blocker |
| ARIC | Atherosclerosis Risk In Communities study |
| ADMA | Assymetrical DiMethylArginine |
| AI | Augmentation Index |
| AKI | Acute Kidney Injury |
| AL | Allison Leslie |
| ANCA | Anti Neutrophil Cytoplasmic Antibody |
| AUC | Area Under Curve |
| B-A | Brachial-Ankle |
| BMD | Bone Mineral Density |
| BMI | Body Mass Index |
| BMP | Bone Morphogenic Protein |
| (NT-pro) BNP | (N terminal pro) Brain Natriuretic Peptide |
| BSUH | Brighton & Sussex University Hospitals NHS Trust |
| CACS | Coronary Artery Calcification Score |
| CACWS | Carotid Artery Circumferential Wall Stress |
| CaRP | Calcification Regulatory Proteins |

| | |
|---|---|
| CCB | Calcium Channel Blocker |
| CESP | Cell-type Exclusive Senescent Phenotype |
| C-F | Carotid-Femoral |
| CHS | Cardiovascular Health Study |
| CI | Confidence Interval |
| CKD(-MBD) | Chronic Kidney Disease (- Mineral and Bone Disorder) |
| CPP | CalciProtein Particle (≡ FMC - Fetuin Mineral Complex) |
| CPHRM | Cox Proportional Hazards Regression Modelling |
| C-R | Carotid-Radial |
| CrCl | Creatinine Clearance |
| CRIB | Chronic Renal Impairment in Birmingham |
| CRIC | Chronic Renal Insufficiency Cohort |
| (hs) CRP | (high sensitivity) C Reactive Peptide |
| CV | Cardiovascular |
| CVr | Coefficient of Variation (standard deviation/mean) |
| CZ | Czech Republic |
| DBP | Diastolic Blood Pressure |
| ECG | Electrocardiograph |
| EDTA | EthyleneDiamineTetraAcetic Acid |
| ELISA | Enzyme Linked ImmunoSorbent Assay |
| EPIC-Norfolk | European Prospective Investigation into Cancer - Norfolk |
| ERA-EDTA | European Renal Association - European Dialysis and Transplant Association |

| | |
|---|---|
| ES | Mr. Edward Smith |
| ESA | Erythropoeisis Stimulating Agent |
| ESRF | End Stage Renal Failure |
| EURECA-M | European Cardiovascular Medicine |
| FGF(-23)(R) | Fibroblast Growth Factor (-23) (Receptor) |
| FMD | Flow Mediated Dilatation |
| (e)GFR | (estimated) Glomerular Filtration Rate |
| H-F | Heart Femoral |
| HR | Hazard Ratio |
| IFN | Interferon |
| IGF1R | Insulin like Growth Factor 1 Receptor |
| IL | Interleukin |
| K-M | Kaplan Meier |
| KDIGO | Kidney Disease Improving Global Outcomes |
| KDOQI | Kidney Disease Outcomes Quality Initiative |
| LDL | Low Density Lipoprotein |
| LgRM | Logistic Regression Model |
| LT | Dr. Laurie Tomlinson |
| LVEF | Left Ventricular Ejection Fraction |
| LVH | Left Ventricular Hypertrophy |
| LVMI | Left Ventricular Mass Index |
| MBP | Mean Blood Pressure |
| MDRD | Modification of Diet in Renal Disease |

| | |
|---|---|
| MF | Dr. Martin Ford |
| MGP | Matrix Gla Protein |
| MLR | Multiple Linear Regression |
| MMKD | Mild to Moderate Kidney Disease |
| MMP | Matrix Metalloproteinase |
| mRNA | Messenger RiboNucleic Acid |
| NF-κB | Nuclear Factor κ B |
| NHS | National Health Service |
| NICE | National Institute for Health and Clinical Excellence |
| NO | Nitric Oxide |
| OPG | Osteoprotegerin |
| OR | Odds Ratio |
| PD | Peritoneal Dialysis |
| PIVUS | Prospective Investigation of the Vasculature in Uppsala Seniors |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| PPi | Pyrophosphate |
| PTH | Parathyroid Hormone |
| PWA | Pulse Wave Analysis |
| PWV | Pulse Wave Velocity |
| QOF | Quality Outcomes Framework |
| RD-P | Radial Dorsalis Pedis |
| RSCH | Royal Sussex County Hospital |
| (Δ)RI | (Change in) Reflection Index |

| | |
|---|---|
| ROC | Receiver Operator Curve |
| RANK(L) | Receptor Activator of Nuclear Factor κ B (Ligand) |
| RRT | Renal Replacement Therapy |
| SASP | Senescence Associated Secretory Phenotype |
| SBP | Systolic Blood Pressure |
| SD | Standard Deviation |
| SHARP | Study of Heart and Renal Protection |
| $SI_{DVP}$ | Stiffness Index of Digital Volume Pulse |
| TIA | Transient Ischaemic Attack |
| TNF | Tumour Necrosis Factor |
| (hs)-cTnT | (highly sensitive) cardiac Troponin T |
| TRAIL | TNF Related Apoptosis Inducing Ligand |
| TREAT | Trail to Reduce cardiovascular Events with Aranesp Therapy |
| uP(F/A)CR | Urine Protein (Fetuin/Albumin) Creatinine Ratio |
| UK | United Kingdom |
| UREKA | UK REsearch alliance into Kidney disease and Arterial stiffness |
| USA | United States of America |
| USRDS | Unites States Renal Data System |
| VSMC | Vascular Smooth Muscle Cell |
| VIF | Variance Inflation Factor |

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## Appendix One

### Peer Reviewed Publications

**Ford ML**, Tomlinson LA, Smith ER, Rajkumar C & Holt SG. Fetuin-A is an independent determinant of change of aortic stiffness over 1 year in non-diabetic patients with CKD stages 3 and 4. NDT 2010; 25:1853-8.

**Ford ML**, Tomlinson LA, Chapman TPE, Rajkumar C, Holt SG. Aortic stiffness is independently associated with rate of renal function decline in chronic kidney disease stages 3 and 4. Hypertension 2010; 55:1110-5.

**Ford ML**, Tomlinson LA, Chapman TPE, Cheek E, Rajkumar C, Holt SG. Macro-meets micro-circulation – a response. Hypertension 2010; 56:e172.

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Smith ER, **Ford ML**, Tomlinson LA, Weaving G, Rocks BF, Rajkumar C, Holt SG. Instability of fibroblast growth factor-23 (FGF-23): implications for clinical studies. Clin Chim Acta 2011; 412:1008-11.

Smith ER, **Ford ML**, Tomlinson LA, Weaving G, Holt SG, Rajkumar C, Chatterjee PK. Factors associated with increasing vascular stiffness in PD. NDT 2011; 26: 2060-1.

**Ford ML**, Smith ER, Tomlinson LA, Chatterjee PK, Rajkumar C, Holt SG. FGF-23 and osteoprotegerin are independently associated with myocardial damage in chronic kidney disease stages 3 and 4. Another link between chronic kidney disease-mineral bone disorder and the heart. NDT 2012; 27:727-33.

Smith ER, **Ford ML**, Tomlinson LA, Rajkumar C & Holt SG. Phosphorylated fetuin-A containing calciprotein particles are associated with aortic stiffness and a procalcific milieu in patients with pre-dialysis CKD. NDT 2011 epub ahead of print Nov. 20.

Smith ER, **Ford ML**, Tomlinson LA, Rajkumar C, McMahon L & Holt SG. Elastin degradation is associated with progressive aortic stiffening and all-cause mortality in pre-dialysis CKD. Hypertension accepted Feb. 2012

## Abstracts and Oral Presentations

Tomlinson LA, **Ford ML**, Leslie AR, Kingswood JC, Holt SG & Rajkumar C. Ambulatory methods of measuring arterial stiffness in a cohort of subjects with CKD 3 & 4. Poster at Renal Association 2008.

**Ford ML**, Tomlinson LA, Smith ER, Rocks BF, Rajkumar C, & Holt SG. Fetuin-A is independently associated with progressive arterial stiffness in patients with CKD. Poster at ASN 2008.

**Ford ML**, Tomlinson LA, Smith ER, Rocks BR, Rajkumar C & Holt SG. Change in fetuin-A is inversely related to change in aortic stiffness in a cohort of patients with CKD stages 3 & 4. Oral Presentation ERA-EDTA V International Symposium Advances in Bone and Mineral Disorders in CKD 2009 and SWEKS 2009.

Chapman TP, Tomlinson LA, **Ford ML**, Rajkumar C & Holt SG. Aortic stiffness is independently associated with rate of decline of renal function in patients with CKD 3 & 4. Oral presentation UK Renal Association, 2009.

**Ford ML**, Tomlinson LA, Smith ER, Rajkumar C, Kingswood JC, Holt SG. Plasma osteoprotegerin concentration is an independent determinant of aortic stiffness in CKD stages 3 & 4. Poster presentation to ASN 2009. Oral presentation to St. George's Cardiorenal Group Symposium, 2009.

Smith ER, **Ford ML**, Tomlinson LA, Rajkumar C & Holt SG. Fetuin-A may not be associated with inflammation in CKD stages 3 & 4. Poster at ERA-EDTA 2010 & ASN 2010.

**Ford ML**, Smith ER, Tomlinson LA, Rajkumar C, & Holt SG. Plasma osteoprotegerin and proteinuria are independently associated with highly sensitive troponin T in CKD stages 3 & 4. Oral presentation to Artery Society Annual Meeting 2010. Artery Research 2010; 4:156 (8.6).

**Ford ML**, Smith ER, Tomlinson LA, Chatterjee PK, Rajkumar C & Holt SG. FGF-23 and osteoprotegerin are independently associated with myocardial damage in

CKD stages 3 & 4. Oral presentation to Australia & New Zealand Society of Nephrology (ANZSN) 2011, Poster presentation UK Renal Association 2011. Nephrology 2011;16:(S1) 68.

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Tomlinson LA, **Ford ML**, Smith ER, Holt SG & Rajkumar C. Blood pressure variability is associated with aortic stiffness and troponin-T in patients with CKD stages 3 and 4. Poster presentation to UK Renal Association and Artery Society 2011.

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Williams SK, Tomlinson LA, **Ford ML**, Cheek E, Leslie AR, Holt SG & Rajkumar C. A nocturnal rise in ambulatory blood pressure is associated with increased aortic stiffness in patients with Chronic Kidney Disease stage 3 and 4. Accepted as oral presentation at European Society of Hypertension 2012.

**Ford ML**, Smith ER, Tomlinson LA, Chatterjee C, Rajkumar C & Holt SG. Troponin-T is independently associated with mortality in patients with CKD stages 3 & 4. Submitted to UK Renal Association 2012.

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## Appendix Two

## Pulse Wave Analysis

### Augmentation Index (AI)

AI is a unitless figure which is derived from analysis of the pressure waveform of the pulse. The measured pressure wave consists of two superimposed waves. A wave generated by the heart travelling towards the periphery and a second wave travelling in the opposite direction. This second wave is formed when the forward wave is reflected from points of impedance mismatch within the systemic circulation. Superimposition of these waves leads to augmentation of the pressure attributable to the forward pressure wave alone. This augmented pressure corresponds to the peak late systolic blood pressure. Augmentation pressure is defined as the pressure increase above the first systolic peak (see fig. A1) [351].

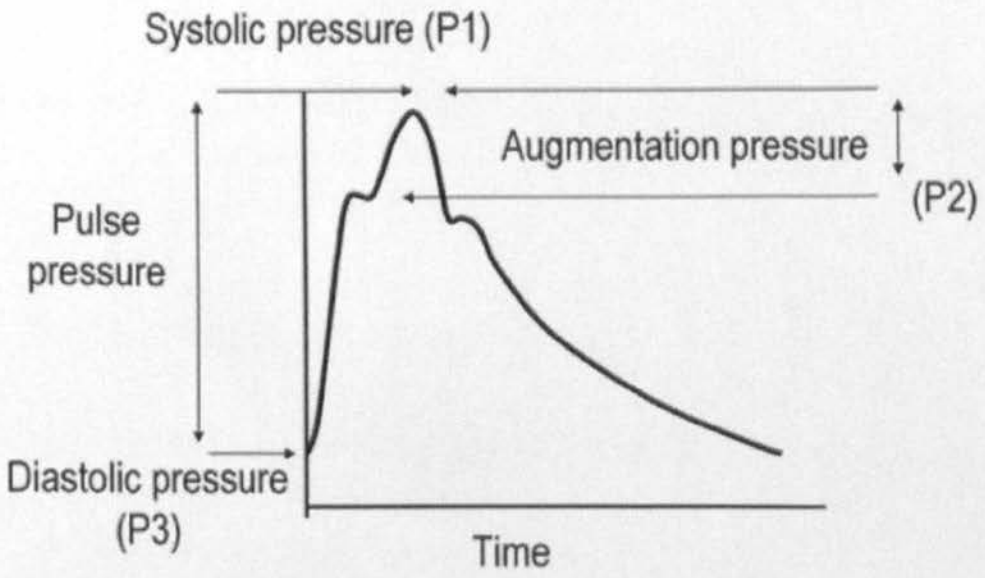


**Figure A1 - Carotid pulse pressure waveform plotted against time of one cardiac cycle [14]**

$$\text{Augmentation Index (AI)} = \frac{\text{Augmentation Pressure (P1-P2)}}{\text{Pulse Pressure (P1-P3)}} \times 100$$

Alterations in the properties of the resistance vessels, for example in response to changes in blood pressure, change the reflection characteristics of the arterial tree. An increase in peripheral resistance will lead to earlier reflection of the arterial pressure wave from the parts of the arterial tree where the resistance is exerted. This causes the reflected wave to arrive earlier in the pressure cycle leading to an increase in AI. Changes in AI are therefore not simply be related to stiffening in the descending aorta, but also incorporate factors related to changes in the resistance vessels. AI, as a measure of stiffness, is therefore confounded by wave reflection.

In addition other sources of error include a greater operator dependence and a reliance upon a general transfer function (mathematical approximation) to derive a central pressure waveform used in some forms of the AI.

**Reflection Index (RI)**

As previously discussed, the height of the systolic peak is determined by the additive effects of two waves. However the time of the arrival of the reflected wave is determined by the point of reflection (i.e. distance travelled) in addition to the speed. The point of wave reflection is, in turn, partially determined by arterial resistance, itself partially determined by endothelial function.

RI is the ratio of the relative height of diastolic inflection point to the pulse pressure (see fig. A2). In the healthy state stimulation of the artery with a β2 agonist, such as terbutaline, causes NO release from the endothelium with associated vasodilatation, reduction in arterial pressure and distal movement of the point of reflection. This results in later arrival of the reflected wave and therefore reduction in RI. In the healthy state therefore a large reduction in RI (ΔRI) would be expected [352]. It has therefore been proposed that ΔRI measures the change in proportion of the pulse pressure which can be attributed to the reflected wave. It is thus primarily a measure of endothelial dependent vasodilation.

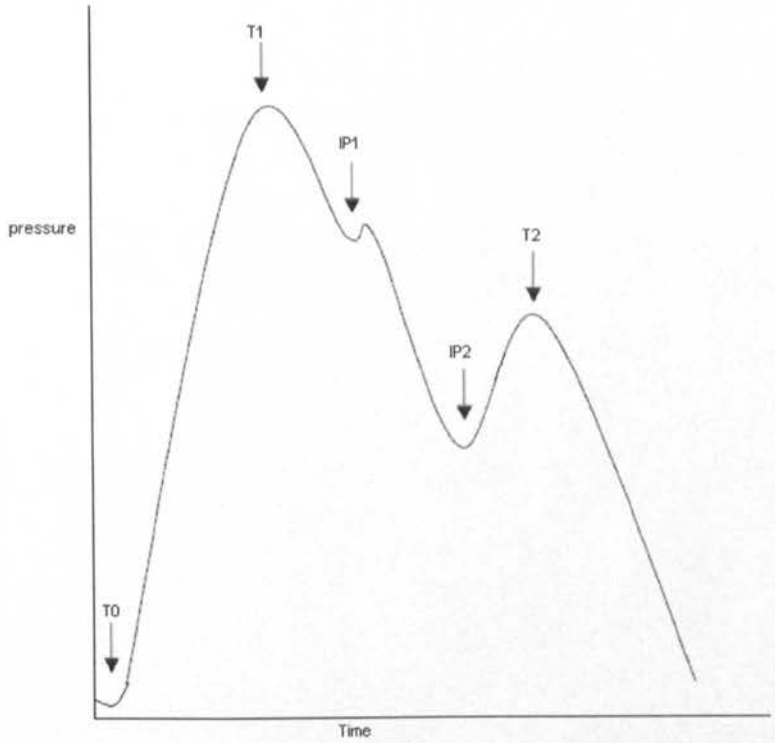


**Figure A2 - Pulse pressure waveform of the radial artery across one cardiac cycle. T0-trough pressure; T1-peak systolic pressure; T2-peak diastolic pressure; IP1-systolic inflection point; IP2-diastolic inflection point (beginning of diastole)** [352(adapted)]

$$\Delta RI = 100 \times \frac{IP2_i - T0_i}{T1_i - T0_i} \Big/ \frac{IP2_0 - T0_0}{T1_0 - T0_0}$$

**IP2-diastolic inflection point, T0-trough pressure; T1-peak systolic pressure; o-baseline measurement, i-post β2 agonist**

## Beta

Beta is a unit-less parameter of stiffness derived from systolic and diastolic pressures, together with echo-tracking measurement of arterial diameter at a specific point in the arterial tree [23].

$$\beta = \ln\left(\frac{Ps}{Pd}\right) \cdot \left(\frac{Dd}{Ds - Dd}\right)$$

**P-pressure; D-diameter; s-systolic; d-diastolic**

Whilst there is some evidence of prognostic value of this measure in dialysis patients [353], it has not been widely adopted outside of one Japanese centre. The limitations of this technically more complex measure include the greater technical skill required and therefore greater operator dependence, the use of brachial blood pressure as a substitute for local blood pressure at the site of measurement, and the localised point of measurement, i.e. carotid. This measure is thought to represent cardiac afterload less well than C-F PWV which encompasses the major capacitance vessel of the body, i.e. the aorta [14].

## Stiffness Index of Digital Volume Pulse ($SI_{DVP}$)

$SI_{DVP}$ is calculated from the ratio of total body height to the time delay between direct and reflected waves in the digital circulation. Whilst this measure is primarily a measure of large artery compliance it is, again, confounded by reflection. In this measure body height is used as a surrogate for the point of reflection, and the time period measured relates to travel of the pulse wave over large and small calibre arteries [354]. Again, this measure of arterial compliance lacks validation by means of outcome data.

$$SI_{DVP} = \frac{h}{T2\text{-}T1}$$

**h-height; T2-time of diastolic peak; T1-time of systolic peak**

# Appendix Three

## Repeatability Sub-Study

In order to understand variation in any measured values the sources of this variation must be appreciated. In addition to the true variation in a parameter, a further potential source of error arises from the technique of measurement. This error may be random or may consist of a systematic bias. Furthermore this error may arise from differences in operator technique (inter-observer error) or from differences between episodes of measurement (intra-observer error).

C-F measurement using Complior™ necessitates two operators. In order to assess the reproducibility of the measurement of arterial stiffness, a study of repeated measurement was performed. The same two operators (MF and AL) were used throughout the repeatability study. This study is therefore a study of intra rather than inter-observer variability.

## Method

Ten healthy individuals who were not known to have cardiovascular disease were recruited. Anthropometry and blood pressure measurement were performed in the same manner as for subjects in the main study. PWV measurements were then performed on two occasions not less than seven days apart. Two sets of data were recorded. The final result taken forward for intra-observer variability analysis was the mean of two data sets. The PWV values were adjusted for MBP prior to subsequent analysis

Intra-observer variability was not performed on ACADEMIC subjects.

### Statistical Analysis of Repeatability

The mean across visit PWV and the difference between the PWV measurements from each visit were calculated. Distribution of data was assessed using the Shapiro-Wilk test. Paired student's t-test was used for comparison between visits.

The differences between visits are displayed using a Bland-Altman plot [355]. This plot has three lines overlaid. These represent the mean difference ±2 SD. The range of mean ± 2SD should include 95% of readings and is termed the 'limits of agreement'.

This method was used rather than correlation coefficients as this allows for examination of the size and distribution of variation, in particular its relationship to the size of the absolute mean value.

## Results

The mean age of the subjects was 34 ± 11years, five were male and five were female. The mean (±SD) interval between visits was 25 ± 21 days. The blood pressure, heart rate and adjusted C-F over the two visits are shown in table A1.

| Parameter | Visit 1 | Visit 2 | Difference | p value |
|---|---|---|---|---|
| SBP (mmHg) | 124 ± 15 | 124 ± 18 | 1 ± 11 | 0.886 |
| DBP (mmHg) | 76 ± 11 | 75 ± 11 | 1 ± 6 | 0.526 |
| Heart rate (bpm) | 69 ± 11 | 66 ± 11 | 3 ± 5 | 0.070 |
| C-F PWV$^{\dagger}$ (m/s) | 9.9 ± 1.3 | 9.4 ± 1.3 | 0.5 ± 0.6 | 0.026$^{*}$ |

**Table A1 - Cardiovascular parameters and adjusted PWV measurements - repeatability study (n=10) - $^{\dagger}$Adjusted for MBP**

There was no significant change in SBP, DBP or HR between visits. There was a small, but statistically significant decrease in C-F PWV from the first visit to the second visit.

The differences between paired and mean measurements are illustrated in fig. A3.

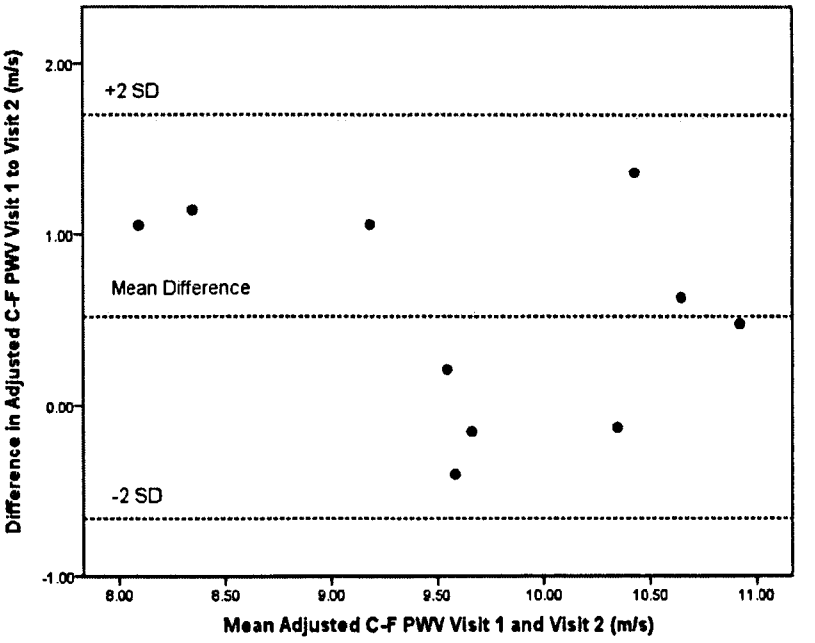


**Figure A3 - Bland-Altman plots of the agreement of C-F PWV (m/s) measurement in 10 healthy volunteers across the range of mean adjusted C-F PWV (m/s) measured on two occasions. Dashed lines indicate limits of agreement at mean difference ± 2SD**

## Discussion

This sub-study shows that values of aortic stiffness measured using this technique in the group of healthy volunteers were significantly lower at the time of second reading compared to the first, despite no significant change in blood pressure.

There are various explanations for this variation. Firstly, the process or knowledge of the first measurement may have altered the subsequent measurement. As illustrated, the process of C-F PWV measurement involves exposing the groin of the subject and palpating the femoral artery. Anecdotally, on the first occasion that this is performed, most patients become slightly anxious. This anxiety is usually much reduced on subsequent visits. It is likely that this anxiety will be associated with an acute rise in blood pressure with associated increase in arterial stiffness. Measurement of the clinic blood pressure precedes measurement of C-F PWV and therefore does not take this into account. Other potential mechanisms include alteration to the physicochemical structure of the artery between the first and second visits. This is unlikely. Alternatively, this could also be a chance finding in a relatively small group.

The PWV values used in the study are automatically generated by the Complior™ software. The role of the operator is to enter blood pressure and demographic data,

and to hold the probe over the corresponding artery. It is therefore unlikely that the variability found in this study is a reflection of operator error. Moreover, the results in this sub-study are consistent with the repeatability results of a similar study of healthy controls, which demonstrated a borderline reduction in C-F PWV (measured using Complior™) with no significant reduction in blood pressure over three months [356]. All recorded C-F PWV values fell within the limits of agreement. There was no systematic bias related to parameter size.

## Conclusion

This sub-study demonstrated a small but statistically significant difference between C-F PWV values between measurements in a cohort of healthy younger volunteers. It is not clear if this variability was also present in the cohort of patients in the main study.

Analysis of the arterial stiffness results for the main study comprises both a study of the baseline results and also of the parameters associated with change in arterial stiffness over time. Intra-observer error must therefore be considered as a potential confounder in this analysis.

## Appendix Four

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I give permission for photographs of myself to be included in the thesis of Dr. Martin Ford. I understand that this thesis is available to the public and that a copy will be kept in the British library.

Signed V. Miles

Name & Address

Valerie Miles

Date

5.4.11

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21 January 2006

Professor C Rajkumar
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Brighton
BN2 5BE

Dear Professor Rajkumar

**Full title of study:** **Arterial Compliance and Oxidant Stress as predictors of rate of loss of renal function, morbidity and mortality in Chronic Kidney Disease**

**REC reference number:** **05/Q1911/89**

Thank you for your letter of 10 January 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

**Ethical review of research sites**

The Committee has designated this study as exempt from site-specific assessment (SSA. There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

**Conditions of approval**

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| *Document* | *Version* | *Date* |
|---|---|---|
| Application Parts a & B only | 2 | 10 January 2006 |
| Investigator CV | | |

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23 October 2008

Professor C Rajkumar
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Dear Professor Rajkumar

| | |
|---|---|
| **Study title:** | **Arterial Compliance and Oxidant Stress as predictors of rate of loss of renal function, morbidity and mortality in Chronic Kidney Disease** |
| **REC reference:** | **05/Q1911/89** |
| **Protocol number:** | **1** |
| **Amendment number:** | **AM02** |
| **Amendment date:** | **16 October 2008** |

**Re: Extension of Study for a further 3 years and addition of Dr Martin Ford to the list of other key investigators.**

Thank you for your letter of 21 October 2008, notifying the Committee of the above amendment.

The Committee does not consider this to be a "substantial amendment" as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

**Documents received**

The documents received were as follows:

| Document | Version | Date |
|---|---|---|
| Notification of a Minor Amendment | AM02 | 16 October 2008 |