

https://dspace.org/

Factors impacting on left ventricular hypertrophy in haemodialysis patients

Chabu, James

2008-10-23T08:35:25Z
http://hdl.handle.net/10539/5802

Downloaded from a DSpace repository. https://dspace.org/

CHAPTER 1

INTRODUCTION

Cardiovascular disease in chronic renal failure: causes and controversies

1.0 INTRODUCTION

With the significant advances in renal replacement therapy (RRT) that have occurred in the recent past, a paradox has emerged in chronic kidney disease (CKD) patients. Patients with end-stage renal disease (ESRD) now die more frequently from cardiovascular disease (CVD) than from uraemia (Agodoa LY and Eggers PW, 1995, McGregor E et al., 1998, Valderrabano F et al., 1995). Cardiovascular disease accounts for 40–55% of deaths in uraemia as reported in international registries (London GM and Fabiani F, 1992, Silberberg JS et al., 1989, McGregor E et al., 1998, U.S. Renal Data System, 2006). Cardiovascular disease mortality is 10-to-30 times higher in patients treated by dialysis than in patients in the general population, independent of sex, race, and the presence of diabetes mellitus (Foley RN et al., 1998). Cardiovascular mortality rates in ESRD are estimated to be between 100 and 1000 times higher than expected in young adults (Middleton RJ et al., 2001). In CKD patients, the risk of death particularly due to cardiovascular disease, is much higher than the risk of eventually requiring RRT (Foley RN et al., 2005, Keith DS et al., 2004). Consequently, practice guidelines from the National Kidney Foundation in 2002 and the American College of Cardiology/American Heart Association task force in 2004 recommend that CKD be considered a coronary artery disease (CAD) risk equivalent (NKF, 2002, Antman EM et al., 2004).

The spectrum of clinically expressed CVD in patients with ESRD includes dilated cardiomyopathy and congestive heart failure (CHF), ischaemic heart disease (IHD) and peripheral vascular disease (PVD)(Foley RN et al., 1998, Sarnak MJ and Levey AS, 2000). Amongst patients with ESRD on haemodialysis or peritoneal dialysis, the prevalence of IHD

and CHF is ~40% as compared to 5-12% in the general population (Foley RN et al., 1998). *De novo* and recurrent CHF occurs in a substantial proportion of patients on dialysis, and impacts on morbidity and mortality, as well as the ability to deliver adequate dialysis (Valderrabano F et al., 1995).

With the awareness of CVD as a major cause of death and morbidity in CKD, attention in more recent years has focussed on the earlier detection of CVD in patients with CKD using intermediate cardiovascular phenotypes, such as left ventricular hypertrophy (LVH) and changes in vascular structure and function. The importance of detecting intermediate cardiovascular phenotypes is related to the substantial evidence that many of these phenotypes refine the ability to predict cardiovascular events independent of conventional cardiovascular risk factors. In this regard, LVH is an important adverse prognostic indicator independent of all other conventional cardiovascular risk factors in the general population and in hypertension (Levy D et al., 1990, Koren MJ et al., 1991). Moreover, changes in large vessel structure and function also predict cardiovascular outcomes independent of all other conventional cardiovascular risk factors in the general population, in hypertensives and in patients with renal failure (Shokawa T et al., 2005, Laurent S et al., 2001, Blacher J et al., 2003).

The excessive prevalence of LVH and structural and functional vascular changes in renal failure are well established. As early as 1836, LVH and other abnormalities were recognized as a frequent occurrence in patients with ESRD (Bright R, 1836), data that has subsequently been supported by many studies (see section 2.0). More recently, with the development of sophisticated methods of assessing large vessels, there is also now substantial evidence that ESRD is associated with abnormal vascular structure and function (see section 3.1). However, what is not entirely clear is exactly what determines these intermediate

cardiovascular changes in chronic renal failure and the extent to which each factor contributes toward LVH and large artery dysfunction. Although there is data to support a number of hypotheses, controversy still remains in this regard. Consequently, this study is aimed at contributing toward our understanding of the pathophysiology of LVH and large artery dysfunction in patients with ESRD on haemodialysis (HD).

The introduction to this thesis will review the evidence to indicate that LVH and large vessel dysfunction are important changes in CKD. Secondly, the potential factors that may determine LVH and large vessel changes in CKD will be discussed. As part of that discussion, I will highlight the controversies surrounding the factors that may determine LVH and large vessel dysfunction in CKD and advance some novel hypotheses tested in the present thesis and justify these hypotheses.

1.1 Left ventricular hypertrophy in chronic kidney disease: prevalence and consequences

1.1.1 Prevalence of left ventricular hypertrophy in chronic kidney disease

There is now substantial evidence to indicate that LVH is a common finding in renal disease (Levin A et al., 1996, Johnstone LM et al., 1996, Mitsnefes MM et al., 2000, Foley RN et al., 1998, London GM and Parfrey PS, 1997). The prevalence of LVH closely correlates with renal function and varies according to glomerular filtration rate (GFR). In this regard, LVH exists in 27% of individuals with a GFR ≥50 ml/min, in 31% of those with a GFR 25-to-49 ml/min, and in 45% of those with a GFR <25 ml/min (Levin A et al., 1996). In

patients with CKD, the prevalence of LVH is approximately 40%, a figure that rises to around 75% at the onset of ESRD (Foley RN et al., 1995, Levin A et al., 1999, Middleton RJ et al., 2001). In the Canadian Prospective Cohort Study of 433 incident dialysis patients, 74% of patients had LVH at baseline, with 44% having concentric LVH and 30% eccentric LVH (Foley RN et al., 1995). The prevalence figures of LVH in ESRD appear to be consistent irrespective of the population group studied. In the Zimbabwe Echocardiographic Study of 60 patients on maintenance haemodialysis (MHD), LVH was found in 68% of patients. However, there is some discrepancy in prevalence figures for LVH in CKD in Africa. In a highly select group of pre-dialysis patients with type -2 diabetes mellitus reaching ESRD in Nigeria (Agaba E I et al., 2004), LVH was reported in only 14% of the patients. Moreover, in a pre-dialysis population of patients with stage 4 and 5 CKD in Nigeria, 95.5% had LVH (Ulasi II et al., 2006).

1.1.2 The relationship between left ventricular hypertrophy and cardiovascular disease in end stage renal disease

As occurs in the general population or in hypertensives (Levy D et al., 1990, Koren MJ et al., 1991), LVH is a key prognostic variable for cardiovascular mortality in ESRD (Middleton RJ et al., 2001). Patients with renal failure and LVH have a two-to-three-fold higher death rate from cardiac causes (Goodman WG and Danovitch GM, 2004). Among patients with LVH, two-thirds die from heart failure or sudden death, while the remaining one-third die from a noncardiovascular event (Stewart GA et al., 2005, Paoletti E et al., 2004). Worsening of LVH in renal failure is a strong predictor of sudden death and arrhythmias, which is also associated with an increased QT interval and dispersal on the electrocardiogram

(2005, Paoletti E et al., 2004). The mechanisms of heart failure in patients with LVH and ESRD are not entirely clear, but heart failure in patients with uraemic-induced LVH may be attributed to adverse cardiac remodelling (cardiac dilatation) and pump dysfunction rather than diastolic dysfunction. In the Zimbabwe Echocardiographic study of sixty patients on MHD, LV diastolic dysfunction was similar between patients with and those without LVH (Hakim JG et al., 1996). In contrast, progressive LV dilation, which becomes less reversible with time, seems to be the most characteristic morphological pattern of patients receiving dialysis who develop CHF (Foley RN et al., 1995, London GM et al., 1987).

The relationship between LVH and CVD in patients with CKD has resulted in The Kidney Disease Quality Outcomes Initiative (K/DOQI) guidelines recommending that, at initiation of dialysis, all patients should undergo baseline echocardiography and electrocardiography (National Kidney Foundation, 2005). Echocardiography should be performed after dry weight is attained (which usually occurs after one to three months), and should be repeated routinely at three yearly intervals thereafter.

1.2.0 Alterations in large artery function in end stage renal disease: Evidence and consequences

1.2.1 Large artery dysfunction in end stage renal disease

Patients with ESRD develop arteriosclerosis and remodelling of large arteries (London GM et al., 2002), changes that lead to alterations in large artery function. There is evidence that large artery stiffness, as indexed by aortic pulse wave velocity (PWV), a measurement that is generally not affected by variable changes in acute haemodynamic factors such as heart

rate (Yasmin and Brown MJ, 1999, Wilkinson IB et al., 2002, Lantelme P et al., 2002), is increased in patients with CKD (Shinohara K et al., 2004). In this regard, as compared to healthy individuals, patients with renal failure have increases in PWV as measured both before and after HD (Covic A et al., 2000). Moreover, increases in PWV in patients on HD are similar and may even exceed those noted to occur in hypertensives (Blacher J et al., 1999, Covic A et al., 2000), a group of patients who are well recognised as developing increased arterial stiffness as a consequence of target organ changes (Roman MJ et al., 1992, Kingwell AB and Cameron JD, 2006).

Chronic HD and renal transplantation reverse increases in PWV. PWV was reported to be higher in 144 patients on MHD (mean systolic blood pressure 153 +/- 26) as compared to 140 control subjects (mean systolic blood pressure 128 +/- 17), but lower than in 71 patients with ESRD prior to HD (Shinohara K et al., 2004). In that study HD was associated with decreased aortic PWV independent of renal failure and other confounding factors. Further analysis of the combined uraemic patients indicated a favourable impact of HD on aortic PWV independent of classical cardiovascular risk factors, or the use of antihypertensive medications. HD is not the only therapeutic modality that modifies PWV in patients with renal failure; successful renal transplantation also reduces the increase in PWV noted to occur in renal failure, with levels of PWV decreasing to lower values (by 7-22%) than those in patients receiving HD (Zoungas S et al., 2004).

1.2.2 Relationship between large artery dysfunction and cardiovascular disease in end stage renal disease

There is presently fairly substantial evidence to indicate that increases in large artery stiffness independently predict CV mortality in renal disease. In a cohort of 242 patients with ESRD undergoing HD for a mean (±SD) duration of 78±46 months, arterial stiffness, as indexed by aortic PWV, was noted to be a strong and independent predictor of both CV and overall mortality, even after a number of adjustments, including for age and time on dialysis (Blacher J et al., 2003). Further, in 241 patients with ESRD undergoing HD and followed up for a mean duration of 72±41 months, age and aortic PWV emerged as the only independent predictors of all-cause and CV mortality (Blacher J et al., 1999). These results have been further substantiated in a study conducted in 265 ESRD patients on HD (Shoji T et al., 2001).

Pulse pressure (PP), which reflects the impact of both LV ejection and aortic stiffness, and is thus a more readily available index of arterial stiffness, has similarly been independently associated with an increased all-cause mortality in patients receiving HD (Tozawa M et al., 2002). In a prospective cohort study of 180 patients with CKD on MHD, followed for a mean duration of 52 ± 36 months, carotid pulse pressure and aortic PWV were strong independent predictors of all-cause (including cardiovascular) mortality. Brachial blood pressure and pulse pressure, had no predictive value for mortality (Safar ME et al., 2002).

There are many potential explanations for the independent relationship between arterial stiffness and CV morbidity and mortality in chronic renal failure. Increases in arterial stiffness may promote adverse CV effects by augmenting central artery systolic blood pressures (BP) and thus increasing central BP, a change that may not be accurately reflected in peripheral BP measurements (Covic A et al., 2000, Wilkinson IB et al., 2001). Central augmentation index (AIc) is similarly an independent predictor for all-cause and CV mortality in patients with

ESRD (London GM et al., 2001) and in a cohort of 51 patients receiving HD, patients whose AIc remained increased after HD had a more dilated LV than those patients for whom HD restored AIc (Covic A et al., 2000). Nevertheless, the independent relationship between arterial stiffness and CV morbidity and mortality in renal failure may still reflect chronic target organ changes, changes that cannot be detected with conventional CV risk factors.

1.3 Causes of left ventricular hypertrophy and large artery dysfunction in chronic renal disease

There are many potential causes of LVH and large artery dysfunction in the general population. However, in renal failure these causes occur more frequently and with increased magnitude. The general causes of these CV target organ changes and the potential causes in chronic renal failure will be outlined in the following sections. The controversies that presently exist regarding the potential causes of LVH and large artery dysfunction in renal disease will be outlined; in addition to this study which attempts to address these controversies.

1.3.1 Causes of left ventricular hypertrophy in general and in chronic renal failure

In the following discussion the general haemodynamic causes of LVH in any population sample will be outlined, restricting the discussion to concepts that are pertinent to LVH in renal failure, and the factors associated with LVH in renal failure will be detailed, outlining the controversies surrounding these theories.

1.3.1.1 Causes of left ventricular hypertrophy in general

Physiologically, LVH is primarily an adaptive remodelling process, compensating for an increase in workload placed on the heart with the aim of minimising ventricular wall stress. Two contrasting models of adaptation may develop depending on the patterns of stress imposed on the heart. These models are based on haemodynamic factors that modify cardiac wall stress. Pressure overload, which is caused, for example, by hypertension or aortic stenosis, requires the generation of greater intracavity pressure during ventricular contraction in order to generate a normal stroke volume. Based on the law of Laplace, to maintain wall stress, an increase in wall thickness and a fall in cavity volume (a reduced internal radius) is the consequence. This form of chamber remodelling is called *concentric hypertrophy*. In contrast, volume overload, which is caused for example, by salt and water retention, anaemia and arteriovenous fistulae (Watkins LO, 1984, United States Renal Data System, 1999., Mann JF et al., 2001), requires the capacity to accommodate greater filling volumes whilst maintaining normal filling pressures. An increase in wall thickness occurs that is nevertheless proportional to the increase in LV diameter and *eccentric hypertrophy* is the consequence (Watkins LO, 1984, United States Renal Data System, 1999., Mann JF et al., 2001). Although these models of cardiac hypertrophy have been considered as discrete entities, there is no doubt that in pressure overload states, concentric LVH may progress to eccentric (Norton et al., 2002). Moreover, these models of LVH do not address the issue of potential neurohumoral, cellular and molecular changes that mediate LVH.

1.3.1.2 Causes of left ventricular hypertrophy in chronic renal failure

The geometric changes associated with LVH in renal failure cast light on the pathogenesis of excessive cardiac growth in renal insufficiency. Concentric LVH is common amongst patients with CKD with a prevalence of approximately 40% in those starting dialysis (United States Renal Data System, 1999., Mann JF et al., 2001). However, as indicated in the aforementioned discussion, in the Canadian Prospective Cohort Study of 433 incident dialysis patients, 74% had LVH at baseline, with 44% having concentric LVH, whilst 30% had LVH with an eccentric LV geometry (Foley RN et al., 1995). These approximate proportions of concentric and eccentric LVH are also noted in paediatric patients at the beginning of dialysis therapy (Mitsnefes MM et al., 2000). Thus, the factors that determine LVH in CKD are likely to generate both pressure and volume overload. Although increasing age is a major risk factor for LVH in dialysis patients (Harnett JD et al., 1994), the mechanisms of this effect are unclear or at least likely to be related to alternative factors not associated with renal pathology. Hence, age will not be discussed. However, a number of haemodynamic and cellular changes have been postulated to cause LVH in renal failure and the evidence and controversies regarding the contribution of each will be described in subsequent discussion. Moreover, new previously unexplored mechanisms studied in the present thesis will be advanced in this section and the justification for these hypotheses outlined.

1.3.1.2.1 Blood pressure effects on left ventricular mass

Hypertension is a well established cause of renal failure, a common complication and an important risk factor for the progression of renal disease (Malvinder S Parmar, 2002, Middleton RJ et al., 2001). A major cause of concentric LVH in chronic renal failure is hypertension. Hypertension is well recognized as contributing to the development of LVH and the associated mortality in renal failure (Zager PG et al., 1998). In the only prospective study performed in a dialysis population, a blood pressure of <140/90 mm Hg was noted to minimise the occurrence of LVH and death (Foley RN et al., 1996).

Hypertension is prevalent amongst patients receiving HD with 50%-60% of 2,535 patients reported as having hypertension, defined as a BP >150/90 mm Hg (Agarwal R et al., 2003). The prevalence is even higher if the Seventh Report of the Joint National Committee for the Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC VII) definitions of hypertension in adults in the general population of systolic BP ≥140 mm Hg and diastolic BP ≥90 mm Hg, and a normal BP <120/80 mm Hg (Chobanian AV et al., 2003) are employed. In a study of 2,535 patients on HD only 14% were normotensive on no drugs, according to these criteria. Moreover, amongst the hypertensive patients, only 30% had BP values that were controlled (Agarwal R et al., 2003). In the Choices for Healthy Outcomes in Caring for ESRD (CHOICE) study, the prevalence of hypertension was 96% in the study participants (Longenecker JC et al., 2002).

The aetiology of hypertension in ESRD is multifactorial and includes expanded extracellular volume, sodium retention, overactivity of the sympathetic nervous system (Morse SA et al., 2003, Zager PG et al., 1998), activation of the renin angiotensin aldosterone system (RAAS), excessive endothelin activation (Schleiffer T et al., 1994) and endothelial

dysfunction, prostaglandin effects, the use of erythropoietin, secondary hyperparathyroidism, and increases in large artery stiffness (Morse SA et al., 2003, Zager PG et al., 1998). However, there is presently controversy over the major factor that mediates hypertension in chronic renal failure, with some postulating that at least 80% of all hypertension in patients on dialysis is due to chronic hypervolaemia (Charra B et al., 1983, Lins RL et al., 1997), with others suggesting that the major factor increasing central BP is an increase in arterial stiffness (London GM et al., 1990); (London GM et al., 1999).

Although hypertension in renal failure is an established major determinant of LVH, the best method of measuring BP to predict LVH in renal disease is still uncertain. Measuring BP using conventional approaches is in any circumstance problematic; with digit-preference, observer bias, “isolated office hypertension” and lability of BP, being a few of the more obvious problems (Pickering TG, 1994). Moreover, a problem of assessing BP in patients on dialysis is that if one relies on BP values obtained at the time of an outpatient dialysis session, there is a very poor correlation between these peri-dialysis values and BP taken away from the dialysis unit during the inter-dialytic interval. In a comparison of pre- and post-HD oscillometric BP readings with 44-hour interdialytic ambulatory BP (ABP) and "home BP" values in 70 chronic HD patients; predialysis systolic BP (SBP) was 13.5 mm Hg higher and post-dialysis BP 3.8 mm Hg higher than ABP (Agarwal R and Lewis RR, 2001). Moreover, as underscored in a recent mini-review, predialysis systolic BP generally overestimated ABP and the limit of agreement between the two BP values was + 41.7 to -25.2 mm Hg (Agarwal R et al., 2006). Predialysis diastolic BP also overestimated ABP with wide limits of agreement (+ 23.7-to-18.9mmHg) (Agarwal R et al., 2006). In contrast, post-dialysis BP underestimated average ABP with wide limits of agreement for both post dialysis systolic BP (+ 33.1 to -36.3 mmHg) and diastolic BP (+ 19.3 to -23.9 mmHg) (Agarwal R et al., 2006). Some have

suggested a combination of predialysis and post dialysis blood pressures as a better measure of mean interdialytic blood pressure (Coomer RW et al., 1997). Thus, there is controversy as to whether pre- or post-dialysis BP would closely correlate with left ventricular mass index (LVMI) in patients with renal failure.

Three studies, with the largest being a study conducted in 64 patients (Zoccali C et al., 1999), have assessed the question of whether multiple pre- or post-dialysis BP measurements are as closely associated with LVMI as ABP. In this study the average of 12 pre-dialysis BP values had the same predictive power for left ventricular mass (LVM) as single 24-hour period of ABP monitoring after adjustments for body mass index (BMI), haematocrit and serum cholesterol concentrations (Zoccali C et al., 1999). Prior studies showing an equivalent effect of multiple conventional measurements on LVMI as compared to 24-hour ABP were performed in smaller study samples and did not adjust for alternative confounders (Conlon PJ et al., 1996, Erturk et al., 1996). Clearly the small sample size may have limited the outcome in these three studies. Consequently, more studies with larger study samples are required to assess this question. This study explores whether 24-hour ABP is more closely associated with LVMI in patients receiving HD than multiple pre- and post-dialysis conventional BP measurements.

The argument that pre- and post-dialysis BP measurements may not be the best assessments for predicting LVMI is further supported by the findings that measurements of nocturnal BP may be essential in predicting adverse effects in renal failure. A reduction in the normal nocturnal decline in BP is observed in CKD and in those undergoing HD (Andersen MJ et al., 2005). In patients undergoing dialysis, the prevalence of an attenuated nocturnal decline in BP varies from 22.2% (Korzets Z et al., 1994) to 80-100% (Ertuk S et al., 1996). An attenuated fall in BP during sleep in patients with chronic renal disease is associated with

eccentric LVH (Covic A et al., 2000). However, alternative studies failed to show a relationship between night-day ratios of ABP and LVMI (Zoccali C et al., 1999, Roman MJ et al., 1997). Thus, further studies are required to assess whether pre- and post-dialysis BP values are as closely associated with LVMI as nocturnal BP in patients with renal failure. As part of study I assessed whether diurnal BP was associated with LVMI in a larger study sample than previously assessed and whether nocturnal BP was more closely associated with LVMI than multiple pre- or post-dialysis conventional BP measurements.

1.3.1.2.2 Arterial stiffness effects on left ventricular mass

A major potential cause of concentric LVH in chronic renal failure could be an increased afterload secondary to increases in large artery stiffness and thus central BP. As central BP is not closely reflected by brachial artery BP measurements (Covic A et al., 2000, Hashimoto J et al., 2007) , it is possible that the impact of uraemic-induced increases in large artery stiffness on LVM cannot be completely accounted for by brachial artery BP measurements. Whether arterial stiffness is associated with LVH in patients with ESRD has not been well established. In 49 patients on chronic HD, PWV was associated with LVH independent of conventional systolic BP measured at the brachial artery (Nitta K et al., 2004). However, in this study neither multiple conventional BP measurements, nor ABP were employed to adjust for the impact of PWV on LVMI. Moreover, in a number of studies performed in populations without renal failure, the conventional brachial artery BP-independent relationship between PWV and LVM has been questioned (Chen CH et al., 1998, Bouthier JD et al., 1985, Baguet JP et al., 2000). Thus, further work is required to establish a relationship between PWV and LVM independent of conventional CV risk factors including

BP measured at the brachial artery. This study therefore explores whether PWV and other indices of large artery dysfunction are associated with LVMI with a view to establish whether the association is independent of conventional office BP, multiple pre- and post-HDs BP measurements and ABP.

1.3.1.2.3 The impact of fluid overload on left ventricular mass

There is no question that overhydration in chronic renal failure contributes to increases in blood volume and cardiac output (Chaignon M et al., 1981). Body fluid volume contraction during dialysis reduces cardiac filling volumes and cardiac output and there is a correlation between interdialytic changes in body weight (which reflects reductions in body fluid volume) and LVM (Harnett JD et al., 1993). Although the impact of fluid overload on LVM may be mediated through BP effects, ultrafiltration and a reduced salt intake in patients on HD reduces LVM independent of BP changes (Özhahya M et al., 1998). However, in assessing the impact of fluid overload on LVM in chronic renal failure, there are no data that have adjusted for fluid overload effects on multiple BP measurements, such as ambulatory or multiple pre- and post-dialysis BP. It is therefore still possible that the LVM changes noted with fluid overload are a consequence of alterations in after- and not preload on the heart, and that multiple measurements of BP are required to assess afterload effects. A further problem with ascertaining whether blood volume and preload are principle determinants of LVM in chronic renal failure is how to most accurately assess fluid overload. In this regard, two of the more recent developments in this assessment are measures of atrial (ANP) or brain (BNP) natriuretic peptides and IVCD. One of the aims of my study was therefore to assess whether an increase in blood volume is a primary determinant of LVM in patients receiving HD independent of

multiple measures of BP (pre- and post-dialysis BP and ABP). ANP and BNP measurements as well as measurements of inferior vena cava diameter (IVCD) were undertaken as possibly more accurate estimates of fluid volume than those traditionally employed (body weight or clinical assessments and BP). The role of ANP and BNP measurements as well as measures of IVCD in assessing body fluid volume status in renal failure will be described in the following subsections.

1.3.1.2.3.1 Measurement of inferior vena cava diameter

The IVC dilates in right-sided cardiac failure (Weil F and Maurat P, 1974) and supine measurements of IVCD taken during expiration (inspiration decreases IVCD) and the IVC collapsibility index (fractional reduction of IVCD during the breathing cycle) correlate with central venous pressures (Tamaki S, 1981). Thus IVCD and the IVC collapsibility index are thought to be appropriate estimates of body fluid status in CKD, as filling pressures in the heart in kidney disease are more likely to reflect fluid overload rather than intrinsic cardiac dysfunction. IVCD has been noted to be a useful estimate of dry weight in patients on HD (Ando Y et al., 1985) and post-dialysis IVCD correlates with right atrial pressure and circulating blood volume (Cheriex EC et al., 1989). Importantly, in this study nearly two-thirds of patients who had met clinical criteria for euhydration were overhydrated. Further studies have shown that post-dialysis IVCD measurements reliably predict haemodynamic changes during dialysis (Leunissen KM et al., 1993, Kouw PM et al., 1993). Therefore, although there are questions regarding intra - and inter-observer variability for IVCD assessments (Jaeger JQ and Mehta RL, 1999), measurement of the IVCD is nevertheless a feasible option for rapid assessment of intravascular volume status in an outpatient dialysis setting by operators with

limited formal training in echocardiography (Brennan JM et al., 2006). The American Society of Echocardiography's guidelines and standards committee and the Chamber Quantification Writing Group in conjunction with the European Association of Echocardiography, have recently recommended that examination of the IVC from the subcostal view should be included as part of the routine trans-thoracic echocardiography examination (Lang R M et al., 2005). Measurements of IVCD and the collapsibility index were therefore selected as the most appropriate indices of body fluid status in patients receiving HD in this present study.

1.3.1.2.3.2 Natriuretic peptides as indicators of volume status in renal failure

This study explored a potential role for both ANP and BNP and their related pro-hormones in the assessment of volume status in patients receiving HD, and subsequently their role in renal failure.

1.3.1.2.3.2.1 The natriuretic peptide family

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) (also known as B-type natriuretic peptide), C-type natriuretic peptide (CNP), D-type natriuretic peptide, and their prohormones, comprise the major members of the natriuretic peptide family (Joffy S and Rosner M H, 2005). The role of C and D-type natriuretic peptides is presently unclear. However, the role of ANP and BNP is fairly well established. Natriuretic peptides respond to increases in extracellular fluid volume sensed by atrial (ANP) and ventricular (BNP) stretch receptors (Humphreys MH and Valentin J-P, 2000). ANP and BNP have a number of beneficial effects on the CV system, including the promotion of natriuresis and diuresis via

activation of cyclic guanosine monophosphate (cGMP) (Humphreys MH and Valentin J-P, 2000), but a detailed discourse on their actions goes beyond the scope of the present thesis.

The precursor protein peptide (pre proANP) is cleaved into a 98 amino acid amino-terminal fragment and mature ANP fragments which circulate in the plasma. ANP is rapidly cleared from the plasma with a half life of 3-4 minutes. ProANP (1-98 amino acids (aa)) has a much longer half-life (60-120 minutes) which leads to significantly higher concentrations in blood compared to ANP. Four peptide hormones comprise the ANP prohormone (126-aa ANP prohormone) including proANP-(1—30); long-acting natriuretic peptide (LANP); vessel dilator proANP-(31—67); kaliuretic proANP-(79—98); and α-ANP. Each of these four peptide hormones circulates in healthy humans, with LANP and vessel dilator concentrations in plasma being 15- to 20-fold higher than ANP and 100-fold higher than BNP (De Palo EF et al., 2000).

Brain natriuretic peptide is synthesized by human cardiac myocytes as a prohormone (proBNP), which is cleaved to BNP and the N-terminal fragment of proBNP (NT-proBNP). The biologically active BNP, the proBNP and the remaining portion of the pro-hormone NT-proBNP all circulate in the plasma. Brain natriuretic peptide is not stored in secretory granules and its release is dependent on continued transcription and translation of its gene (de Bold AJ et al., 2001). The major stimulus for ANP secretion is atrial stretch or distention which stimulates secretion of previously synthesized ANP stored in secretory granules and atrial stretch also quickly leads to an increase in ANP gene transcription and messenger RNA abundance (Gardner DG et al., 1992). The difference in secretory patterns of ANP and BNP is reflected in the differing values of these hormones in patients with heart

failure, for whom BNP provides a better index of LVM and load than ANP level, which is more reflective of volume status (Nakao K et al., 1992).

In normal subjects the plasma concentrations of NT-proBNP and BNP are similar. However, in patients with pathology, NT-proBNP concentrations rise more than BNP with plasma concentrations increasing 2–10 times higher than BNP (Vanderheyden M et al., 2004). BNP concentrations increase in both LVH and with heart failure.

Atrial natriuretic peptide and BNP are removed from the circulation by binding to natriuretic peptide receptors and through degradation by neutral endopeptidases present on the surface of many endothelial cells (Panteghini M and Clerico A, 2004). However, NT-proBNP does not bind to either natriuretic peptide receptors and is not degraded by neutral endopeptidases; therefore, clearance is believed to occur solely in the kidney (Panteghini M and Clerico A, 2004). The lower affinity of BNP for natriuretic peptide receptors partly accounts for the longer plasma half-life of BNP as compared to ANP (Kone BC, 2001).

1.3.1.2.3.2.2 Natriuretic peptides in renal failure

The measurement of ANP and related hormone concentrations has been suggested as an indicator of volume status in patients receiving HD (Rascher W et al., 1985). Many studies have shown elevated plasma concentrations of natriuretic peptides and their prohormones in patients with chronic renal failure. In this regard, a 7-fold elevation in plasma BNP concentrations and a 4-fold elevation in plasma ANP levels occur in patients with ESRD before HD compared with plasma BNP and ANP concentrations in healthy volunteers (Kohse K et al., 1993). With respect to ANP specifically, ANP concentrations are reduced after both

haemofiltration (Zoccali C et al., 1986) and HD (Anderson JV et al., 1986). To decrease ANP concentrations in chronic renal failure may however require ultrafiltration and not just dialysis alone (Shiota J et al., 1990, Wahl H et al., 2004). In patients with ESRD a correlation between the percentage decrease in plasma ANP concentrations and loss of body weight during the dialysis session has been noted (Hasegawa K et al., 1986). There is however a drawback in the use of ANP concentrations as a predictor of volume status. Atrial natriuretic peptide concentrations may be persistently elevated after HD in patients with altered left atrial haemodynamics as compared to those with normal left atrial haemodynamics (Leunissen KM et al., 1989). Consequently, ANP concentrations may often remain elevated in "dry" individuals (Jaeger JQ and Mehta RL, 1999). Thus, alternative indices of volume status may be required to predict volume status in chronic renal failure.

Although increases in BNP concentrations appear to be greater than the increases in ANP concentrations noted in patients with ESRD (Kohse K et al., 1993), ANP concentrations decrease to a much greater extent during HD than BNP concentrations, and ANP concentrations correlate better with changes in volume status. This is consistent with the notion that BNP concentrations are determined more by changes in LVM and afterload than volume status (Joffy S and Rosner M H, 2005). However, the interpretation of these studies is hampered by a lack of rigorous measurement of volume status. Nevertheless, in 15 patients post-dialysis, decreases in ANP, but not BNP concentrations were associated with reductions in left atrial dimensions and IVCD (Ishikura F et al., 1996). These data are however in contrast to the relationship between pre-HD IVCD and BNP concentrations in another study of 49 patients (Lee SW et al., 2003). Moreover, in a separate study, volume reductions with HD were associated with changes in BNP concentration (Fagugli RM et al., 2003).

Relationships between volume status and BNP concentrations in chronic renal failure are partly confounded by increases in LVM, which in itself is associated with elevated plasma BNP concentrations. BNP concentrations correlated directly with the presence of LVH in 164 patients receiving HD, (Naganuma T et al., 2002). Nevertheless, in 213 predialysis patients with CKD, estimated GFR and LVMI were independently related to plasma BNP and NT-proBNP concentrations, but NT-proBNP appeared to be affected more by declining kidney function than LVMI (Vickery S et al., 2005). The lack of clarity regarding the role of ANP and BNP in predicting volume status in chronic renal failure, prompted me to study IVCD as an index of volume status and adjust for the confounding relationship between either ANP or BNP and LVMI.

With respect to alternative measurements that could be employed to predict volume status in chronic renal failure, cGMP, which is generated when ANP activates membrane-bound guanylate cyclase, has been proposed as a potential index of fluid status. In this regard, plasma cGMP concentrations of 20 pmol/L immediately post dialysis correlate with the achievement of clinical dry weight; the majority of those with post dialysis levels greater than 20 pmol/L have evidence of fluid overload; and reductions in dry weight are associated with both a reduction in cGMP concentrations to approximately 20 pmol/L and clinical resolution of the fluid overloaded state (Lauster F et al., 1990, Lauster F et al., 1993, Lauster F et al., 1992). However, some authors have not confirmed these findings (Jaeger JQ and Mehta RL, 1999, Franz M et al., 2000). Consequently, in the present thesis plasma cGMP concentrations were not measured.

1.3.1.2.4. **Parathyroid hormone and left ventricular mass**

Renal failure, through a number of effects, including an inability to convert vitamin D to its active form, results in secondary hyperparathyroidism. Parathyroid hormone (PTH) is now widely acknowledged as a permissive factor for the development of cardiac hypertrophy (Amann K et al., 1994). By virtue of its effect on calcium metabolism, it is known to promote intramyocyte calcium overload, a change that could directly contribute toward the development of LVH (Massry SG and Smogorzweski M, 1994, Middleton RJ et al., 2001). Importantly, myocardial calcium deposition is common in ESRD (Rostand SG et al., 1988). Alternatively, PTH can promote aortic valve calcification and thus increase LVMI through increases in LV afterload (Raine AE, 1994). Despite some data to suggest that PTH contributes to LVM in renal disease, (Amann K et al., 1994, Randon RB et al., 2005, Nasri H et al., 2004, Wanic-Kossowska M et al., 2002, Strózecki P et al., 2001); (Stack AG and Saran R, 2002) other studies have failed to show similar findings (London et al 1997;(Fujii H et al., 2007) except in patients who had markedly elevated PTH concentrations (Fujii H et al., 2007). However, in favour of a role for calcium metabolism as a determinant of LVH in chronic renal disease, is evidence that suggests that vitamin D therapy, which would alleviate secondary hyperparathyroidism, has beneficial effects on LVM (Achinger SG and Ayus JC, 2005, Kim HW et al., 2006). Nevertheless, because of the controversy surrounding a role for PTH in mediating LVMI, in the present study this issue was not addressed. However, I assessed whether independent of vitamin D, PTH concentrations are associated with cardiovascular target organ changes, in order to determine whether PTH should be employed as an adjustor in subsequent analysis.

1.3.1.2.5 Anaemia and left ventricular mass

Anaemia is another potential cause of eccentric LVH in CKD. Anaemia in CKD is primarily caused by a decreased production of erythropoietin by the failing kidney (Eschbach JW, 1989). Anaemia produces a number of effects that mediate LVH. Anaemia results in systemic arterial dilatation, a decrease in peripheral vascular resistance, and hence both an increased venous return and an increased preload as well as a reduced afterload (Pereira AA and Sarnak MJ, 2003, Sarnak MJ and Levey AS, 2000). Anaemia also results in a decreased blood viscosity which leads to increase in venous return and an increased preload. Moreover, anaemia results in an increased sympathetic activity which results in increase in heart rate, myocardial contractility and venous tone. An increased venous return, heart rate, contractility and venous tone with a decreased afterload all raise cardiac output (Pereira AA and Sarnak MJ, 2003) and hence promote LVH. Thus, anaemia has independently been associated with the development of LVH (Foley RN et al., 1996).

The role of anaemia in LVH is underscored by the ability of erythropoietin administration to partly regress LVH in both experimental models of uraemia-induced LVH (Tyralla K and Amann K, 2003) and in humans with renal failure (Dahan M et al., 1997, Eschbach JW et al., 1992, Portoles J et al., 1997). However, this has not been consistent across all studies. In the Canadian Normalization of Haemoglobin Trial (Foley RN et al., 2000), patients with either asymptomatic concentric LVH or LV dilatation were randomly assigned to receive doses of epoeitin (erythropoietin) designed to achieve a target haemoglobin level of either 10gm/dl or 13gm/dl. There was no change in LVMI in both groups at the end of 48 weeks. Nevertheless, a potential explanation for the lack of benefit of erythropoietin on LVM in this study is that normalisation of haemoglobin may not impact on LVM when cardiac disease is already established. In the Anaemia CORrection in Diabetes (ACORD) study, in

patients with diabetes mellitus with mild-to-moderate anaemia and moderate LVH, correction of anaemia to haemoglobin target concentrations of 13 to 15 g/dL (130 to 150 g/L) did not decrease LVMI. However, normalisation of haemoglobin concentrations prevented an additional increase in LVH (Ritz E et al., 2007). While the role of anaemia as a predictor of LVMI was not primarily explored in the present study, I nevertheless assessed whether haemoglobin concentrations were associated with LVMI and arterial stiffness, in order to determine whether haemoglobin should be employed as an adjustor in subsequent analysis.

1.3.1.2.6 Arterio-venous fistulae and left ventricular mass

The creation of a HD arterio-venous access (AVA) results in an increase in preload, and hence, like volume retention and anaemia, could promote further increases in LVM. The creation of a HD AVA is independently associated with further progression of already existing LVH (Ori Y et al., 2002). The increase in LVM associated with the creation of an AVA is accounted for mostly by an increase in interventricular septal thickness, whereas LV end-diastolic diameter and posterior wall thickness do not change (Ori Y et al., 2002). Bradycardia during fistula or graft occlusion by finger pressure suggests that the AV shunt is importantly and pathologically contributing to an increased cardiac output (Branham's sign) (Weiner DE et al., 2007). Although the role of an AVA in uraemia-induced LVH was not specifically explored in the present study, branham's sign was however negative in all 42 patients with AVAs.

1.3.1.2.7 Neurohumoral, autocrine and metabolic effects on left ventricular mass

In animal models of uraemic LVH, while LVH is not associated with hypertension, it may be prevented by angiotensin-converting enzyme (ACE) inhibitors (Tornig J et al., 1996), agents that reduce central sympathetic outflow (Tornig J et al., 1996), and endothelin receptor antagonists (Nabokov AV et al., 1999). Thus, there is evidence that neurohumoral and autocrine changes may contribute to LVH in uraemia apparently independent of haemodynamic changes. With respect to autocrine effects, bradykinin may contribute to the effect of ACE inhibition in ameliorating uraemic LVH (Amann K et al., 2000, Middleton RJ et al., 2001).

With respect to potential metabolic effects on LVM, there appears to be an interaction between nephropathy and diabetes mellitus. LVMI increases in proportion to the progression of diabetic nephropathy in type 2 diabetic patients (Suzuki K et al., 2001) and diabetes mellitus has been identified as a predictor of LVH in patients on dialysis (Parfrey PS et al., 1996). Patients with diabetes mellitus were excluded from this study in order to avoid a potential confounding effect of diabetes mellitus on LVM.

1.3.1.2.8 Ischaemic heart disease and left ventricular mass

Ischaemic heart disease is an established cause of LVH in hypertension and uraemia. In 963 hypertensive patients (mean age 66 ± 7 years, 41% women) with electrocardiographic LV hypertrophy, and divided into 149 with and 814 without clinical (prior myocardial infarction or angina pectoris) or electrocardiographic evidence of CAD, patients with CAD had larger LV internal dimensionsions and LV mass (Zabalgoitia M et al., 2001). Ischaemic heart disease was an independent and significant predictor of the presence of LV dilatation in the Canadian prospective study (Parfrey PS et al., 1996). The prevalence in the black race is

low. A study in Soweto in the 1960s found CAD accounted for less than 1% deaths (Gill GV and Ouwerkerk J, 1995). The Haemodialyis Study reported black race was associated with a 36% reduction in CAD (Cheung AK et al., 2000). Although this was not studied in the present thesis, importantly no participants had evidence of ischaemic heart disease and none were on therapy for this condition.

1.3.1.2.9 Collagens and collagenases: Role in left ventricular structure

A number of recent studies have demonstrated relationships between markers of interstitial changes and alterations in cardiac structure in population groups other than patients with chronic renal failure. These changes could also explain some of the cardiac structural changes noted to occur in renal failure and this was a hypothesis explored in the present thesis. Thus, the following section reviews the substances responsible for interstitial changes in the heart and the evidence to suggest that some of these substances when measured in the circulation may index cardiac structural abnormalities.

1.3.1.2.9.1 Collagen and collagenases

Fibrillar collagen facilitates the arrangement of the cardiomyocytes, thus maintaining the integrity of the myocardium (Spinale F G, 2002). Myocardial collagen undergoes continual turnover due to a balance of collagen synthesis and degradation by the proteolytic action of matrix metalloproteinases (MMPs) (MacKenna D et al., 2000). Collagens regulate tissue remodelling during growth, differentiation, and morphogenesis and wound healing. The fibril-forming collagens are synthesized as procollagen molecules, which have propeptides at the N and C-terminal ends of their polypeptide chains. Five different collagen subtypes are present

in the human myocardium, but the predominant subtypes are types I (stiff; 80% of the total collagen content) and III (elastic; 10% of the total collagen content) (Weber KT et al., 1994). Various factors such as interleukin (IL)-1-β (Siwik DA et al., 2000), platelet-derived growth factor (PDGF) (Simm A et al., 1998), transforming growth factor (TGF)-β (Eghbali M et al., 1991), angiotensin II (Lijnen PJ et al., 2001), aldosterone (Neumann S et al., 2002) and mechanical stretch (Lee AA et al., 1999) influence collagen synthesis. Growth factors and cytokines are also known to influence collagen degradation by modulating the expression of MMPs and their tissue inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

Humans have 23 MMPs, the activities of which are very low or negligible in normal steady-state tissue, but expression of which is upregulated in disease states by inflammatory cytokines, growth factors, hormones, cell–cell and cell–matrix interactions (Nagase H and Woessner JF Jr, 1999). Only those MMPs that have been associated with cardiovascular abnormalities in previous studies will be discussed. Matrix metaloproteinase-1 (collagenase) is able to cleave interstitial collagens I, II, and III and additionally acts on collagen VII, VIII, X, pro-MMP-2, and pro-MMP-9 (Gueders M M et al., 2006). Gelatinase A (MMP-2) and B (MMP-9) readily digest denatured collagens, called gelatins. Matrix metalloproteinase-2, but not 9, also digests type I, II, and III collagens as with collagenase (Aimes RT and Quigley JP, 1995, Patterson ML et al., 2001). In addition, the gelatinases digest collagen IV,V, X and XIV (Gueders M M et al., 2006). Matrix metalloproteinase-2 and 9 can be distinguished by the fact that MMP-2 binds preferentially to TIMP-2, which is required for its activation, whereas MMP-9 is preferentially inhibited by TIMP-1 (Baker A H et al., 2002).

1.3.1.2.9.2 Plasma Collagen and collagenases as potential markers of cardiac structural abnormalities

From data obtained in preclinical studies, increases in myocardial collagen content and alterations in MMP and TIMP expression are well recognized changes that occur in association with LVH in hypertension (Laviades C et al., 1998). Thus it is reasonable to hypothesise that changes in plasma concentrations of substances indicative of myocardial interstitial turnover could be used as markers of adverse effects on the heart. In a study of 699 Framingham Study participants who had no history of heart failure or myocardial infarction, plasma MMP 9 concentrations were associated with increased LV dimensions and increased LV wall thickness in men (Sundström J, 2004). Moreover, in hypertensives with LVH, plasma MMP 9 concentrations were noted to be increased and plasma MMP 2 concentrations decreased (Ahmed et al 2006). In addition, hypertensives with LVH have decreased plasma MMP 1 and carboxy-terminal propeptide of procollagen type I concentrations (PIP) (marker of collagen synthesis), and higher concentrations of TIMP-1 (Laviades et al 1998) and plasma PIP concentrations are associated with myocardial fibrosis in hypertension (Querejeta R et al., 2000). Taken together, these data provide evidence to suggest that a substantial proportion of the variability of LVM changes in pathology may be accounted for by indices of interstitial myocardial changes. However, this hypothesis has never been tested in patients with chronic renal failure. Consequently, as part of the present thesis I evaluated whether plasma concentrations of PIP, MMP1, MMP-2, MMP-9, TIMP-1,TIMP-2 and C-terminal telopeptide of type I collagen (ICTP) are associated with LVMI in patients on HD.

1.3.2 Causes of large artery dysfunction in chronic renal failure

As discussed in the earlier sections of this chapter, a target organ change that occurs in chronic renal failure that heralds early CV events is an increase in large artery stiffness. Large artery stiffness is in-turn best assessed from measures of aortic PWV. In the following section the potential factors that may cause increases in PWV and alternative large artery changes in renal failure are reviewed, highlighting the outstanding issues that still need to be addressed in the context of the study described in this thesis.

Increased arterial stiffness in patients receiving dialysis is likely to be the consequence of a number of factors. At a histological level fibroelastic intimal thickening, an increased extracellular matrix, and vascular medial calcification are implicated in the pathogenesis of large artery dysfunction. From a pathophysiological perspective, in addition to conventional CV risk factors, the causal factors that have been implicated in chronic renal failure include excessive increases in BP, a chronic flow-volume overload and the effects of salt retention, vascular calcification, inflammatory changes, activation of the renin-angiotensin system and effects of lipid accumulation on the vascular wall (London GM et al., 2002).

1.3.2.1 Blood pressure and arterial stiffness

Increases in BP are independently associated with indices of large artery stiffness in chronic renal failure (Blacher J et al., 1999). Further, PWV frequently improves when BP is reduced (Asmar RG et al., 1988). In well-controlled, treated hypertensives the annual progression in PWV is similar to that of normotensives and significantly lower than in treated

hypertensives with poor BP control (Benetos A et al., 2002). Although BP is undoubtedly a major factor involved in mediating increases in arterial stiffness in renal failure, what has not been evaluated in this regard is the BP measurement that most appropriately predicts arterial stiffness as a target organ change. Consequently, I explored whether multiple conventional pre-dialysis and post-dialysis BP measurements were as closely associated with arterial stiffness measurements as 24-hour ambulatory BP. Furthermore, I evaluated whether night BP was a better predictor of arterial stiffness than day BP or multiple pre- and post-dialysis BP measurements.

1.3.2.2 Fluid overload and arterial stiffness

Rats receiving a high-salt diet display an increased vascular stiffness and altered arterial wall composition that precedes BP increases by weeks (Limas C et al., 1980). Thus, volume overload may contribute to increases in vascular stiffness, a change that may in-part explain vascular stiffening in renal failure; in patients with renal failure on HD, arterial stiffness is related to hydration status (Lin YP, 2003). Increased extracellular-to-intracellular fluid ratio measured by bioimpedance spectroscopy is associated with an increased PWV, common carotid artery incremental modulus, AI and common carotid artery diameter in patients receiving HD (Lin YP, 2003). Moreover, volume status as determined from clinical criteria (BP, oedema, etc) has previously been associated with PWV (Tycho Vuurmans JL et al., 2002). However, whether other predictors of hydration status which are more objective, such as IVCD are associated with indices of arterial stiffness still requires further study. Hence

the present study examines the relationship between IVCD and indices of arterial stiffness in patients with chronic renal failure on HD.

1.3.2.3 Vascular calcification and arterial stiffness

Several clinical and autopsy studies provide strong evidence to indicate that vascular calcification is prevalent in patients with renal failure and particularly in patients with diabetes mellitus on dialysis (Clyne N et al., 1986). The increased arterial stiffness noted to occur in chronic renal failure is strongly related to mediacalcinosis (Nitta K et al., 2004), even after correcting for age, duration of dialysis therapy, prescribed dose of calcium-containing phosphate binders, and microinflammatory status (Haydar AA et al., 2004). In addition, both plasma calcium and alkaline phosphatase concentrations are associated with large artery stiffness (Seyrek N et al., 2003), changes which may be accounted for by calcification of large arteries. Moreover, the relationship between increases in aortic stiffness and CV mortality in chronic renal failure may be explained by vascular medial calcification (Guerin AP et al., 2000). The exact mechanisms of vascular calcification in chronic renal failure are uncertain; however a potential mechanism may be via secondary hyperparathyroidism. This study explores the possibility that plasma PTH concentrations are associated with PWV and other indices of large artery dysfunction.

1.3.2.4 Inflammation and arterial stiffness

There is modest evidence to support a view that inflammatory changes may result in increases in arterial stiffness in chronic renal failure. In this regard, in patients receiving HD, PWV was associated with C-reactive protein concentrations (CRP) and after adjustments for confounding factors, improvements in aortic stiffness after long-term HD were negatively associated with the serum CRP concentrations (London GM et al., 2003). Moreover, in a separate study, plasma CRP concentrations were independently associated with large artery stiffness in 32 patients receiving HD (Seyrek N et al., 2003). As a potential relationship between inflammatory changes and large artery dysfunction in chronic renal failure was not explored, I cannot exclude this potential mechanism as a contributory factor in mediating large artery changes in chronic renal failure.

1.3.2.5 Neurohumoral activation and large artery stiffness

Neurohumoral activation in chronic renal failure may promote increases in large artery stiffness. Plasma angiotensin II concentrations are associated with PWV in patients receiving HD (Tycho Vuurmans JL et al., 2002). Moreover, RAAS inhibition reduces arterial stiffness beyond BP control (Asmar RG et al., 1988). Combined volume reduction and ACE inhibition similarly has an enhanced effect on arterial stiffness as compared to volume reduction alone (Tycho Vuurmans JL et al., 2002).

1.3.2.6 Lipid effects on arterial stiffness

There is some evidence that accelerated atherogenesis, mediated by lipid effects, contributes toward large artery dysfunction in chronic renal failure. The administration of lipid lowering agents (statins) was noted to modify large artery changes in 22 patients with diabetes mellitus receiving HD (Ichihara A, 2002). However, the effects of statins on arterial compliance are more pronounced in muscular arteries than in the aorta or carotid artery (Smilde TJ et al., 2000). Therefore, there is some question as to whether lipid abnormalities in chronic renal failure modify central as opposed to peripheral large artery stiffness to a clinically significant degree. Changes in peripheral artery stiffness are unlikely to produce major effects on central BP.

1.3.2.7 Collagens, collagenases and arterial stiffness

The predominant collagen subtypes found in the normal human arterial wall are type-I (70–75%) and type-III (20–25%) collagen, with type-V collagen comprising 1–2% (Barnes MJ and Farndale RW, 1999). A change in the proportions of collagen subtypes may markedly affect the mechanical properties of the vessel wall. Alterations in the composition of the collagen subtypes occur in hypertensive rats, a change that is associated with stiffer arteries (Chamiot Clerc P et al., 1999). With respect to changes in circulating markers of interstitial fibrosis in 46 normotensive and hypertensive subjects, plasma concentrations of MMP-1, the enzyme responsible for collagen type-I degradation, was positively related to both large elastic and muscular artery stiffness in normotensive and hypertensive subjects (McNulty M et al., 2006). Moreover, in 116 subjects with isolated systolic hypertension and 114 matched

controls, as well as 447 individuals free from CVD, plasma MMP-9 concentrations correlated with aortic PWV (r=0.45; p=0.001) even after adjustments for confounding variables (Yasmin et al., 2005). A potential explanation for this association is that an increased MMP-9 activity promotes destruction of the elastic laminae of arteries (Longo GM et al., 2002). Thus data exist suggesting that changes in plasma MMP concentrations may predict large artery changes. However, this hypothesis has not been tested in patients with chronic renal failure. Consequently, as part of my thesis I examined the relationship between plasma procollagen, MMP and TIMP concentrations and indices of large artery stiffness in patients with chronic renal failure receiving HD.

1.3.2.8 Anaemia and large artery dysfunction

Chronic anaemia may adversely affect large blood vessels through arterial hypertrophy and remodelling, a change that is likely to occur as a consequence of increases in cardiac output (Gibbins and Dzau 1994, London and Parfrey 1997). The arterial remodelling that occurs as a result of anaemia may initially be reversible, but if sustained in chronic kidney disease, could become permanent (Metivier et al 2000). One mechanism responsible for anaemia-induced arterial remodelling is through enhanced blood flow resulting in increased circumferential wall stress, which is then compensated for by an augmented wall thickness. The increased wall thickness maintains a normal wall stress, but this could potentially be at the expense of a reduced arterial compliance or increased arterial stiffness. An alternative mechanism responsible for anaemic-induced arterial remodelling is through increases in arterial blood flow resulting in a greater shear stress on the vascular wall. A reduced vascular radius and decreased blood viscosity in chronic anaemia may initially keep shear stress within

normal ranges despite increases in flow, but eventually, as the wall thickens, reductions in radius may occur and shear stress may increase. Although anaemia as a cause of large artery dysfunction was not specifically addressed in this thesis, exploratory analysis was performed to determine whether correlations were present. This analysis was performed in order to determine whether haemoglobin should be employed as an adjustor in subsequent analysis.

1.4 Aims

The aim of this study was to investigate factors impacting on left ventricular hypertrophy in haemodialysis patients.

1.4.1 Objectives

1. To estimate the prevalence of LVH and its geometric models in patients with ESRD on HD.
2. To investigate the risk factors of myocardial remodelling in ESRD with established LVH-echocardiographically verified.
3. To assess the risk factors impacting on LVH in these chronic HD patients.
4. To investigate the contribution of serum MMPs to development of LVH and dysfunction and to evaluate the association between collagen metabolism markers and PWV in HD patients with LVH.
5. To use this information to recommend interventions for prevention of progression of LVH.

The following questions were therefore addressed in this thesis:

1. The question of whether multiple pre- and post-dialysis conventional BP measurements are equally as effective as 24-hour BP measurements in predicting either LVMI or large artery stiffness in patients with chronic renal failure receiving HD was addressed in chapter 2 of the present thesis.

2. The relationship between indices of large artery function (carotid-femoral pulse wave velocity, augmentation index and pulse pressure) and LVMI in a sample of patients with renal failure receiving HD was addressed in chapter 3 of the present thesis.

3. Evaluation of whether an indicator of volume status (inferior vena cava diameter) is associated with LVMI and geometry and indices of arterial stiffness or wave reflection, independent of 24-hour ambulatory or multiple pre- and post-dialysis BP measurements in a sample of patients with renal failure receiving HD was addressed in chapter 4 of the present thesis.

4. Determination of whether natriuretic peptide concentrations predict volume status as determined from inferior vena cava diameter, independent of LVMI in patients with chronic renal failure receiving HD and whether natriuretic peptides predict LVMI independent of volume status as determined from inferior vena cava diameter in these patients were addressed in chapter 5 of the present thesis.

5. The association of plasma concentrations of markers of interstitial changes, including procollagen I C-peptide, matrix metalloproteinases (MMP) 1, 2, and 9, tissue inhibitors of MMPs-1 and 2 and C-terminal telopeptide of type I collagen with LVMI or large artery dysfunction in patients with chronic renal failure receiving HD was addressed in chapter 6..

CHAPTER 2

Prediction of left ventricular mass and arterial stiffness changes in haemodialysis patients: multiple dialysis blood pressures versus ambulatory blood pressures

Abstract

The value of multiple pre- and post-dialysis BP measurements in chronic renal failure is uncertain. This study assessed whether 24-hour BP predicts target organ changes better than pre-, post- and averaged dialysis BP in 79 patients without diabetes mellitus receiving MHD for an average of ~49 (3-300) months. 3 Pre- and 3 post-dialysis BPs were determined over 3 sessions of dialysis per week for 4 weeks and the average calculated from the mean of these measurements. Pulse wave analysis performed at the carotid, femoral and radial artery was employed to determine carotid-femoral PWV and AIc. Echocardiography was performed to determine LVM which was indexed to body surface area (LVMI). Using multivariate regression analysis with adjustments for potential confounders, pre- ($p \leq 0.005$), post- ($p<0.05$) and averaged dialysis ($p < 0.015$) systolic BP were associated with LVMI and PWV. Neither 24 hour ($r = 0.260$, $p < 0.05$), day ($r = 0.25$, $p< 0.05$), nor night ($r= 0.24$, $p <0.05$) systolic BP were more closely associated with LVMI than the averaged dialysis systolic BP ($r = 0.27$, $p < 0.02$). Moreover, neither 24 hour ($r = 0.41$, $p = 0.0003$), day ($r=0.400$, $p = 0.0005$), nor night ($r =0.41$, $p< 0.0005$) systolic BP were more closely associated with PWV than the post-dialysis systolic BP ($r=0.39$, $p=0.0001$). In conclusion, these results indicate that the average of multiple pre- and post-dialysis BP measurements are equally effective in predicting cardiovascular target organ changes (LVMI and PWV) as 24-hour ambulatory BP values in patients receiving HD.

2.0 INTRODUCTION

Although hypertension is an established major determinant of cardiovascular target organ changes in chronic renal failure, the best method of measuring blood pressure (BP) when predicting cardiovascular damage in renal disease is uncertain. In patients on dialysis, BP values obtained at the time of an outpatient dialysis session are very poorly correlated with BP values obtained away from hospital during the inter-dialytic interval (Agarwal R and Lewis RR, 2001). Moreover, pre- and post-dialysis BP values considerably over- and under-estimate ambulatory BP with wide limits of agreement between the measurements (Agarwal R et al., 2006).

Three studies (Conlon PJ et al., 1996); (Erturk et al., 1996); (Zoccali C et al., 1999), with the largest being the study of Zoccali et al conducted in 64 patients, have assessed the question of whether multiple pre- or post-dialysis BP measurements are as closely associated with LVM as 24-hour ambulatory BP measurements. Despite the poor relationship between pre- or post-dialysis BP and 24 hour BP values, in a study conducted in 64 patients, the average of 12 pre-dialysis BP values had the same predictive power for LVMI as a single 24-hour period of ambulatory BP monitoring after adjustments for BMI, haematocrit and serum cholesterol concentrations (Zoccali C et al., 1999). Prior studies, also showing an equivalent effect of multiple conventional measurements on LVMI as compared to 24-hour BP were performed in even smaller study samples and did not adjust for alternative confounders (Conlon PJ et al., 1996, Erturk et al., 1996). In all of these studies only LVMI was assessed as a target organ change. Clearly more studies are required to elucidate the value of multiple pre- and post-dialysis BP measurements in patients on HD.

The present study therefore explored whether multiple pre- and post-dialysis conventional BP measurements are equally effective as 24-hour BP measurements in predicting either LVMI or large artery stiffness, a common target organ change noted to occur in patients with chronic renal failure (Blacher J et al., 1999); (Shoji T et al., 2001).

2.1 Methods

The study protocol was approved by the Human Ethics Committee of the University of the Witwatersrand (clearance number M060324). All participants provided written informed consent for participation in the study.

2.1.1 Study population

A convenience sample of 94 patients with chronic renal failure receiving haemodialysis therapy thrice weekly at the Johannesburg Hospital (n=59), Donald Gordon Medical Centre (n=6), Helen Joseph Hospital (n=19), Milpark Hospital (n=5), Linksfield Hospital (n=3) and Glynnwood Hospitals' (n=3) haemodialysis units in Johannesburg, South Africa, were studied. Patients who had been on haemodialysis for at least three months and consented to participate were eligible. All patients with diabetes mellitus or who were less than 18 years of age were excluded from the study, as were patients with human immunodeficiency virus infection. All but four eligible patients at Johannesburg hospital who met inclusion criteria participated.

2.1.2 Demographic and clinical data

A structured medical questionnaire was completed for each participant to gather information on their illness and treatment. Age, ethnicity, gender, aetiology of renal failure, duration of HD and the history of previous peritoneal dialysis or renal transplantation was noted. The use of antihypertensive medications, intravenous iron and erythropoietin, calcium containing and other phosphate binders was also noted. Height and weight were measured using the Detecto scale (New York) and BMI was calculated as weight to height squared. A full clinical examination was performed with particular attention given to the presence of pedal oedema, the level of the jugular venous pressure (JVP), the apex beat location and quality, the presence of basal crepitations, heart rate, the presence of a recent onset of cough and the presence of hepatomegaly.

2.1.3 Blood Pressures and the diagnosis of hypertension

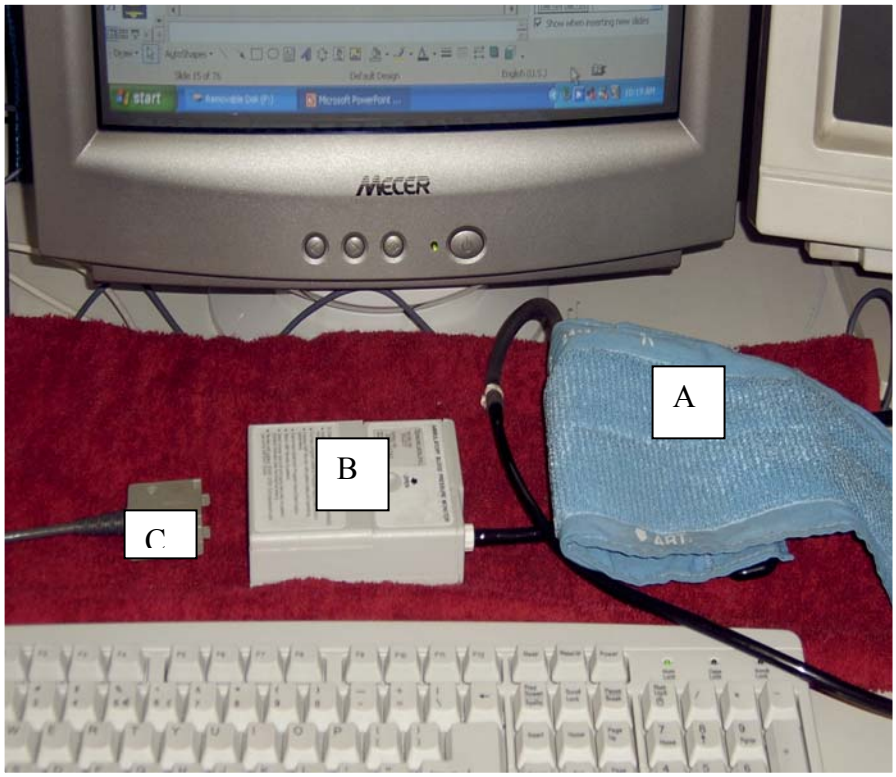
Pre- and post-haemodialysis blood pressure. Brachial artery BP was measured after 15 minutes in the semi-recumbent position using the arm contralateral to the arteriovenous fistula either immediately before or after completion of a dialysis session. Blood pressures before or after dialysis sessions were monitored using oscillometric devices incorporated in Fresenius HD devices (model BPM 4008B) or with the use of a Dinamap (model critikon 8100) oscillometric device in patients using B Braun (model Dialog+) or Baxter (model Tina, Aurora) dialysis devices. Cuffs with a 22 x 12 cm bladder were employed, or a 31 x 15 cm bladder if arm circumference exceeded 31 cm. Blood pressures were obtained before and after each dialysis session conducted 3 times per week for a month. Pre- and post- dialysis BP was calculated as the average value of all recordings (12 measurements [3/wk]). Mean arterial BP

(MAP) was calculated as MAP=DBP + [(SBP-DBP)/3]. The mean value of all pre- and post dialysis BP values was calculated for each patient for statistical purposes.

Office blood pressure measurements. A trained observer measured brachial artery systolic and diastolic (phase V) BP to the nearest 2 mm Hg according to the recommendations of the European Society of Hypertension (Mancia G et al., 2007). BP was measured five times consecutively after the subjects had rested for 5 minutes in the sitting position. Again cuffs with a 22 x 12 cm bladder were employed, or a 31 x 15 cm bladder if arm circumference exceeded 31 cm. For analysis, the 5 BP readings were averaged.

Diagnosis of hypertension. Hypertension was determined according to the World Health Organisation criteria (office BP> or =140/90 and/or the use of antihypertensive therapy).

24 Hour ambulatory blood pressure monitoring. Spacelabs model 90207 ambulatory BP monitors (Figure 2.1) were utilised to determine 24 hour ambulatory BP profiles. 24-Hour BP monitoring was performed between two dialysis sessions. The monitors were programmed to measure BP at 15-minute intervals from 06:00 to 22:00 h and at 30-minute intervals from 22:00 to 06:00 h. Subjects kept a diary card for the duration of the recordings to note the time of going to bed in the evening and getting up in the morning. Subjects were also asked to record the time when taking medication in those receiving medication. Daytime and night-time periods were defined as ranging from 09:00 to 19:00 h and from 23:00 to 05:00 h, respectively in line with pattern of daily activity. This approach eliminates the transition periods (evening and morning) during which BP changes rapidly in most subjects. Only data with at least 20 hours of recordings and more than 10 and 5 readings for the computation of daytime and night-time means was analysed.



A Ambulatory blood pressure cuff.

B Spacelabs model 90207 ambulatory blood pressure monitor

C Interface between monitor and computer to initialise and download Data

Figure 2.1 Hardware used to perform 24-hour ambulatory blood pressure monitoring

2.1.4 Biochemistry

Haemoglobin, albumin, urea, creatinine serum calcium and phosphate were determined each month (mid-week pre-dialysis values). Parathyroid hormone (PTH) concentrations were

assessed every 3 months in patients at the Johannesburg hospital, and every 6 months in those from the other hospitals. For the study we used time-averaged serum calcium, phosphate, urea, and creatinine concentrations and blood haemoglobin concentrations. The mean of three measurements taken over 3-months from the time of recruitment including the month of entry into the study were used for analysis. Haemoglobin was determined using standard procedures of spectrophotometry using Beckman coulter analyser. Serum albumin was measured by a colorimetric assay. Urea determination was done using a commercial (Roche) assay based on Talke and Schubert's method optimised for analysers permitting kinetic (fixed-time) measurements. Serum creatinine was measured by the modified Jaffe method using a kinetic colorimetric assay principle. Serum calcium was determined using the complexometric method. Serum phosphate was measured using the principle of endpoint method with sample blanking. Inorganic phosphate forms an ammonium phosphomolybdate complex with ammonium molybdate in the presence of sulphuric acid. The complex is determined photometrically in the ultraviolet region (340nm). Intact PTH was measured using the ADVIA Centaur intact PTH assay (Bayer Diagnostics United Kingdom), using direct chemiluminometric technology which uses constant amounts of two anti-human PTH antibodies in the Lite Reagent. The amount of PTH in the patient's sample is equivalent to the amount of relative light units (RLU) detected by the system.

2.1.5 Echocardiography

Echocardiography was performed in the Cardiology Unit of the Johannesburg Hospital with a GE Vingmed Vivid 7 echocardiograph (Figure 2.2) with 1.5-4.0 MHz M3S matrix phased array adult cardiac probe allowing M-mode, two dimensional and pulsed Doppler

measurements. Echocardiography was performed according to the American Society of Echocardiography convention (Sahn DJ et al., 1978) by a single observer (Clinical Technologist) blinded to the clinical status of the patient. All data was recorded and reviewed off-line by a single senior cardiologist to minimise intra- and inter-observer variability. These studies were performed on a non-dialysis day except in 7 patients when echocardiography was performed at least one hour after dialysis. Scans were performed with the head of the table inclined at an angle of 15 degrees and the participant rotated 30–45 degrees in the left lateral decubitus position at end-expiration. Left ventricular internal dimensions in end-diastole (LVEDD), and end-systole (LVESD) and the interventricular wall thickness (IVSTd) and posterior wall thickness (PWTd) during diastole were measured at the level of the mitral valve leaflet tips in the parasternal long-axis view. From these measurements, LVM was calculated according to the formula of Devereux and Reichek (Devereux RB et al., 1986) as follows:
LVM = ([LVEDD + IVSTd + PWTd] ^{3} x 1.04) - ([LVEDD] ^{3} - 13.6). Left ventricular mass was divided by body surface area (BSA) to calculate the LVMI (Sahn DJ et al., 1978). LVH was diagnosed when LVMI was >134 g/m^2 in men and >110 g/m^2 in women (Abergel E et al., 1995, Devereaux RB et al., 1984). Left ventricular relative wall thickness (RWT) was calculated as follows: RWT = (2 x PWTd)/ LVEDD, and LVH was characterised as eccentric if RWT <0.45 and concentric if RWT ≥ 0.45 (Savage DD et al., 1987).

Left ventricular diastolic function was assessed using peak early (e) and atrial (a) transmitral Doppler velocities and deceleration time of (e). The approximate values for age and gender specific normal ranges for mitral flow-derived indices of LV diastolic function in a general population were used i.e. E-wave deceleration time 0.21 +/- 0.04 in males, 0.19 +/- 0.04 in females and E/A ratio of 1.04 +/- 0.38 and 1.03 +/- 0.34 in males and females respectively (Schirmer H et al., 2000). Systolic dysfunction was defined as left ventricular

endocardial shortening fraction less than 28% (Devereux RB et al., 2000) and left ventricular ejection fraction less than 50% (Andrew P, 2003, Kaddoura S, 2002, McGregor E et al., 1998).

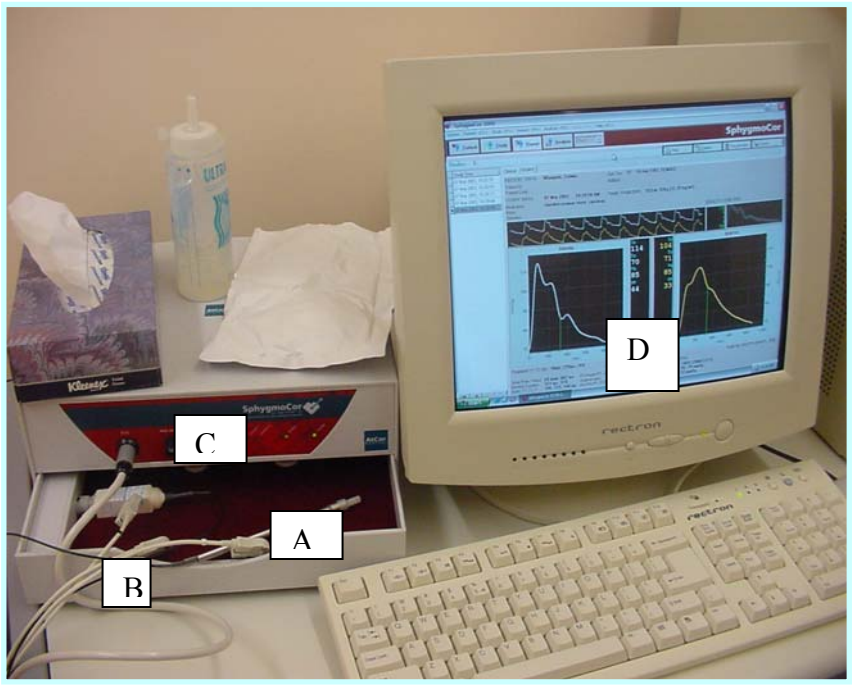


Figure 2.2 Echocardiograph used to determine left ventricular dimensions in the present study.

2.1.6 Pulse wave analysis

Pulse wave analysis to determine large artery function was assessed with applanation tonometry performed at the radial, carotid and femoral arteries using a SphygmoCor device (PWV Inc., Sydney, Australia) (Figure 2.3). The measurements were taken by a single observer. After resting for 15-minutes in the supine position, the radial waveform at the nonvascular access arm was recorded during an 8 second period using a high fidelity SPC-301 micro manometer (Millar Instrument, Inc., Houston, Texas) interfaced with a computer employing SphygmoCor, version 6.21 software (AtCor Medical Pty Ltd., West Ryde, New South Wales, Australia). Recordings were discarded when the systolic or diastolic variability

of consecutive waveforms exceeded 6%, or the amplitude of the pulse wave signal was less than 80 mV. The pulse wave was calibrated by manual measurement (auscultation) of BP immediately before recordings were obtained. The average of 5 readings was taken for pulse wave analysis. From the radial signal, SphygmoCor software calculates the aortic pulse wave by means of a validated and population-based generalized transfer function (Nichols WW and O'Rourke MF, 1998). The central augmentations index (AIc) was calculated as difference between the second and first systolic peak given as a percentage of aortic pulse pressure (Figure 2.4, lower panel). Central pulse pressure (PPc) was defined as the difference between central systolic and central diastolic BP.



A Applanation tonometer

B ECG electrodes

C SphygmoCor device

D Computer

Figure 2.3 Hardware used to perform pulse wave analysis

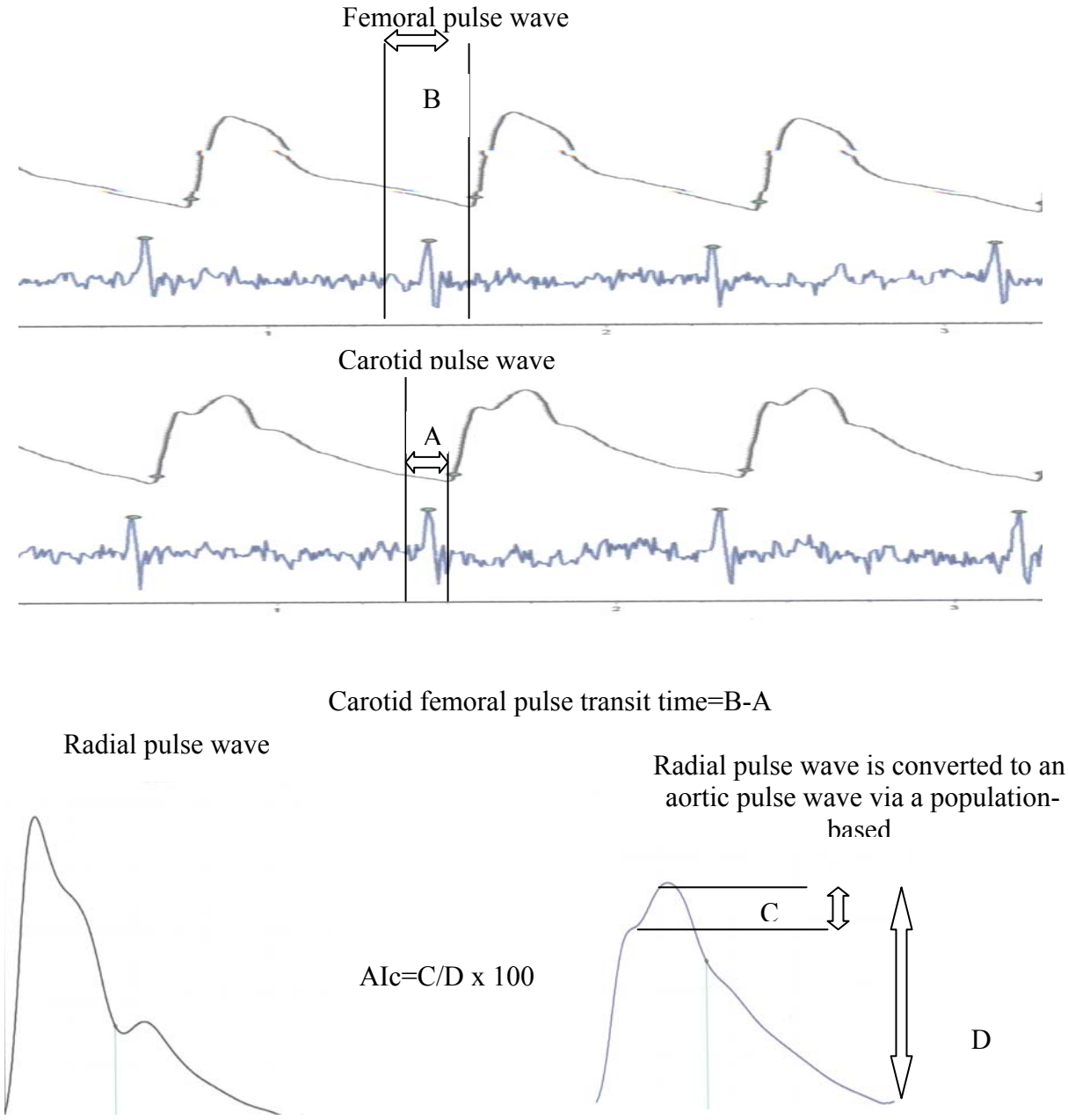


Figure 2.4 Shows the derivation of pulse transit time and central augmentation index (AIc) obtained from pulse wave analysis performed at the carotid, femoral and radial arteries.

Pulse wave velocity (PWV) was determined from carotid and femoral waveform measurements. The distance travelled by the pulse wave was measured over the body surface as the distance between the recording sites at the femoral artery to the suprasternal notch minus the distance from the recording site at the carotid artery to the suprasternal notch (D). The time (t) taken for the pulse wave to travel from the carotid to the femoral site (pulse transit time) was determined from the differences in the times taken to generate the femoral and carotid pulse waveforms (Figure 2.4). To assess time differences in the generation of carotid and femoral waveforms, these waveforms were matched to a simultaneous electrocardiograph recording (Figure 2.4). Pulse transit times were determined from an average of 10 consecutive beats aortic PWV was calculated as PWV = D/t.

2.1.7 Electrocardiography

Twelve lead electrocardiography (ECG) was done on all the patients. Most patients had computerised ECG done using NIHON KOHDEN Cardiofax GEM device (Tokyo,Japan). In the 5 patients who had ECG using the Hewlette Packard machine (Palo Alto, USA) LVH was assessed using Sokolow-Lyon voltage criteria (Sum of S wave in V1 and R wave in V5 or V6 $\geq$3.5 mV [35 mm]).

2.1.8 Statistics

Data base management and statistical analyses were performed with SAS software, version 9.1 (The SAS Institute Inc., Cary, North Carolina, USA). Results are expressed as mean +/- standard deviation (SD) or median +/-range or number (%). Multivariate regression analysis was performed to determine the relationships between BP (pre- and post dialysis, averaged office, averaged dialysis, 24-hour, day and night) and either LVMI, PWV or AIc before and after adjustments for age, sex, body mass index, number of antihypertensive agents

used and smoking. To determine whether one BP predicted target organ changes (LVMI, PWV or AIc) independent of another BP, the latter was included as an additional adjustor in the multivariate model. Statistical significance was determined with a p value <0.05.

2.2 Results

2.2.1 Causes of renal failure

A total of 94 participants were studied. The causes of ESRD included hypertension in 46 participants (48.9%), obstructive uropathy in 6 (6.4%), reflux nephropathy in 6 (6.4%), glomerulonephritis in 5 (5.3%), lupus nephritis in 4 (4.3%), polycystic kidney disease in 3 (3.2%), congenital abnormalities of the kidney in 3 (3.2%), Alports disease in 2 (2.1%), analgesic nephropathy in 2 (2.1%), ethylene glycol poisoning in 1 (1.1%), ischaemic nephropathy in 1 (1.1%), sickle cell disease in 1 (1.1%), systemic sclerosis in 1 (1.1%), trauma in 1 (1.1%) and unknown causes in 12 (12.77%).

2.2.2 Dialysis programs

All patients at the Johannesburg Hospital were being treated three times a week using Fresenius model 4008 machines and two Dialog machines from B Braun with standard bicarbonate dialysis (sodium 138mmol/L, HCO_3 35 mmol/L, potassium 1.5 mmol/L, calcium 1.50 mmol/L, and magnesium 0.75 mmol/L). The majority of patients were dialysed with the Fresenius hollow fibre polysulfone dialysers (F5 to F10HPS, surface area 1.0 to 2.2m^2 Fresenius Medical Care Bad Homburg Germany); 71 (75.53%) patients were using high flux

dialysers (Exeltra 170, Exeltra 150 or 170, FX80) while 23 (24.47%) were using low flux dialysers (F6, F6HPS, F8, F8HPS, F10, F10HPS).

2.2.3 General demographic and clinical data

Table 2.1 summarises general demographic and clinical data of the study population The mean age of the study group was 41.1±11.8 years and the mean HD duration was 49.3±50.9 months. Male patients comprised 61.7% of the study population. Of the participants 70 (74.47%) were black, 12 white (12.77%), 4 (4.26%) Indian and 8 (8.51%) mixed race. Three quarters of the patients had never smoked and an average body size was noted in the whole group (body mass index 25±6). 48(51%) patients had anaemia (haemoglobin less than 11g/dl). 27(29%).patients had hypocalcaemia and 13 (14%) hypophosphataemia. 88 (93.62%) patients were receiving calcium carbonate containing phosphate binders and 58 (61.7%) patients were receiving 1α Vitamin D. 15 (16.3%) patients were hypoalbuminaemic (albumin less than 34g/l). 59 (64.84%) participants had parathyroid hormone levels above 300pg/ml while 23 (25.27%) had levels less than 150pg/ml.

Table 2.1 General demographic and clinical characteristics of the study population

Age (years)(n=94)	41.1± 11.8 (20-72).
Male (%)	58 (61.7%)
Haemodialysis duration (months) (n=94)	49.3±50.9 (3-300)
Ever smoked (yes/former/no) (n=94)	12 (12.77%), 9 (9.57%), 73 (77.66%)
Body height (cm) (n=94)	164.8±10.1
Body weight (kg) (n=94)	67.9±17.5
Body mass index (kg/m^2) (n=94)	25.0± 6.0
Kt/V urea* (n=90)	1.35±0.28
Albumin (g/dl) (n=92)	39.1±5.0
Haemoglobin (g/dl) (n=94)	11.1± 1.6
Calcium (mmol/l) (n=93)	2.27±0.24
Phosphate (mmol/l) (n=93)	1.54±0.56
Calcium x Phosphate ($mmol^2/l^2$) (n=93)	3.48±1.30
Parathyroid hormone (pg/ml) (n=91)	637±578 (2.83 – 1900)
Patients presenting with LVH on ECG (n=91)	31(34%)

* indicates dialysis dose (Kt/V averaged equilibrated urea clearance)

2.2.4 Treatment of hypertension and blood pressures in the group

Only 17 (18.1%) participants were not receiving antihypertensive agents. 14 (14.9%) participants were receiving 1 class of antihypertensive agent; 21 (22.3%) were receiving 2 classes of antihypertensive agents; 20 (21.3%) were receiving 3 classes of antihypertensive agents, 18 (19.15%) were receiving 4 classes of antihypertensive agents, whilst 2 (2.13%) were receiving 5 classes of antihypertensive agents and 2 (2.13%) were receiving 6 classes of antihypertensive agents. 67 patients (71.3%) were receiving calcium channel blockers; 43 (45.7%) beta blockers; and 38 (40.4%) angiotensin-converting enzyme inhibitors. A quarter of the patients (25 [26.60%]) were receiving diuretic agents; 14 (14.89%) were receiving alpha blockers; 11 (11.70%) were receiving vasodilators; whilst 7 (7.4%) were receiving alpha-methyldopa (centrally acting). Only 3 (3.19%) participants were receiving an angiotensin II receptor blocker.

The mean BP values for the different methods of measurement are shown in Table 2.2. As would be expected, irrespective of whether office, pre-, post- or mean dialysis systolic BP values were assessed, these values were higher than the 24 hour systolic BP values. However, diastolic BP values were similar between the groups. Also as expected night BP values were considerably lower than either ambulatory day BP or office, pre-, post- or mean dialysis systolic and diastolic BP.

Table 2.2 Blood pressures of study patients

Office BP (n=92)	
Systolic/diastolic BP (mmHg)	136±29/88±17
Pulse pressure (mmHg)	48±19
Mean arterial pressure (mmHg)	104±20
Pre-dialysis BP (n=94)	
Systolic/diastolic BP(mmHg)	141±18/83±12
Pulse pressure (mmHg)	58±12
Mean arterial pressure (mmHg)	102±13
Post-dialysis BP (n=94)	
Systolic./diastolic BP(mmHg)	138±21/79±14
Pulse pressure (mmHg)	59±13
Mean arterial pressure (mmHg)	99±15
Mean-dialysis BP (n=94)	
Systolic/diastolic BP (mmHg)	140±19/82±13
Pulse pressure (mmHg)	58±12
Mean arterial pressure (mmHg)	101±14
24-Hour BP (n=80)	
Systolic/diastolic BP (mmHg)	131±23/83±16
Pulse pressure	49±13
Mean arterial BP (mmHg)	100±17
Day BP (n=79)	
Systolic/diastolic BP (mmHg)	134±23/86±15
Pulse pressure	48±13
Mean arterial BP (mmHg)	102±17
Night BP (n=79)	
Systolic/diastolic BP (mmHg)	128±24/78±16
Pulse pressure	49±14
Mean arterial BP (mmHg)	96±18

2.2.5 Cardiovascular target organ changes

Table 2.3 summarises the LV dimensions and function obtained in the study group. Importantly mean LV posterior and septal wall thickness values indicated the presence of marked LV hypertrophy in the study group. Concentric LVH was noted in 39 (42.4%) patients, eccentric LVH in 28 (30.4%) patients, a normal LVM in 10 (10.9%) patients, and concentric LV remodelling in 10 (10.9%) patients. Moreover, the mean LV ejection fraction of the study group was below 50%, indicating the presence of LV systolic dysfunction in a significant number of patients. Using the classification by Foley and Parfrey (1996), LV systolic dysfunction was present in 53 (57.6%) patients. Of the patients with LV systolic dysfunction, 15 had LV dilatation (LV end diastolic volume/body surface area>90mls) with 12 of these having LVH (LVMI>134 g/m^2 and >110g/m^2 in males and females respectively). 26 patients with LV systolic dysfunction but without dilatation also had LVH.

Table 2.3 also summarizes large artery function in the study group. Although no thresholds for AIc and PPc have been agreed on, some guidelines cite a PWV of >12 cm/sec as being elevated (Mancia et al 2007). In this regard only ~1% of patients had evidence of large artery dysfunction.

Table 2.3 Left ventricular (LV) structure and function and large vessel function in study group

LV interventricular septal thickness (mm) (n=92)	12.4±3.7
LV posterior wall thickness (mm) (n=92)	12.5±3.3
LV end diastolic diameter (mm) (n=92)	51.5±8.6
LV mass (g) (n=92)	323±159
LV mass index (g/m^2) (n=92)	184±85
LV ejection fraction (%) (n=92)	47±13
E/A ratio (n=93)	1.30±0.42
Aortic augmentation index (%) (n=90)	26.8±14.5
Aortic augmentation index at a heart rate of 75 (%) (n=89)	24±13
Aortic pulse wave velocity (m/sec) (n=90)	7.04± 1.97
Central pulse pressure (mm Hg) (n=90)	37±16
Peripheral pulse pressure (mm Hg) (n=92)	48±19

Thresholds defined in healthy individuals from the African Program on Genes in Hypertension, suggest that an abnormal PWV in the groups of African descent may be >8 cm/sec in age groups similar to that noted in the present study (Shiburi et al 2006). Based on these thresholds 19% of patients had evidence of large artery dysfunction.

2.2.6 Non haemodynamic factors associated with target organ changes

To identify whether haemoglobin concentrations or PTH should be included in the multivariate regression analysis together with standard factors known to determine LVMI or large artery stiffness (age, sex, body mass index, treatment, smoking), I first performed regression analysis assessing the relationships between haemoglobin or PTH and LVMI and large artery function. Haemoglobin was not correlated with either LVMI (r=-0.001, p=0.99) or with PWV (r=-0.018, p=0.87). Furthermore, after adjustments for vitamin D therapy, PTH was not associated with either LVMI (r=0.15, p=0.18) or with PWV (r=-0.04, p=0.689). Thus, neither haemoglobin concentrations, nor PTH were included as adjustors in subsequent analysis.

2.2.7 Association between dialysis blood pressures and left ventricular mass index

On univariate analysis pre-, post- and mean dialysis systolic BP were associated with LVMI (Figure 2.5). However, neither pre- nor post- dialysis diastolic BP (r=0.128, p=0.224, n=92, and r=0.079, p=0.452, n=92) were associated with LVMI.

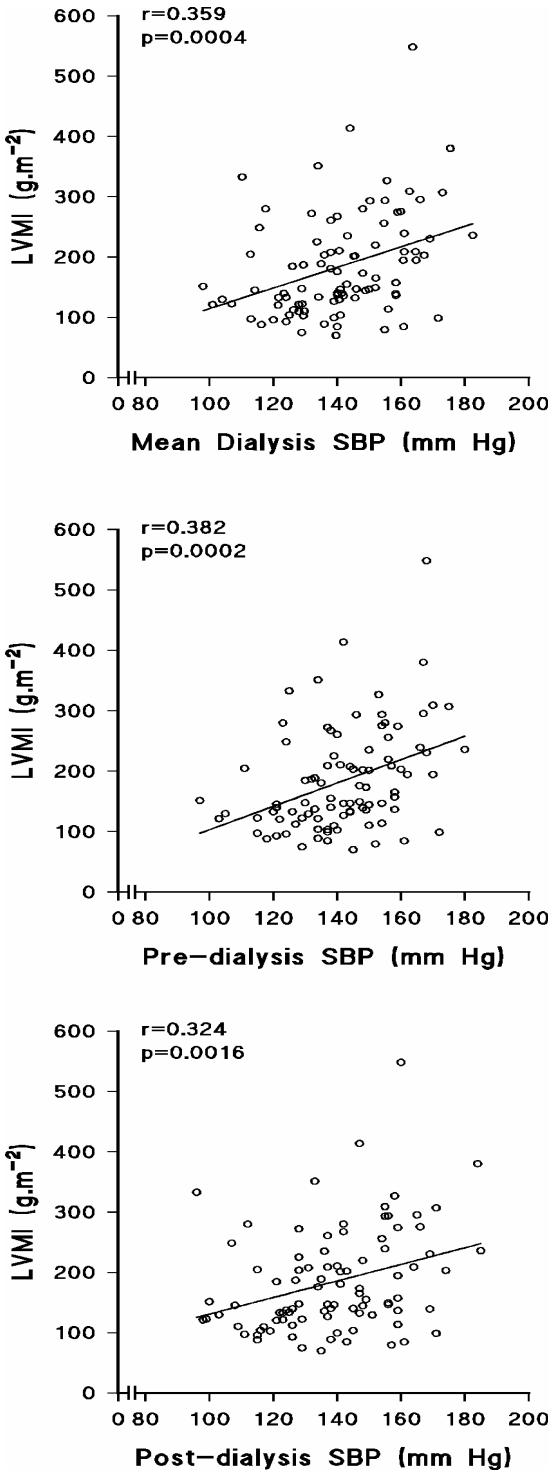


Figure 2.5 Correlations between dialysis blood pressures and left ventricular mass index (LVMI)

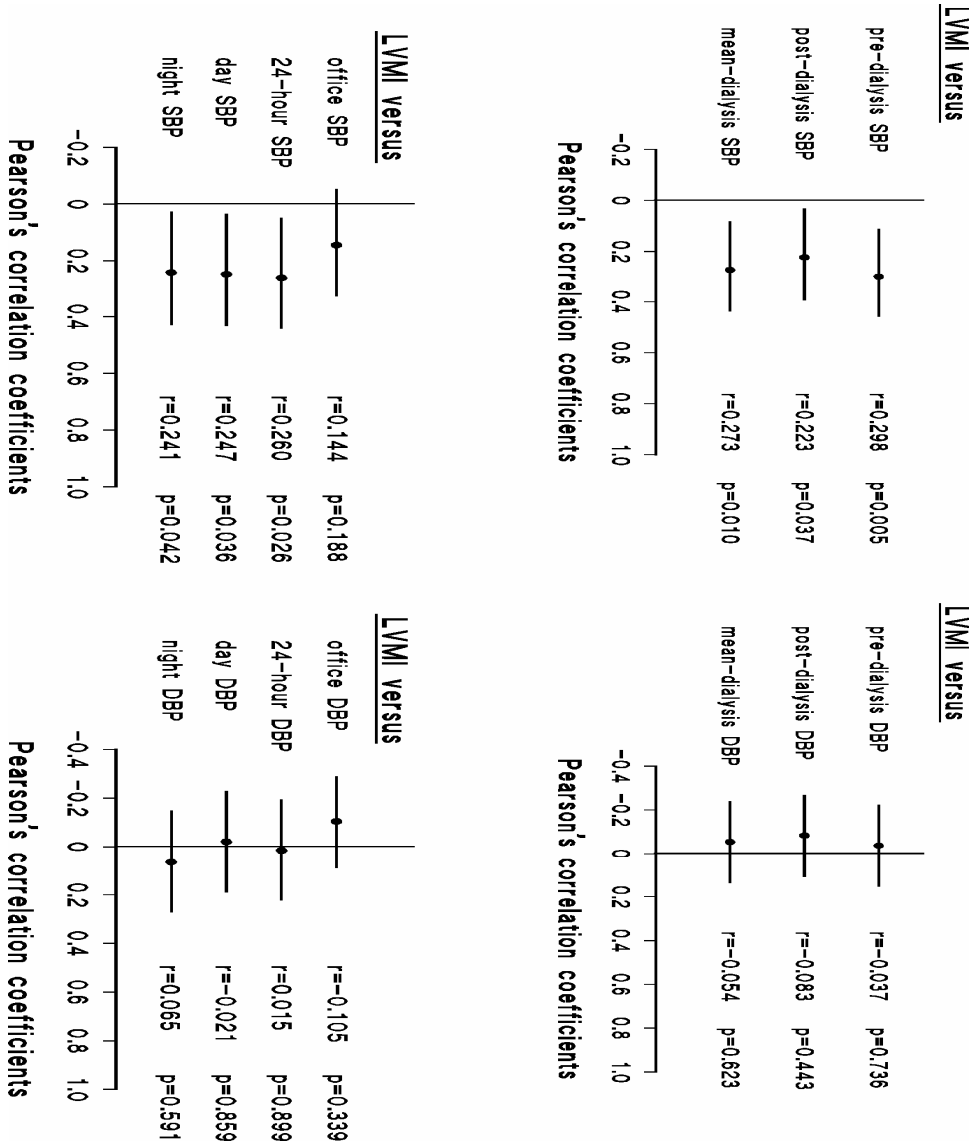


Figure 2.6 Multivariate adjusted partial correlation coefficients and the 95% confidence intervals between blood pressures and left ventricular mass index (LVMI). Adjustments were for age, sex, body mass index, number of antihypertensive agents, and smoking.

On multivariate regression analysis (adjusting for age, sex, BMI, smoking and number of anti hypertensive agents) pre, post- and mean dialysis systolic, but not diastolic BP were associated with LVMI (Figure 2.6, upper panel). When both pre- and post- dialysis systolic BP were included in the same model with the other adjustors, pre-dialysis, but not post-dialysis systolic BP showed a trend for an association with LVMI independent of post-dialysis systolic BP (partial r=0.204, p=0.059, n=92).

2.2.8 Comparison of the association between dialysis, office and 24-hour ambulatory blood pressures and left ventricular mass index

Figure 2.6 (lower panel) shows the relationships, after adjustments for age, sex, BMI, number of antihypertensive agents, and smoking, between conventional office, 24-hour, day and night systolic BP and LVMI. Average office, 24-hour, day and night SBP were independently associated with LVMI. Importantly, no differences were noted in the partial correlation coefficients between pre-, post- and averaged dialysis BP (upper panels) and either 24-hour, day or night systolic BP (lower panels). Moreover, no differences in the partial correlation coefficients between night and day systolic BP and LVMI were noted (lower panels)

2.2.9 Association between dialysis blood pressures and pulse wave velocity

On univariate analysis post-dialysis systolic (r=0.24, p=0.023, n=90) and diastolic BP (r=0.29, p=0.007, n=90) BP were associated with PWV (Figure 2.7). However, neither pre-dialysis systolic (r=0.17, p=0.10, n=90) nor diastolic (r=0.16, p=0.12, n=90) BP were

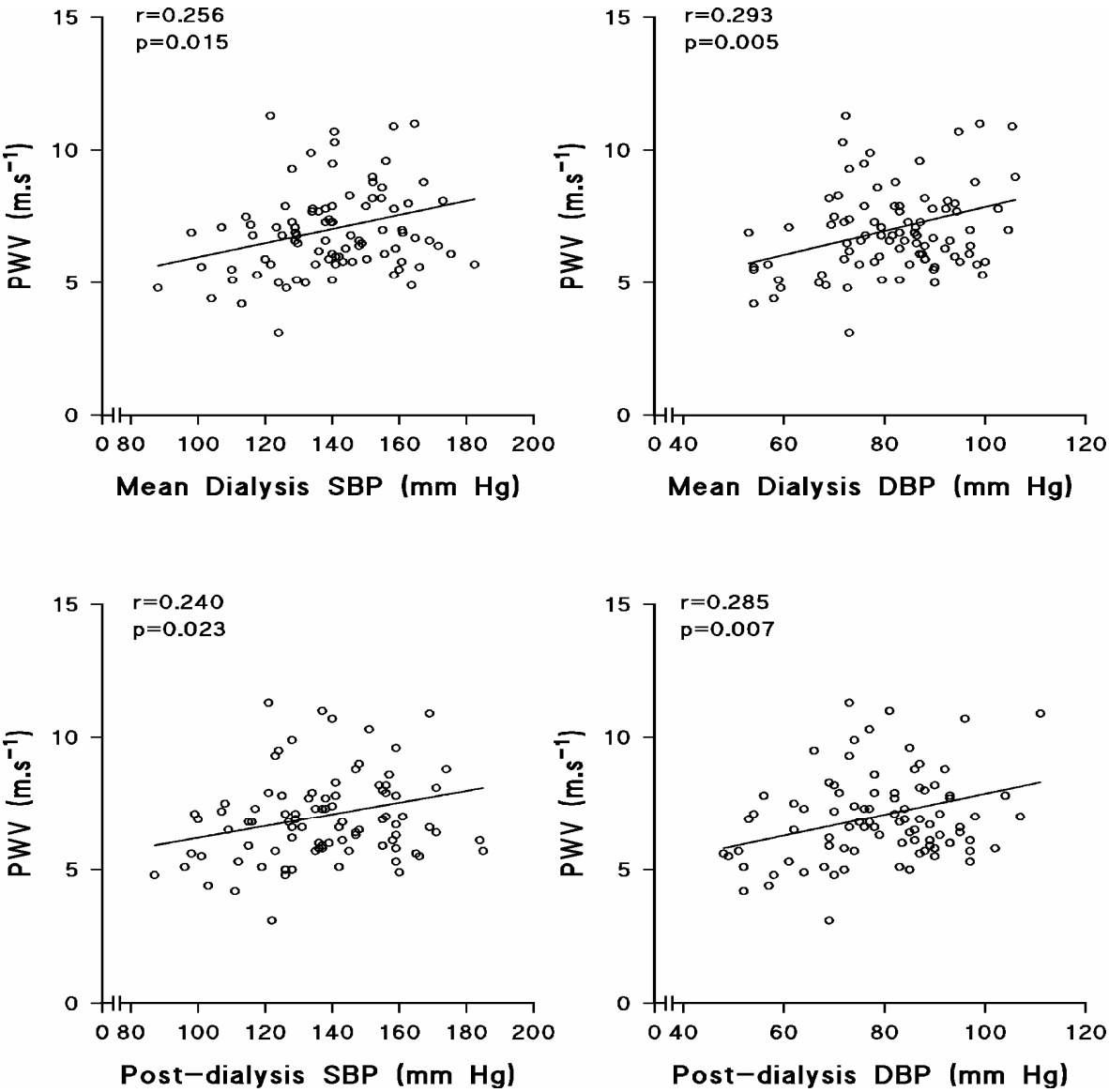


Figure 2.7 Correlations between dialysis blood pressures and pulse wave velocity (PWV) in haemodialysis patients.

Figure 2.8 Multivariate adjusted partial correlation coefficients and the 95% confidence intervals between blood pressures and pulse wave velocity (PWV). Adjustments were for age, sex, body mass index, number of antihypertensive agents, and smoking correlated with PWV.

On multivariate regression analysis (adjusting for age, sex, BMI, smoking and number of anti hypertensives), post-dialysis systolic and diastolic BP were associated with PWV (Figure 2.8).

Pre-dialysis BP was not associated with PWV even after adjustments for potential confounders (data not shown). When both pre- and post-dialysis systolic BP were included in the same regression model with all the other adjustors, post-dialysis systolic BP was associated with PWV independent of pre-dialysis systolic BP (r=0.22, p=0.04, n= 90) and post-dialysis diastolic BP is significantly associated with PWV independent of pre-dialysis diastolic BP (r = 0.30, p = 0.007, n = 90).

2.2.10 Comparison of the association between dialysis, office and 24-hour ambulatory blood pressures and pulse wave velocity

Figure 2.8 (lower panel) shows the relationships, after adjustments for age, sex, BMI, number of antihypertensive agents, and smoking, between conventional office, 24-hour, day and night systolic and diastolic BP and PWV. All conventional and ambulatory BP values were associated with PWV. Importantly, however, the partial correlation coefficients for the relations between post-dialysis or averaged dialysis BP and PWV were not significantly less than those for the relations between 24-hour, day or night BP and PWV.

2.2.11 Association between dialysis blood pressures and central and peripheral augmentation index

On univariate analysis post-dialysis systolic and diastolic BP were associated with AIc (Figure 2.9).

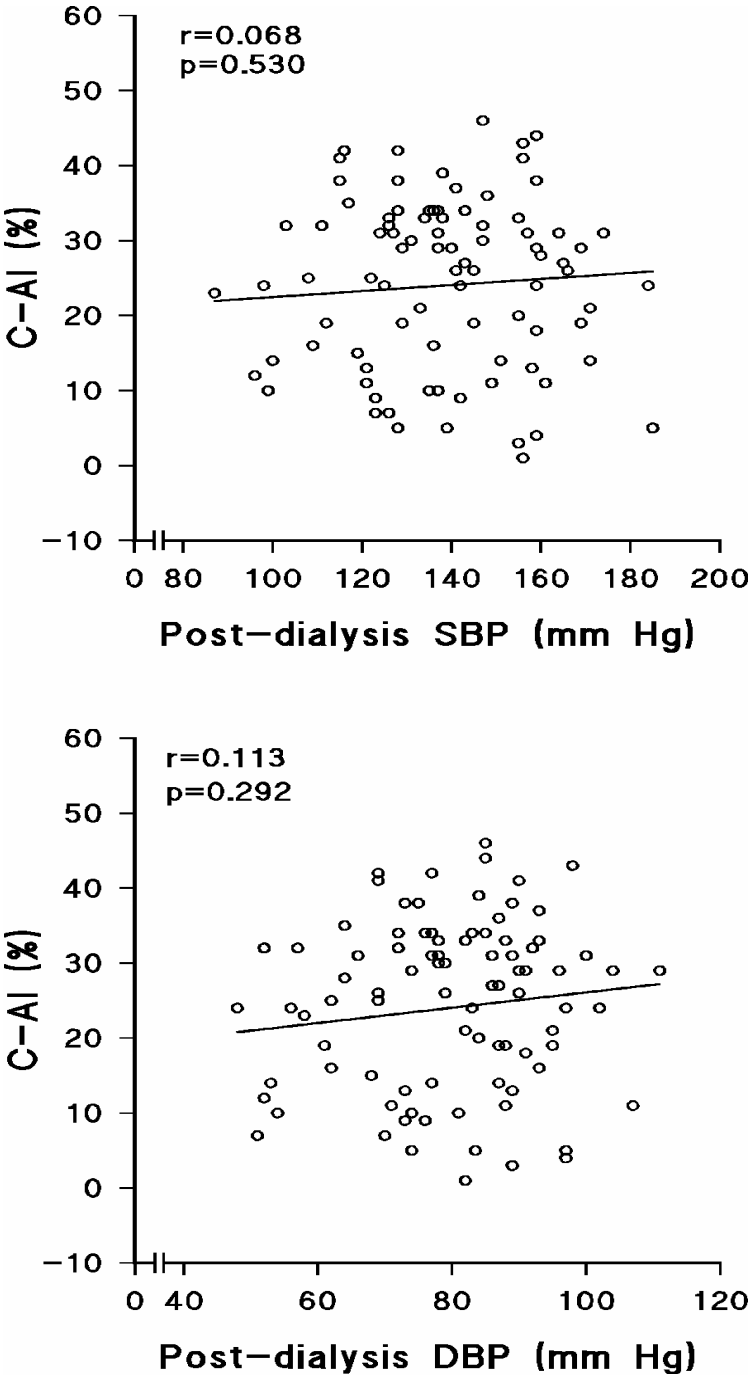


Figure 2.9 Correlations between dialysis blood pressures and central(C-AI) augmentation index in haemodialysis patients

2.2.12 Comparison of the association between dialysis, office and 24-hour ambulatory blood pressures and augmentation index

Figure 2.10 (lower panel) shows the relationships, after adjustments for age, sex, BMI, number of antihypertensive agents, and smoking, between conventional office, 24-hour, day and night systolic and diastolic BP and AIc. Only office systolic and diastolic BP and 24-hour and day diastolic BP were associated with AIc. Neither 24-hour nor night systolic BP, nor night diastolic BP were associated with AIc. Importantly, however, the partial correlation coefficients for the relations between pre- or post-dialysis or averaged dialysis diastolic BP and AIc were not significantly less than those for the relations between 24-hour or day diastolic BP and AIc.

2.3 Discussion

The main finding of the present study is that the average of 12 BP recordings obtained both prior to and after 12 dialysis sessions was as closely associated with LVMI and PWV as 24-hour, day and night ambulatory BP in 79 patients receiving haemodialysis.

The finding that multiple pre- and post-dialysis BP recordings are as closely associated with LVMI as 24-hour BP values in patients with renal failure has previously been reported on in only three studies (Ertuk S et al., 1996, Zoccali C et al., 1999, Conlon PJ et al., 1996). In this regard only one study adjusted for potential confounders (Ertuk S et al., 1996, Zoccali C et al., 1999, Conlon PJ et al., 1996) and in that study, after adjustments for all confounders, none of the ambulatory BP values retained an independent relationship with LVMI. In contrast, in the present study, after adjustments for age, sex, BMI, number of antihypertensives and

smoking, averaged dialysis, 24-hour, day and night BP were all independently associated with LVMI.

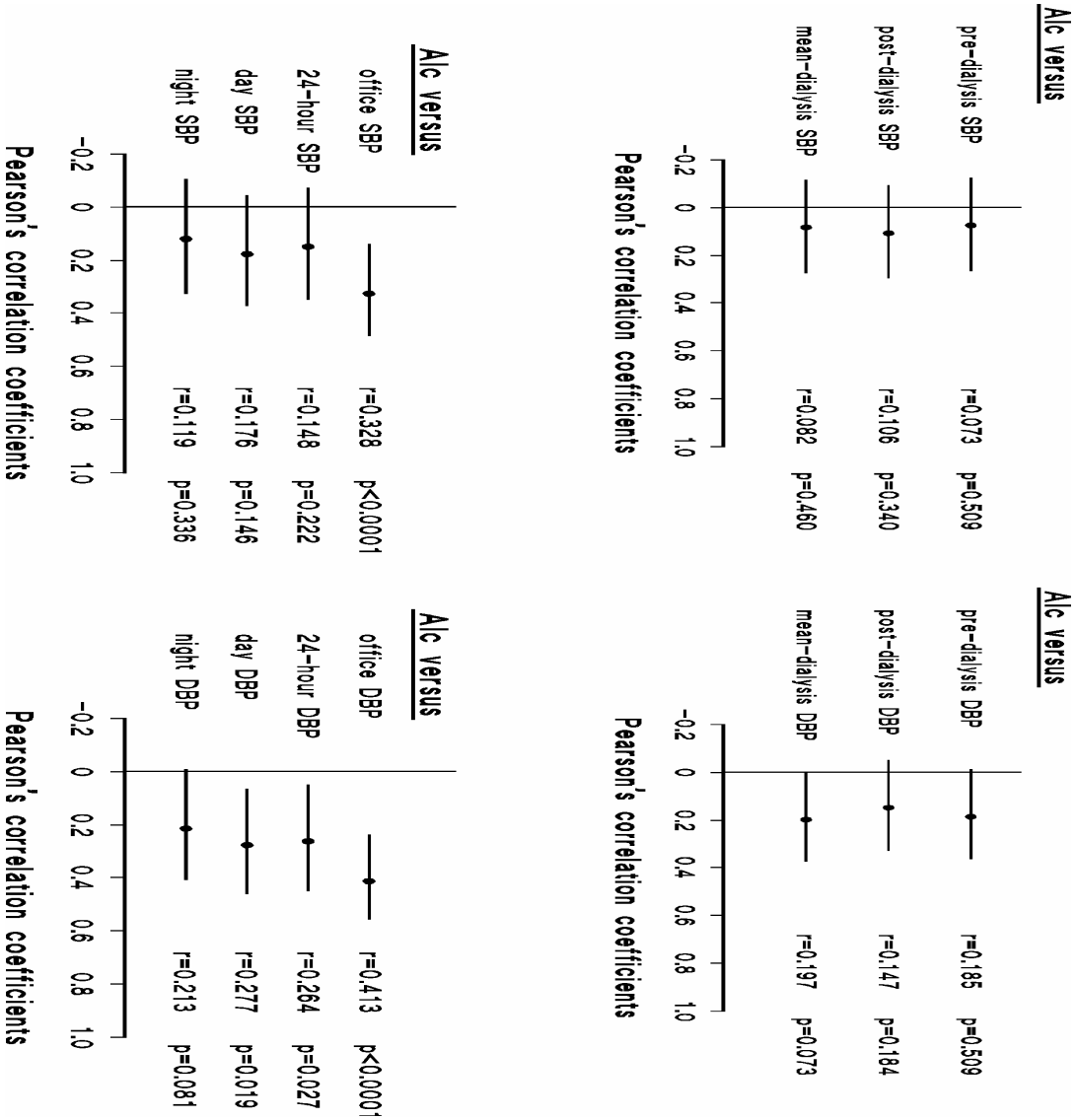


Figure 2.10 Multivariate adjusted partial correlation coefficients and the 95% confidence intervals for relations between blood pressures and AIc and AIp. Adjustments were for age, sex, body mass index, number of antihypertensive agents, and smoking

Clearly, the present study not only provides substantial data to support the notion that multiple pre- and post-dialysis BP values are equally as closely associated with LVMI, but also data to indicate that the strength of the relationship between peri-dialysis BP values and other target organs (as assessed from large artery function) is as close as ambulatory BP.

In the present study night BP was associated with neither LVMI nor indices of large artery dysfunction more closely than either day BP or dialysis BP. In this regard, an attenuated fall in BP during sleep in patients with chronic renal disease may be associated with LVH (Covic A et al., 2000). However, there are a number of potential explanations for the apparent discrepancy between the present and the previous (Covic A et al., 2000) study. One explanation is that the patients assessed in the present study may not have an attenuated nocturnal decline in BP. Indeed, in patients undergoing dialysis, the prevalence of an attenuated nocturnal decline in BP may vary from 22% (Korzets Z et al., 1994) to 80-100% (Ertuk S et al., 1996). As thresholds for a normal nocturnal decline in BP have not been defined in South African groups, and these may differ from other groups (Fumo et al 1992, Mayet et al 1998), I could not assess the prevalence of an attenuated nocturnal decline in BP in the present study.

In the present study, although post-dialysis BP was associated with PWV, pre-dialysis BP was not. An explanation for this finding is not apparent. However, this finding cannot be attributed to poor quality BP measurements obtained in the pre-dialysis period, as pre-dialysis BP if anything had higher correlation coefficients for relationships with LVMI than did post-dialysis BP. It is also possible that prior to dialysis, BP is determined to a greater extent by expanded plasma volume (fluid overload) than after dialysis and that blood volume-induced effects on large artery changes are not as critical as other effects in renal failure.

In contrast to the present study, where occasional day BP measurements (dialysis BP) were as closely correlated with LVMI and other target organ changes as 24-hour, day and night BP, as previously reviewed (Mancia G and Parati G, 2000); (Fagard R et al., 1995) a number of studies have shown that ambulatory BP shows significantly better correlations with LVMI and other target organ changes than conventional BP (Mancia and Parati 2000). However, as previously underscored (Fagard R et al., 1995) these findings are not supported by all studies. In this regard, a lack of standardisation and inappropriate quality control of occasional daytime BP measurements may explain the better correlations between ambulatory as compared to other BP measurements with target organ changes (Fagard R et al., 1995). Nevertheless, the similarity of the relations between occasional day BP and ambulatory BP and target organ changes in the present study could be specific to patients with renal failure. Moreover, few studies that have compared the relationships between occasional day BP values versus ambulatory BP and target organ changes have employed 12 non-ambulatory BP recordings. In this regard, the greater the number of recordings obtained the closer non-ambulatory BP recordings reflect the impact of ambulatory BP recordings on target organ changes (Fagard RH et al., 1997).

In the present study multiple (5 measurements) carefully conducted conventional BP measurements obtained in a single office visit were not significantly associated with LVMI. These data support the view that BP measurements obtained on multiple visits are required to appropriately predict all target organ changes in patients receiving haemodialysis. This may however, not necessarily apply to all target organ changes as multiple carefully conducted conventional BP measurements obtained in a single office visit were associated with decreases in large artery function. Moreover, the partial correlation coefficient for the relationship between multiple carefully conducted conventional BP measurements obtained in a single

office visit and PWV (r=0.44) was quantitatively similar to that obtained between 24-hour BP and PWV (r=0.41). However, the closeness of these relationships may in-part be for different reasons. The office BP measurements may have been closely related to PWV as they were obtained on the same day and at a similar time as the pulse wave analysis. In contrast, the 24-hour BP measurements may be closely related to PWV as they more closely represent a 24-hour BP load.

In the present study conventional office, 24-hour and day diastolic BP, but neither pre-, nor post-dialysis diastolic BP values were associated with AIc. As conventional office BP was associated with AIc, these data do not support the notion that ambulatory BP is a more appropriate BP measurement than conventional BP when predicting large artery dysfunction. The reason for the inability of pre- and post-dialysis diastolic BP to predict AIc, despite conventional diastolic office BPs association with AIc is not apparent. It is possible that AIc is determined by acute BP effects and hence same-day BP measurements are more likely to be associated with AIc. The relationship between diastolic BP and AIc cannot be attributed to an impact of AIc on diastolic BP rather than an effect of diastolic BP on AIc, as the relationship was a positive relationship, and augmentation of central artery BP is likely to decrease and not increase diastolic BP.

The strengths of the present study include the following: First, as highlighted above, in the present study a comparison of the relationships between dialysis BP and target organ changes and ambulatory BP and target organ changes was determined using two, rather than one target organ, as employed in prior studies (Ertuk S et al., 1996, Zoccali C et al., 1999, Conlon PJ et al., 1996). Second, unlike these prior studies (Ertuk S et al., 1996, Zoccali C et al., 1999, Conlon PJ et al., 1996) we could determine the relative impact of dialysis and ambulatory BP on CV target organ changes after adjustment for a number of confounders.

Third, we excluded patients with diabetes mellitus from the study as diabetes mellitus is associated with LVH and large artery stiffness.

The limitations of the present study are as follows: First, this was a cross-sectional study and hence we cannot exclude the possibility that BP-target organ relations are simply epiphenomena. However, in this regard this is unlikely as there are numerous studies, too numerous to cite in the present thesis, that support the notion that BP causes an increased LVM and large artery dysfunction. Second, the limited study sample size (n=79) may have prevented us from detecting stronger ambulatory BP-target organ than dialysis BP-target organ relations. However, even after multivariate analysis, the partial correlation coefficients for post dialysis BP-LVMI (r=0.30) and post dialysis BP-PWV (r=0.39) relations were remarkably similar to that for the 24-hour BP-LVMI (r=0.26), and 24-hour-PWV (r=0.41) relations. Hence it is unlikely that a larger study sample would have demonstrated a closer relationship between ambulatory BP and target organ changes as compared to dialysis BP and target organ changes.

In conclusion, the present study provides evidence to indicate that multiple BP measurements taken before and after dialysis sessions were as closely associated with both LVMI and large artery dysfunction as 24-hour ambulatory BP values in patients with renal failure. These data suggest a cost-effective approach to predicting the impact of BP on cardiovascular target organ changes that appears to be as effective as ambulatory BP monitoring. These data provide evidence to indicate that further studies are required with cardiovascular morbidity and mortality as the main outcome to confirm this finding.

CHAPTER 3

Is there an association between indices of arterial stiffness/wave reflection and left ventricular mass index in patients receiving haemodialysis?

Abstract

The relationship between aortic stiffness and function and LVM in patients on HD is uncertain. This study assessed whether large artery function is associated with LVMI in 94 non-diabetic patients receiving MHD for an average of ~49 (3-300) months. Pulse wave analysis performed at the carotid, femoral and radial arteries was employed to determine carotid-femoral PWV and AIc. Echocardiography was performed to determine LVM which was indexed to body surface area (LVMI). Despite relations noted between systolic blood pressure and LVMI ($r=0.36$, $p<0.0005$) and pulse pressure and LVMI ($r=0.44$, $p<0.0001$), on univariate analysis no relationship between either PWV ($r=-0.08$), or AIc ($r=-0.10$) and LVMI was noted. Further, despite significant relations noted between systolic blood pressure and the mean of LV posterior and septal wall thickness (LV mean wall thickness-MWT) ($r=28$, $p<0.01$) and pulse pressure and LV MWT ($r=0.27$, $p<0.02$), on univariate analysis no relationship between PWV ($r=-0.11$), or AIc ($r=0.03$) and LV MWT was noted. Adjustments for potential confounders did not reveal a relationship between large artery function and either LVMI, or LV MWT. In conclusion, these results suggest that large artery dysfunction plays little role in contributing toward LVM or wall thickness in patients receiving chronic HD.

3.0 INTRODUCTION

Renal failure is associated with a high prevalence of left ventricular hypertrophy (LVH) (see section 1.1.1 of chapter 1). Left ventricular mass is a key prognostic variable for cardiovascular mortality in ESRD (Middleton RJ et al., 2001), (Goodman WG and Danovitch GM, 2004), (Stewart GA et al., 2005, Paoletti E et al., 2004). However, the explanation for the relationship between LVM and vascular events still remains uncertain. One potential explanation is that large artery pathology results in increases in aortic stiffness and arterial stiffness may enhance LVM through the impact of early reflected waves on central aortic pressures. In this regard, a number of studies conducted in hypertensives and in the general population have provided evidence to indicate that indices of arterial stiffness are indeed associated with LVM (Boutouyrie P et al., 1995); (Lekakis JP et al., 2004); (Leoncini G et al., 2006); (Gates PE et al., 2003); (Bouthier JD et al., 1985); (Roman MJ et al., 1996); (Chen CH et al., 1998); (Roman MJ et al., 2000);(Baguet JP et al., 2000); (Deague JA et al., 2001); (Iketani T et al., 2000). However, it is uncertain whether changes in aortic stiffness are associated with LVM in renal failure. In this regard, there is now substantial evidence to indicate that renal failure is associated with large artery dysfunction and that this predicts cardiovascular outcomes independent of conventional cardiovascular risk factors (Blacher J et al., 1999); (Shoji T et al., 2001); (Blacher J et al., 2003). However, whether large artery dysfunction is independently associated with LVM in patients with renal failure is presently controversial (London GM et al., 1996); (Lin YP et al., 2002); (Nitta K et al., 2004). The aim of the present study was therefore to clarify whether a relationship exists between commonly employed indices of large artery function (pulse wave velocity, augmentation index and pulse pressure) and LVM in a sample of patients with chronic renal failure receiving HD.

3.1 Methods

The study population, demographic and clinical assessments, blood pressure measurements, echocardiography techniques, pulse wave analysis and statistical procedures have been described in chapter 2.

3.2 Results

3.2.1 Characteristics of study population

The aetiology of ESRD, the management of these patients, and the demographic and clinical characteristics of these patients are outlined in chapter 2.

3.2.2 Factors associated with indices of arterial stiffness/wave reflection

The factors associated with arterial stiffness and wave reflection were determined in a multivariate stepwise regression model with age, sex, BMI, mean arterial pressure (MAP) (average dialysis MAP), number of antihypertensive agents, and smoking forced into the model (Table 3.1). In these models, age, MAP, and number of antihypertensive agents were associated with PWV, whereas MAP and male gender were associated with AIc.

3.2.3 Association between indices of arterial stiffness/wave reflection and left ventricular mass index or LV geometry

Despite strong correlations noted between systolic blood pressure (BP) and LVMI ($r=0.036$, $p<<0.0005$) and pulse pressure and LVMI (Figure 3.1), on univariate analysis no relationship between either PWV or AIc and LVMI was noted (Figure 3.2). Further, despite strong relations noted between systolic BP and the mean of LV posterior and septal wall thickness (LV mean wall thickness-MWT) ($r=0.28$, $p<0.03$) and pulse pressure and LV MWT (Figure 3.3), on univariate analysis no relationship between PWV (Figure 3.4), or AIc ($r=-0.03$, $p=0.75$) and LV MWT was noted. Similarly, no correlations were noted between PWV ($r= -0.15$, $p=0.16$), or AIc ($r=0.10$, $p=0.35$) and LV relative wall thickness or between PWV ($r=0.16$, $p=0.14$) and LV end diastolic diameter. However, AIc was correlated with LV end diastolic diameter ($r=0.33$, $p<0.005$).

After age and MAP adjustments there were still no relations between either PWV ($p=0.32$), or AIc ($p=0.21$) and LVMI. After age and MAP adjustments there was a trend for a relationship between PWV ($p=0.06$), but not AIc ($p=0.57$) and LV MWT. Similarly, a trend for an age and MAP-adjusted correlations was noted between PWV ($p=0.06$), but not AIc ($p=0.45$) and LV relative wall thickness. No relationship was noted after age and MAP adjustments between PWV and LV end diastolic diameter ($p=0.44$). However, after age and MAP adjustments, AIc was correlated with LV end diastolic diameter ($p<0.002$).

After adjustments for age, MAP, sex, smoking, body mass index, and number of antihypertensives, again there were no relations between either PWV ($p=0.48$), or AIc $p=0.22$) and LVMI. After adjustments for age, MAP, sex, smoking, body mass index, and number of antihypertensives, there was no relationship between either PWV ($p=0.11$), or AIc ($p=0.75$)

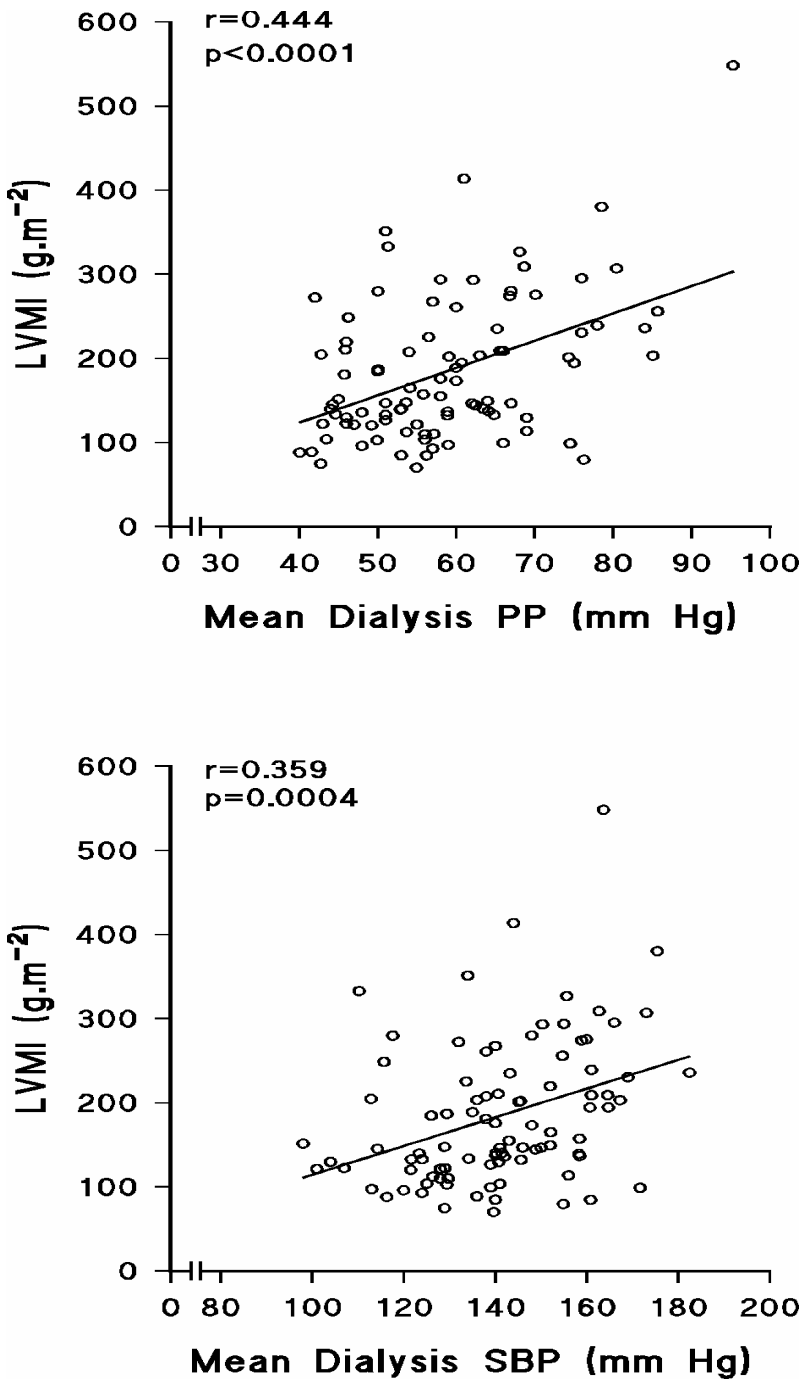


Figure 3.1 Association between pulse pressure (PP) or systolic blood pressure (SBP) and left ventricular mass index (LVMI) in patients receiving haemodialysis

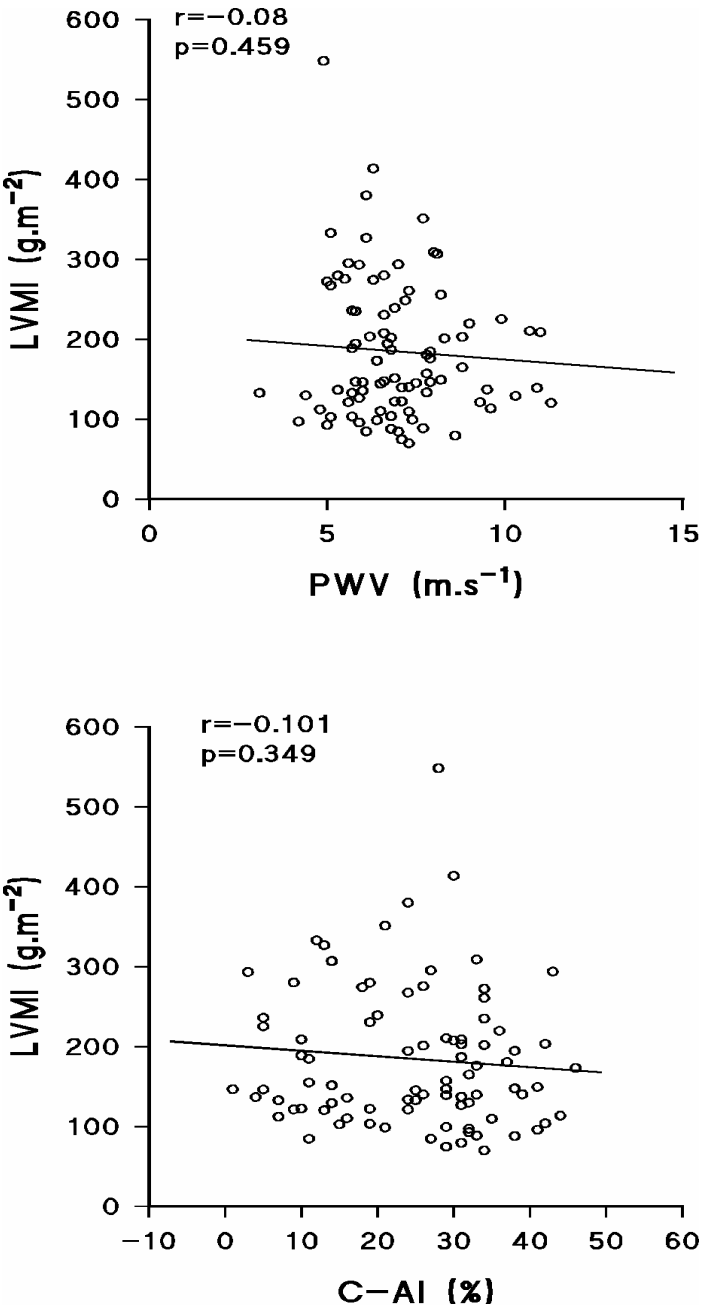


Figure 3.2 Association between pulse wave velocity (PWV) or central augmentation index (C-AI) and left ventricular mass index (LVMI) in patients receiving haemodialysis

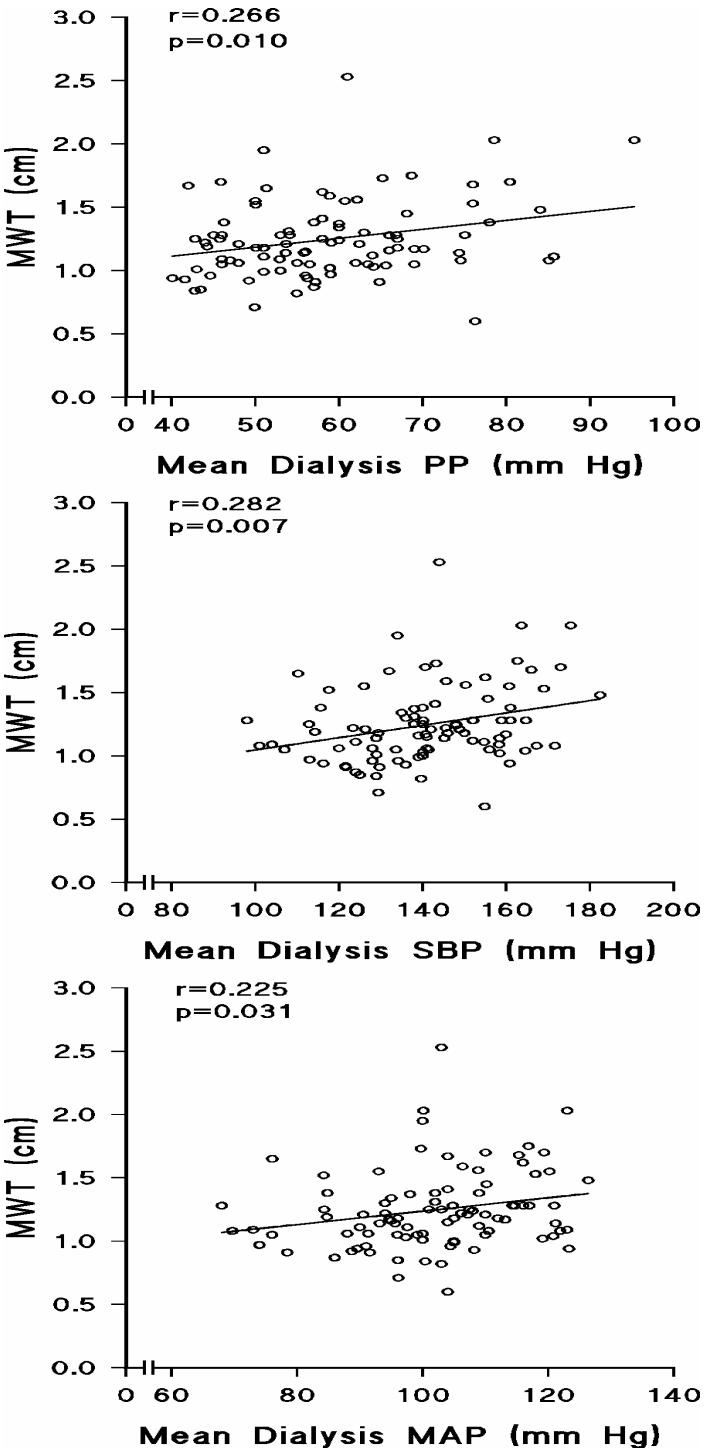


Figure 3.3 Association between pulse pressure (PP), systolic blood pressure (SBP) or mean arterial pressure (MAP) and left ventricular mean wall thickness (MWT) in patients receiving haemodialysis

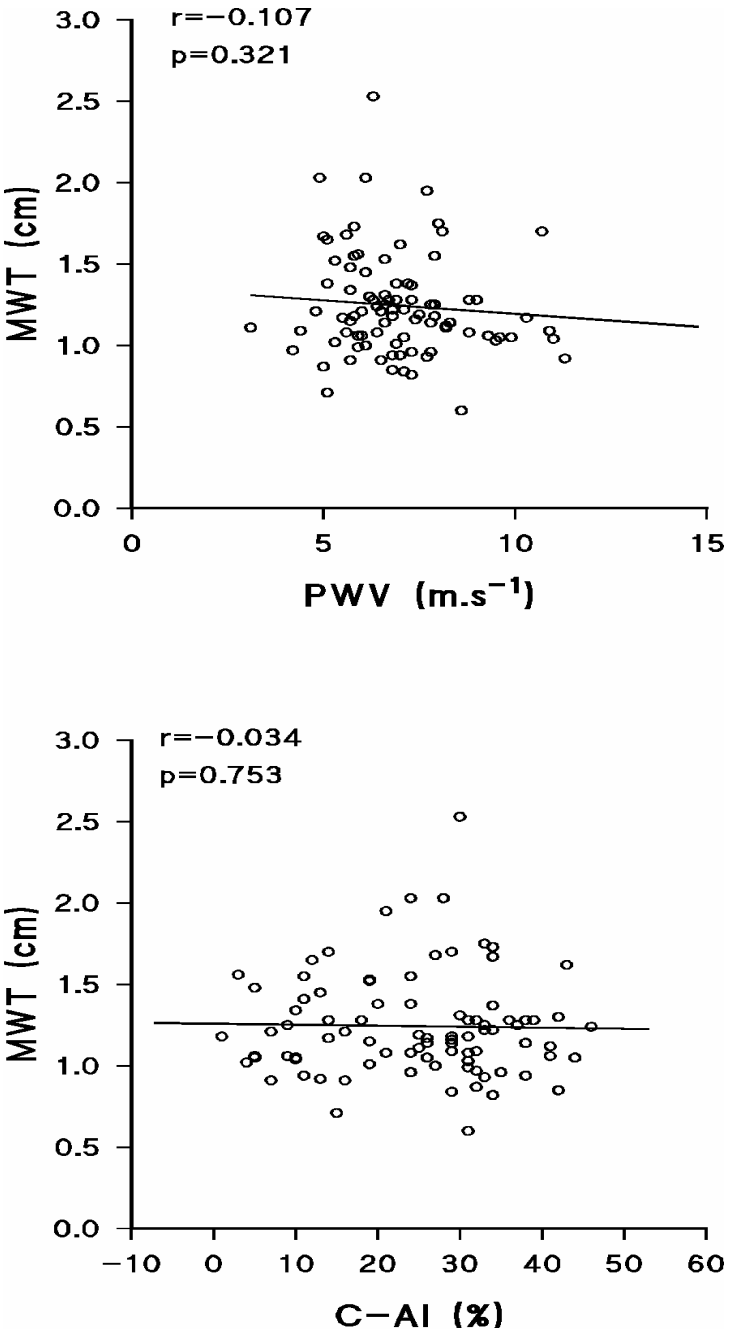


Figure 3.4 Association between pulse wave velocity (PWV) central or augmentation index (C-AI) and left ventricular mean wall thickness (LV MWT) in patients receiving haemodialysis

and LV MWT. However, after adjustments for age, MAP, sex, smoking, body mass index, and number of antihypertensives, there was a significant correlation noted between PWV ($p<0.05$), but not AIc ($p=0.39$) and LV relative wall thickness. No relationship was noted after adjustments for age, MAP, sex, smoking, body mass index, and number of antihypertensives between PWV and LV end diastolic diameter ($p=0.14$). However, after adjustments for age, MAP, sex, smoking, body mass index, and number of antihypertensives, AIc was correlated with LV end diastolic diameter ($p<0.01$).

3.3 Discussion

The results of the present study are in apparent contrast to previous studies demonstrating correlations between PWV and LVMI in patients with ESRD (Matsumoto Y et al., 2000, London GM et al., 1990); (Nitta K et al., 2004). However, in one study, although carotid artery thickness and dimensions were associated with LV dimensions and LVMI, PWV was not independently associated with LV dimensions and LVMI (London GM et al., 1996) Moreover, in another study the association between PWV and LVMI was in a much older sample (mean age ~60 years) of 49 patients on chronic HD, 17 of whom had diabetes mellitus (Nitta K et al., 2004). In this regard an older age group, and the presence of diabetes mellitus, both of which are well established risk factors for large artery stiffness (King GL and Wakasaki H, 1999) may enhance the ability to show relations between aortic stiffness and LVMI. Nevertheless, as suggested by the present study, this relationship is unlikely to be through an impact of renal failure on aortic stiffness and consequently LVMI. . In a follow-up study conducted in patients undergoing HD a third group of investigators demonstrated an independent relationship

between changes in aortic stiffness and LVMI (Matsumoto Y et al., 2000). However, the small sample size of that study (n=21) could have contributed toward a false positive outcome (Matsumoto Y et al., 2000).

The results of the present study are partly in support of one study, where a correlation between PWV and LVMI was only noted in normotensive, but not in hypertensive patients receiving HD (Lin YP et al., 2002). This finding is nevertheless difficult to explain considering that aortic stiffness effects on LVMI are thought to be mediated through central BP effects, which are correlated with peripheral BP.

An increase in arterial stiffness is well recognized to occur in patients with renal failure (Shinohara K et al., 2004). In the present study the mean carotid-femoral PWV reported on (see chapter 2) was ~7.0 cm/sec. For the same age range as the patients receiving HD in the present study, in a normal, healthy population sample in South Africa the mean PWV was reported to be 6.2 cm/sec (Shiburi CP et al., 2006). Consequently, as would be expected, in the present study there is evidence of an increased PWV. However, the upper threshold of a normal carotid-femoral PWV in healthy South Africans is reported to be ≤8.0 cm/sec (Shiburi CP et al., 2006). Using this threshold of normality 19% of the patients in the present study had an increased PWV. Whether this proportion is comparable with other studies cannot be determined as thresholds for PWV have not been determined in most populations. However, it is possible that in the present study, a lack of independent relationship between indices of arterial stiffness and LVMI could be explained by a relatively low proportion of subjects with an increased PWV. Indeed, some guidelines (Mancia G et al., 2007) cite cut-offs of 12 cm/sec for carotid-femoral PWV. Based on these thresholds, only ~1% of patients had an elevated carotid-femoral PWV. The reason for the relatively low proportion of subjects with an

increased PWV in the present study could relate to the relatively low mean age of the group studied (~41 years).

Although neither PWV nor augmentation index were associated with LVMI or LV MWT in the present study, these results do not preclude the possibility of increases in aortic stiffness contributing toward LVMI in other population samples. A number of studies conducted in the general population and in hypertensives show consistent relations between PWV and/or AIc and LVMI or LV geometry (Boutouyrie P et al., 1995); (Lekakis JP et al., 2004); (Leoncini G et al., 2006); (Gates PE et al., 2003); (Bouthier JD et al., 1985);(Roman MJ et al., 1996); (Chen CH et al., 1998); (Roman MJ et al., 2000);(Baguet JP et al., 2000); (Deague JA et al., 2001); (Iketani T et al., 2000)). However, even in these studies there is some argument as to whether this relationship is because of colinearity between BP, PWV and LVMI. Indeed, the majority of these previous studies have observed no additional predictive power of measures of arterial stiffness in relation to LVM beyond that provided by BP measurements. (Leoncini G et al., 2006); (Gates PE et al., 2003); (Bouthier JD et al., 1985); (Roman MJ et al., 1996); (Chen CH et al., 1998); (Roman MJ et al., 2000); (Baguet JP et al., 2000); (Deague JA et al., 2001).

The strong relationship between pulse pressure and LVMI, but not PWV or AI and LVMI in the present study may appear surprising. However, it is well recognized that pulse pressure is determined by stroke volume and large artery stiffness. Stroke volume is in-turn determined by cardiac filling volumes (Frank-Starling effect) and cardiac contractility. Filling volumes are largely driven by blood volume, whereas cardiac contractility is determined by a range of factors including sympathetic nervous system activity. Hence factors other than arterial distensibility are likely to predict pulse pressure and consequently contribute toward increases in LVMI in patients receiving HD. In the present study stroke volume was not determined and

hence the relative contribution of stroke volume and arterial distensibility to pulse pressure and, hence LVMI, could not be assessed.

In the present study an independent relationship was noted between AIc and LV end diastolic diameter, suggesting that cardiac cavity dimensions in chronic renal failure are associated with an enhanced wave reflection. This relationship, although fairly robust, is difficult to explain. It is possible that increased filling volumes in the heart may enhance stroke volume and thus the speed of wave reflection. However, this should have resulted in an association between LV end diastolic diameter and PWV. Alternatively, an increased wave reflection could have limited ventricular ejection and thus increased LV end diastolic diameter. However, no relationship between pump function and AIc was noted ($r=0.07$, $p=0.53$). Thus, further work is required to attempt to explain the relationship between AIc and LV end diastolic diameter in patients with chronic renal failure.

The limitations of the present study have largely been addressed in chapter 2 but are worth emphasising. First, the present and most previous studies (Matsumoto Y et al., 2000, London GM et al., 1990, Nitta K et al., 2004); (Lin YP et al., 2002) exploring the relationship between PWV and LVMI in patients receiving HD have been cross-sectional studies. The only prospective study conducted to-date was performed in 21 patients only (Matsumoto Y et al., 2000). Further prospective studies are required to better understand this potential relationship. Second, the limited study sample size ($n=79$) in the present study may have prevented us from detect ing an arterial function-LVMI relationship. However, with a correlation coefficient of -0.08 for the PWV-LVMI relationship, the chance of a larger sample size improving the sensitivity to detect a clinically meaningful positive as opposed to negative relationship is small. Another potential limitation of the study is that analysis of the impact of each class of antihypertensive agents could not be performed due to limited study sample size.

In conclusion, the present study indicates that neither PWV, nor central AI are determinants of LVMI, or LV mean wall thickness in all population samples of patients receiving HD. Although the present study therefore suggests that the relationship between LVMI and cardiovascular mortality in ESRD (Middleton RJ et al., 2001, Goodman WG and Danovitch GM, 2004);(Stewart GA et al., 2005, Paoletti E et al., 2004) is unlikely to be explained by an impact of large vessel changes on LVMI, further studies are required to clarify this issue in older groups of patients with ESRD.

CHAPTER 4

Inferior vena cava diameter as a blood pressure-independent determinant of left ventricular mass index and arterial stiffness in haemodialysis

Abstract

Although volume status contributes toward cardiovascular target organ changes in chronic renal failure, the relative contribution of blood pressure (BP)-dependent and -independent mechanisms has not been determined. This study assessed whether IVCD, an index of volume status was associated with LVM and geometry and large artery dysfunction independent of multiple pre- and post- dialysis BP measurements and 24-hour BP in 94 non-diabetic patients receiving maintenance haemodialysis for an average of ~49 (3-300) months. Pulse wave analysis performed at the carotid, femoral and radial artery was employed to determine carotid-femoral PWV and AIc. Echocardiography was performed to determine LVM which was indexed to body surface area (LVMI) and LV geometry and IVCD was determined using ultrasound techniques. After adjustments for a number of potential confounders as well as the average of pre- and post-dialysis systolic BP (SBP) values or 24-hour SBP, IVCD was independently associated with LVMI (partial r adjusted for average dialysis SBP=0.27, $p=0.014$; partial r adjusted for 24-hour SBP=0.29, $p=0.013$), and LV mean wall thickness ($p<0.01$), but not with either LV relative wall thickness ($p=0.18$), or LV end diastolic diameter ($p=0.88$). Moreover, after adjustments for a number of potential confounders as well as the average of pre- and post-dialysis systolic BP (SBP) values or 24-hour SBP, an association between IVCD and AIc (partial r adjusted for average dialysis SBP=0.21, $p<0.05$), but not PWV was noted. These data support the notion that in patients receiving HD, volume-overload produces effects on cardiovascular target organs that are not predicted by BP effects alone.

4.0 INTRODUCTION

As highlighted in chapter 1, there is substantial evidence to indicate that increases in LVM (Middleton RJ et al., 2001); (Goodman WG and Danovitch GM, 2004);(Stewart GA et al., 2005, Paoletti E et al., 2004) and in aortic stiffness (Blacher J et al., 1999, Shoji T et al., 2001); (Blacher J et al., 2003) predict cardiovascular outcomes independent of conventional risk factors in chronic renal failure. However, the principal factors responsible for increases in LVM and arterial stiffness in ESRD are uncertain. In this regard, there is a correlation between interdialytic changes in body weight, which reflect reductions in body fluid volume, and LVM (Harnett JD et al., 1993). Moreover, in patients with chronic renal failure receiving HD, extracellular-to-intracellular fluid ratio and the extent of oedema is associated with increases in arterial stiffness (Lin YP, 2003);(Tycho Vuurmans JL et al., 2002). As increases in body fluid volume result in increases in blood pressure (BP), volume overload in renal failure could impact on cardiovascular target organ changes through effects that are nevertheless predicted by BP values. However, ultrafiltration and a reduced salt intake in patients receiving HD reduces LVM independent of BP changes (Özhahya M et al., 1998). Nevertheless, as indicated in chapter 2, the strength of the relationship between BP and cardiovascular target organ changes is to some extent dependent on the BP measurement. In this regard, to our knowledge, there are no studies that have adjusted for multiple BP measurements, such as ambulatory or multiple pre- and post-dialysis BP when assessing the impact of fluid overload on LVM in chronic renal failure patients on MHD. The aim of the present study was therefore to assess whether an indicator of volume status [IVCD], previously shown to accurately and reliably predict right atrial pressures, circulating blood volume and haemodynamic changes during dialysis (Ando Y et al., 1985); (Cheriex EC et al., 1989); (Leunissen KM et al., 1993,

Kouw PM et al., 1993), was associated with LVMI and geometry and indices of arterial stiffness or wave reflection, independent of ambulatory or multiple pre- and post-dialysis BP measurements.

4.1 Methods

The study population, demographic and clinical assessments, BP measurements, echocardiographic techniques, pulse wave analysis and statistical procedures have been described in chapter 2.

4.1.1 Clinical assessment of hydration status

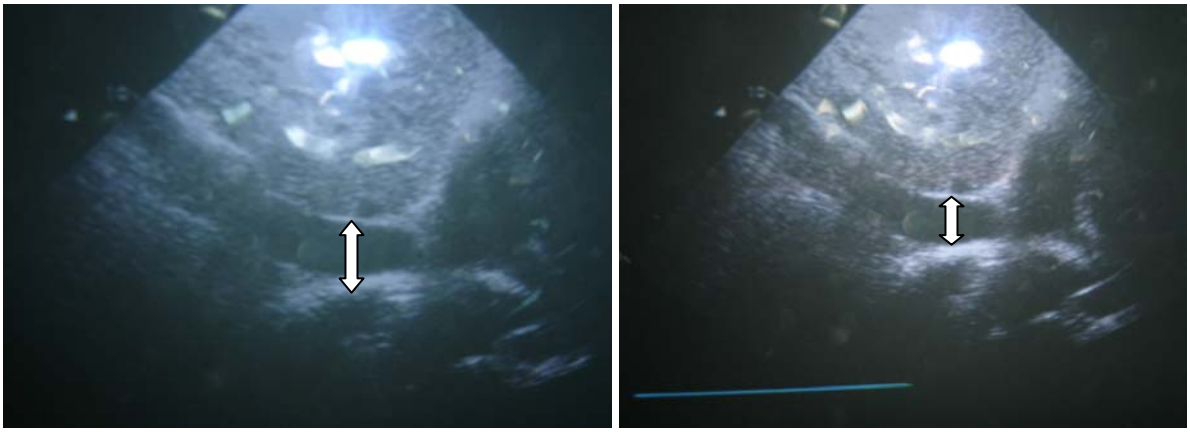
A clinical score of volume status was obtained as described by Kraemer et al and listed in Table 4.1 (Kraemer M et al., 2006). This was undertaken by completing a questionnaire on the same non-dialysis day prior to sonar examination (in all patients, save the 7 who had it at least one hour after dialysis) regarding symptoms observed by the patients since the last dialysis treatment. All symptoms were recorded by the investigator to standardise the process.

Table 4.1 Clinical score of volume state (Kraemer M et al., 2006):

Symptoms of hypovolaemia	
Thirst directly after HD	-1
Symptoms of hypotension, position change	-1
Symptoms of hypotension, requiring saline infusion	-2
Muscle cramps; moderate (calf)	-2
Muscle cramps, severe (calf)	-3
Limpness/tiredness between dialyses	-3
Dizziness between dialyses	-4
Symptoms of hypotension, vomiting	-6
Indication of euvolaemia	
Absence of symptoms given in this table	0
Symptoms of hypervolaemia	
Blood pressure increase during UF	+2
Pretibial oedema, weak	+2
Chronic coughing (new)	+2
Dyspnoea at rest, recumbent	+2
Pretibial oedema, severe	+3
Dyspnoea at rest, one cushion	+3
Dyspnoea at rest, two cushions	+4
Dyspnoea at rest, sitting	+6

4.1.2 Inferior vena cava diameter

Ultrasonography of the IVC was performed as described elsewhere (Brennan JM et al., 2006). This was performed on a non-dialysis day, at the same time as echocardiography in all patients with the exception of the seven patients who had it at least one hour after dialysis.This was done with patients in the supine position after l0 minutes of rest. The transducer was placed in the sub-xiphoidal region and long and short axis views of the IVC were obtained just below the diaphragm in the hepatic segment (Figure 4.1). Images were then frozen and scrolled to find the maximal IVC diameter during passive respiration within 2.5 cm of the IVC-right atrial junction. Patients then were asked to take a short, quick inspiratory effort or "sniff." Minimum IVC diameter (IVCmin) was measured as the smallest IVC size recorded during inspiration. IVCD was calculated at expiration and was corrected for body surface area (IVCD/BSA). The collapsibility index (Ci) was defined as [(maximal diameter on expiration-minimal diameter on deep inspiration)/maximal diameter on expiration] x 100. Patients with an IVCD less than 8 mm/m^2 and a collapsibility index of greater than 75% were considered underhydrated, and patients with an IVCD greater than 11.5 mm/m^2 or a collapsibility index less than 40% were considered overhydrated (Cheriex EC et al., 1989). Body surface area (BSA) was calculated using the Mosteller equation i.e. BSA (m^2) = ([Height (cm) x Weight (kg)]/ 3600)^{½} i.e. BSA = square root (SQRT) ((cm*kg)/3600) (Mosteller RD, 1987). The average of 2 readings taken in one sitting were used in the study. All studies were recorded and measurements were assessed off-line by an experienced consultant cardiologist.



Expiration phase (IVCmax) Inspiration phase (IVCmin)

Figure 4.1 Representative image of the inferior vena cava obtained using ultrasonography and the measurements thereof, in one of the patients

4.2 Results

4.2.1 Characteristics of study population

The aetiology of ESRD, the management of these patients, and the demographic and clinical characteristics of these patients are outlined in chapter 2. The mean IVCD/BSA was 11.4±2.1 mm/m^2.

4.2.2 Hydration status

The average interdialytic weight gain was 1.94±0.63 kg (range 0.39-3.88 kg) while the mean weight change during dialysis was 1.9±0.6 kg (range 0.42-3.74kg). Table 4.1 gives the clinical scores for hydration status of the patients. By clinical score 57 (61.3%) were euvolaemic (normal hydration status) as assessed by a score of 0, whilst an equal number 18

(19.4%) were either hypovolaemic (underhydrated) (negative score) or hypervolaemic (positive score). However, by inferior vena cava diameter assessment and Collapsibility index 49 (53%) and 53 (57%) patients were fluid overloaded, while 38(41%) and 40 (43%) respectively were euvolaemic.

Table 4.2 Clinical scores for hydration status of the study patients
(n = 93)

Total score	Frequency	Percent
-7	1	1.08
-4	1	1.08
-3	7	7.53
-2	2	2.15
-1	7	7.53
0	57	61.29
1	2	2.15
2	14	15.05
4	2	2.15

4.2.3 Association between inferior vena cava diameter and left ventricular mass and geometry

On univariate analysis IVCD was positively correlated to LVMI (Figure 4.2), the mean of LV posterior and septal wall thickness (LV mean wall thickness-MWT)(Figure 4.2), but not with LV relative wall thickness (RWT) (Figure 4.3) and LV end diastolic diameter (LVEDD)(Figure 4.3) .There was however no correlation between collapsibility index (Ci)

and either LVMI (r=0.07, p=0.54), LV MWT (r=0.05, p=0.63), or with LV RWT or LVEDD. Figures 4.4 and 4.5 show the partial correlations coefficients for the adjusted relations between IVCD and either LVMI, LV MWT, LV RWT or LVEDD. Adjustments were for age, sex, BMI, number of antihypertensive agents, and smoking and in addition either the average of pre- and post-dialysis, 24-hour, day, or night systolic BP (Figure 4.4) or pulse pressure (Figure 4.5). Importantly, even after adjustments for a number of different measures of BP and additional confounders, IVCD was still strongly related to LVMI, and LV MWT, but was not associated with either LV RWT or LVEDD.

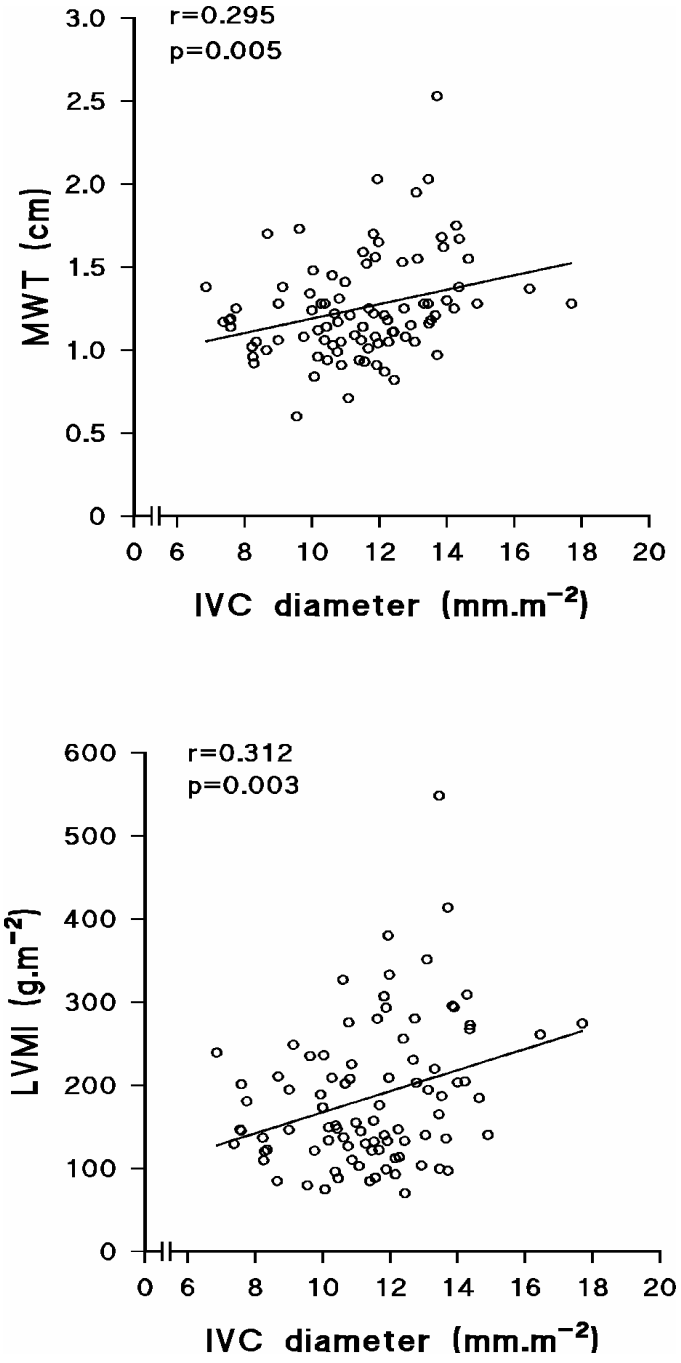


Figure 4.2 Relationship between inferior vena cava diameter and left ventricular mass index (LVMI) and LV mean wall thickness (MWT) in patients receiving haemodialysis

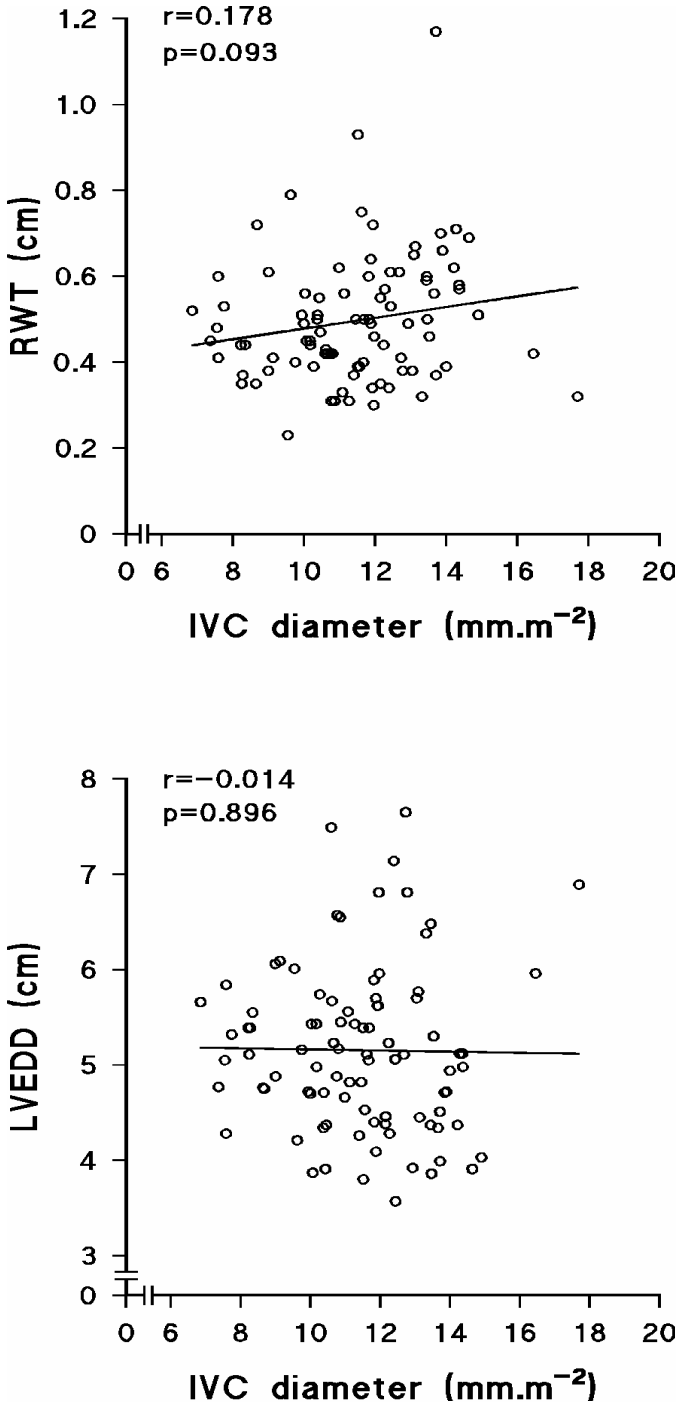


Figure 4.3 Relationship between inferior vena cava diameter and left ventricular relative wall thickness (RWT) and LV end diastolic diameter (LVEDD) in patients receiving haemodialysis

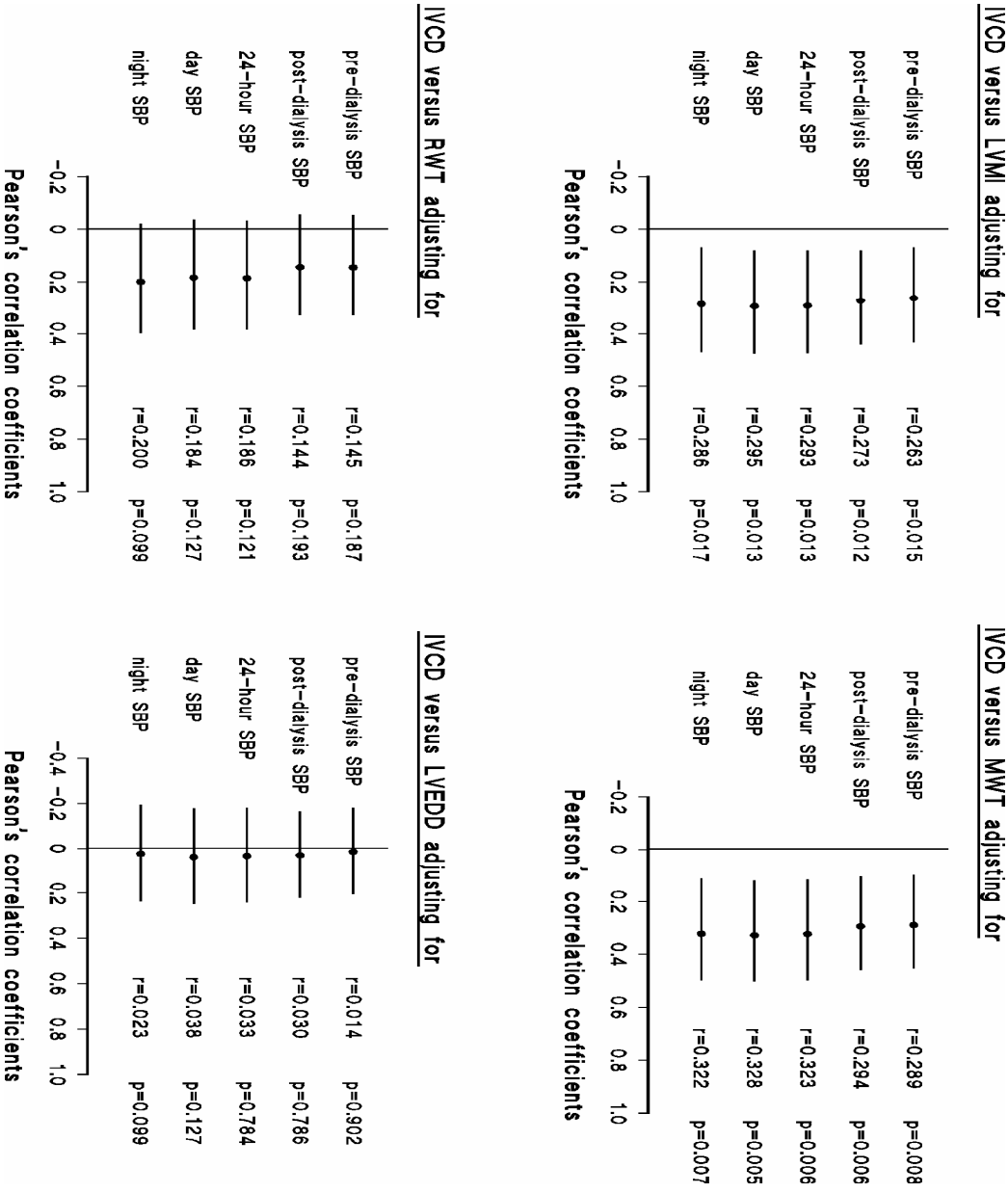


Figure 4.4 Partial correlation coefficients and the 95% confidence intervals for the adjusted relations between inferior vena cava diameter and left ventricular mass index (LVMI), LV mean wall thickness (LV MWT), LV relative wall thickness (LV RWT) and LV end diastolic diameter (LVEDD) in patients receiving haemodialysis. The data shown are after adjustments for age, sex, BMI, number of antihypertensive agents, and smoking and in addition either the average of pre- and post-dialysis, 24-hour, day, or night systolic BP (SBP).

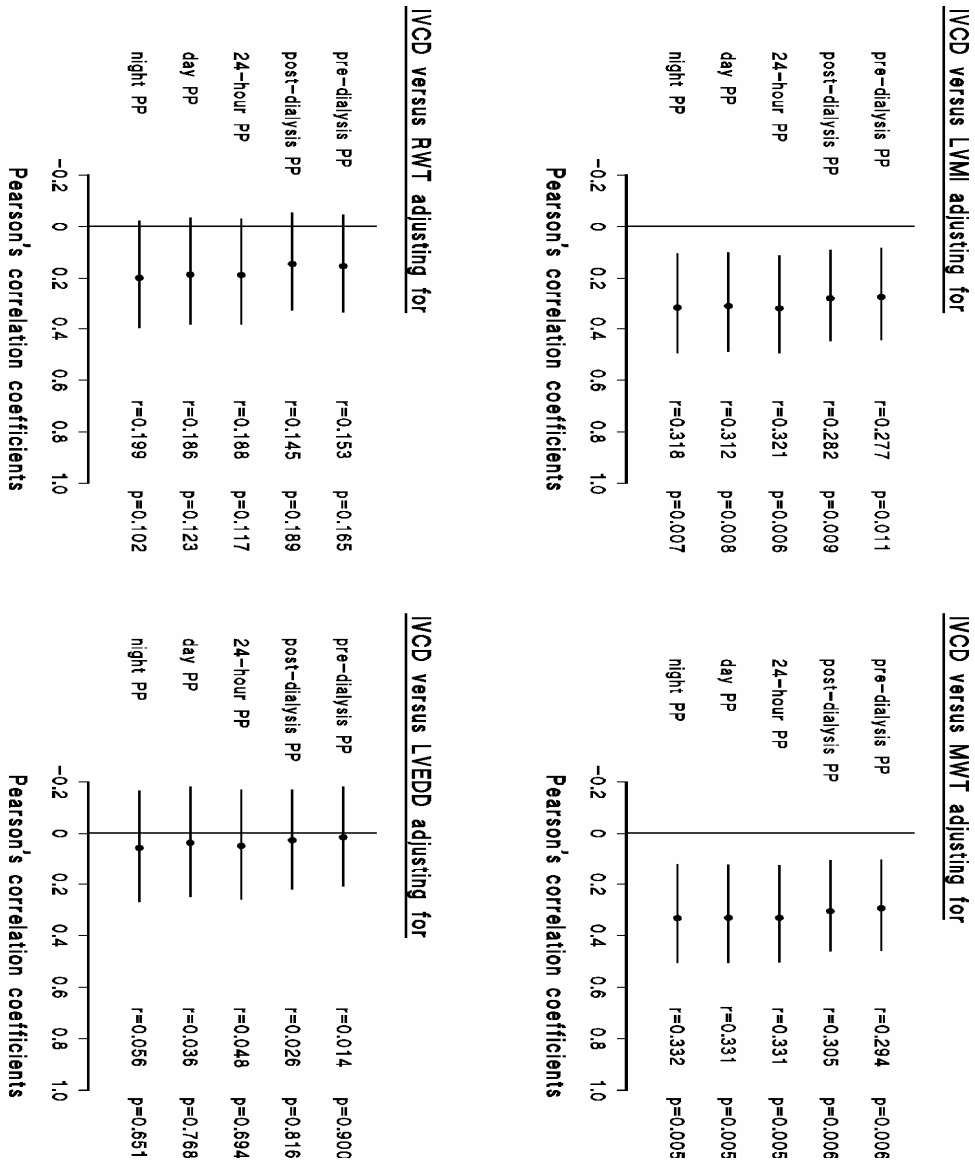


Figure 4.5 Partial correlation coefficients and the 95% confidence intervals for the adjusted relations between inferior vena cava diameter and left ventricular mass index (LVMI), LV mean wall thickness (LV MWT), LV relative wall thickness (LV RWT) and LV end diastolic diameter (LVEDD) in patients receiving haemodialysis. The data shown are after adjustments for age, sex, BMI, number of antihypertensive agents, and smoking and in addition either the average of pre- and post-dialysis, 24-hour, day, or night pulse pressure (PP).

4.2.4 Association between inferior vena cava diameter and arterial stiffness/wave reflection

On univariate analysis, IVCD showed a trend for a negative association with PWV (r=-0.21, p=0.056) and a trend for a positive association with AIc (r=0.20, p=0.06). On multivariate regression analysis with adjustments for age, sex, BMI, number of antihypertensive agents, and smoking, IVCD was no longer associated with PWV. Similarly on multivariate regression analysis with adjustments for all of the aforementioned confounders as well as average dialysis systolic BP, IVCD was not associated with PWV (r =-0.12, n=88, p= 0.27).

On multivariate regression analysis adjusting for age, BMI, smoking, and number of antihypertensives, IVCD was significantly associated with AIc (partial r=0.22, 95% confidence interval=0.002 to 0.416, p=0.047, n=87). When average dialysis SBP was included in the regression model, IVCD still retained a trend for an association with AIc with a similar regression coefficient as noted before adjustments for dialysis BP (r=0.21, 95% confidence interval= -0.008 to 0.411, p=0.057, n=87). Moreover, when 24-hour pulse pressure was included in the regression model, IVCD still retained a trend for an association with AIc with a similar regression coefficient as noted before adjustments for 24-hour pulse pressure (r= 0.24, 95% confidence interval= -0.002 to 0.45, p=0.05, n=74).

4.3 Discussion

The main findings of the present study are as follows. First, IVCD, which is an index of volume status, was associated with LVMI, LV mean wall thickness, LV relative wall

thickness and LV end diastolic diameter independent of both the average of multiple dialysis BP measurements and 24-hour BP measurements in patients receiving HD. Second, a trend for a relationship between IVCD and augmentation index independent of both the average of multiple dialysis BP measurements and 24-hour BP measurements was also noted.

The results of the present study are in agreement with the view that volume-overload in chronic renal failure is an important determinant of LVMI. Indeed, a correlation exists between interdialytic changes in body weight, which reflects reductions in body fluid volume, and LVM (Harnett JD et al., 1993). Moreover, the results of the present study are in agreement with the view that volume-induced changes in LVMI are partly independent of BP. Ultrafiltration and a reduced salt intake in patients receiving HD reduce LVM independent of BP changes (Özhahya M et al., 1998). The present study extends these previous studies (Harnett JD et al., 1993) by providing evidence to show that the volume-induced effects on LVMI are independent of multiple BP measurements obtained both before and after dialysis periods and independent of ambulatory BP, a finding that has not been previously reported, to the best of our knowledge. Thus, in the present study, a measurement of volume status (IVCD) predicted ~7% of the variability in LVMI in patients receiving HD beyond multiple BP values obtained in the interdialytic periods.

The results of the present study are in contrast to previous studies that suggest that volume-overload mediates increases in arterial stiffness in patients receiving HD. Indeed, the extracellular-to-intracellular fluid ratio and the extent of oedema have previously been shown to be associated with increases in arterial stiffness (Lin YP, 2003); (Tycho Vuurmans JL et al., 2002). In contrast, in the present study we were unable to show a positive relationship between IVCD and PWV. Nevertheless, as pointed out in chapter 3, although an increase in arterial stiffness is well recognized to occur in patients with renal failure (Shinohara K et al., 2004) ,

based on thresholds of a normal PWV in normal, healthy South Africans of 8.0 cm/sec (Shiburi CP et al., 2006), only 19.% of the patients in the present study had an increased PWV and based on thresholds for PWV of 12 cm/sec cited in guidelines (Mancia G et al., 2007) only ~1% of the patients in the present study had an increased PWV. It is therefore possible that in the present study, a lack of relationship between IVCD and PWV could be explained by a relatively low proportion of subjects with an increased PWV.

Although a relationship between IVCD and PWV could not be demonstrated in the present study, a trend for a relationship between IVCD and central AI was noted. Moreover, this relationship tended to persist despite adjusting for a number of potential confounders including the average of multiple pre- and post-dialysis BPs and ambulatory BP. These data therefore suggest that volume-overload may indeed mediate large artery changes beyond that produced by BP effects. The fact that this trend effect was noted for AI, but not for PWV provides further evidence to indicate that these measures cannot be used as surrogates of each other (Lemogoum et al 2007). The potential explanation for the contrasting relationship between IVCD and either PWV or AI is not however evident from the present study.

In the present study, although IVCD was clearly independently associated with LVMI and wall thickness, it was not associated with LV end diastolic diameter. These data suggest that volume-overload in chronic renal failure may not promote eccentric LVH alone. The impact of filling volumes on the heart may largely induce cardiac growth, with chamber dimensions being determined by alternative factors such as structural remodelling (right shifts in diastolic pressure-volume relations), pump dysfunction, or heart rate. These data also indicate that LV end diastolic diameter may not be a good index of cardiac volume status in chronic renal disease.

The clinical implications of the present study are that IVCD measurements may be clinically useful to predict target organ changes beyond conventional assessments of haemodynamic status, such as BP, in patients receiving HD. However, the fact that these measurements may reflect more acute, rather than chronic volume status should be considered as a potential confounding effect. To address this issue, further large prospective studies are required to assess whether IVCD is associated with changes in cardiovascular target organ effects over time.

The limitations of the present study have largely been addressed in chapters 2 and 3. First, the present study is a cross-sectional study. As indicated, further prospective analysis is still required to assess the value of IVCD measurements in predicting subsequent changes in cardiovascular target organ changes. Second, the study sample size may have prevented the detection of a statistically significant relationship between IVCD and AIc.

In conclusion, the present study supports the notion that volume status is an independent predictor of cardiovascular target organ changes in patients receiving HD. These data support the design and execution of prospective studies to assess whether IVCD is a useful clinical measurement in patients receiving HD.

CHAPTER 5

Natriuretic peptides in haemodialysis patients: Predictors of volume overload or left ventricular mass?

Abstract

Increased plasma concentrations of natriuretic peptides are associated with both increases in cardiac preload and in LVM. In chronic renal failure, there is uncertainty as to whether plasma natriuretic peptide concentrations predict volume status independent of LVMI. The association between natriuretic peptides and IVCD independent of LVM was assessed in 94 non-diabetic patients receiving MHD for an average of ~49 (3-300) months. Echocardiography was performed to determine LVM which was indexed to body surface area and IVCD was determined using ultrasound techniques. ANP, NT-proANP, BNP and NT-proBNP were measured in blood samples taken on the same interdialytic day as echocardiographic measurements. On univariate analysis, natriuretic peptides were correlated with LVMI and IVCD. On multivariate analysis, adjusting for age, sex, BMI, smoking, number of antihypertensive agents and IVCD, both NT-proANP and NT-proBNP were independently associated with LVMI ($p<0.0001$). Neither NT-proANP nor NT-proBNP were associated with IVCD independent of LVMI and additional confounders, although a trend effect for NT-proANP was still noted (partial $r=0.22$, $p= 0.074$, $n=76$). These findings suggest that plasma natriuretic peptide concentrations are closely associated with LVMI after adjusting for volume status in patients receiving HD.

5.0 INTRODUCTION

Although the measurement of circulating natriuretic peptides has an established role in the diagnosis and management of patients with heart failure (Cowie MR et al., 1997) and left-ventricular systolic dysfunction (McDonagh TA et al., 1998), the utility of natriuretic peptide measurement in CKD and ESRD is unclear. The measurement of ANP and related hormone concentrations has been suggested as an indicator of volume status in patients receiving HD (Rascher W et al., 1985). Elevated plasma concentrations of natriuretic peptides and their prohormones occur in patients with chronic renal failure (Kohse K et al., 1993) and plasma concentrations are reduced after haemofiltration (Zoccali C et al., 1986), HD (Anderson JV et al., 1986) and ultrafiltration (Shiota J et al., 1990, Wahl H et al., 2004). Moreover, in patients with chronic renal disease, natriuretic peptide concentrations are associated with increased IVCD (Ishikura F et al., 1996); (Metry G et al., 2001), an index of volume status shown to accurately and reliably predict right atrial pressures, circulating blood volume and haemodynamic changes during dialysis (Ando Y et al., 1985); (Cheriex EC et al., 1989), (Leunissen KM et al., 1993, Kouw PM et al., 1993). In addition, decreases in ANP concentrations are correlated with IVCD post-dialysis (Lee SW et al., 2003). However, relationships between plasma natriuretic peptide concentrations and volume status in chronic renal failure are partly confounded by increases in LVM, a change which in itself is associated with elevated plasma BNP concentrations (Naganuma T et al., 2002) (Joffy S and Rosner M H, 2005). To my knowledge there are no other studies that have assessed whether plasma natriuretic peptide concentrations are associated with more accurate measures of volume status such as IVCD independent of the confounding influence of LVM. Moreover, there are no studies that have assessed whether plasma natriuretic peptide concentrations are associated

with LVMI independent of the confounding influence of volume status (such as IVCD). Hence, the aim of the present study was to determine whether natriuretic peptide concentrations predict IVCD independent of LVMI and whether natriuretic peptides predict LVMI independent of IVCD in patients with CKD receiving MHD.

5.1 Methods

The study population, demographic and clinical assessments, BP measurements, echocardiographic techniques, and statistical procedures have been described in chapter 2. The measurement of IVCD has been described in chapter 4.

5.1.1 Plasma natriuretic peptides

Blood samples for ANP and BNP measurements were collected in vacutainer tubes containing EDTA in patients who had rested for 20 minutes prior to venepuncture.These were collected on the same interdialytic day as echocardiographic measurements. The sample was gently rocked several times immediately after collection for anti-coagulation and transferred to centrifuge tubes containing aprotinin (0.6TIU/ml of blood) to inhibit the activity of proteinases. Plasma ANP and BNP concentrations were determined in 36 patients. Blood samples were centrifuged at 1,600 x g for 15 minutes at 4°C and plasma was stored at -70°C**.** Plasma ANP concentrations were determined using an α-ANP (1-28) (Canine, Human, Porcine, Bovine) enzyme immunoassay (EIA) commercial kit (Phoenix Pharmaceuticals, Belmont, CA USA). The specificity of the assay to α-ANP (1-28) (Human, Canine, Porcine, Bovine) is 100%, to β-ANP (Human) is 100%, to rat ANP is 0%, and to porcine BNP-26 is <0.1%. The sensitivity (minimum detectable concentration) of the assay is 0.09 ng/ml. Plasma

BNP concentrations were determined using a BNP-32 (Human) (BNP-32, Human) EIA commercial kit (Phoenix Pharmaceuticals, Belmont, CA USA). The sensitivity (minimum detectable concentration) of the assay is 0.25 ng/ml.

Plasma N-terminal (NT)-proANP and serum NT-proBNP concentrations were determined in 76 and 92 patients respectively. To determine NT-proANP and NT-proBNP concentrations, centrifugation of blood samples was performed for 20 min at 2,000 x g, at 4°C and plasma and serum stored at –70°C. Plasma NT-proANP concentrations were determined using a proANP (1-98) EIA commercial kit (Biomedica Gruppe, Slovakia). The detection limit of the assay is 0.05 nmol/l with a standard range of 0-10 nmol/l. To determine NT proBNP an assay system based on a Sandwich ELISA principle (electrochemiluminescence immunoassay) was employed using Elecsys proBNP reagent kit (Roche). The sensitivity (minimum detectable concentration) of the assay is 5 pg/mL. All assays were performed in duplicate.

5.2 Results

In the group, plasma concentrations of ANP, NT-proANP, BNP, and NT-proBNP were 0.73±0.22 ng/ml (n=36), 46.2±0.22 ng/ml (n=76), 2.43±1.16 ng/ml (n=36) and 12.5±12.8 ng/ml respectively (n=92).

5.2.1 Association of natriuretic peptides with left ventricular mass

On univariate analysis conducted in those patients who had all of the natriuretic peptide data available, neither plasma ANP (r=0.03, p=0.86, n=36), BNP (r=0.04, p=0.81, n=36), nor NT-proBNP (r=0.16, p=0.35, n=36) concentrations were associated with LVM

index (LVMI). However, plasma NT-proANP concentrations were associated with LVMI ($r=0.38$, $p=0.02$, $n=36$). On multivariate analysis adjusting for age, sex, BMI, smoking, number of antihypertensives, in addition to IVCD, plasma NT-proANP concentrations remained significantly associated with LVMI (partial $r=0.37$, $p<0.05$). However, neither plasma ANP, BNP, nor NT-proBNP concentrations were independently associated with LVMI.

When assessing the relationship between plasma NT-proANP or NT-proBNP concentrations and LVMI in all patients who had these measurements ($n=76$ for NT-proANP and $n=92$ for NT-proBNP), marked unadjusted correlations were noted (Figure 5.1). On multivariate regression analysis, after adjusting for age, sex, BMI, smoking, number of antihypertensives and IVCD, both plasma NT-proANP and plasma NT-proBNP concentrations were still strongly and independently associated with LVMI (Figure 5.2).

5.2.2 Association of natriuretic peptides with inferior vena cava diameter

On univariate analysis plasma NT-proANP concentrations were strongly correlated with IVCD (Figure 5.3). A trend for a relationship between plasma NT-proBNP concentrations and IVCD was also noted (Figure 5.3). However, neither plasma ANP nor plasma BNP concentrations were correlated with IVCD (data not shown).

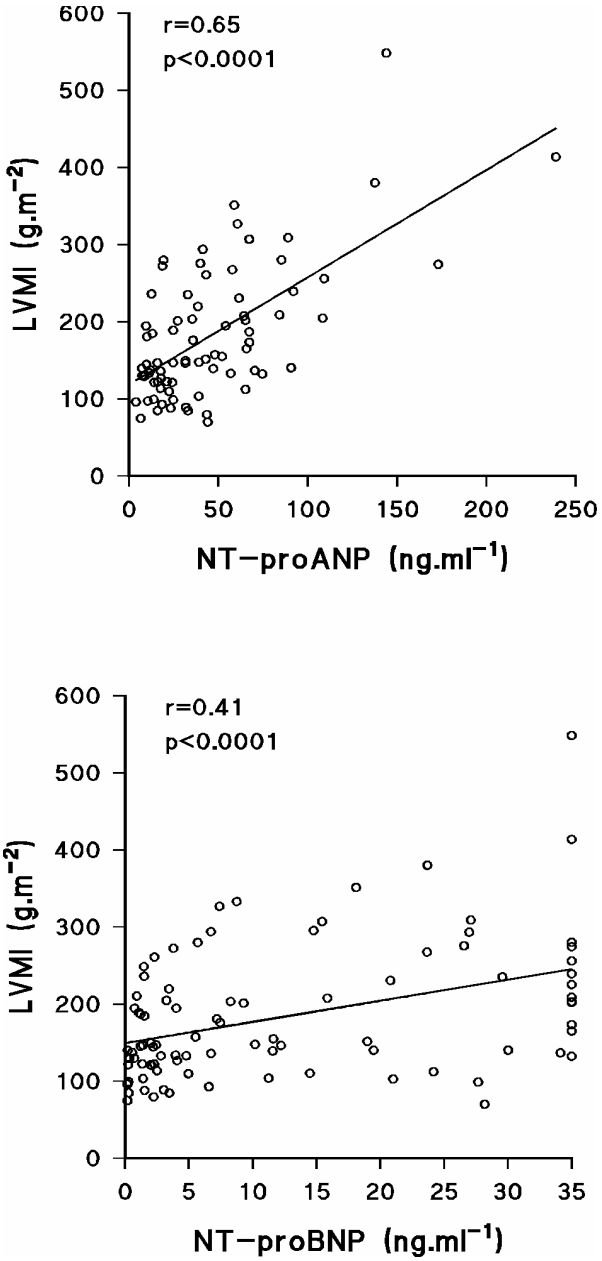


Figure 5.1 Correlations between N-terminal pro atrial natriuretic peptide (NT-proANP) or NT-proB-type natriuretic peptide (NT-proBNP) and left ventricular mass index (LVMI) in patients receiving haemodialysis

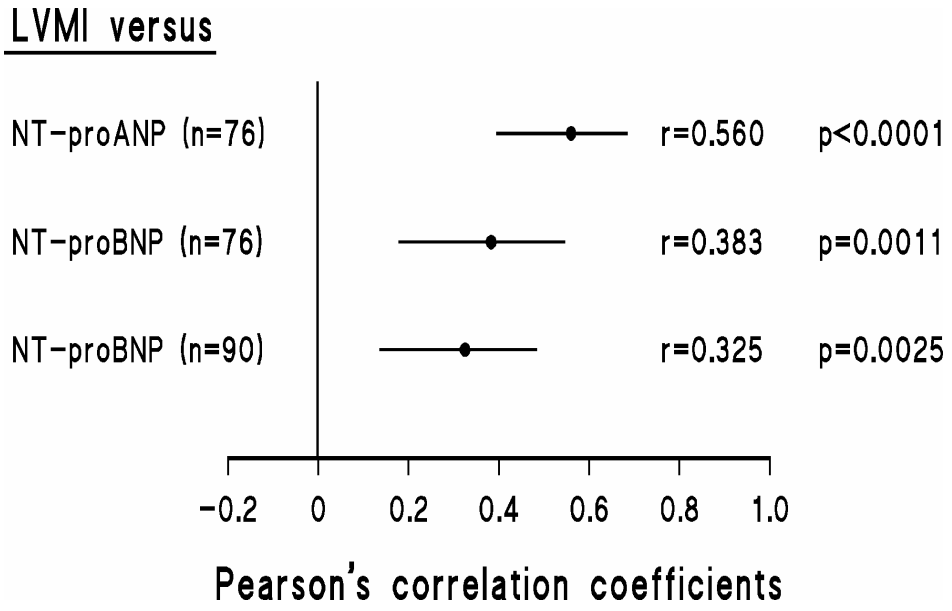


Figure 5.2 Partial correlation coefficients and 95% confidence intervals for the relationships between N-terminal pro atrial natriuretic peptide (NT-proANP) or NT-proB-type natriuretic peptide (NT-proBNP) and left ventricular mass index (LVMI) in patients receiving haemodialysis. Adjustments were for age, sex, BMI, smoking, number of antihypertensives and inferior vena cava diameter

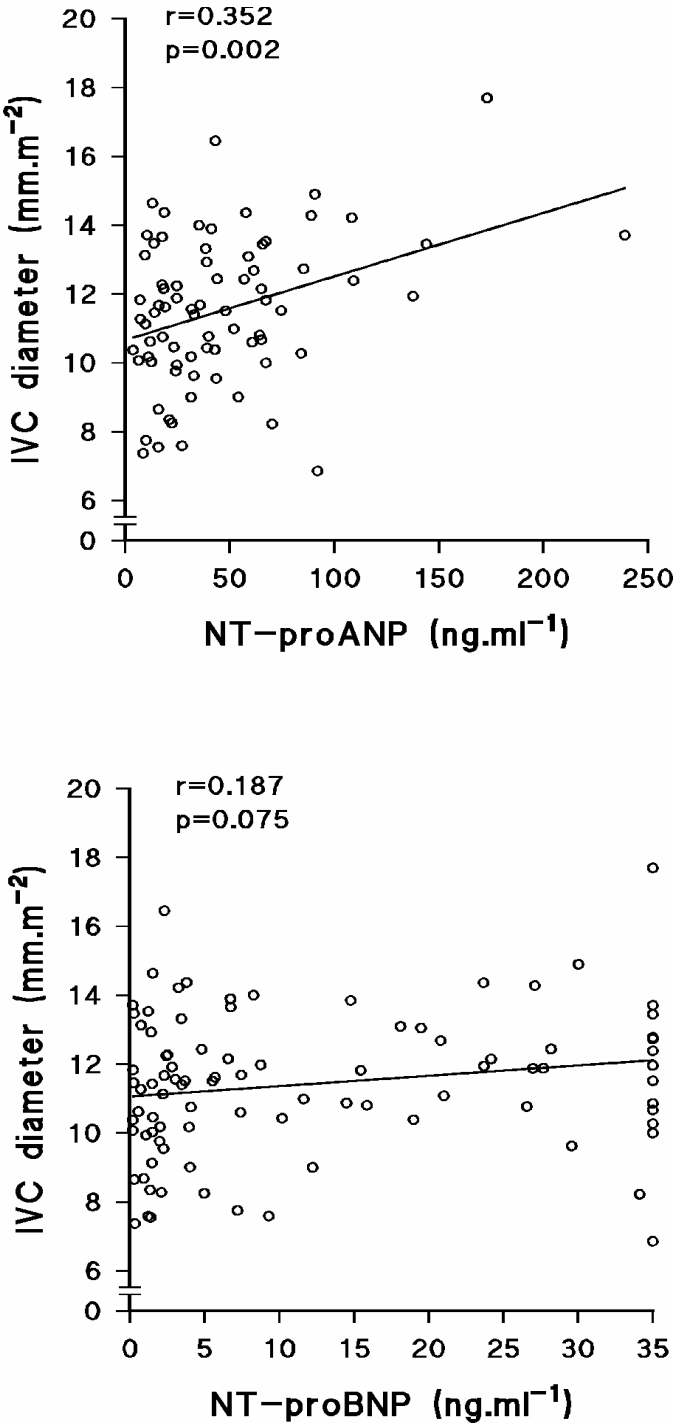


Figure 5.3 Correlations between N-terminal pro atrial natriuretic peptide (NT-proANP) or N-terminal brain natriuretic peptide (NT-proBNP) and inferior vena cava diameter (IVCD) in patients receiving haemodialysis

On multivariate regression analysis, after adjustments for age, sex, BMI, smoking, and number of antihypertensives, plasma NT-proANP remained independently associated with IVCD (partial r=0.35, confidence intervals=0.126 to 0.539, p<0.003, n=76). However, with the inclusion of LVMI in the regression model together with the other adjustors, plasma NT-proANP concentrations were no longer significantly associated with IVCD, although a trend effect still remained (partial r=0.22, p= 0.074, n=76). NT-proBNP similarly did not predict IVCD independent of LVMI and alternative potential confounders (partial r=0.04, p=0.72, n=90).

5.3 Discussion

The main findings of the present study in patients receiving chronic HD are as follows: First, both NT-proANP and NT-proBNP are associated with LVMI independent of a number of confounders including an index of volume status, namely IVCD. Second, although NT-proANP, but not NT-proBNP is associated with IVCD, this relationship is markedly diminished after adjustments for LVMI. Third, NT-proBNP is not associated with IVCD. Fourth, ANP and BNP were not associated with ICVD.

Although it is recognized that in CKD, estimated GFR and LVMI are independently related to plasma BNP and NT-proBNP concentrations (Vickery S et al., 2005), the present study is the first to address the question as to whether natriuretic peptide concentrations are associated with volume status independent of LVMI in patients receiving HD. Although a number of studies have reported on elevated natriuretic peptide concentrations in chronic renal

failure and changes in these concentrations with volume reduction, none of these studies considered the possibility that volume status is an independent predictor of LVMI (see chapter 4), and hence that relationships between natriuretic peptides and volume status could be accounted for by the well recognized relationship between LVMI and natriuretic peptides (Naganuma et al 2002; (Joffy S and Rosner M H, 2005). In this regard, the present study provides strong evidence to indicate that although NT-proANP is associated with IVCD, this relationship is markedly influenced by adjustments for LVMI. Indeed, with LVMI included as an adjustor in the model, the partial r value for the relationship between IVCD and NT-proANP concentrations was diminished from 0.35 to 0.22, with the statistical significance of the association diminishing from $p<0.005$ to $p=0.07$.

The measurement of ANP and related hormone concentrations has been suggested by some authors as a useful clinical indicator of assessing volume status in patients receiving HD (Rascher W et al., 1985). In this regard, plasma ANP and BNP concentrations may be increased by 4-7-fold in patients with ESRD before HD as compared to plasma concentrations noted in healthy volunteers (Kohse K et al., 1993) and pre-HD, IVCD is associated with natriuretic peptide concentrations (Lee SW et al., 2003). Further, an association between volume reductions produced by HD and changes in BNP concentrations have been reported (Fagugli RM et al., 2003) and ANP concentrations are reduced after both haemofiltration (Zoccali C et al., 1986), HD (Anderson JV et al., 1986) and ultrafiltration (Shiota J et al., 1990, Wahl H et al., 2004). In addition, decreases in ANP concentrations are correlated with IVCD post-dialysis (Lee SW et al., 2003). Although the present study does not preclude an independent association between NT-proANP and volume status in patients receiving HD, the present study does provide evidence to indicate that NT-proANP and related measurements need to be interpreted with caution when they are employed to determine volume status, due to

the strong relationship that exists between these measures and LVMI. Thus, although changes in NT-proANP concentrations over a period of HD may accurately reflect changes in volume status, a single measurement of NT-proANP concentrations may more closely reflect an increased LVMI, rather than volume status.

In the present study although NT-proANP concentrations were fairly closely associated with IVCD, ANP concentrations were not associated with IVCD. Although the sample of patients with ANP measurements was limited in size, similar outcomes were noted for the relationship between NT-proANP concentrations and IVCD in those patients with ANP measurements available, as was noted in the larger sample. Consequently, a limited sample size cannot explain this apparent discrepancy. This difference in the relationship between ANP concentrations and IVCD as compared to between NT-proANP concentrations and IVCD could be explained by the rapid clearance from plasma of ANP which has a half life of 3-4 minutes and hence limits the reproducibility of the measurement. In contrast, proANP has a much longer half-life (60-120 minutes) which leads to more stable concentrations in blood as compared to ANP and hence may produce values from single measurements as in the present study, which more closely resemble circulating concentrations over longer periods.

The association between NT-proANP with IVCD, but not between either BNP or NT-proBNP and ICVD in the present study is to some extent consistent with one previous study conducted in 15 patients, where post-dialysis, decreases in ANP, but not BNP concentrations were associated with reductions in left atrial dimensions and IVCD (Ishikura F et al., 1996). These data (Ishikura et al 1996) and the findings of the present study are nevertheless in contrast with the findings of a relationship between pre-HD IVCD and BNP concentrations in 49 patients (Lee SW et al., 2003) and with the association of volume reductions with HD and changes in BNP concentrations (Fagugli RM et al., 2003). Thus, further work is required to

attempt to explain the relationship between NT-proANP and IVCD, but not between either BNP or NT-proBNP and ICVD in the present study.

Unlike ANP, BNP is not stored in secretory granules and its release is dependent on continued transcription and translation of its gene (de Bold AJ et al., 2001). The difference in secretory patterns between ANP and BNP is thought to result in BNP providing a better index of LVM than ANP, which is thought to be more reflective of volume status (Nakao K et al., 1992). However, in the present study both NT-proANP and NT-proBNP were associated with LVMI independent of IVCD with the partial correlation being marginally higher for the NT-proANP relationship (r=0.56) than the NT-proBNP relationship (r=0.33-0.38). The present study therefore does not support the notion that NT-proBNP provides a better index of LVM than NT-proANP.

As with all studies the data should be interpreted in the context of the potential limitations of the study. As indicated in chapter 4, only a small proportion of patients were clinically overhydrated (~20%) by clinical score at the time of performing the measurements described in the present study. Thus, on this basis it is possible that the present study was not powered enough to detect a relationship between natriuretic peptides and IVCD independent of LVMI. However, by IVCD and Ci which are more sensitive, 49 and 53 patients were fluid overloaded, thus it is unlikely the number of patients with fluid overload would have influenced the outcome in this study. Further studies in a larger study sample are required to clarify this issue. Second, a limited number of patients had ANP and BNP measurements performed on them. This is obviously likely to limit the statistical power of relations between these values and either LVMI or IVCD. Further prospective studies are therefore still required.

In conclusion, the present study indicates that NT-proANP is independently associated with LVMI in patients receiving HD and hence that the relationship between NT-proANP and

volume status (as determined from IVCD), is markedly diminished by adjustments for LVMI. These data therefore suggest that the use of measurements of plasma concentrations of pro-natriuretic peptides to predict volume status in patients receiving HD needs to be interpreted with caution due to the confounding relationship between pro-natriuretic peptides and LVMI.

CHAPTER 6

Collagens and collagenases and LVMI and PWV in patients receiving haemodialysis:

The role of matrix metalloproteinase 1, 2 and 9; tissue inhibitor of matrix metalloproteinase 1 and 2; C-terminal telopeptide of type I collagen and carboxy-terminal peptide of procollagen type-I

Abstract

Recent studies have suggested that a number of markers of interstitial changes (collagens and collagenases) may predict pathological changes in the cardiovascular system. However, this hypothesis has not been tested in patients with renal failure, except for a study on intima media thickness. This study assessed whether (matrix metalloproteinase 1, 2 and 9; tissue inhibitor of matrix metalloproteinase 1 and 2; C-terminal telopeptide of type I collagen and carboxy-terminal peptide of procollagen type-I) these blood markers of interstitial changes were associated with left ventricular mass (LVM) and geometry and large artery dysfunction in 40 non-diabetic patients receiving maintenance haemodialysis for an average of ~49 (3-300) months. Pulse wave analysis performed at the carotid, femoral and radial artery was employed to determine carotid-femoral pulse wave velocity (PWV) and central augmentation index (AIc). Echocardiography was performed to determine LVM which was indexed to body surface area (LVMI). In either univariate or multivariate regression analysis, neither plasma concentrations of matrix metalloproteinases 1, 2 and 9, nor their tissue inhibitors (tissue inhibitor of metalloproteinases 1 and 2) were associated with LVMI, LV end diastolic diameter, LV mean wall thickness or PWV. Furthermore, neither procollagen I nor the C-terminal telopeptide of type I collagen (ICTP) was associated with LVMI, LV end diastolic diameter, LV mean wall thickness or PWV. In conclusion, the present study suggests that circulating measures of tissue interstitial changes may not predict cardiovascular target organ changes in patients with chronic renal failure receiving haemodialysis.

6.0 INTRODUCTION

Increases in myocardial and vascular collagen content, or alterations in either the subtypes of myocardial collagen found in the myocardial or vessel wall or the enzymes responsible for collagen degradation, are well recognized changes that occur in association with hypertension (Laviades C et al., 1998); (Chamiot Clerc P et al., 1999). Based on these findings it has been hypothesized that circulating concentrations of interstitial collagen turnover may predict cardiac and vascular pathology. In this regard, in the Framingham Heart Study plasma matrix metalloproteinase (MMP) 9 concentrations were associated with increased LV dimensions and LV wall thickness in men (Sundström J, 2004). Moreover, plasma MMP-9 and tissue inhibitor of MMP-1 (TIMP-1) concentrations were noted to be increased and MMP-1 and 2 and carboxy-terminal propeptide of procollagen type I (PIP) concentrations to be decreased in hypertensives with LVH (Ahmed SH et al., 2006, Laviades C et al., 1998). Further, plasma concentrations of MMP-1 were positively related to both large elastic and muscular artery stiffness in normotensive and hypertensive subjects (McNulty M et al., 2006) and plasma MMP-9 concentrations were correlated with aortic PWV even after adjustments for confounding variables (Yasmin et al., 2005). Despite these studies suggesting that measures of tissue collagen turnover in the circulation can predict cardiovascular target organ changes, whether similar relations are noted in chronic renal failure, which is characterised by cardiovascular damage, has not been assessed. Studies of circulating levels of MMPs and their inhibitors have been conducted previously during haemodialysis(Chou F-P et al., 2002) and in CKD (Chang H-R et al., 2006). After haemodialysis process, MMP-9 and MMP-2 levels were significantly different (lower) from predialysis values, while TIMP-2 increased and TIMP-1 was not affected by HD (Chou F-P et al., 2002). Moreover, in CKD,

serum creatinine concentrations and MMP-2 activity were higher while MMP-9 activity and creatinine clearance were lower than controls (Chang H-R et al., 2006). More recently, Pawlak et al have shown that intima media thickness exceeded controls in HD patients with and without atherosclerosis and that pre HD values of MMP-2, TIMP-1 and TIMP-2 were significantly increased in HD patients and associated with increased oxidative stress and increased intima media thickness prevalence. Consequently, as part of the present study we evaluated, on a dialysis free day,whether serum concentrations of PIP, MMP1, MMP-2, MMP-9, TIMP-1, TIMP-2 and C-terminal telopeptide of type I collagen (ICTP) are associated with LVMI and dimensions or large artery dysfunction in patients with chronic renal failure receiving HD.

6.1 Methods

The study population, demographic and clinical assessments, BP measurements, echocardiographic techniques, pulse wave analysis and statistical procedures have been described in chapter 2. Additionally all patients with atrial fibrillation, wall motion abnormalities and all the 7 patients who had echocardiography and IVCD measurements and blood tests on the same day were excluded.

6.1.1 Blood markers of interstitial changes

The blood samples for the measurement of MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, PIP and ICTP were obtained within a week of echocardiography or pulse wave analysis and centrifuged at 3000 rpm for 15 minutes. The serum was stored at -70°C until analysis.

Human matrix metalloproteinase (MMP) enzyme-linked immunosorbent assays. An *in vitro* enzyme-linked immunosorbent assay (ELISA) was employed to determine serum MMP-1, 2 and 9 concentrations (Raybiotech). These assay systems are designed for the quantitative measurement of human MMP-1, 2 and 9 pro- and active forms in serum, plasma (heparin), cell culture supernatants and in urine. These assays employ an antibody specific for either human MMP-1, 2 or 9 coated on 96-well plates. Samples were diluted 2-fold for the purposes of these assays. The results were obtained from an EL x 800 plate reader. The minimum detectable concentration of MMP-1 is <8 pg/ml, MMP-2 is less than 80 pg/ml and MMP-9 is less than 10 pg/ml. The intra- assay and inter-assay coefficients of variation are <10% and <12% respectively. For the MMP-2 assay only 17 samples were above blank absorbance despite repeating the assay on three occasions.

Human tissue inhibitors of matrix metalloproteinase (TIMP) enzyme-linked immunosorbent assays. An in vitro ELISA was also employed to determine circulating TIMP-1 and 2 concentrations (Raybiotech). The assay systems are designed for the quantitative measurement of human TIMP-1 and 2 in serum, plasma (heparin), cell culture supernatants and urine. These assays similarly employ an antibody specific for human TIMP-1 or TIMP-2 coated on 96-well plates. The samples were diluted 150-fold for the TIMP-1 assay and 25-fold for the TIMP-2 assay. The results were obtained using an EL x 800 plate reader. The

minimum detectable concentration of TIMP-1 is <40 pg/ml and for TIMP-2 is <10 pg/ml. The intra-assay and inter-assay coefficients of variation are <10% and <12% respectively.

C-terminal telopeptide of type I collagen (ICTP) enzyme immunoassay. Serum ICTP concentrations were determined using an enzyme immunoassay system (The Orion Diagnostica UniQ ICTP, Orion Diagnostics). This assay system is based on a competitive immunoassay technique where after washing away the free antigen, the amount of labelled ICTP in the well is inversely proportional to the amount of ICTP in the sample. The results were obtained using an EL x 800 plate reader. The measurement range for the assay is 1.0-50 μg/l, and the detection limit ~0.3 μg/l.

Procollagen type I C-peptide (PIP) enzyme immunoassay. Serum PIP concentrations were also determined using an enzyme immunoassay (Procollagen Type I C-peptide EIA Kit, Takara Bio Inc., Japan). The PIP enzyme immunoassay is a solid phase immunoassay based on a sandwich method that utilizes two mouse monoclonal anti-PIP antibodies to detect PIP by a one-step procedure. The samples were diluted 5-fold and the results were obtained using an EL x 800 plate reader. The detection limit for the assay is 10 ng/ml. The intra-assay and inter-assay coefficients of variation are 4.5-7.4% and 4.3-6.3% respectively.

6.2 Results

6.2.1 Characteristics of patients in whom interstitial markers were measured versus those in whom they were not

Only data from ~40 samples could be analysed as insufficient resources were available to complete this aspect of the study. Table 6.1 compares the demographic and clinical data

between patients in whom interstitial marker measurements could be obtained versus those in whom data could not be obtained. Table 6.1 also shows the mean±SD values for interstitial markers. Importantly, the characteristics of patients in whom serum concentrations of interstitial markers were measured were comparable with the characteristics of the group in whom they were not measured. Not indicated in Table 6.1 are the causes of renal failure. No significant differences were noted in the causes of renal failure between the two groups.

6.2.2 Relationships between serum concentrations of tissue interstitial changes

Figure 6.1 shows correlations between some serum concentrations of markers of tissue interstitial changes. Importantly, all the MMPs were correlated with each other. Further, MMP-9 was correlated with TIMP-1 and PIP correlated with TIMP-2. However, PIP was negatively correlated with MMP-2.

Table 6.1 General demographic and clinical characteristics of patients who had (with) versus those that did not have (without) measures of interstitial markers

	With (n=40)	Without (n=54)	p value
Age(years)	38.4±11.2	43.0±12.0	0.062
Male (%)	65	60	0.67
Haemodialysis duration (months)	38.9±57.6	57.1±44.2	0.08
Ever smoked (%)	19.5	15.0	0.58
Body height (cm)	163±11	166±10	0.17
Body weight (kg)	65±15	70±19	0.17
Body mass index (kg/m2)	24.4±4.9	25.3±6.6	0.47
Kt/V urea*	1.28±0.30	1.39±0.27	0.07
Pre-dialysis blood pressure (mm Hg)	145±15/88±10	138±19/79±16	0.06/0.001
Post-dialysis blood pressure (mm Hg)	141±18/84±13	135±23/76±14	0.17/0.006
Left ventricular mass index (g/m^2)	163±67	201±95	0.03
Pulse wave velocity (m/sec)	6.87±1.32	7.17±2.35	0.5
Procollagen type I C-peptide	1319±570		
C-terminal telopeptide of type I collagen	22.4±15.2 (n=36)		
Matrix metalloproteinase (MMP)-1 (mg/ml)	0.29±0.30 (n=35)		
Matrix metalloproteinase-2 (mg/ml)	0.25±0.45 (n=17)**		
Matrix metalloproteinase-9 (mg/ml)	5.06±3.82 (n=36)		
Tissue inhibitor of MMP-1 (mg/ml)	2.80±8.94 (n=36)		
Tissue inhibitor of MMP-2 (mg/ml)	0.27±0.25 (n=40)		

* indicates dialysis dose (* indicates dialysis dose (Kt/V averaged equilibrated urea clearance)

** Matrix metalloproteinase-2 studied in 36 patients but results detectable in only 17

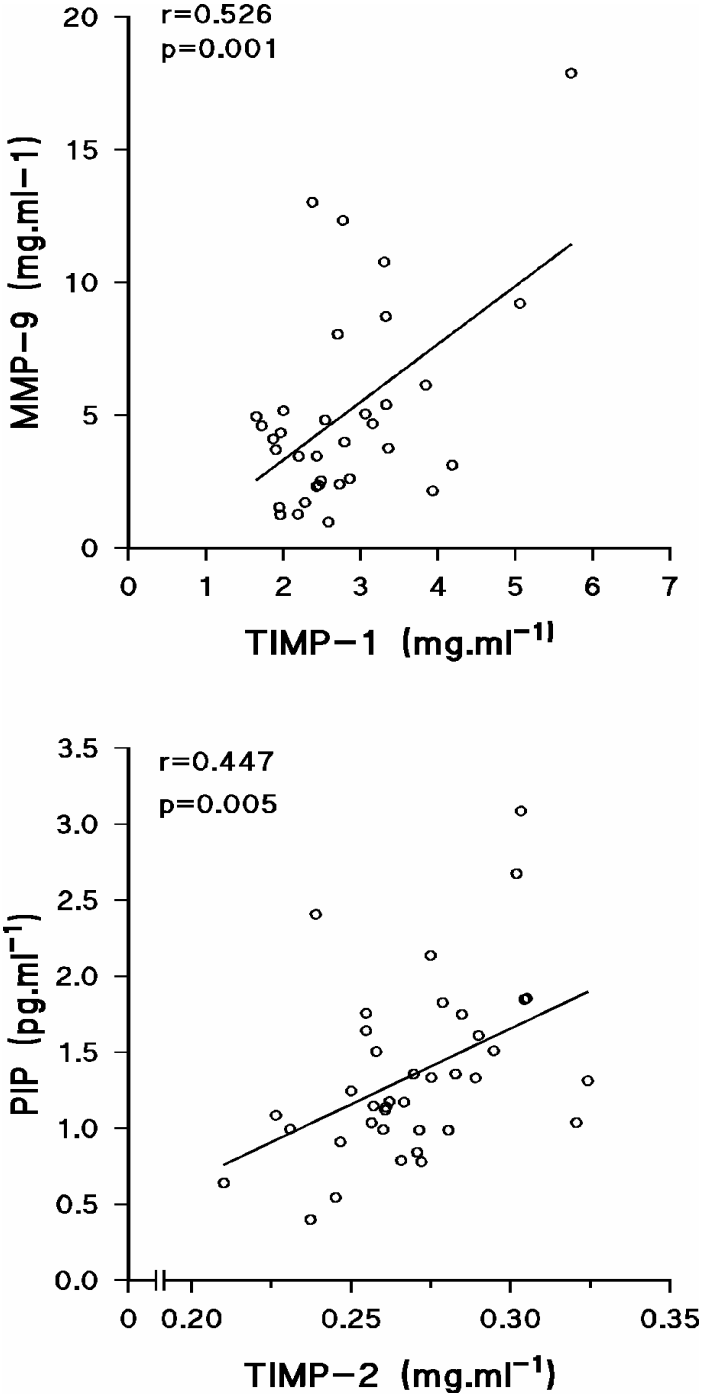


Figure 6.1 Relations between serum concentrations of markers of tissue interstitial changes. PIP, procollagen type I C-peptide; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP

6.2.3 Relationships between serum concentrations of tissue interstitial changes and LVMI

Table 6.2 summarises the univariate relationships between serum concentrations of markers of interstitial collagen changes and LVMI and between ratios of these markers and LVMI. No correlations between serum concentrations of markers of tissue interstitial changes and LVMI or between ratios of these markers and LVMI were noted. Moreover, on multivariate regression analysis with age, sex, the average of dialysis systolic BP values, and number of antihypertensives or smoking included in the regression analysis revealed no independent relationship between serum concentrations of markers of tissue interstitial changes and LVMI or between ratios of these markers and LVMI.

6.2.4 Relationships between serum concentrations of tissue interstitial changes and LV end diastolic diameter

Table 6.3 summarises the univariate relationships between serum concentrations of markers of interstitial collagen changes and LV end diastolic diameter and between ratios of these markers and LV end diastolic diameter. No correlations between serum concentrations of markers of tissue interstitial changes and LV end diastolic diameter or between ratios of these markers and LV end diastolic diameter were noted. Moreover, on multivariate regression analysis with age, sex, the average of dialysis systolic BP values, and number of antihypertensives or smoking included in the regression analysis revealed no independent relationship between serum concentrations of markers of tissue interstitial changes and LV end diastolic diameter or between ratios of these markers and LV end diastolic diameter.

Table 6.2 Pearson's correlation coefficients between serum concentrations of markers of interstitial collagen changes and left ventricular mass index in patients with chronic renal failure receiving haemodialysis

	R value	p value	Number
MMP-1	-0.152	0.39	35
MMP-2	0.067	0.80	17
MMP-9	0.061	0.73	34
TIMP-1	0.231	0.18	36
TIMP-2	0.185	0.25	40
PIP	0.119	0.46	40
ICTP	0.102	0.56	36
MMP-1/TIMP-1	-0.202	0.25	34
MMP-2/TIMP-2	0.082	0.76	17
MMP-9/TIMP-1	-0.137	0.44	34
MMP-9/TIMP-2	0.013	0.94	34
MMP-2/TIMP-1	0.011	0.97	17
MMP-1/TIMP-2	-0.166	0.35	34
PIP/ICTP	-0.041	0.81	36

Table 6.3 Pearson's correlation coefficients between serum concentrations of markers of interstitial collagen changes and left ventricular end diastolic diameter in patients with chronic renal failure receiving haemodialysis

	R value	p value	Number
MMP-1	0.14	0.42	34
MMP-2	0.14	0.60	17
MMP-9	-0.10	0.58	34
TIMP-1	0.11	0.52	36
TIMP-2	0.14	0.41	40
PIP	-0.06	0.70	40
ICTP	-0.10	0.55	36
MMP-1/TIMP-1	0.11	0.53	34
MMP-2/TIMP-2	0.15	0.56	17
MMP-9/TIMP-1	-0.21	0.25	34
MMP-9/TIMP-2	-0.11	0.52	34
MMP-2/TIMP-1	0.14	0.60	17
MMP-1/TIMP-2	0.14	0.43	34
PIP/ICTP	0.01	0.97	36

6.2.5 Relationships between serum concentrations of tissue interstitial changes and LV mean wall thickness

Table 6.4 summarises the univariate relationships between serum concentrations of markers of interstitial collagen changes and LV mean wall thickness (MWT) and between ratios of these markers and LV MWT. No correlations between serum concentrations of markers of tissue interstitial changes and LV MWT or between ratios of these markers and LV MWT were noted. Moreover, on multivariate regression analysis with age, sex, the average of dialysis systolic BP values, and number of antihypertensives or smoking included in the regression analysis revealed no independent relationship between serum concentrations of markers of tissue interstitial changes and LV MWT or between ratios of these markers and LV MWT.

6.2.6 Relationships between serum concentrations of tissue interstitial changes and PWV

Table 6.5 summarises the univariate relationships between serum concentrations of markers of interstitial collagen changes and PWV and between ratios of these markers and PWV. No correlations between serum concentrations of markers of tissue interstitial changes and PWV or between ratios of these markers and PWV were noted. Moreover, on multivariate regression analysis with age, sex, the average of dialysis systolic BP values, and number of antihypertensives or smoking included in the regression analysis revealed no independent relationship between serum concentrations of markers of tissue interstitial changes and PWV or between ratios of these markers and PWV.

Table 6.4 Pearson's correlation coefficients between serum concentrations of markers of interstitial collagen changes and left ventricular mean wall thickness in patients with chronic renal failure receiving haemodialysis

	R value	p value	Number
MMP-1	-0.26	0.13	35
MMP-2	-0.08	0.77	17
MMP-9	0.10	0.56	34
TIMP-1	0.20	0.25	36
TIMP-2	0.13	0.41	40
PIP	0.17	0.28	40
ICTP	0.04	0.82	36
MMP-1/TIMP-1	-0.31	0.08	34
MMP-2/TIMP-2	-0.07	0.80	17
MMP-9/TIMP-1	-0.09	0.61	34
MMP-9/TIMP-2	0.06	0.75	34
MMP-2/TIMP-1	-0.13	0.61	17
MMP-1/TIMP-2	-0.28	0.11	34
PIP/ICTP	0.07	0.70	36

Table 6.5 Pearson's correlation coefficients between serum concentrations of markers of interstitial collagen changes and carotid-femoral pulse wave velocity in patients with chronic renal failure receiving haemodialysis

	R value	P value	Number
MMP-1	-0.156	0.38	34
MMP-2	-0.230	0.38	17
MMP-9	-0.250	0.16	33
TIMP-1	-0.289	0.09	35
TIMP-2	0.150	0.36	39
PIP	0.025	0.88	39
ICTP	-0.251	0.15	35
MMP-2/TIMP-1	-0.229	0.38	17
MMP-9/TIMP-2	-0.220	0.22	33
MMP-1/TIMP-1	-0.095	0.60	33
MMP-2/TIMP-2	-0.216	0.41	17
MMP-9/TIMP-1	-0.074	0.68	33
MMP-1/TIP-2	-0.173	0.34	33
PIP/ICTP	0.190	0.28	35

6.3 Discussion

The main findings of the present study are that circulating concentrations of markers of cardiovascular collagen remodelling are not correlated with either LVMI, LV end diastolic diameter, LV mean wall thickness or PWV in a sample of patients with chronic renal failure receiving HD.

The present study is the first to my knowledge that has assessed whether circulating concentrations of markers of cardiovascular collagen remodelling are related to cardiovascular target organ changes (LVM and PWV) in patients with CKD. Previous studies have addressed this question in other groups. In this regard in a study of 699 Framingham Heart Study participants who had no history of heart failure or myocardial infarction, plasma MMP-9 concentrations were associated with increased LV dimensions and LV wall thickness in men (Sundström J, 2004). In the present study, the study sample was too small to assess sex-specific relations and hence I cannot exclude the possibility that MMP-9 is associated with LV end diastolic diameter and wall thickness in men with CKD. In this regard, the present study is being extended to allow for sex-specific analysis. Further, in the present study the majority of patients were receiving antihypertensive therapy, and this could have modified the relationship between MMP-9 concentrations and LVMI, LV end diastolic diameter or LV wall thickness.

Plasma MMP-9 concentrations have previously been reported to be increased and plasma MMP-2 concentrations decreased in hypertensives with LVH (Ahmed et al 2006). In addition, hypertensives with LVH have decreased plasma MMP-1 and PIP concentrations and higher concentrations of TIMP-1 (Laviades et al 1998). In the present study ~73% of patients had LVH. It is therefore possible that the high prevalence of LVH in the present study together

with the impact of concomitant antihypertensive therapy may have reduced the sensitivity to detect relations between MMP-1, 2 and 9, TIMP-1 and PIP concentrations and LVMI. In this regard, as indicated above, the present study is being extended to increase the study sample size. However, it is unlikely that the present study can be conducted in drug naïve patients.

With respect to studies showing relations between circulating markers of interstitial changes and changes in large artery function, in 46 normotensive and hypertensive subjects, plasma concentrations of MMP-1 were positively related to both large elastic and muscular artery stiffness (McNulty M et al., 2006). Moreover, in 116 subjects with isolated systolic hypertension and 114 matched controls, as well as 447 individuals free from CVD, plasma MMP-9 concentrations correlated with aortic PWV even after adjustments for confounding variables (Yasmin et al., 2005). In the present study no relationship between MMP-1 or MMP-9 and carotid-femoral PWV was noted in 33-34 patients with CKD receiving HD. However, again the lack of relationship between interstitial markers and large artery dysfunction in the present study could be explained by the fact that the majority of patients in the present study were receiving antihypertensive therapy. In this regard, antihypertensive therapy could have modified the relationship between interstitial markers and large artery function.

As indicated in previous chapters, the limitations of the present study are the cross-sectional nature of the study design and the relatively small study sample. However, in addition, as pointed out in the above discussion, the majority of patients were receiving antihypertensive therapy which may have modified the relationship between interstitial markers and target organ changes. Further, due to a limitation in resources, a large group of patients did not have measurements of interstitial markers available, thus further limiting the statistical power of the study and potentially biasing the outcome of the study toward the characteristics of patients in whom data on interstitial markers was available. However,

patients with and without measurements of interstitial markers had similar characteristics, and no trend effect for relations between interstitial markers and cardiovascular target organ changes was noted.

In conclusion, the present study does not support the use of measures of circulating concentrations of markers of cardiovascular interstitial changes as predictors of target organ changes in patients with CKD receiving HD. However, a larger study sample is required to improve on statistical power.

CHAPTER 7

Conclusion

Cardiovascular disease is a leading cause of death in ESRD. Early detection of CVD in patients with CKD using intermediate cardiovascular phenotypes, such as left ventricular hypertrophy (LVH) and changes in vascular structure and function is important as these have been shown to be important adverse prognostic indicators independent of all other conventional cardiovascular risk factors in the general population and in hypertensive populations. However, what is not entirely clear in literature is exactly what determines these intermediate cardiovascular changes in chronic renal failure and the extent to which each factor contributes toward LVH and large artery dysfunction. Controversy still remains in this regard. Moreover, Africa lacks reliable, comprehensive statistics and database containing profiles and outcomes in HD. Consequently, this study was aimed at contributing towards our understanding of the pathophysiology of LVH and large artery dysfunction in patients with ESRD on HD. We investigated the factors impacting on left ventricular hypertrophy and large artery stiffness in haemodialysis patients in a cross-sectional study. Potential factors that may determine LVH and large vessel changes in CKD were studied in a convenient sample of 94 patients undergoing MHD at various units in Johannesburg, South Africa. Pulse wave analysis performed at the carotid, femoral and radial artery was employed to determine carotid-femoral PWV and AIc. Echocardiography was performed to determine LVM which was indexed to body surface area (LVMI). Additionally, this study analysed the role for matrix metalloproteinases 1, 2 and 9 and their tissue inhibitors (TIMP1, 2) and markers of collagen 1 synthesis and degradation (PIP, ICTP) in these patients. These have been shown to affect cardiac and vascular remodelling in hypertensive and heart failure patients and animal studies but whose role in renal failure patients on HD is still unknown.

The value of multiple pre- and post-dialysis BP measurements in chronic renal failure is uncertain. In this study the average of multiple pre- and post-dialysis BP

measurements are equally effective in predicting cardiovascular target organ changes (LVMI and PWV) as 24-hour ambulatory BP values. This has clinical implications, particularly in Africa where 24 hour ABPM and echocardiography may not be widely available.

No relationship was found between large artery function and either LVMI, LV MWT, LV relative wall thickness or LV end diastolic diameter, the present study indicates that neither PWV, nor central or peripheral augmentation index are determinants of LVMI, LV mean wall thickness or LV relative wall thickness in this population sample of patients receiving HD, probably due to the relatively young age of the study population..

In this study we further showed after adjustments for a number of potential confounders as well as the average of pre- and post-dialysis SBP values or 24-hour SBP, IVCD is independently associated with LVMI, data supporting the view that in patients receiving HD, volume-overload produces effects on cardiovascular target organs that are not predicted by BP effects alone.

Natriuretic peptide concentrations are closely associated with LVMI after adjusting for volume status in patients receiving HD. However, after adjusting for LVMI, natriuretic peptides may only be weakly associated with volume status and hence have little role in predicting hydration status. We found no significant correlation between LVMI and the metalloproteinases, tissue inhibitors, and collagen type1synthesis and degradation in this study group, these need to be confirmed in a larger study.

LIMITATIONS OF THIS STUDY

The main limitation of the study was the cross sectional design.. Additionally the small sample size for the ANP, BNP, collagen and collagenase measurements affected the outcomes.

.

RECOMMENDATIONS

1. As LVH is a common occurrence in HD and has prognostic significance, it is recommended that, at initiation of dialysis, all patients should undergo baseline echocardiography and electrocardiography; these should be repeated routinely at three yearly intervals thereafter (National Kidney Foundation, 2005). Echocardiography should be performed after dry weight is attained.

2. Strict control of blood pressure is recommended in HD patients as it affects cardiac and vascular target organs contributing to the high cardiovascular mortality and morbidity.

3. Fluid overload is a major problem in haemodialysis. It is recommended that strict control of fluids and ideal dry weight be adhered to beyond control of blood pressure, as our study suggests that fluid overload has cardiovascular target organ effects beyond the effects of blood pressure.

4. Multiple pre, post and averaged dialysis blood pressures predict LVM and PWV just as well as does 24 hour ABPM. It is recommended that this may be a cost-effective approach to predicting the impact of BP on cardiovascular target organ changes that appears to be as effective as ambulatory BP monitoring, particularly in resource poor Africa where echocardiography and ABPM may not be widely available.

5. Further research in a larger sample is recommended to evaluate the role of MMPs, TIMPs and markers of collagen synthesis and degradation in cardiovascular target organ changes.

6. Further research is recommended in a larger and older HD population to clarify the relationship between LVM and arterial stiffness.

APPENDIX 1

Alpha-Atrial Natriuretic Polypeptide (1-28) (Canine, Human, Porcine, Bovine) EIA

Dilute the 20x assay buffer concentrate with 950ml of distilled water. This 1x assay buffer will be used to reconstitute all of the other compounds in this kit and samples.

Centrifuge and dilute standard peptide with 1 ml of 1x assay buffer. The concentration of this stock solution is 1,000ng/ml. Allow the standard to sit at least 10 minutes at room temperature to completely dissolve in solution. Centrifuge and vortex immediately before use.

Prepare peptide standard solutions as follows:

Standard No.	Std. volume	Assay Buffer	Concentrations
Stock	1,000µl	----	1,000ng/ml
#1	25µl of stock	975µl	25ng/ml
#2	200µl of #1	800µl	5ng/ml
#3	200µl of #2	800µl	1ng/ml
#4	200µl of #3	800µl	0.2ng/ml
#5	200µl of #4	800µl	0.04ng/ml

Rehydrate primary antiserum with 5ml of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.

Rehydrate biotinylated peptide with 5ml of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.

Centrifuge and rehydrate the positive control with 200µl of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.

Leave wells A-1 and A-2 empty as Blank.

Add 50µl of 1x assay buffer into wells B-1 and B-2 as Total Binding.

Add 50µl of the prepared peptide standard solutions from #5 to #1 (reverse order of serial dilution) into the wells from C-1 and C-2 to G-1 and G-2 respectively.

Add 50µl of rehydrated positive control into wells H-1 and H-2.

Add 50µl samples into their designated wells in duplicate.

Add 25µl rehydrated primary antiserum into each well except the Blank well.

Add 25µl rehydrated biotinylated peptide into each well except the Blank well.

Seal the immunoplate with acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C). Orbital shaking at 300-400 r.p.m.is recommended for the duration of the incubation.

Centrifuge the SA-HRP vial provided in this kit @ (3,000-5,000r.p.m., 5 seconds) and pipette 12µl SA-HRP into 12ml assay buffer to make SA-HRP solution. Vortex thoroughly.

Remove APS from the immunoplate. Discard contents of wells.

Wash each well with 350ml 1x assay buffer, discard the buffer,invert and blot dry plate. Repeat 4 times.

Add 100µl SA-HRP solution into each well.

Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C). Orbital shaking at 300-400 r.p.m.is recommended for the duration of the incubation.

Remove APS from the immunoplate. Wash and blot dry the immunoplate 4 times with 1x assay buffer as described above in step 16.

Add 100µl of the TMB substrate solution provided in this kit into each well including the Blank well. Orbital shaking at 300-400 r.p.m.is recommended for the duration of the incubation. After the addition of TMB it is strongly recommended to cover the immunoplate to protect from light.

Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C).

Remove APS from the immunoplate. Add 100µl 2N HCl into each well to stop the reaction. The colour in the well should change from blue to yellow. If colour change does not appear to be uniform gently tap the plate to ensure thorough mixing. Proceed to the next step within 20 minutes.

Remove APS and load the immunoplate onto a Microtiter Plate Reader.

Load the immunoplate onto a microtitre plate reader. Read absorbance O.D. at 450nm. The acceptable value of positive controls was given as 0.15-0.35ng/ml.

CALCULATIONS:

Plot the standard curve on semi-log graph paper. Known concentrations of standard peptide and its corresponding O.D. reading is plotted on the log scale (X-axis) and the linear scale (Y-axis) respectively. The standard curve shows an inverse relationship between peptide concentrations and the corresponding O.D. absorbances. As the standard concentration increases, the intensity of the yellow colour, and in turn the O.D. absorbance, decreases.

The concentration of peptide in a sample is determined by locating the sample's O.D reading on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line drawn from this point will intersect the X-axis at a coordinate corresponding to the peptide concentration in the sample. Because samples have been diluted prior to assay, the measured concentration must be multiplied by their respective dilution factors.

SUMMARY OF ASSAY PROTOCOL:

Add 50µl/well of standard or sample, 25µl primary antiserum and 25µl biotinylated peptide.

Incubate at room temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 350µl/well of 1x assay buffer

Add 100µl/well of SA-HRP solution

Incubate at room temperature (20-23°C) for 1 hour

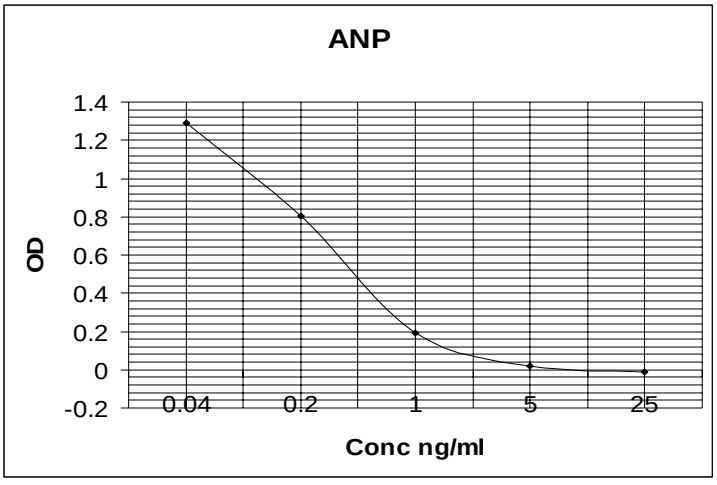
Wash immunoplate 4 times with 350µl/well of assay buffer

Add 100µl/well of TMB substrate solution

Incubate at room temperature (20-23°C) for 1 hour

Terminate reaction with 100µl/well of 2N HCl

Read absorbance O.D. at 450nM and calculate results



OD adsorbance, Conc concentration,

Graph showing the ANP concentration plot obtained in study patients

APPENDIX 2

Enzyme Immunoassay For The Quantitative Determination Of Human Proanp (1-98)
REAGENTS AND SAMPLE PREPARATION

This assay is suitable for the use of EDTA- or Heparinised plasma, urine or cell culture supernatants. ProANP in freshly collected blood samples is stable for at least 2.5 hrs at RT (18-26°C). Nevertheless it is recommended to perform plasma separation by centrifugation as soon as possible (e.g. 20 min at 2,000 x g, preferably at 4°C). Aliquot the acquired plasma samples and store them at –20°C or –70°C. Samples can be subjected to 4 freeze/thaw cycles without any loss of immune reactivity. Lipemic or haemolysed samples may give erroneous results. Urine or cell culture supernatants are used neat, without any further treatment. Samples should be mixed well before assaying. Duplicates are recommended for all values. If samples read higher than the top standard, it is recommended to dilute with ASYBUF (dilution buffer) (e.g.:1+4 and 1+9) and to re-measure the samples.

Reconstitute as follows:

 WASHBUF (Wash buffer): Dilute the concentrate 1:20 (1+19) e.g. 50 ml WASHBUF + 950 ml distilled water. Crystals in the buffer concentrate will dissolve at room temperature. Buffer is stable at 2-8°C until expiry date stated on label. Use only diluted WASHBUF (Wash buffer) to perform the assay:

 STD (Standard) and CTRL (Control): Pipette 250 µl of distilled or deionised water into the vial. Leave at room temperature (18-26°C) for 10 min. Reconstituted standard and control are stable at –20°C/-70°C until expiry date on label. Avoid freeze –thaw cycles.

ASSAY PROTOCOL

All reagents and samples must be at room temperature (18-26°C) before use in the assay.

Mark position for Blank/Standard/Sample/Control) on the protocol sheet.

Take microtiter strips out of the alu bag, take a minimum of one well as Blank. Store unused strips with desiccant at 2-8° C in the alu bag. Strips are stable until expiry date stated on the label.

Add 10 µl STD/SAMPLE/CTRL (Standards/Sample/Control) in duplicate into respective well, except blank.

Add 200µl CONJ (Conjugate) into each well except blank, swirl gently.

Cover tightly and incubate at room temperature (18-26°C) for 3 hrs in the dark.

Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer);

remove remaining WASHBUF by hitting plate against paper towel after the latest wash.

Add 200 µl SUB (Substrate) into each well.

Incubate for 30 min at room temperature (18-26°C) in the dark.

Add 50 µl STOP (Stop solution) into each well.

Measure absorbance immediately at 450 nm with reference 620 nm, if available.

CALCULATION OF RESULTS

Subtract the blank extinction from all other values. Construct the Standard curve from the Standard values. Use commercially available software or graph paper. Obtain sample concentration from this Standard curve. The assay has been evaluated using a 4PL algorithm. Different curve fitting methods need to be evaluated by the user. Respective dilution factors have to be considered.

ASSAY CHARACTERISTICS

Reference data: Plasma: median = 1.45 nmol/l (n=53). Each laboratory should establish own reference values.

Standard range: 0-10 nmol/l

Sample volume: 10µl plasma, urine or cell culture supernatant.

Detection Limit: (0 nmol/l + 3 SD): 0.050 nmol/l

Incubation time: 3 hours / 30 min

Cross reactivity: proANP (1-30) <1 %, proANP (31-67) <1%, ProANP (79-98) <1% , alpha ANP (99-126)<1%,proBNP (8-29) <1%, proBNP (32-57) <1%, proCNP (1-19) <1%, proCNP (30-50) <1%, proCNP(51-97) <1%.The assay also detects mouse and rat proANP (1-98).

No Hook-effect was observed up to a concentration of 80 nmol/l.

PRECISION

Intra-Assay (n=10)	Inter-Assay (n=5)
Mean (nmol/ml) 0.66	Mean (nmol/ml) 0.88
SD 0.013	SD 0.035
CV% 2%	CV% 4%

APPENDIX 3

Brain Natriuretic Peptide-32 (Human) (BNP-32, Human) EIA Kit

Dilute the 20x assay buffer concentrate with 950ml of distilled water. This 1x assay buffer will be used to reconstitute all of the other compounds in this kit and samples.

Centrifuge and dilute standard peptide with 1 ml of 1x assay buffer. The concentration of this stock solution is 1,000ng/ml.

Prepare peptide standard solutions as follows:

Standard No.	Std. volume	Assay Buffer	Concentrations
Stock	1,000µl	----	1,000ng/ml
#1	100µl	900µl	100ng/ml
#2	100µl of #1	900µl	10ng/ml
#3	100µl of #2	900µl	1ng/ml
#4	100µl of #3	900µl	0.1ng/ml
#5	100µl of #4	900µl	0.01ng/ml

Rehydrate primary antiserum with 5ml of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.

Rehydrate biotinylated peptide with 5ml of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.

Centrifuge and rehydrate the positive control with 200µl of 1x assay buffer. Allow to sit for at least 5 minutes to completely dissolve. Mix thoroughly.

Leave wells A-1 and A-2 empty as Blank.

Add 50µl of 1x assay buffer into wells B-1 and B-2 as Total Binding.

Add 50µl of the prepared peptide standard solutions from #5 to #1 (reverse order of serial dilution) into the wells from C-1 and C-2 to G-1 and G-2 respectively.

Add 50µl of rehydrated positive control into wells H-1 and H-2.

Add 50µl of prepared samples into their designated wells in duplicate.

Add 25µl rehydrated primary antiserum into each well except the Blank well.

Add 25µl rehydrated biotinylated peptide into each well except the Blank well.

Seal the immunoplate with acetate plate sealer (APS). Incubate the immunoplate for 2 hours at room temperature (20-23°C). Orbital shaking at 300-400 r.p.m.is recommended for the duration of the incubation.

Centrifuge the SA-HRP vial provided in this kit @ (3,000-5,000rpm, 5 seconds) and pipette 12µl SA-HRP into 12ml assay buffer to make SA-HRP solution, vortex thoroughly.

Remove APS from the immunoplate. Discard contents of wells.

Wash each well with 350ml 1x assay buffer, discard the buffer,invert and blot dry plate. Repeat 4 times.

Add 100µl SA-HRP solution into each well.

Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C). Orbital shaking at 300-400 rpm is recommended for the duration of the incubation.

Remove APS from the immunoplate. Wash and blot dry the immunoplate 4 times with 1x assay buffer as described above in step 17.

Add 100µl of the TMB substrate solution provided in this kit into each well including the Blank well. Orbital shaking at 300-400 rpm is recommended for the duration of the incubation. After the addition of TMB it is strongly recommended to cover the immunoplate to protect from light.

Reseal the immunoplate with APS. Incubate for 1 hour at room temperature (20-23°C).

Remove APS from the immunoplate. Add 100µl 2N HCl into each well to stop the reaction. The colour in the well should change from blue to yellow. If colour change does not appear to be uniform gently tap the plate to ensure thorough mixing. Proceed to the next step within 20 minutes.

Load the immunoplate onto a Microtitre Plate Reader. Read absorbance O.D. at 450nm.

CALCULATIONS:

Plot the standard curve on semi-log graph paper. Known concentrations of standard peptide and its corresponding O.D. reading is plotted on the log scale (X-axis) and the linear scale (Y-axis) respectively. The standard curve shows an inverse relationship between peptide concentrations and the corresponding O.D. absorbances. As the standard concentration increases, the intensity of the yellow colour, and in turn the O.D. absorbance, decreases. The concentration of peptide in a sample is determined by locating the sample's O.D reading on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line drawn from this point will intersect the X-axis at a coordinate corresponding to the peptide concentration in the sample. Because samples have been diluted prior to assay, the measured concentration must be multiplied by their respective dilution factors. The standard curve will be a reverse sigmoidal shape.

The acceptable value of positive controls was given as 0.55-0.75ng/ml.

SUMMARY OF ASSAY PROTOCOL:

Add 50µl/well of standard or sample, 25µl primary antiserum and 25µl biotinylated peptide.

Incubate at room temperature (20-23˚C) for 2 hours

Wash immunoplate 4 times with 350µl/well of 1x assay buffer

Add 100µl/well of SA-HRP solution

Incubate at room temperature (20-23˚C) for 1 hour

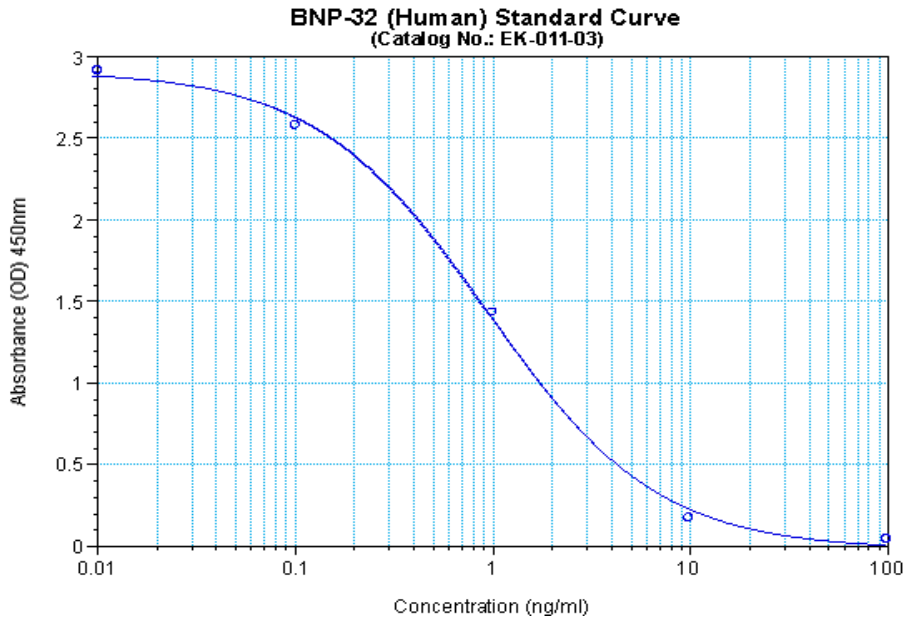
Wash immunoplate 4 times with 350µl/well of assay buffer

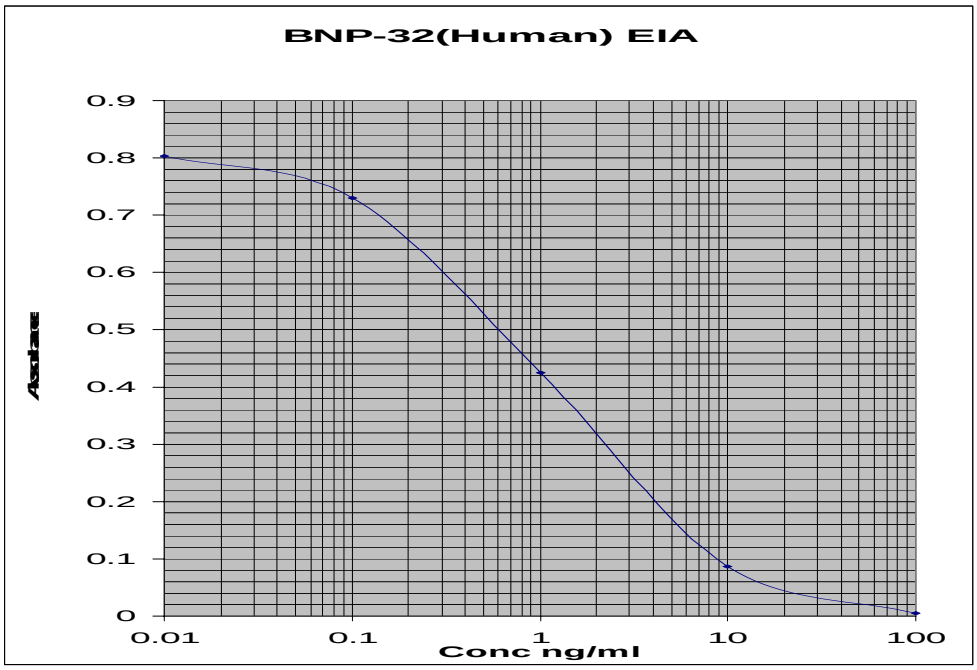
Add 100µl/well of TMB substrate solution

Incubate at room temperature (20-23˚C) for 1 hour

Terminate reaction with 100µl/well of 2N HCl

Read absorbance O.D. at 450nM and calculate results





Standard curve for BNP study participants

APPENDIX 4

NT-proBNP Elecsys proBNP reagent kit, Cat. No. 03121640122

Immunoassay for the in vitro quantitative determination of N-terminal pro B-type natriuretic peptide in human serum and plasma.

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable. Serum collected using standard sampling tubes or tubes containing separating gel. Li- and NH+4 -heparin plasma. When K3-EDTA plasma is used, the values found are by approximately 10% lower. (A representative result is: 95% confidence range for the recovery of the serum value = 74-94 %.)

Criterion: Recovery within 90-110% of serum value or slope 0.9-1.1 + intercept within <± 2x analytical sensitivity (LDL) + coefficient of correlation > 0.95. Stable for 3 days at 20-25°C, 3 days at 2-8°C, and 12 months at -20°C. When processing samples in primary tubes, follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay. Do not use samples and controls stabilized with azide. Ensure the patients' samples, calibrators, and controls are at ambient temperature (20-25°C) before measurement. Because of possible evaporation effects, samples, calibrators, and controls on the analyzers should be measured within 2 hours.

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

• 1st incubation: Antigen in the sample (20 µL), a biotinylated polyclonal NT-proBNP-specific antibody, and a polyclonal NT-proBNP-specific antibody labelled with a ruthenium complex form a sandwich complex.

• 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.

• The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

• Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Calculation

The analyser automatically calculates the analyte concentration of each sample (either in pmol/L or pg/mL).Conversion factors: pmol/L x 8.457 = pg/mL, pg/mL x 0.118 = pmol/L

Limitations - interference

The assay is unaffected by icterus (bilirubin < 599 µmol/L or < 35 mg/dL), haemolysis (Hb < 0.869 mmol/L or < 1.4 g/dL), lipemia (triglycerides < 45.6 mmol/L or < 4000 mg/dL), and biotin < 123 nmol/L or < 30 ng/mL. In patients receiving therapy with high biotin doses (i.e. > 5 mg/day), no sample should be taken until at least 8 hours after the last biotin administration. No interference was observed from rheumatoid factors up to a concentration of 1500 IU/mL. There is no high-dose hook effect at NT-proBNP concentrations up to 33,400 pmol/L (300,000 pg/mL). In vitro tests were performed on 51 commonly used pharmaceuticals. No interference with the assay was found.

Measuring range

0.6-4130 pmol/L or 5-35,000 pg/mL (defined by the lower detection limit and the maximum of the master curve). Values below the detection limit are reported as < 0.6 pmol/L (< 5 pg/mL). Values above the measuring range are reported as > 4130 pmol/L (> 35,000 pg/mL) or up to 8277 pmol/L (70,000 pg/mL) for 2-fold diluted samples.

Dilution

Samples with NT-proBNP concentrations above the measuring range can be diluted with Elecsys Diluent Universal. The recommended dilution is 1:2 (either automatically by the E170/Elecsys 1010/2010 analyzer or manually). The concentration of the diluted sample must be > 1770 pmol/L or > 15,000 g/mL. After manual dilution, multiply the result by the dilution factor. After dilution by the analysers, the E170/Elecsys 1010/2010 software automatically takes the dilution into account when calculating the sample concentration. Dilutions of up to 1:10 may entail maximum deviations of 25% from the theoretical value.

APPENDIX 5

ORION DIAGNOSTICA UNIQ ICTP EIA

REAGENTS

Materials provided

REAGENTS	QUANTITY	STORAGE
ICTP EIA MICROTITRE PLATE 96 wells, coated with goat anti-rabbit antibodies.	12x8 wells	2...8 °C in the original package until expiry date. Close the package tightly after use.
ICTP EIA ENZYME CONJUGATE Ready to use peroxidase labelled ICTP. Red colour additive.	1 vial 7 mL	2...8 °C until expiry date
ICTP EIA ANTISERUM Ready to use rabbit antiserum. Blue colour additive.	1 vial 7 mL	2...8 °C until expiry date
ICTP EIA CALIBRATORS Lyophilized 0, 1.0, 2.5, 5.0, 10, 25 and 50 µg/L in phosphate buffer. *Calibrated against an in-house master calibrator set*	7 vials Reconstitute with 0.5 mL of distilled water	2...8 °C until expiry date 2...8 °C 7 weeks after reconstitution.
ICTP EIA CONTROLS 1&2 Lyophilized, in human serum. Expected values are indicated on a separate sheet.	2 vials Reconstitute with 0.5 mL of distilled water	2...8 °C until expiry date 2...8 °C 1 week after reconstitution. Longer storage at -20 °C. Avoid repeated freezing and thawing.
ICTP EIA WASH CONCENTRATE 12.5 x concentrated	1 bottle 80 mL Dilute to 1000 mL with distilled water	2...8 °C until expiry date 2...25°C 7 weeks after dilution.
ICTP EIA SUBSTRATE Ready to use 3,3'5,5'-Tetramethylbenzidine in aqueous buffer.	1 bottle 17 mL	2...8 °C until expiry date
ICTP EIA STOPPING SOLUTION: Ready to use 0.5 M H_2SO_4	1 bottle 13 mL	2...8 °C until the expiry date

SPECIMEN HANDLING AND STORAGE

Although serum samples are recommended, the kit may also be used with EDTA-plasma samples. Do not use heparin or citrate plasma samples. Serum samples may be stored for up to 5 days at 2…8 °C, and for longer periods at least at -20 °C. Repeated freezing and thawing should be avoided.

Reconstitution of reagents

Controls:

Allow the vials to reach equilibrium at room temperature (18…25 °C) before opening. **Reconstitute** the controls by adding 500 µl of distilled water to the vials. Cap and mix well by gentle swirling or inversion to avoid foaming. Allow to stand for 30 minutes before use.

Wash concentrate:

Dilute the wash concentrate to 1000 ml (80 ml + 920 ml) with distilled water.

Details of the procedure

1. **Bring** all reagents, controls and patient samples to room temperature (18...25 °C) at least 30 minutes before use.
2. **Remove** excess strips from the plate frame, **return** them to the pouch and close tightly.
3. **Pipette** 50 µl of calibrator, control and patient sample in duplicate into appropriate microtitre wells. Reserve two wells for the substrate blank.
4. **Pipette** 50 µl of ICTP enzyme conjugate (red) into all wells except blanks.
5. **Pipette** 50 µl of ICTP antiserum (blue) into all wells except blanks. The antiserum must be applied to all wells within 3 minutes. The use of an electronic dispenser or multichannel pipette is recommended.
6. **Incubate** on a plate shaker at 18...25°C for 2 hours. Use a shaking speed of 600-1000 rpm.
7. **Wash** the strips 4 times with the wash solution on a plate washer. The wash solution must be dispensed above the wells and it is recommended to use 600µL of wash solution per well/cycle (overfill mode), otherwise up to 8 washing cycles may be required. After washing **remove any remaining moisture** from the wells by tapping the strips firmly against absorbent paper. Press the long sides of the holder firmly, so that the strips do not drop off. **Note: Proper washing is crucial for the assay performance.**
8. **Pipette** 100 µl of ICTP substrate into all wells.
9. **Incubate** on a plate shaker at 18...25 °C for 30 minutes.
10. **Stop** the enzyme reaction by adding 100 µl of stopping solution into all wells. **Shake** for 15-30 seconds to mix the reagents.
11. **Read** the absorbances of all wells at 450 nm on a plate reader within 10 minutes.

CALCULATION OF RESULTS

For automatic result processing spline function curve fitting is recommended. Results can also be produced manually on semi-log graph paper.

Calculate the mean absorbances for all calibrators, samples and controls.

Calculate the $\%B/B_o$ from:

$$\%B/B_o = \frac{\text{(calibrator or sample absorbance - blank)} \times 100}{\text{(0-calibrator absorbance - blank)}}$$

Draw a calibration curve on semi-log graph paper with $\%B/B_o$ values on the ordinate and the ICTP concentrations (µg/l) of the calibrators on the abscissa.
Read the ICTP concentrations of the unknowns from the calibration curve.

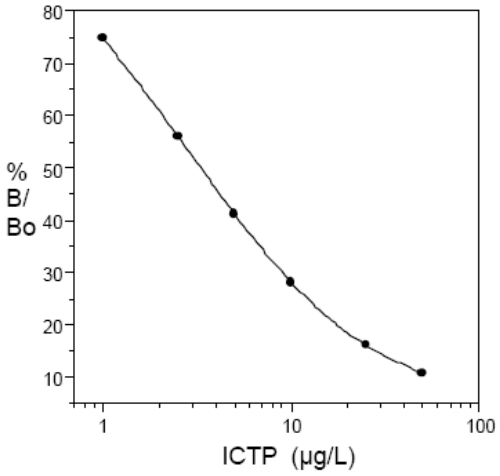


Figure
Typical calibration curve. Example.

Well	mean absor-bance	absor-bance - blank	$\%B/B_o$	ICTP (µg/L)
Blank	0,072			
Cal 0	1.857	1.785		
Cal 1.0	1.407	1.335	74.8	
Cal 2.5	1.073	1.001	56.1	
Cal 5.0	0.808	0.736	41.2	
Cal 10.0	0.575	0.503	28.2	
Cal 25.0	0.360	0.288	16.1	
Cal 50.0	0.264	0.192	10.8	
Unkn 1	0.818	0.746	41.8	4.8
Unkn 2	0.484	0.412	23.1	13.9

Table
Calculation of results using typical data. Example.

REFERENCE LIMITS

Due to ethnic, dietary and age variations, the reference limits given may not apply to all populations. Therefore each laboratory should establish its own representative reference limits. ICTP values of apparently healthy adults (19-74 years) were measured with the ICTP EIA kit. The non-parametric method recommended by IFCC was used to calculate the reference intervals for the upper and lower limits.
Serum ICTP reference limits

	Female	Male
Number of subjects	146	127
Mean	2.6 µg/L	2.7 µg/L
Reference interval*	**1.6 - 4.2 µg/L**	**1.5 - 4.3 µg/L**
0.90-confidence intervals		
for lower reference limit	1.5 - 1.8 µg/L	1.2 - 1.6 µg/L
for upper reference limit	3.8 - 5.2 µg/L	3.9 - 5.3 µg/L

* Reference interval = 0.025 & 0.975 fractiles

PERFORMANCE CHARACTERISTICS

The results presented below have been obtained at Orion Diagnostica and represent typical performance data.
Measurement range
1.0 - 50 µg/L.

Detection limit
Approximately 0.3 µg/L, defined as twice the standard deviation of the 0-binding value.
Dilution
Samples with high ICTP concentrations may be diluted using saline or the 0- calibrator of the kit.
Recovery
Known amounts of ICTP were added to five patient serum samples containing 3.4 – 6.2 µg/L of ICTP. Recoveries were in the range of 88 - 95 % with a mean value of 93 %.
Precision
Intra- and inter-assay variations were determined using native and spiked serum pools containing different concentrations of the ICTP antigen.
Table Intra-assay / Inter-assay precision

Intra-assay precision of 10 replicates			Inter-assay precision of 10 duplicate determinations		
Sample	Mean (µg/L)	CV (%)	Sample	Mean (µg/L)	CV (%)
1	2.9	11.3	*6*	3.2	6.4
2	4.7	13.2	*7*	4.8	7.6
3	5.6	8.1	*8*	7.1	7.5
4	13.7	8.9	*9*	13.1	9.8
5	26.4	7.6	*10*	28.2	6.4

Interfering substances
Serum bilirubin concentration < 340 µmol/l does not interfere. Serum haemoglobin concentration up to 5 g/l does not interfere. Triglycerides up to 30 g/l do not interfere.
Specificity
The antigenic determinant of the antibody is within the two hydrophobic phenylalanine-rich sequences of the trivalently cross-linked ICTP. A trivalent cross-link is necessary, the divalently cross-linked and monomeric peptides containing only one phenylalanine-rich sequence show poor immunoreaction. The ICTP EIA antiserum does not cross-react with antigens used in other UniQ Collagen assays.

APPENDIX 6

Procollagen Type I C-Peptide (PIP) EIA Kit (Precoated)

Kit components

1.	Antibody Coated Microtiter plate	1 plate (8 well × 12 strips)
2.	Antibody-POD Conjugate	1 vial (for 11 ml × 1)
3.	Standard	1 vial (for 1 ml × 1)
4.	Sample Diluent	2 vials (11 ml × 2)
5.	Substrate Solution	1 vial (12 ml × 1)

One-step sandwich-type EIA

Specimen collection and handling

Venous blood samples are collected aseptically. Serum is suitable for use in the assay, however, plasma, cultured cell extracts or cell culture supernatant can be also used. Remove the serum or plasma from the clot or red cells, respectively, soon after clotting and separation. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly haemolysed or lexemic specimens.
Samples may be stored up to 12 hours at 2-10°C. If the length of time between sample collection and assay is to exceed 12 hours, samples should be stored frozen under -20°C for optimal results. Excessive freeze-thaw cycles should be avoided. Prior to assay, frozen samples should be brought to room temperature slowly, and gently mixed by hand. Do not thaw samples in a hot bath. Do not vortex or sharply agitate. PBS containing 0.5% Triton X-100, 1mM EDTA and 1mM Phenylmethylsulfonyl fluoride (pH7.2) should be used for preparation of cell extracts. It is necessary to dilute human blood sample refer to sample dilution curve in basal data. (Probably 5-10 folds)

Preparation of solutions

Note: The following solutions should be prepared directly before use.
Solution 1. Antibody-POD Conjugate Solution
Dissolve the contents of Vial 2 in 11 ml distilled water and mix gently followed by 10 minutes slowly rolling or occasional mixing, avoiding foam formation.
Solution 2. Standard Solution
Rehydrate Standard (Vial 3) with 1 ml distilled water. Slowly roll for approximately 10 minutes or let stand and sporadically mix gently.

The standard solution contains 640 ng PIP/ml. A dilution series can be formed by mixing the standard solution and Sample Diluent (Vial 4) for establishing the calibration curve, e.g.:

Final conc. (ng/ml)	0	10	20	40	80	160	320	640
Sample Diluent (Vial 4)	400µl	393.75µl	387.5µl	375µl	350µl	300µl	200µl	-
Standard Solution (Vial 3; 640 ng/ml)	-	6.25µl	12.5µl	25µl	50µl	100µl	200µl	400µl

Solution 3 Stop Solution (1N H2SO4)

Add 5.8 ml concentrated H2SO4 carefully to approximately 180 ml of distilled water (acid MUST be added to water, not vice versa). Add distilled water to a final volume of 200 ml. Mix well. Store at 2 - 26°C for up to 6 months.

Stability of solutions

Solution 1. The reconstituted lyophilisate is stable for 1 week stored at 4°C,or for 1 month stored at -20 °C. Do not repeat freeze-thaw cycle.

Solution 2. The reconstituted lyophilisate is stable for 1 week stored at 4°C,or for 1 month stored at -20 °C. Do not repeat freeze-thaw cycle.

Procedure

Double determinations of all samples and standards should be performed. All of the Kit's content should be brought to room temperature before use ! For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand.

1. Enzyme immunoassay

- Immunological reaction: Transfer 100 µl of antibody-POD conjugate solution (Solution 1) into one well, and subsequently add 20 µl sample or standard (Solution 2). Mix, seal the microtiter plate (e.g. with a foil) and incubate 3 hours at 37°C. A sample and standard solution should be added within 5 minutes per well.

- Remove contents by suction and wash the wells 4 times with ca.400 µl of PBS; between the separate washing steps empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.

- Substrate incubation: Add 100 µl of Substrate Solution (vial 5) into each well and incubate at room temperature (20 - 30°C) for 15 minutes.

- Add 100 µl of Stop Solution (Solution 3) into each well in same order as for substrate. Tap plate gently to mix.

- Measure the absorbance at 450 nm with a plate reader. The absorbance should

be read as soon as possible after the completion of the assay. It may be read up

to 1 hour after addition of Stop Solution if wells are protected from light at room

temperature.

Results

1. Standard curve

- Record the absorbance at 450 nm for each standard well.

- Average the duplicate values and record the averages.

- Plot the absorbance (vertical axis) versus the PIP concentration in ng/ml (horizontal axis) for the standards using optimal fitting curve.

2. Samples

- Record the absorbance at 450 nm for each sample well.

- Average the duplicate values and record the averages.

- Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the PIP concentration (ng/ml) from the horizontal axis.

Performance characteristics

1. **Range of standard curve:** 10 - 640 ng/ml.

2. **Specificity:** This kit specifically measures PIP with no detectable cross reaction

with human fibronectin, vitronectin, laminin, collagen type I, or collagen type III.

This kit cannot be used to measure mouse PIP.

Note

The antibodies used in this kit cross react with procollagen type I C-peptides from bovine, horse and dog. The measurement may be disturbed, when it is performed with samples that include animal serum (ex. Foetal Bovine Serum (FBS) and Horse Serum). It is recommended to perform the measurement with the kit under the serum-free condition.

3. **Assay duration:** Three and a half hour.

4. **Total assay capacity:** 96 assays.

5. **Assay capacity for test samples:** If all assay wells (including standards and

test samples) are run in duplicate, 40 test samples can be run in duplicate per kit.

6. **Test specimen type:** Human bovine or canine serum or plasma; culture

supernatants, cell extracts.

7. **Specimen volume required:** If each test sample is run in duplicate,

approximately 50 µl (i.e., 20 µl per assay well plus ~10 µl for each sample

transfer) is required.

8. **Limitation:** Since conditions may vary from assay to assay, a standard curve must be established for every run. Since cross contamination between reagents will invalidate the test, disposable pipette tips should be used.

Thorough washing of the wells between incubations is required:

1) Completely empty out the remaining fluid from the well before dispensing fresh wash solution.

2) Use sufficient wash solution for each wash cycle (approximately 400 µl).

3) Do not allow wells to sit uncovered for extended periods between incubation steps.

Only samples with absorbance values falling within the range of the standard curve should be assigned a PIP concentration from the curve.

Basal data

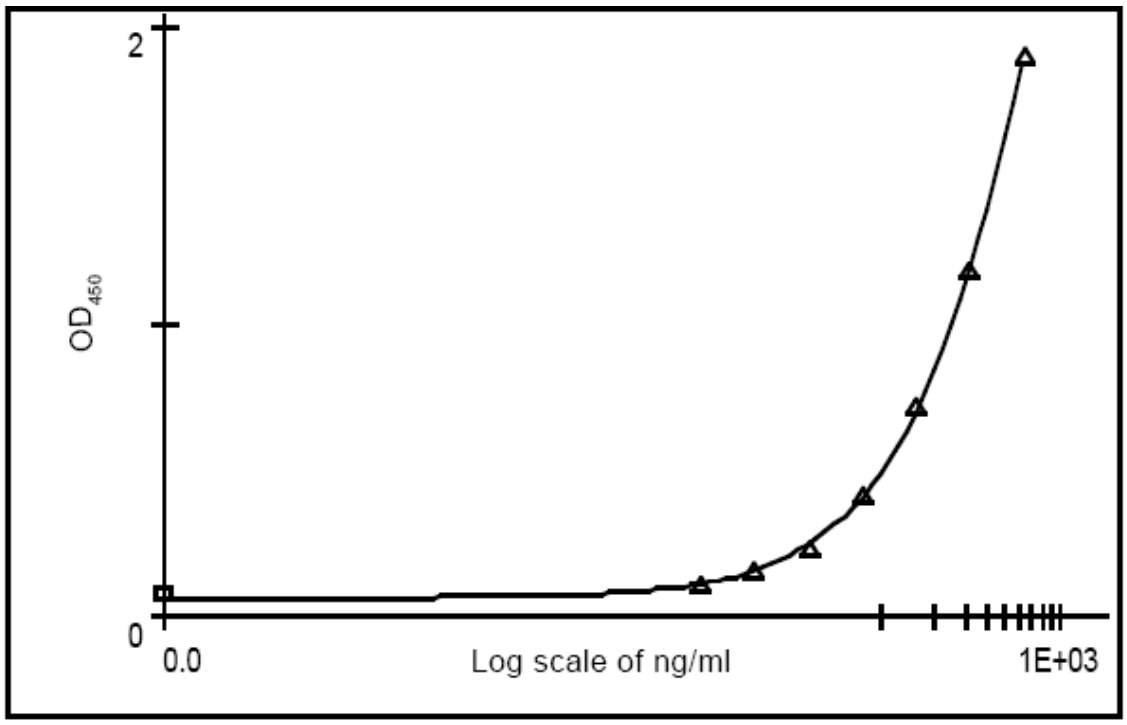
1. **Typical standard curve**

Detection limit is 10 ng/ml

Curve Fit: 4-Parameter Corr. Coeff: -1.00

y= (A-D) / (1 + (x/CB)) + D A = 0.0710 B = 0.983 C = 1.18E + 03 D = 5.23

PIP (ng/ml)	640	320	160	80	40	20	10	0
A_{450}	1.903	1.185	0.720	0.413	0.242	0.162	0.120	0.074



Intra-assay precision (n=16)

Assay was carried out with 16 replicates of 3 samples containing different concentrations of PIP.

	Ave. (ng/ml)	S.D.(ng/ml)	CV(%)
Sample A	484.8	35.64	7.4
Sample B	87.3	6.282	7.2
Sample C	31.7	1.411	4.5

Inter-assay precision (performance 3 times)

Assay to assay precision with one laboratory was evaluated in three independent experiments.

	Ave. (ng/ml)	S.D.(ng/ml)	CV(%)
Sample A	466.1	20.25	4.3
Sample B	90.7	4.349	4.8
Sample C	29.6	1.873	6.3

APPENDIX 7

The RayBio® Human Matrix Metalloproteinase -1(MMP-1) Enzyme-Linked Immunosorbent Assay (ELISA) kit

REAGENTS

1. MMP-1 Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human MMP-1.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials, recombinant human MMP-1.
4. Assay Diluent Buffer (Item E): 15 ml of 5x concentrated buffered. For Standard/Sample (serum/plasma/cell culture medium/urine) diluent.
5. Detection Antibody MMP-1 (Item F): 2 vial of biotinylated anti-human MMP-1 (each vial is enough to assay half microplate).
6. HRP-Streptavidin Concentrate (Item G): 8 µl 22,000x concentrated HRP-conjugated streptavidin.
7. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'-tetramethylbenzidine (TMB) in buffered solution.
8. Stop Solution (Item I): 8 ml of 2 M sulphuric acid.

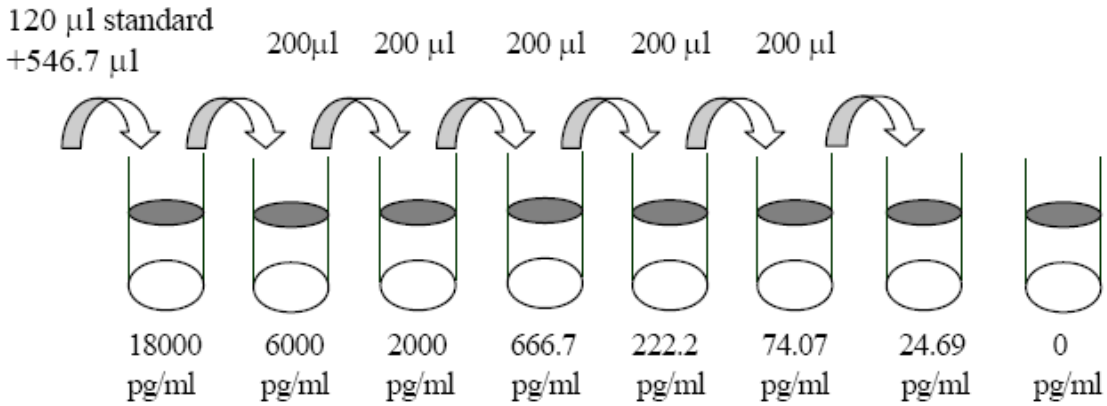
STORAGE

May be stored for up to 5 months at 2^{o} to 8^{o}C from the date of shipment. Standard (recombinant protein) should be stored at -20 ^{o}C or -80 ^{o}C (recommended at –80 ^{o}C) after reconstitution. Opened Microplate Wells and reagents may be store for up to 1 month at 2^{o} to 8^{o}C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge of zip seal.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Preparation of standard: **Briefly spin the vial of Item C** and then add 400 ml 1x Assay Diluent Buffer (Item E) into Item C vial to prepare a 0.1 µg/ml standard. **Dissolve the powder thoroughly** by a gentle mix. Add 120 ml MMP-1 standard from the vial of tem C, into a tube with 546.7 µl 1x Assay Diluent Buffer (for serum/plasma/cell culture medium/urine) to prepare a 18000 pg/ml stock standard solution. Pipette 400µl 1x Assay Diluent into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Gently vortex to mix. 1x Assay Diluent Buffer serves as the zero standard (0 pg/ml).
3. Sample dilution: If your samples need to be diluted, Assay Diluent Buffer (Item E) is used for dilution of serum/plasma/culture supernatants/urine.
4. Assay Diluent (Item E) should be diluted 5-fold with deionised or distilled water before use.
5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionised or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate

should be diluted 80-fold with 1x Assay Diluent and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 22,000-fold with 1x Assay Diluent.



ASSAY PROCEDURE:

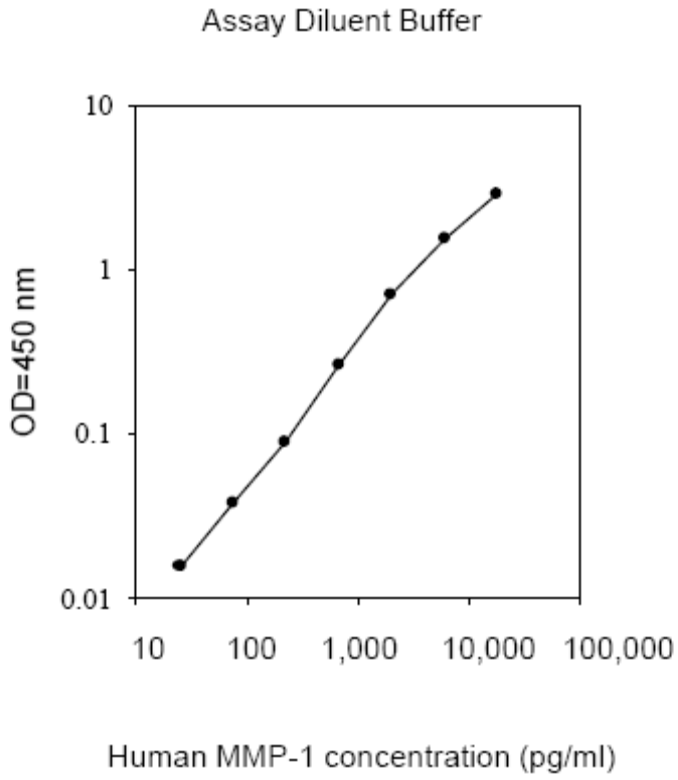
1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C.
3. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature.
5. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
6. Add 100 ml of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature.
7. Discard the solution and wash 5 times with 1x Wash Solution (200 µl each).
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



SENSITIVITY

The minimum detectable dose of MMP-1 is typically less than 8 pg/ml.

RECOVERY

Recovery was determined by spiking various levels of human MMP-1 into human serum, plasma and cell culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	97.78	88-106
Plasma	99.59	90-107
Cell culture media	98.66	90-106

REPRODUCIBILITY

Intra-Assay: CV<10% Inter-Assay: CV<12%

SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested *(e.g.*, human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN-g, Leptin (OB), MCP-1, MCP-3, MDC, MIP-1a, MIP-1 b, MIP-1d, MMP-2, - 3, -9, -10, PARC, RANTES, SCF, TARC, TGF-b, TIMP-1, TIMP-2, TNF-a, TNF-b, TPO, VEGF).

APPENDIX 8

RayBio® Human MMP-2 ELISA Kit Protocol

REAGENTS

1. MMP-2 Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human MMP-2.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution
3. Standards (Item C): 2 vials, recombinant human MMP-2.
4. Assay Diluent Buffer (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (serum/plasma samples/cell culture medium/urine) diluent.
5. Detection Antibody MMP-2 (Item F): 2 vial of biotinylated anti-human MMP-2 (each vial is enough to assay half microplate).
6. HRP-Streptavidin Concentrate (Item G): 8 µl of 15,000x concentrated HRP-conjugated streptavidin.
7. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
8. Stop Solution (Item I): 8 ml of 2 M sulphuric acid.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Preparation of standard: **Briefly spin the vial of Item C** and then add 400 µl 1x Assay Diluent Buffer (Item E) into Item C vial to prepare a 0.1 µg/ml standard. **Dissolve the powder thoroughly by a gentle mix.** Add 120 µl MMP-2 standard from the vial of tem C, into a tube with 546.7 µl 1x Assay Diluent Buffer (for serum/plasma samples/cell culture medium/urine) to prepare a 18,000 pg/ml stock standard solution. Pipette 300µl 1x Assay Diluent Buffer into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Gently vortex to mix. 1x Assay Diluent Buffer serves as the zero standard (0 pg/ml).
3. Sample dilution: If your samples need to be diluted, Assay Diluent (Item E) is used for dilution of serum/plasma/culture supernatants/urine.
4. Assay Diluent (Item E) should be diluted 5-fold with deionised or distilled water before use.
5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionised or distilled water to yield 400 ml of 1x Wash Buffer.
6 Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent and used in step 4 of Part VI Assay Procedure.
7 Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 15,000-fold with 1x Assay Diluent.

ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C.
3. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature.
5. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
6. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature.
7. Discard the solution and wash 5 times with 1x Wash Solution (200 µl each).
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

SENSITIVITY

The minimum detectable dose of MMP-2 is typically less than 80 pg/ml.

REPRODUCIBILITY

Intra-Assay: CV<10% Inter-Assay: CV<12%

SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested *(e.g.*, human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN-γ, Leptin (OB), MCP-1, MCP-3, MDC, MIP-1α, MIP-1 β, MIP-1δ, MMP-1, - 2, -3, -7, -8, -10, -12, -13, PARC, RANTES, SCF, TARC, TGF-β, TIMP-1, TIMP-2, TNF-α, TNF-β, TPO, VEGF).

APPENDIX 9

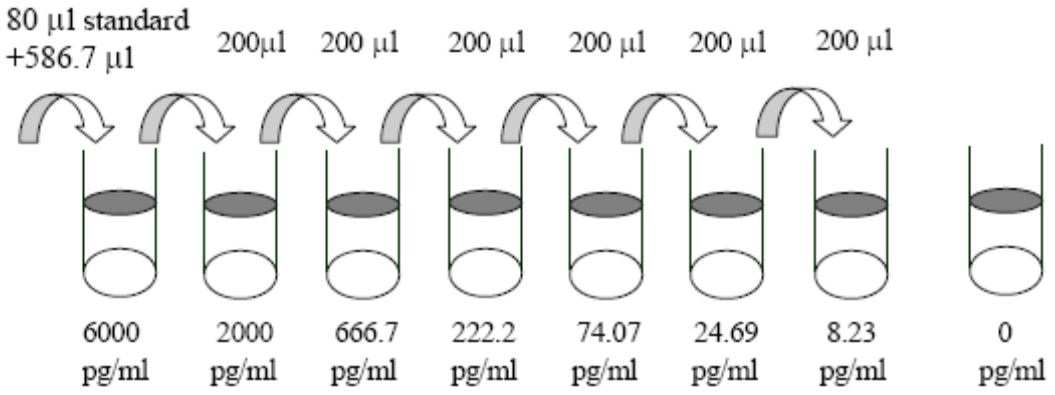
RayBio® Human MMP-9 ELISA Kit Protocol

REAGENTS

1. MMP-9 Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human MMP-9.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution
3. Standards (Item C): 2 vials, recombinant human MMP-9.
4. Assay Diluent Buffer (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (serum/plasma samples/cell culture medium/urine) diluent.
5. Detection Antibody MMP-9 (Item F): 2 vial of biotinylated anti-human MMP-9 (each vial is enough to assay half microplate).
6. HRP-Streptavidin concentrate (Item G): 8 µl 20,000x concentrated HRP-conjugated streptavidin.
7. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
8. Stop Solution (Item I): 8 ml of 2 M sulphuric acid.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Preparation of standard: **Briefly spin the vial of Item C**. Add 400 µl 1x Assay Diluent (Item E) into Item C vial to prepare a 50 ng/ml standard. **Dissolve the powder thoroughly by a gentle mix**. Add 80 µl MMP-9 standard from the vial of tem C, into a tube with 586.7 µl 1x Assay Diluent Buffer to prepare a 6000 pg/ml stock standard solution. Pipette 400µl 1x Assay Diluent into each tube. Use the stock standard solution to produce a Dilution series (shown below). Mix each tube thoroughly before the next transfer.
Gently vortex to mix. 1x Assay Diluent serves as the zero standard (0 pg/ml).



3. Sample dilution: If your samples need to be diluted, Assay Diluent Buffer (Item E) is used for dilution of serum/plasma/culture supernatants/urine.
4. Assay Diluent (Item E) should be diluted 5-fold with deionised or distilled water before use.

5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionised or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 100-fold with 1x Assay Diluent and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 20,000-fold with 1x Assay Diluent.

ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C.
3. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature.
5. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
6. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature.
7. Discard the solution and wash 5 times with 1x Wash Solution (200 µl each).
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 50 ml of Stop Solution (Item I) to each well. Read at 450 nm immediately.

CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

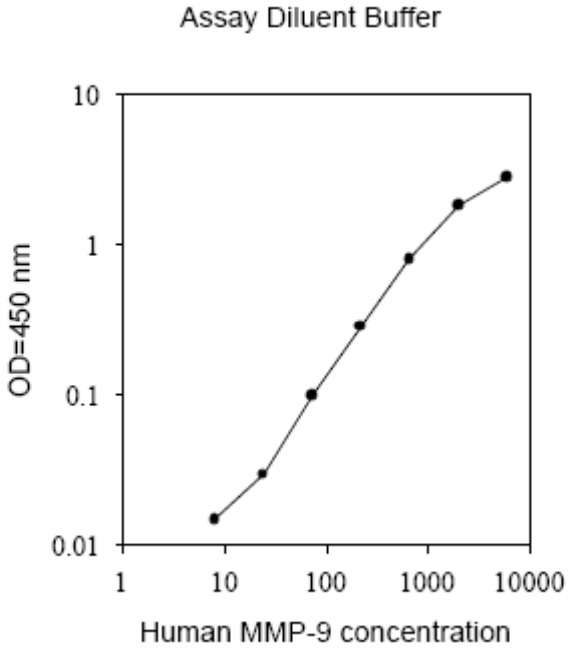
SENSITIVITY

The minimum detectable dose of MMP-9 is typically less than 10 pg/ml.

RECOVERY

Recovery was determined by spiking various levels of human MMP-9 into normal human serum, plasma and cell culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	96.23	84-103
Plasma	94.64	83-102
Cell culture media	95.38	84-104



TYPICAL DATA

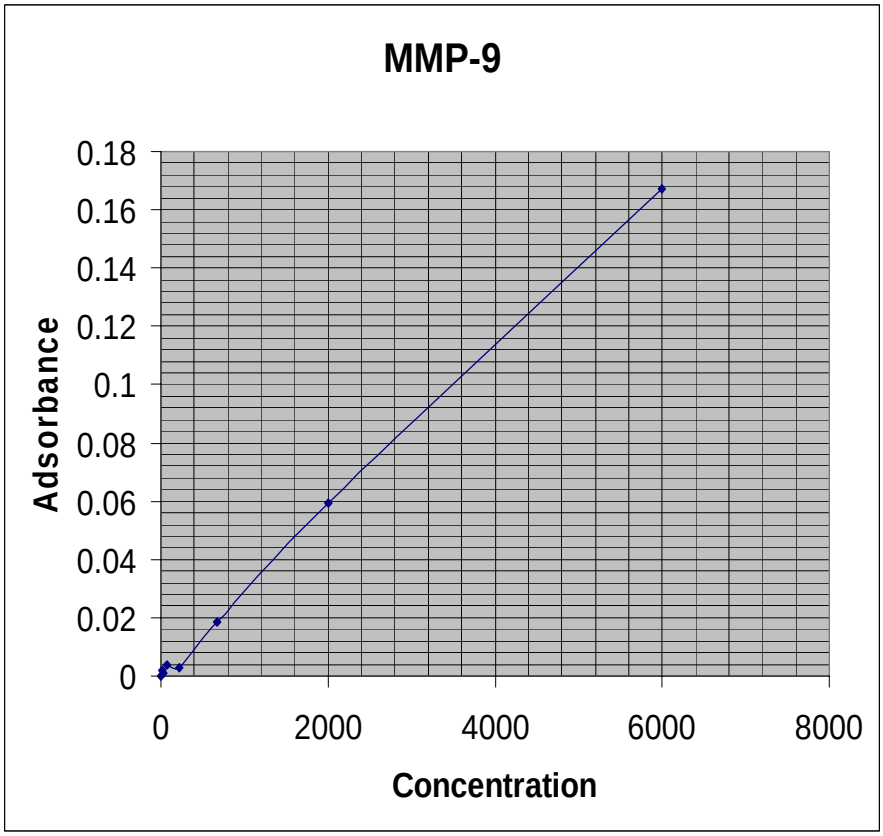
These standard curves are for demonstration only. A standard curve must be run with each assay.

REPRODUCIBILITY

Intra-Assay: CV<10% Inter-Assay: CV<12%

SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested *(e.g.*, human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN-g, Leptin (OB), MCP-1, MCP-3, MDC, MIP-1a, MIP-1 b, MIP-1d, MMP-1, - 2, -3, -10, PARC, RANTES, SCF, TARC, TGF-b, TIMP-1, TIMP-2, TNF-a, TNF-β, TPO, VEGF).



Standard Curve obtained from estimations of MMP-9 in our study participants

APPENDIX 10

RayBiotech Human TIMP-1 ELISA Kit Protocol

REAGENTS

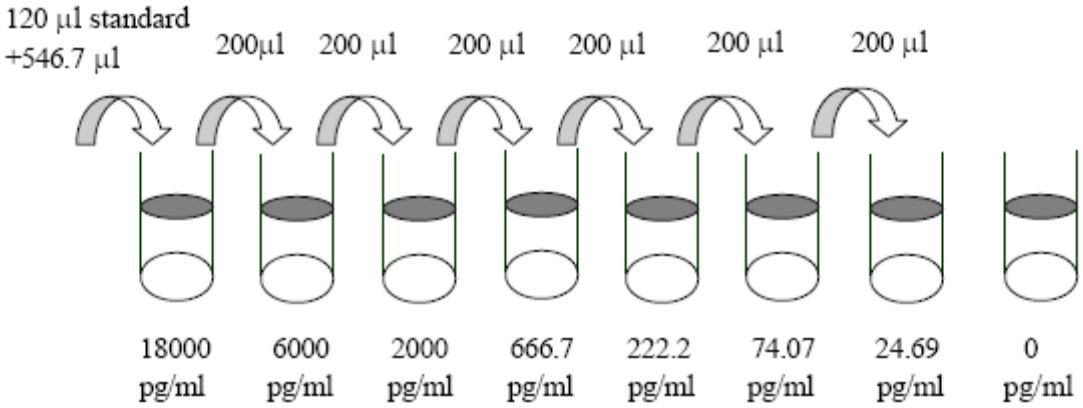
1. TIMP-1 Microplate (Item A):96 wells (12 strips x 8 wells) coated with anti-human TIMP-1.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution
3. Standards (Item C): 2 vials, recombinant human TIMP-1.
4. Assay Diluent A (Item D): 30 ml of animal serum with 0.09% sodium azide as preservative. For Standard/Sample (serum/plasma) diluent.
5. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.
6. Detection Antibody TIMP-1 (Item F): 2 vial of biotinylated anti-human TIMP-1 (each vial is enough to assay half microplate).
7. HRP-Streptavidin Concentrate (Item G): 8 µl of 15,000x concentrated HRP-conjugated streptavidin.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 2 M sulphuric acid.

STORAGE

May be stored for up to 5 months at 2^{o} to 8^{o}C from the date of shipment. Standard (recombinant protein) should be stored at -20 ^{o}C or -80 ^{o}C (recommended at –80 ^{o}C) after reconstitution. Opened Microplate Wells and reagents may be store for up to 1 month at 2^{o} to 8^{o}C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge of zipseal.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Preparation of standard: **Briefly spin the vial of Item C** and then add 400 µl Assay Diluent A (for serum/plasma samples) or 1x Assay Diluent B (for cell culture medium and urine) into Item C vial to prepare a 0.1 µg/ml standard. **Dissolve the powder thoroughly by a gentle mix.** Add 120 µl TIMP-1 standard from the vial of Item C, into a tube with 546.7 µl Assay Diluent A or 1x Assay Diluent B to prepare a 18000 pg/ml stock standard solution. Pipette 400 µl Assay Diluent A or 1x Assay Diluent B into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent A or 1x Assay Diluent B serves as the zero standard (0 pg/ml).



3. Sample dilution: If your samples need to be diluted, Assay Diluent A (Item D) is used for dilution of serum/plasma samples, and Assay Diluent B (Item E) is used for dilution of culture supernatants and urine.
4. Assay Diluent B should be diluted 5-fold with deionised or distilled water.
5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionised or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) before use. HRP-Streptavidin concentrate should be diluted 15,000-fold with 1x Assay Diluent B.

ASSAY PROCEDURE:

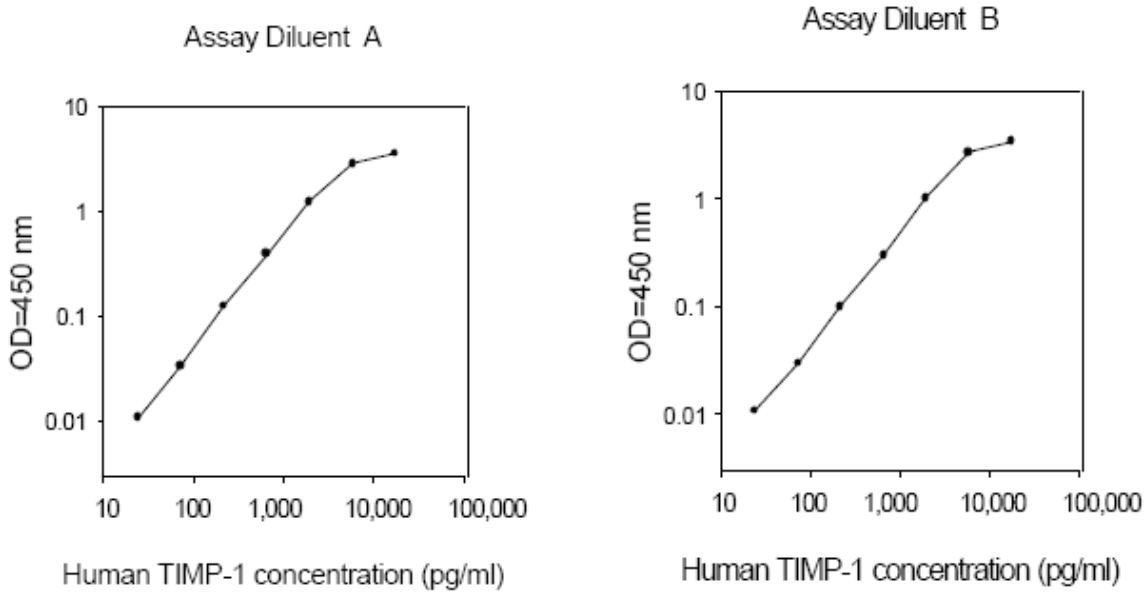
1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C.
3. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature.
5. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
6. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature.
7. Discard the solution and wash 5 times with 1x Wash Solution (200 µl each).
8. Add 100 ml of TMB One- Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



SENSITIVITY

The minimum detectable dose of TIMP-1 is typically less than 40 pg/ml.

RECOVERY

Recovery was determined by spiking various levels of human TIMP-1 into human serum, plasma and cell culture media. Mean recoveries are as follows:

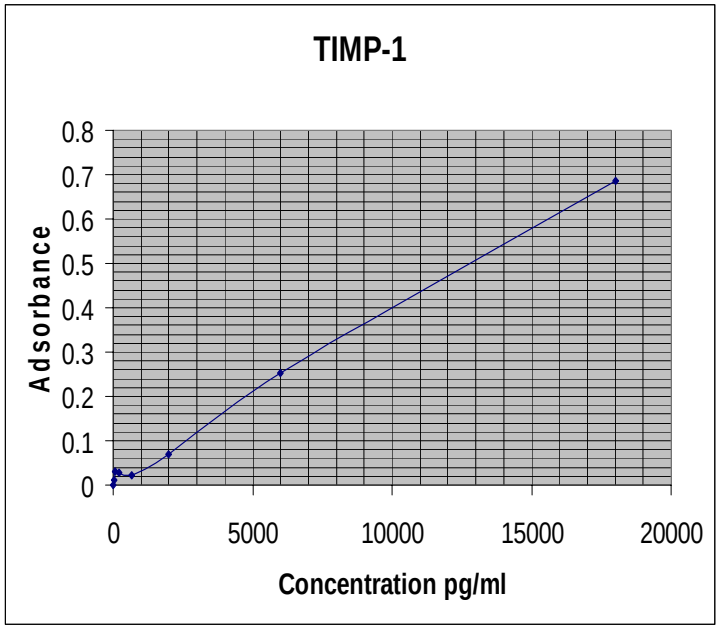
Sample Type	Average % Recovery	Range (%)
Serum	92.43	81-102
Plasma	93.67	83-102
Cell culture media	94.49	84-104

REPRODUCIBILITY

Intra-Assay: CV<10% Inter-Assay: CV<12%

SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested *(e.g.*, human ANG, CD23, Eotaxin, GCSF, GM-CSF, GRO- a, GRO-b, GRO-g, I-309, IFN-g, IL-1a, IL-1b, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-15, IL-16, IP-10, MCP-1, MCP-2, MCP-3, MCP-4, MCSF, MIG, MIP-1a, MIP-1b, NAP-2, PDGF, PF-4, PARC, SCF, SDF-1a, Tiff, TGFb1, TGFb2, TGFb3, VEGF). Less than 0.2 % cross-reactivity with rm TIMP-1 and rh TIMP-2 was observed.



Standard curve obtained from estimations of TIMP-1 in our study population

APPENDIX 11

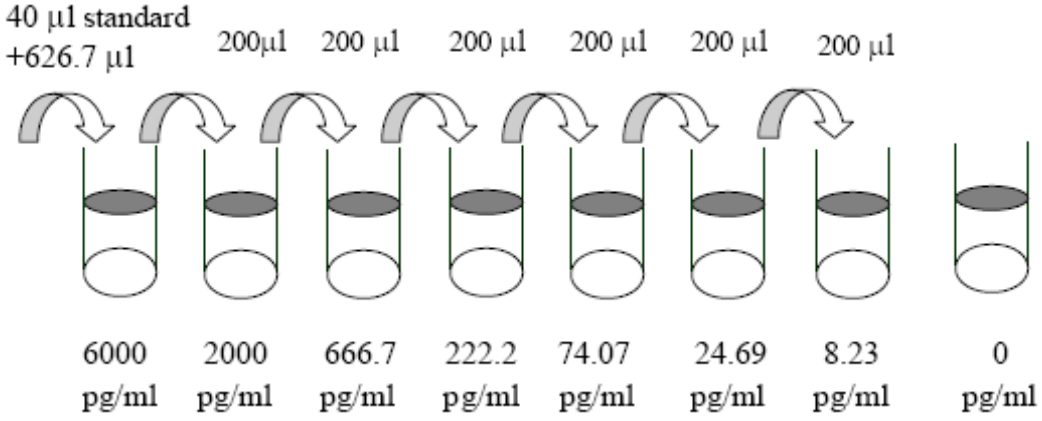
RayBiotech Human TIMP-2 ELISA Kit Protocol

REAGENTS

1. TIMP-2 Microplate (Item A):96 wells (12 strips x 8 wells) coated with anti-human TIMP-2.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials, recombinant human TIMP-2.
4. Assay Diluent A (Item D): 30 ml of animal serum with 0.09% sodium azide as a preservative. For Standard/Sample (serum/plasma) diluent.
5. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (cell culture medium/urine) diluent.
6. Detection Antibody TIMP-2 (Item F): 2 vial of biotinylated anti-human TIMP-2 (each vial is enough to assay half microplate).
7. HRP-Streptavidin concentrate (Item G): 8 µl of 25,000x concentrated HRP-conjugated streptavidin.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 2 M sulphuric acid.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18-25°C) before use.
2. Preparation of standard: **Briefly spin the vial of Item C**. Add 200 µl Assay Diluent A (for serum/plasma samples) or 1x Assay Diluent B (for cell culture medium and urine) into Item C vial to prepare a 0.1 µg/ml standard. **Dissolve the powder thoroughly by a gentle mix**.
Add 40 µl TIMP-2 standard from the vial of Item C, into a tube with 626.7 µl Assay Diluent A or 1x Assay Diluent B to prepare a 6000 pg/ml stock standard solution. Pipette 400 µl Assay Diluent A or 1x Assay Diluent B into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Gently vortex to mix. Assay Diluent A or 1x Assay Diluent B serves as the zero standard (0 pg/ml).



3. Sample dilution: If your samples need to be diluted, Assay Diluent A (Item D) is used for dilution of serum/plasma samples and Assay Diluent B (Item E) is used for dilution of culture supernatants and urine.
4. Assay Diluent B should be diluted 5-fold with deionised or distilled water.
5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionised or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 ml of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 25,000-fold with 1x Assay Diluent B.

ASSAY PROCEDURE:

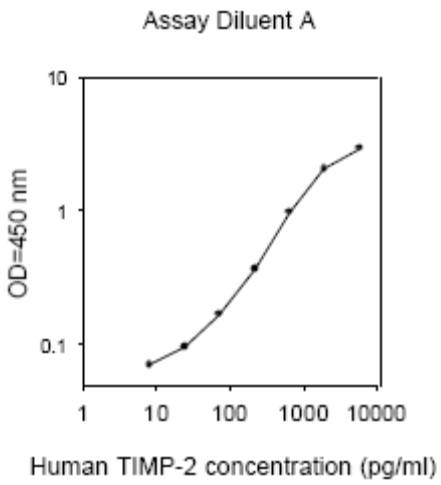
1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C.
3. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
4. Add 100 µl of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature.
5. Discard the solution and wash 4 times with 1x Wash Solution (200 µl each).
6. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature.
7. Discard the solution and wash 5 times with 1x Wash Solution (200 µl each).
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

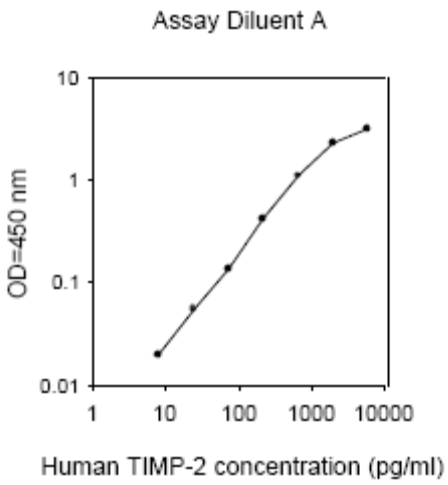
CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.





SENSITIVITY

The minimum detectable dose of TIMP-2 is typically less than 10 pg/ml.

RECOVERY

Recovery was determined by spiking various levels of human TIMP-2 into human serum, plasma and cell culture media. Mean recoveries are as follows:

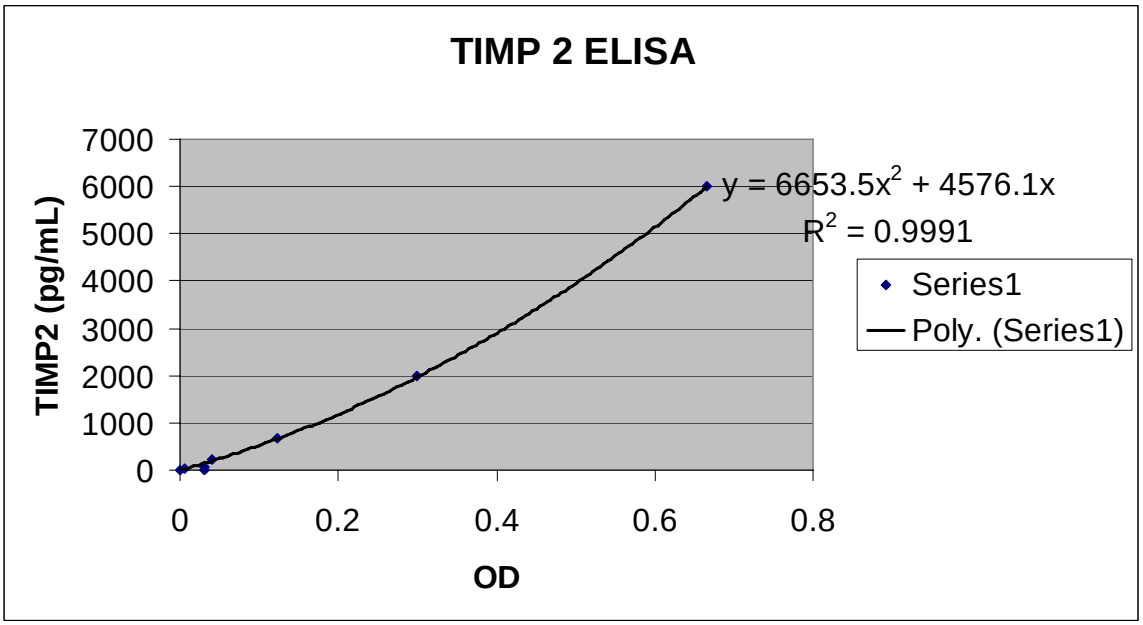
Sample Type	Average % Recovery	Range (%)
Serum	94.28	83-103
Plasma	93.69	82-102
Cell culture media	94.53	84-103

REPRODUCIBILITY

Intra-Assay: CV<10% Inter-Assay: CV<12%

SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested *(e.g.*, human IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-7, IL- 8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, G-CSF, GM-CSF, IFN-g, MCP-1, MCP-2, MCP-3, MDC, MIP-1a, MIP-1 b, PDGF**,** SCF, TNF-a, TNF-b, TPO, VEGF).



Standard curve obtained from estimations of TIMP-2 in our study patients

APPENDIX 12 Demographic data and CKD duration

No.	age	sex	race	Causes	Height (cm)	Weight (kg)	BMI(Kg/m^2)	CKD duration	HD Duration(months)	Access code
1	37	0	1	1	171	53	23.26	1.58	19	4
2	45	0	1	1	170	75	25.95	17	144	3
3	33	0	1	1	161	56	21.6	3.5	38	1
4	52	1	1	2	152	107	46.31	2.58	31	1
5	46	1	1	1	163	73	27.48	13.92	167	1
6	36	0	1	4	174	74	24.44	4.25	43	2
7	49	1	1	1	164	110	40.9	10	60	1
8	54	0	2	3	176	77	24.86	19.5	120	1
9	30	0	2	9	180	93	28.7	13.5	30	1
10	47	0	1	1	166	77	27.94	9.42	5	4
11	50	1	1	1	152	85	36.79	1.67	20	5
12	40	1	1	1	152	53	28.57	3	36	1
13	41	1	1	1	152	53	22.94	2.75	33	3
14	45	0	1	8	185	102	29.8	2.08	25	3
15	47	0	1	1	173	90	30.07	2.75	25	1
16	29	0	1	1	168	104	36.85	3.67	20	2
17	37	1	1	6	165	61	22.41	2.08	14	4
18	46	0	1	4	172	83	28.06	2	5	4
19	37	0	1	1	167	58	20.8	3.42	8	4
20	59	0	1	4	163	70	26.35	0.33	4	4
21	31	0	4	7	159	59	23.34	20.33	180	1
22	55	1	1	2	150	72	32	2	24	3
23	39	1	2	3	158	53	21.63	25.67	44	4
24	52	0	1	1	158	53	21.23	3.25	27	1
25	20	1	1	1	159	52	20.57	1	12	4
26	53	1	1	1	154	72	30.36	0.92	11	4
27	41	0	1	1	181	85	25.95	14.42	173	2

28	43	0	1	1	165	61	22.41	2.75	33	3
29	40	1	1	1	157	53	21.91	3.58	43	1
30	61	0	1	1	180	67	20.68	1.17	14	4
31	57	1	1	1	159	54	21.36	1.58	19	4
32	23	0	1	5	154	46	19.4	1.42	17	3
33	29	0	1	9	149	45	20.27	14.85	69	1
34	23	0	1	2	148	43	19.63	10.42	41	3
35	42	0	1	1	169	88	30.81	4	24	1
36	38	0	1	1	178	65	20.52	3.67	32	1
37	52	1	1	4	168	77	27.28	4.75	21	1
38	28	0	3	5	155	59	24.56	7.17	84	3
39	52	1	1	4	159	96	37.97	10	120	1
40	30	0	1	5	157	47	19.07	22	120	2
41	34	0	1	2	175	61	19.92	4.67	32	4
42	53	1	2	6	159	73	28.88	18	156	1
43	29	1	1	1	179	67	20.91	2.58	13	4
44	21	0	1	4	167	64	22.95	0.58	7	4
45	48	1	1	9	156	54	22.19	4.83	44	3
46	39	1	1	1	149	72	32.43	0.83	10	1
47	31	0	2	4	172	84	28.39	8.42	41	4
48	23	0	1	4	151	36	15.79	2.25	3	4
49	21	1	1	7	137	35	18.65	2	48	1
50	24	1	1	9	158	52	20.83	3.42	41	1
51	49	1	1	1	154	63	26.56	6.67	80	4
52	44	0	1	1	177	80	25.54	2	24	2
53	39	0	4	1	168	55	19.49	12.75	129	1
54	60	1	1	1	160	86	33.59	3.83	46	1
55	23	0	2	9	173	76	25.39	7.33	88	1

56	22	1	1	1	172	59	19.94	3.83	46	1
57	31	0	1	5	148	32.2	14.7	21	108	3
58	41	0	1	9	166	67	24.31	1.08	12	4
59	28	0	1	1	179	58	18.1	1.17	6	4
60	41	0	1	1	165	48	17.63	6.42	77	4
61	26	0	4	6	158	56	22.43	11.42	137	4
62	28	0	1	1	182	67	20.23	1.92	23	1
63	39	0	1	1	175	67	21.88	4.08	49	2
64	38	0	1	4	169	59	20.66	0.75	9	2
65	46	0	4	1	171	72	24.62	1.46	8	4
66	38	1	1	9	154	45	18.97	3.17	26	4
67	24	0	1	1	163	53	19.95	3.33	28	1
68	29	1	1	6	150	51	22.67	5.33	16	3
69	38	0	1	1	172	109	27.04	3.42	41	2
70	48	1	1	1	153	54	23.07	0.25	3	4
71	31	0	4	2	168	71	25.16	5.08	61	1
72	69	0	3	2	165	60	22.04	5.75	69	2
73	66	0	3	9	186	70	20.23	0.83	10	2
74	43	0	1	1	181	85	25.95	1.08	13	2
75	58	1	3	5	160	81	31.64	10	24	1
76	57	0	1	1	171	91	31.12	1.17	14	1
77	45	0	1	1	173	99	33.08	9.58	43	1
78	50	1	1	4	167	99	35.5	8	96	4
79	55	1	4	1	150	98	43.56	1	12	1
80	45	1	2	8	161	51	19.68	4.67	56	1
81	50	1	4	9	174	67	22.13	0.5	6	4
82	52	0	1	1	178	60	18.94	6.5	54	3

83	51	0	2	8	172	88	29.75	3.08	37	2
84	34	0	2	3	176	82	26.47	18.33	136	2
85	32	0	1	1	165	46	16.9	9.08	13	1
86	45	0	2	3	174	55	18.17	17.08	121	2
87	35	1	1	1	154	50	21.08	3.83	22	4
88	56	1	2	5	163	69	25.97	12	96	3
89	26	0	2	7	163	66	24.84	17.58	51	2
90	31	1	1	9	161	57	21.99	1.33	16	3
91	50	0	1	1	171	68	23.26	4.25	39	3
92	38	0	1	4	177	70	22.34	18.17	32	1
93	44	0	4	3	164	66	24.54	25	300	2
94	72	1	1	1	164	77	28.63	0.92	11	1

Access codes

arm arteriovenous fistula =1
forearm arteriovenous fistula =2
arteriovenous graft =3
permanent catheter = 4
permanent catheter with arteriovenous fistula = 5

Causes

Hypertension=1
Obstructive uropathy= 2
Glomerulonephritis= 3
Unknown =4
Pyelonephritis/reflux =5
Lupus nephritis =6
Congenital abnormalities =7
Polycystic kidney disease= 8
Miscellaneous causes= 9
Sex: males= 0, females= 1
Race: black= 1, white= 2, Indian =3, mixed race= 4