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Cardiac Biomarkers for The Diagnosis and Monitoring of Cardiovascular Disease in The Dialysis Population

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Abstract

Dialysis patients have a 10-50 fold increased risk of cardiac death compared to the general population. Key factors underpinning this heightened risk are fluid overload-induced cardiomyopathy and ischaemic heart disease. Progress in modifying these outcomes has been hampered by our inability to assess patients' fluid status and cardiac risk in an accurate and dynamic manner. The overarching aim of this thesis is to advance our understanding of the roles of two cardiac biomarkers, the amino terminal fragment of pro-B-type natriuretic peptide (NT-proBNP) and high sensitivity cardiac troponin-T (hs-cTnT), in diagnosing and monitoring cardiovascular disease in the dialysis population.

Two multi-centre, prospective cohort studies were performed. The aim of the first study was to estimate the biological variation of NT-proBNP and plasma hs-cTnT in 55 prevalent haemodialysis and peritoneal-dialysis patients, and to use these estimates to derive the critical difference between serial measurements needed to detect a clinically significant change with 90% confidence. Patients were reviewed weekly for 4 consecutive weeks then monthly for a further 4 months. Assessments were conducted at the same dialysis-cycle time point and entailed clinical review, bioimpedance spectroscopy, electrocardiography, hs-cTnT and NT-proBNP testing. Patients were excluded if they underwent a change in cardiac medication, dialysis prescription, ischaemic symptomatology, extracellular volume >1L, new arrhythmia or hospitalisation between visits.

137 weekly and 114 monthly NT-proBNP and hs-cTnT measurements from 42 stable patients were analysed. Between-person(CV_G) and within-person(CV_I) coefficients of variation were estimated using nested analysis of variance. For weekly measurements CV_G, CV_I, and the critical difference were 153%, 27%, and -46% and +84% respectively for NT-proBNP, and 83%, 7.9%, and -25% and +33% respectively for hs-cTnT. For monthly measurements CV_G, CV_I, and the critical difference were 148%, 35%, and -54% and +119% respectively for NT-proBNP, and 79%, 12.6%, 2.4% and -37% to +58% respectively for hs-cTnT. The CV_I:CV_G ratios for weekly and monthly measurements were 0.18 and 0.24 respectively for NT-proBNP, and 0.10 and 0.16 respectively for hs-cTnT. CV_G was not significantly different by dialysis modality, hydration status, history of ischaemic heart disease, NT-proBNP or hs-cTnT concentration, severity of left ventricular hypertrophy, systolic or diastolic dysfunction. Thus, serial NT-proBNP levels need to double or halve and hs-cTnT levels need to increase by at least 20-34% or fall by 17-25% to confidently exclude change due to analytical & biological variation alone. The low CV_I:CV_G implies the best strategy for applying

these biomarkers in dialysis is relative change monitoring after a baseline estimate rather than comparing results to reference intervals.

The second study performed was a longitudinal cohort study of 103 prevalent haemodialysis and peritoneal dialysis patients, of whom 78 patients also participated in an echocardiographic sub-study. The aim of the echocardiographic sub-study was to determine the cross-sectional associations between each of plasma NT-proBNP and hs-cTnT, and measures of hydration status, and functional and structural cardiovascular indices. Patients underwent clinical review, bioimpedance spectroscopy, electro- and echo-cardiography, hs-cTnT and NT-proBNP testing. Multivariable analysis found that plasma NT-proBNP was independently associated with hydration status (standardised β=0.313, P<0.01), left ventricular mass indexed to body surface area (standardised β=0.238, P<0.01), residual renal function (standardised β=-0.086, P=0.05), and left ventricular systolic dysfunction assessed using global longitudinal strain (standardised β=0.233, P<0.01) but not using ejection fraction (standardised β=-0.038, P=0.71). Plasma hs-cTnT was independently associated with hydration status (standardised β=0.379, P<0.01), and with pulse wave velocity (standardised β=0.250, P=0.04) in multivariable analysis. These findings suggest that NT-proBNP testing may have a role in monitoring the risk of adverse cardiac events related to hydration state and cardiomyopathy in the dialysis population, and that hs-cTnT testing may have role either alone or together with plasma NT-proBNP in monitoring hydration status in the dialysis population.

The aim of the longitudinal cohort study was to determine the longitudinal correlation of plasma NT-proBNP and hydration status assessed using bioimpedance spectroscopy. Patients were reviewed monthly at the same dialysis-cycle time point and underwent clinical review, bioimpedance spectroscopy, electrocardiography, and NT-proBNP testing. 103 patients were assessed for a median of 14-months (IQR 9-22 months) yielding 1431 paired bioimpedance and plasma NT-proBNP measurements. The correlation coefficient between plasma NT-proBNP and hydration status was estimated for each study participant, followed by meta-analysis of the correlation coefficients across the entire study cohort. Plasma NT-proBNP and hydration status were found to be independently correlated across time (r=0.273 (95% CI 0.200-0.342, I^2=48.3%), and meta-regression found that the significant correlation between plasma NT-proBNP and hydration did not differ between subgroups of dialysis modality, left ventricular hypertrophy, systolic or diastolic function. These findings support the use of plasma NT-proBNP testing as a means of monitoring hydration state in the dialysis population.

The findings presented in this thesis advance understanding of the pathophysiological factors underpinning plasma NT-proBNP and hs-cTnT in the dialysis population and improve interpretation of serial measurements of these biomarkers in clinical practice.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

ORIGINAL RESEARCH

1. **Fahim M**, Hawley CM, McDonald SP, Brown FG, Rosman JB, Wiggins KJ, Bannister KM, Johnson DW: Coagulase-negative staphylococcal peritonitis in Australian peritoneal dialysis patients: predictors, treatment and outcomes in 936 cases. *Nephrol Dial Transplant,* 25**:** 3386-3392, 2010
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6. **Fahim MA**, Hayen A, Horvath AR, Dimeski G, Coburn A, Johnson DW, Hawley CM, Campbell SB, Craig JC: N-Terminal Pro-B-Type Natriuretic Peptide Variability in Stable Dialysis Patients. *Clin J Am Soc Nephrol*, 2015 DOI:10.2215/CJN.09060914

BOOK CHAPTERS (PEER-REVIEWED)

1. **Fahim, MA** and Johnson, DW. Hepatitis C Infection in Dialysis . *Advances in Medicine and Biology*. Berhardt LV (Ed). pp 111-134. Hauppauge, NY: Nova Science Publishers, 2010.
2. **Fahim, MA**, Campbell SB, Johnson DW, Hawley CM. B-Type Natriuretic Peptides in The Dialysis Population – Current Knowledge and Future Directions. *Research Advances in Nephrology, Dialysis and Transplantation*. Dr R. M. Mohan (Ed). Global Research Network, 2012.

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CONFERENCE ABSTRACTS

1. Coagulase-negative staphylococcal peritonitis in Australian peritoneal dialysis patients: predictors, treatment and outcomes in 936 cases. **Fahim M**, Hawley CM, McDonald SP, Brown FG, Rosman JB, Wiggins KJ, Bannister KM, Johnson DW. Presented at the 13th congress of The International Society Of Peritoneal Dialysis (ISPD), Mexico City, Mexico (2010)
2. Biological Variation of N-Terminal B-type Natriuretic Peptide (NT-proBNP) in The Stable Dialysis Population. **Fahim M**, Hayen A, Coburn A, Dimeski G, Johnson D, Craig J, Horvath A, Campbell S, Hawley C. Presented at American Society of Nephrology Kidney Week, San Diego, United States of America (2012)
3. Short and Long Term Biological Variation of high sensitivity Troponin T (hs-cTnT) and N-Terminal B-type Natriuretic Peptide (NT-proBNP) in The Stable Dialysis Population. **Fahim M**, Hayen A, Coburn A, Dimeski G, Johnson D, Craig J, Horvath A, Campbell S, Hawley C. Presented at Young Investigator Award Session, Australia and New Zealand Society of Nephrology Annual Scientific Meeting, Brisbane, Australia (2013)

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Contributions by others to the thesis

Mrs Amanda Coburn – research nurse. Assisted with conduct of study visits (30%) and data entry

Dr Brian Haluska – echocardiographer. Assisted with design of echocardiography protocol and performed cardiac and vascular imaging.

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Dialysis, cardiomyopathy, cardiovascular mortality, monitoring, amino terminal fragment of pro-B-type natriuretic peptide (NT-proBNP), high sensitivity cardiac troponin-T (hs-cTnT), extracellular fluid, bioimpedance

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List of Abbreviations used in the thesis

ACEI	Angiotensin converting enzyme inhibitor
ARB	Angiotensin type-1 receptor blocker
BNP	B-type natriuretic peptide
CRP	C-reactive protein
ECW	Extracellular water volume
EF	Ejection fraction
GLS	Global longitudinal strain
hs-cTnT	High sensitivity cardiac troponin-T
LV	Left ventricular
LVMI	Left ventricular mass indexed to body surface area
NT-proBNP	Amino terminal fragment of pro-B-type natriuretic peptide
TBW	Total body water

Chapter 1 – Introduction.

The overarching aim of this thesis is to advance understanding of the roles of two cardiac biomarkers, the amino terminal fragment of pro-B-type natriuretic peptide (NT-proBNP) and high sensitivity cardiac troponin-T (hs-cTnT), in diagnosing and monitoring cardiovascular disease in the dialysis population.

Cardiovascular disease is the leading cause of death among patients on dialysis (6-11 deaths per 100 patient years)(1). Indeed, observed cardiovascular mortality rates in dialysis patients are 10-60 times greater than those of the non-dialysis population. Of particular concern is the fact that cardiovascular mortality in the dialysis population has declined by no more than 5% over the past decade whereas cardiovascular mortality in the non-dialysis population has fallen by 27-33% during the same period(2). A key factor underpinning the poor progress in combating the heightened cardiovascular risk among dialysis patients is an inability to identify patients at increased risk of cardiac morbidity and/or mortality in a timely manner or to monitor the efficacy of cardiac interventions in either the clinical or research settings(3).

Both NT-proBNP and hs-cTnT have garnered considerable interest as candidate biomarkers for the diagnosis and monitoring of cardiovascular disease in the dialysis population. Numerous cohort studies have demonstrated an association between plasma concentrations of both NT-proBNP and hs-cTnT, and the risk of adverse cardiovascular outcomes in the dialysis population(4, 5). However, understanding of the pathophysiological factors underpinning these biomarkers and the interpretation of serial measurements in clinical practice remains poor, thereby hampering the adoption of these biomarkers in both the clinical and research settings.

This thesis aims to improve overall understanding of these biomarkers by first undertaking a review of the burden and pathophysiology of cardiovascular disease in the dialysis population to identify key targets for monitoring, followed by a critical review of the published literature surrounding NT-proBNP in the dialysis population. Thereafter, the rationale and design of two cohort studies are presented with the aims of answering four crucial questions needed for the interpretation of these cardiac biomarkers in clinical practice:

1. How much change in serial measurements of plasma NT-proBNP and hs-cTnT can be attributed to biological variation and is therefore of no clinical significance?

2. What are the pathophysiological associations of plasma NT-proBNP and hs-cTnT in the dialysis population and which of these serve as potential targets for intervention?
3. How accurate is serial testing of plasma NT-proBNP and hs-cTnT for monitoring cardiovascular risk and the pathophysiological factors underpinning this risk in the dialysis population?
4. How often should these biomarkers be measured and what is their associated time to event?

The results of the biological variation studies, the pathophysiological associations of the cardiac biomarkers from cross-sectional analyses, and the longitudinal correlation of plasma NT-proBNP and hydration status in the dialysis population are then presented. The ultimate aim of this research is to address all of the research questions posed above to develop a cardiac biomarker-based monitoring strategy, which can then be tested to determine if it improves patient-related outcomes in the dialysis population compared with current standard care.

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Chapter 2

Cardiovascular Disease in the Chronic Kidney Disease Population – Burden, Pathophysiology & End-organ Manifestations.

This chapter reviews the epidemiology and pathophysiology of cardiovascular disease in the chronic kidney disease population with a particular focus on those mechanisms unique to chronic kidney disease, their physiological consequences and end-organ effects. An understanding of these mechanisms is the first step to developing effective cardiac risk monitoring strategies and therapies for this group.

The following chapter was published as the peer reviewed book chapter:

MA, Fahim and Hawley, CM**.** Cardiovascular Disease in the Chronic Kidney Disease Population – Burden, Pathophysiology & End-organ Manifestations. Colin Hutchinson (Ed). *Renal Failure: Prevention, Causes and Treatment*. Hauppauge, NY: Nova Science Publishers 2013.

Chapter 2

Cardiovascular Disease in the Chronic Kidney Disease Population – Burden, Pathophysiology & End-organ Manifestations.

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ABSTRACT

Individuals with chronic kidney disease have a disproportionately elevated risk of cardiovascular disease which accounts for the majority of the morbidity and mortality in this population. While traditional cardiovascular risk factors are overrepresented in the chronic kidney disease population, this alone is not sufficient to explain the excess risk observed. Chronic kidney disease is also associated with a number of novel pathophysiological mechanisms including salt and water retention, bone and mineral disorders, sympathetic overactivity and oxidative stress which combine to cause a number of pathological cardiovascular changes unique to chronic kidney disease. A greater understanding of these mechanisms is the first step to developing effective cardiac risk monitoring strategies and therapies. This chapter reviews the pathophysiology of cardiovascular disease in the chronic kidney disease population with a particular focus on those mechanisms unique to chronic kidney disease, their physiological consequences and end-organ effects.

THE BURDEN OF CARDIOVASCULAR DISEASE IN CHRONIC KIDNEY DISEASE

Cardiovascular disease is the leading cause of death in patients with chronic kidney disease who have a 1.5 - 30 fold increased risk of cardiovascular mortality compared with their non-dialysis counterparts(1-3). This is particularly evident in patients with end-stage renal disease on dialysis therapy among whom cardiovascular mortality accounts for 43% of all deaths or 6.6 - 9.6 deaths per 100 patient years(1, 4). Cardiovascular disease is also a major source of morbidity in this population accounting for 34.7 - 56 hospitalizations per 100 patient years(4, 5).

Unfortunately, the past decade has seen little(2) or no improvement(6) in cardiovascular mortality rates among patients with end-stage renal disease while cardiovascular mortality rates among the general population have fallen by 33%(7).

Among the various causes of cardiovascular mortality, sudden cardiac death accounts for 15-30% of all deaths in the dialysis population making it the single leading cause of death in this group(1, 2, 5, 8). In contrast to the general population, cardiomyopathy and not coronary artery disease is reported to be the primary pathology underlying sudden cardiac death in the dialysis population(1, 2, 9-11). This fact combined with the high prevalence of cardiomyopathy in the dialysis population (61.8 – 84.5%)(10, 12) have positioned sudden cardiac death and cardiomyopathy as key targets of efforts to improve current poor outcomes in dialysis.

The excess burden of cardiovascular disease in populations with renal disease is explained by the fact that cardiovascular disease is both an important cause *and* consequence of chronic kidney disease. Compared with the general population, individuals with chronic kidney disease have a higher incidence and prevalence of cardiovascular risk factors especially diabetes and hypertension(13),(14), as well as established end-organ disease(5, 10, 13-15) which may be partly or wholly implicated in the aetiology and/or progression of their renal disease. Indeed over 80% of incident and prevalent chronic kidney disease patients treated with dialysis have at least one cardiovascular diagnosis(5, 10). In addition, end-stage kidney disease is itself an independent risk factor for cardiovascular disease(16),(17), mediated through a variety of pathophysiological mechanisms including volume overload, sympathetic overactivity, hyperphosphataemia, hyperparathyroidism, oxidative stress, and anaemia. This chapter reviews the pathophysiology of cardiovascular disease in the chronic kidney disease population with a particular focus on those mechanisms unique to chronic kidney disease. The physiological derangements and end-organ pathologies that characterize cardiovascular disease in chronic kidney disease result from a confluence of multiple inciting mechanisms (Figure 1). The physiological aberrations and their end-organ consequences will be discussed first before detailing the novel mechanisms that result in these changes.

END-ORGAN PATHOLOGY

Cardiomyopathy

Cardiomyopathy encompassing left ventricular hypertrophy, dilatation, systolic and/or diastolic dysfunction is highly prevalent in the dialysis population (61.8 - 84.5%)(10, 18-20) and carries a poor prognosis. A prospective cohort study of 433 incident dialysis patients followed for 41-months found that the only independent predictors of overall mortality at ≥ 2-years were a diagnosis of cardiac failure and cardiomyopathic indices after adjusting for demographic, comorbid, traditional and non-traditional vascular risk factors(10). These results were echoed by a prospective cohort study of 220 prevalent peritoneal-dialysis patients followed for 5-years which found that the only independent predictors of sudden cardiac death were left ventricular systolic dysfunction, systolic and diastolic blood pressure(21).

Heart failure is the most common non-fatal manifestation of cardiomyopathy in dialysis; is highly prevalent (30-40%) and portends an increased risk of overall mortality of 80-100%(10, 12). A prospective cohort study of 230 prevalent peritoneal dialysis patients followed for 4-years(12), found that the only independent predictors of new-onset heart failure were cardiomyopathy and diabetes mellitus, while cardiomyopathy and low serum albumin (possibly reflecting fluid overload) were the only independent predictors of recurrent heart failure.

Left ventricular hypertrophy may be classified morphologically into either concentric or eccentric hypertrophy. Concentric hypertrophy is characterized by the addition of new sarcomeres in a parallel formation leading to an increase in ventricular wall thickness without an increase in ventricular luminal diameter and usually occurs in response to pressure overload. In contrast, eccentric hypertrophy represents an adaptive response to volume overload in which sarcomeres are added in a serial formation leading to an increase in both ventricular luminal diameter and wall thickness(22, 23). In individuals with non-dialysis dependent chronic kidney disease eccentric hypertrophy is the predominant pattern observed(24), while both patterns are equally represented among the dialysis population(10).

Cardiomyocyte hypertrophy is the histological hallmark of left ventricular hypertrophy irrespective of renal disease; however, the cardiomyopathy of chronic kidney disease is additionally characterized by excessive intermyocardic fibrosis. This was illustrated in a post-mortem series with a case-control design comparing the cardiac fibrosis scores between

individuals with renal disease and matched individuals with normal renal function. The myocardium of individuals with renal disease demonstrated the highest fibrosis scores and renal disease was found to be an independent predictor of intermyocardic fibrosis even after adjusting for demographic factors and co-morbid conditions. Furthermore dialysis duration was found to be strongly correlated with the severity of fibrosis(25). The fibrosis observed in these cases is due to the deposition of type I collagen fibres in between cardiomyocytes at the expense of myocardial capillaries resulting in impaired myocardial perfusion, reduced ventricular compliance and variances in electrical conduction and resistance which in turn predisposes to arrhythmia(26).

A number of factors contribute to the genesis and evolution of cardiomyopathy in dialysis including hypertension, volume overload, vascular stiffness, sympathetic overactivity, cardiotonic steroids, direct fibrotic effects of angiotensin II and aldosterone, hyperparathyroidism and cardiac ischaemia. These factors are discussed in detail in subsequent sections of this chapter.

Coronary Atherosclerosis and Microvessel Disease

Individuals with chronic kidney disease have an increased incidence and prevalence of coronary artery disease compared to matched individuals in the general population(5, 13, 15, 27). Coronary atherosclerosis associated with chronic kidney disease is characterized histologically by plaque calcification, and concurrent medial hypertrophy and calcification(28).

While myocardial ischaemia is more prevalent among the dialysis population, a disproportionate number of individuals on dialysis present with either symptomatic myocardial ischaemia or myocardial infarction but do not have a significant coronary artery stenosis on angiography(29). This finding is thought to be related to reduced myocardial capillary density caused by cardiac fibrosis, and to thickening of the walls of post-epicardial intramyocardial vessels – so called microvessel disease. Such vessel wall thickening has been documented in animal models(30) and in post-mortem myocardial biopsies from individuals with end-stage renal disease but not in biopsies taken from hypertensive individuals with normal renal function(25). Post-epicardial vessel wall thickening occurs in the absence of an increase in luminal area reducing myocardial perfusion and impairing oxygen diffusion into the surrounding myocardium.

Vascular Calcification

Vascular calcification refers to ectopic accumulation of calcium-phosphate mineral in the arterial vasculature, myocardium and cardiac valves. This pathological phenomenon is frequently observed among individuals with chronic kidney disease and has been associated with an increased risk of cardiovascular mortality. A prospective cohort study of 439 individuals with a mean estimated glomerular filtration rate (MDRD eGFR) of 50.6 ml/min reported that the prevalence of any calcification in the coronary arteries, descending thoracic aorta, aortic valve, and mitral valve was 67, 49, 25, and 20% respectively(31). The prevalence of vascular calcification is even greater among individuals with end-stage renal disease on dialysis; a randomised controlled trial of 360 haemodialysis patients investigating the impact of two therapies on vascular calcification progression reported that the prevalence of calcification in the thoracic aorta, mitral valve and aortic valve at enrollment was 91, 50, and 46% respectively(32).

The poor prognosis portended by vascular calcification is highlighted by the findings of a prospective cohort study of 110 haemodialysis patients followed for a mean duration of 53±21 months assessing the association between the presence and severity of vascular calcification, and all-cause and cardiovascular mortality. The study reported a significant, graded increase in the risk of all-cause mortality with increasing numbers of vascular sites involved with calcification (Risk of all-cause mortality for 0 to 4 sites involved was 3, 17, 31, 50 and 73% respectively). Furthermore, a multivariate analysis adjusting for demographic factors, co-morbidities and vascular functional indices found that vascular calcification severity assessed using a calcification score was an independent predictor of both all-cause mortality (HR = 1.9 per unit increase in calcification score) and cardiovascular mortality (HR = 2.6 per unit increase in calcification score)(33).

The mortality risk conveyed by vascular calcification is thought to be mediated by increased vascular stiffness which acts to promote left ventricular hypertrophy and reduce myocardial perfusion. This hypothesis is supported by findings of a prospective longitudinal study of 134 dialysis and non-dialysis dependent chronic kidney disease patients assessing the association between change in vascular calcification severity and both vascular stiffness assessed using pulse wave velocity and pulse pressure, and all-cause mortality. The study demonstrated a significant correlation between progressive vascular calcification and increasing pulse pressure and pulse wave velocity, in addition to a significant association between increasing vascular

calcification and the risk of all-cause mortality(34). These findings were echoed by a prospective cohort study of 110 haemodialysis patients which demonstrated a significant association between measures of increasing vascular stiffness and increasing severity of vascular calcification(33).

Vascular calcification associated with chronic kidney disease is characterized by accumulation of calcium phosphate mineral in the medial rather than the intimal layer of arterial vessels. Ultrastructural examination of vessels from patients end-stage renal disease undergoing renal transplantation reveals calcium-phosphate mineral in extracellular loci as either hydroxyapatite [$Ca_{10}(PO_4)_6OH_2$] and/or whitlockite [$(Ca,Mg)_3(PO_4)_2$] crystals co-localizing to areas of damaged vascular smooth muscle cells, extracellular vesicles, and bone proteins including Type I collagen fibrils, osteopontin, bone sialoprotein and alkaline phosphatase(35-37). There is also inconclusive evidence that arterial elastin fibrils may be disrupted and/or calcified as part of the vascular calcification process(38). The pathogenesis of vascular calcification is discussed in the subsequent section entitled 'Mineral & Bone Disorders and The Pathophysiology of Vascular Calcification'.

PHYSIOLOGICAL DERANGEMENTS

Hypertension

Hypertension is highly prevalent in the dialysis population (75 – 86 %)(14, 39, 40), is principally related to volume overload and can be remedied in over 90% of cases by ultrafiltration(41, 42). Other contributing factors include sympathetic overactivity, activation of the renin-angiotensin axis, and vascular stiffness which are discussed in more detail below.

Early studies examining the association between blood pressure and survival in the dialysis population suggested a 'U' shaped relationship whereby mortality risk was increased not only with extremely high post-dialysis blood pressures (systolic > 180 mm Hg or diastolic > 90 mm Hg), but also with blood pressures targets often prescribed in dialysis units (pre- or post- dialysis blood pressure < 110 mm Hg)(43, 44). These findings contrast with those of a longitudinal cohort study demonstrating that baseline hypertension at dialysis inception and residual hypertension at 1-year post-dialysis commencement were associated with a significantly increased risk of mortality without any survival disadvantage associated with low blood pressure(45), and those of a prospective cohort study of 692 haemodialysis patients which

reported that a mean arterial blood pressure > 110 mmHg was associated with an increased risk of all-cause and cardiovascular death after adjusting for demographic factors and co-morbid conditions(46). Finally, a recent meta-analysis of 8 randomised controlled trials of antihypertensive therapy in dialysis found significant reductions in the risks of fatal and non-fatal cardiovascular events and all-cause mortality in actively treated patients compared with controls supporting the hypothesis that hypertension portends an adverse prognosis in the dialysis population(47). The discrepancy between early and contemporary studies regarding the association between hypertension and mortality is likely explained by a lack of adjustment for important confounding factors, particularly cardiac systolic dysfunction in which low blood pressure increases the risk of mortality(48).

Vascular Stiffness

Vascular stiffness refers to a reduction in the compliance and elastic properties of central arteries such as the thoracic aorta. The reduced compliance of these vessels impairs propagation of the cardiac pressure wave generated during systole, necessitating an increase in systolic blood pressure in order to maintain systemic perfusion. This increased myocardial work stimulates myocardial hypertrophy and increases myocardial oxygen demand. Furthermore, increased vascular stiffness accelerates the propagation of the pulse wave and earlier return of the reflected wave towards the myocardium which reduces diastolic blood pressure compromising coronary perfusion which occurs predominantly during diastole(49, 50).

Vascular stiffness is most often measured as central pulse pressure and pulse wave velocity. This latter technique measures the rate of propagation of the pulse wave from the heart to a central artery using tonometry and oscillometric pulse recognition algorithms; increasing pulse wave velocity reflects worsening vascular stiffness(50).

The adverse prognosis conveyed by vascular stiffness was demonstrated in a prospective cohort study of 265 haemodialysis patients followed for 63±23 months investigating the association between pulse wave velocity, and all-cause and cardiovascular mortality. When stratified around the cohort's median value, increasing pulse wave velocity was shown to be an independent predictor both all-cause and cardiovascular mortality after adjusting for demographic and co-morbid conditions including diabetes and hypertension(51).

Vascular stiffness observed in chronic kidney disease populations results from an interplay between several pathologic mechanisms including vascular calcification(52), reduction in the elastin content of central vessels(38), and vascular smooth muscle hypertrophy secondary to hypertension and the trophic effects of catecholamines, angiotensin II and aldosterone(53).

Myocardial Ischaemia

Ischaemic heart disease has a prevalence of 30 – 40 % in the dialysis population(5, 20) and is related to accelerated atherosclerosis of epicardial coronary arteries due to a higher prevalence of traditional ischaemic risk factors(13) and novel mechanisms including oxidative stress, sympathetic overactivity, and mineral and bone disorder. However, approximately 30% of dialysis patients presenting with symptomatic ischaemic heart disease (myocardial infarction or angina) do not have evidence of epicardial coronary stenosis(29). Myocardial ischaemia in these individuals is thought to be due to reduced myocardial oxygen reserve secondary to the reduction in myocardial capillary density that accompanies myocardial fibrosis and thickening of the vessel wall of post-epicardial arterioles(25, 30). Cardiac hypoperfusion may also be exacerbated by vascular stiffness which impairs coronary and myocardial perfusion during diastole as has been discussed previously.

INCITING PATHOLOGICAL MECHANISMS

Sodium & Water Excess

Extracellular volume expansion as a result of sodium and water retention is common both among individuals with early stage chronic kidney disease(54) and those with end-stage renal disease on dialysis therapy(55, 56). Several studies have identified volume overload as a critical risk factor for both morbidity and mortality in the dialysis population. A cohort study of 269 prevalent haemodialysis patients followed for 3.5-years found that overhydration assessed by bioimpedance spectroscopy (an instrument to objectively assess volume state) was associated with an independent relative risk of all-cause mortality of 2.1 in multivariate analysis(56). Similarly, a cohort study of 3009 prevalent haemodialysis patients followed for 1-year demonstrated that increasing volume state conveyed a significant, graded increase in the risk of death. Indeed, the relative risk of death was as high as 2.83 for the most overhydrated patients following adjustment for demographic factors, co-morbid conditions and novel cardiovascular risk factors(55). A number of cohort studies have also demonstrated a significant association

between clinical and surrogate biochemical measures of overhydration, and cardiovascular mortality(55-59) and sudden cardiac death(21, 60).

Sodium and water accumulation in chronic kidney disease results from hormonally induced adaptations to renal injury, a progressive overwhelming of tubular solute excretory capacity, and excessive sodium intake. Glomerular injury and subsequent nephron loss from any aetiology induces a compensatory response whereby the single nephron glomerular filtration rate (GFR) of remaining functioning nephrons increases to compensate for the reduction in absolute numbers of nephrons. This glomerular hyperfiltration is effected by an increase in renal perfusion due to extracellular fluid volume expansion, and concurrent dilatation of the afferent arteriole and relative vasoconstriction of the efferent arteriole which have the net effect of increasing glomerular transcapillary pressure and single nephron GFR. These adaptations are mediated in large part by activation of the intra-renal renin-angiotensin-aldosterone axis in response to the renal hypoperfusion caused by any aetiology. Angiotensin II plays a critical role in inducing the changes in arteriolar caliber described above, while aldosterone stimulates an increase in tubular sodium and hence water reabsorption producing extracellular volume expansion(61-63).

Progressive chronic kidney disease is accompanied by tubular adaptations which increase fractional sodium excretion per nephron in order to maintain sodium homeostasis despite a reduction in functional nephron mass. However, despite this increase in relative sodium excretion per nephron, *absolute* sodium excretion is reduced contributing to excess sodium and water retention particularly in the latter stages of chronic kidney disease(54, 62).

Extracellular volume expansion induced by sodium and water retention plays an important role in the genesis of hypertension in chronic kidney disease. This is particularly true of the end-stage renal disease population in whom hypertension can be attributed to volume overload in over 90% of cases(41, 64) and can be effectively remedied using ultrafiltration(42). Volume expansion has been shown to be an independent predictor of cardiomyopathic changes including left ventricular hypertrophy, left atrial dilatation, and left ventricular systolic dysfunction in all stages of chronic kidney disease(10, 12, 18, 54), while control of volume state in dialysis has been shown to improve echocardiographic indices(65).

In addition to its role in volume expansion, sodium excess has a number of independent adverse physiological effects including the direct stimulation of angiotensin II by vascular tissue(66) and

stimulating the secretion of the a cardiotonic marinobufagenin-like steroid by adrenal cortical cells(67, 68). This latter hormone has been implicated in the genesis of cardiomyopathy and cardiac fibrosis in animal models of chronic kidney disease(69, 70).

Mineral and Bone Disorders & The Pathophysiology of Vascular Calcification

Progressive chronic kidney disease and the consequent fall in GFR reduces the filtered phosphate load and increases plasma phosphate concentrations(71). The resulting hyperphosphataemia has a number of important consequences including (A) antagonizing 1α-hydroxylase activity; reducing circulating concentrations of $1,25(OH)_2$ Vitamin D (calcitriol)(72) (B) inducing hypocalcaemia due to its effect on reducing circulating calcitriol concentrations and to a much lesser degree by directly binding ionized calcium to form calcium hydroxyphosphate(73) (C) inducing secondary hyperparathyroidism by directly upregulating parathyroid hormone synthesis(74, 75) and and through the effects of hyperphosphataemia on reducing calcium and $1,25\ (OH)_2$ Vitamin D concentrations which in turn upregulate parathyroid hormone synthesis.

The association between bone mineral disturbances and mortality is highlighted by a recent systematic review of 35 prospective, observational and interventional studies which examined the association between all-cause and cardiovascular mortality, and hyperphosphataemia, hypercalcaemia and hyperparathyroidism. Heterogeneity between studies with respect to participant characteristics, method of mineral parameter assessment (continuous vs. dichotomous vs. categorical) and control for confounding precluded meta-analysis of the studies' findings, however, the studies were consistent in finding a significant association between hyperphosphataemia and both all-cause and cardiovascular mortality albeit of varying magnitudes. In addition, the majority of studies demonstrated a significant increase in the risk of all-cause mortality in association with hypercalcaemia and hyperparathyroidism(76). The increased risk of mortality conveyed by these mineral disturbances is thought to be mediated through their role in initiating and promoting vascular calcification and subsequent vascular stiffness. This is particularly true of hyperphosphataemia which has been shown to be an independent predictor of vascular calcification even after adjustment for CKD severity, demographic factors, co-morbidities, parathyroid hormone and $1,25(OH)_2$ Vitamin-D concentrations(31).

Vascular calcification represents an interplay between a number of pathological processes including the (1) osteogenic / chondrogenic differentiation of vascular smooth muscle cells (VSMC), (2) extracellular release of calcium-phosphate rich vesicles by VSMC (3) a reduction in circulating factors that inhibit calcification, and (4) apoptosis of vascular smooth muscle cells. Two receptors – Pit-1 and Pit-2 – located on VSMC play differing but complementary roles in vascular calcification. In the presence of hyperphosphataemia, the Pit-1 receptor increases intracellular phosphate concentrations in VSMC and induces osteogenic / chondrogenic differentiation through the upregulation of the osteogenic transcription factor Runx2. This transcription factor induces the synthesis of extracellular matrix bone proteins such collagen type I, osteopontin, and bone sialoprotein which are able to undergo calcification(77, 78). In contrast, the Pit-2 receptor plays a critical role in loading intracellular VSMC vesicles with calcium and phosphate(52). These vesicles are subsequently secreted and act as nidus for extracellular medial calcification(36). Vascular calcification is also promoted by a reduction in concentration of circulating calcification inhibitors such as fetuin-A and pyrophosphate which are reduced in chronic kidney disease its associated chronic inflammatory state(77, 79, 80). In addition to its metaplastic effect, phosphate is also able to induce apoptosis of VSMC with the resulting apoptotic bodies acting as nuclei for calcification(52).

Sympathetic Overactivity

Sympathetic activity measured in the form of circulating norepinephrine concentrations or using microneurography of the peroneal nerve (muscle sympathetic nerve activity) has been shown to be markedly elevated in patients with dialysis-(81, 82) and non-dialysis(83, 84) dependent chronic kidney disease and correlates with the severity of renal impairment(84) suggesting that chronic renal injury may stimulate sympathetic hyperactivity. This hypothesis is supported by the observations that patients with end-stage renal disease on dialysis who have undergone bilateral, but not unilateral, nephrectomy have sympathetic activity in keeping with that of healthy controls(82).

The adverse prognosis portended by sympathetic hyperactivity was highlighted by a prospective cohort study of 228 haemodialysis patients followed for 34±15 months, 45% of whom had elevated circulating norepinephrine concentrations. After adjusting for demographic features, co-morbidities and concurrent medication use, the authors reported a significant, graded association between plasma norepinephrine concentrations and the risk of fatal and non-fatal cardiovascular events, and all-cause mortality(85). These findings are also circumstantially supported by the

results of two pilot randomised controlled trials in dialysis demonstrating a reduction in mortality(86) and improvement in cardiac diastolic function with the use of the beta-blocker carvidelol(87), however the beneficial effects observed may be partly or wholly related to concurrent improvements in blood pressure.

Sympathetic overactivity associated with renal injury is explained both by an increase in sympathetic outflow and reduced catecholamine breakdown. Animal studies have demonstrated that acute and chronic renal injury stimulate autonomic centers in the hypothalamus via afferent nerves in the spinothalamic tract resulting in increased sympathetic outflow(88) and that this sympathetic hyperactivity can abolished by denervation of the injured kidney(89).

Angiotensin II and adenosine have been implicated as potential mediators between the kidney and the autonomic nervous system. As discussed previously both acute and chronic renal injury are characterized by upregulation of the renin-angiotensin axis. The increase in circulating concentrations of angiotensin II stimulates angiotensin receptors in the medulla oblongata which in turn stimulate the posterior hypothalamic nuclei leading to an increase in sympathetic efferent activity(90). The importance of this pathway in promoting sympathetic hyperactivity was elegantly demonstrated in animal models in which sympathetic overactivity associated with renal injury could be abolished by either intravenous or targeted intracranial injection of an angiotensin receptor blocker(91). Angiotensin II is able to upregulate sympathetic nervous activity through direct peripheral, pre-synaptic binding which increases the secretion and reduces the reuptake of norepinephrine(90). Adenosine is released from the injured kidney following minor ischaemic insults and has also been implicated as an important mediator of sympathetic excitation. This was illustrated by experiments in 1-clip, 2-kidney dogs in which intrarenal adenosine infusion increased sympathetic activity and circulating norepinephrine concentrations which could be abolished by renal denervation or ganglionic blockade(92).

In addition to increased stimulation of efferent activity, it is now also recognized that sympathetic hyperactivity associated with chronic kidney disease is related to reduced clearance of circulating catecholamines(93). The kidney has been identified as the synthetic site of the proenzyme prorenalase(94, 95), which in the presence of high circulating concentrations of catecholamines is activated to renalase which in turn metabolizes and inactivates catecholamines including dopamine, epinephrine and norepinephrine(96). Renalase synthesis and activity are

reduced in individuals with chronic kidney disease(94) which contributes to the increase in circulating catecholamine concentrations.

Sympathetic hyperactivity has a number of direct and indirect adverse cardiovascular consequences. It contributes significantly to hypertension associated with chronic renal disease as is illustrated by the correlation between blood pressure and sympathetic nerve activity(97) and the profound blood pressure response to sympatholytic agents in haemodialysis(98). Norepinephrine is able to induce left ventricular hypertrophy directly through a trophic effect on cardiac myocytes(99, 100), contributes to increased vascular stiffness by promoting hypertrophy of vascular smooth muscle in the peripheral vasculature(101), and may play a role in triggering arrhythmias(102).

Oxidative Stress & Inflammation

Oxidative stress is defined as tissue injury caused by excess circulating anionic free radicals called reactive oxygen species (ROS). The increased concentration of these injurious free radicals is caused both by the increased generation of ROS and reduced synthesis of neutralizing anti-oxidant molecules(103). ROS cause cellular injury both directly and indirectly through the activation of cellular and humoral inflammatory processes. Increased concentrations of ROS and inflammatory cytokines, and reduced levels of antioxidants have been documented in the chronic kidney disease population and are associated with a poor prognosis. A prospective cohort study of 19 patients with stage 5 CKD, 15 of whom were on dialysis, reported significantly higher concentrations of two oxidative species (Carbonyls and F_2-isoprostanes) and numerous inflammatory markers in the end-stage renal disease group compared to healthy controls. In addition, there was a significant positive correlation between the concentrations of F_2-isoprostanes and C-reactive protein(104). The adverse prognosis associated with oxidative species and inflammation is illustrated by a cohort study of 94 prevalent haemodialysis patients followed for 2-years which reported a significant direct association between the concentrations of both C-reactive protein and anti-oxidized LDL antibody (a marker of oxidative stress) and the risk of all-cause mortality in a multivariate analysis(105). These findings were echoed by a retrospective cohort study of 105 haemodialysis patients followed for 9-years which reported a significant inverse association between concentrations of anti-oxidant proteins and the risk of cardiovascular mortality(106). The possible role of renal dysfunction per se in promoting oxidative stress is supported by the finding that concentrations of oxidative species and

inflammatory markers fall following successful renal transplantation and approximate those of healthy controls(104).

The mechanisms underlying the association between oxidative stress and heightened cardiovascular risk have not been fully elucidated but may include accelerated atherosclerosis related to oxidized LDL, carbonyl proteins and advanced glycation end-products(107, 108) or endothelial injury and dysfunction leading to hypertension, vascular stiffness and promoting atherosclerosis(109).

Increased concentrations of oxidant species in chronic kidney disease may be due to an increase in the generation of ROS through reactions involving nitrogenous waste products including urea(110), derangements of the mitochondrial respiratory chain(111), and exposure to artificial dialysis membranes. In addition, chronic kidney disease is associated with reduction in concentration of antioxidant molecules such as thiols(112) and antioxidant enzymes such as superoxide dismutase which is normally expressed in renal tubules(113).

CONCLUSION

Individuals with chronic kidney disease have a disproportionate risk of cardiovascular disease which accounts for the majority of the morbidity and mortality in this population. While traditional cardiovascular risk factors are overrepresented in the chronic kidney disease population, this alone is not sufficient to explain the excess risk observed. Chronic kidney disease is also associated with a number of novel pathophysiological mechanisms including salt and water retention, bone and mineral disorders, sympathetic overactivity and oxidative stress which combine to cause a number of pathological cardiovascular changes unique to chronic kidney disease. A greater understanding of these mechanisms is the first step to developing effective cardiac risk monitoring strategies and therapies.

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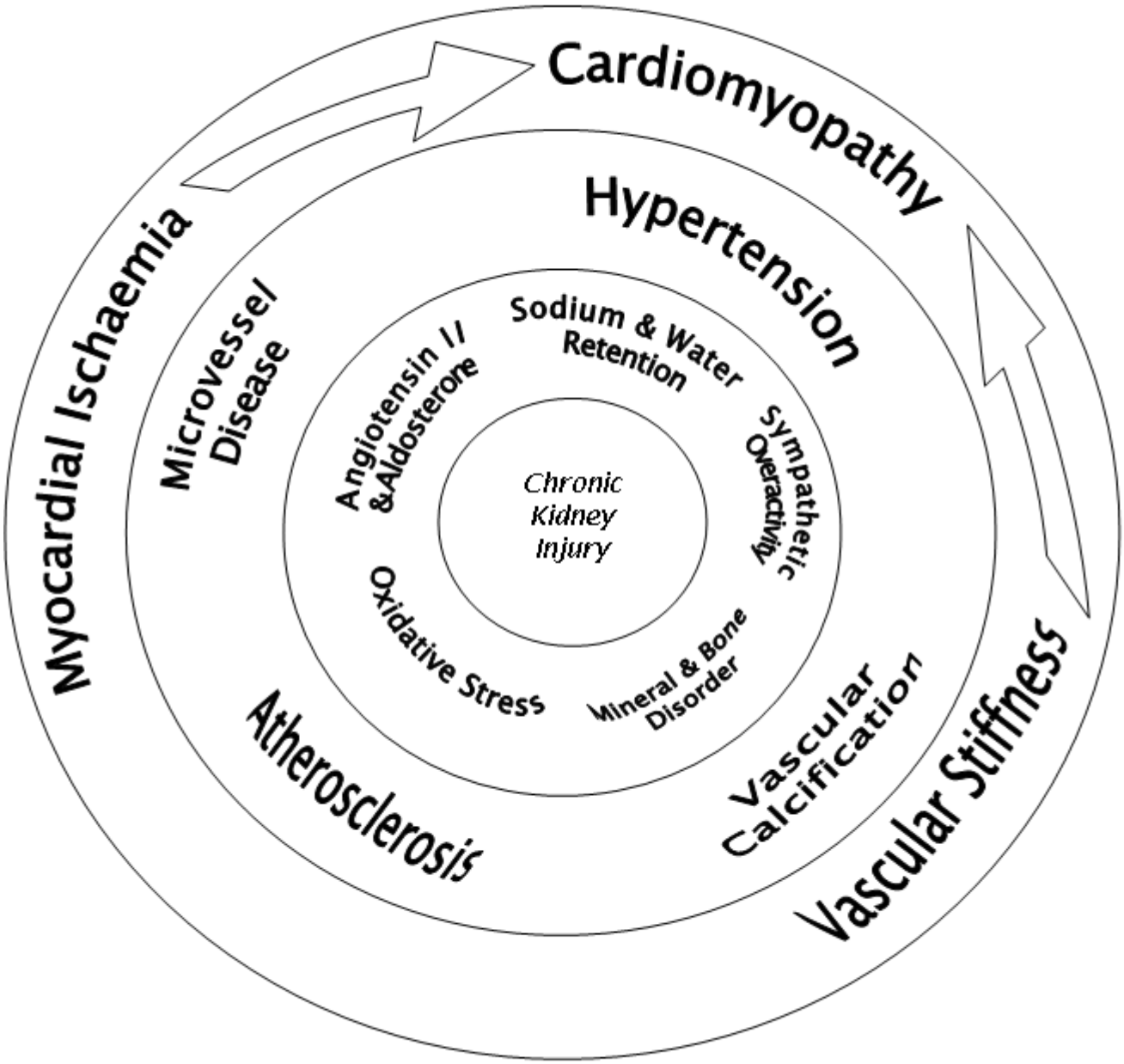
Figure Legends

Figure 1. The pathophysiology of cardiovascular disease in association with chronic kidney disease. Chronic renal injury incites several pathological mechanisms including sodium and water retention, sympathetic overactivity, mineral & bone disorders, oxidative stress and activation of the renin-angiotensin-aldosterone axis. An interplay between these pathological mechanisms produces a number of adverse physiological changes including hypertension, volume overload, vascular calcification and stiffness, coronary and peripheral atherosclerosis, and coronary microvessel disease which result in cardiomyopathy, and cardiac and peripheral ischaemia.

Figure 1


Cardiomyopathy
Hypertension
Vascular Stiffness
Vascular Calcification
Atherosclerosis
Myocardial Ischaemia
Microvessel Disease
Angiotensin II &Aldosterone
Sodium & Water Retention
Sympathetic Overactivity
Mineral & Bone Disorder
Oxidative Stress
Chronic Kidney Injury

Chapter 3

B-type Natriuretic Peptides for Monitoring Cardiac Risk in Dialysis: Current Role and Future Directions.

The following chapter discusses the emerging role of the B-type natriuretic peptides in monitoring volume state, cardiomyopathy and cardiac risk in the renal dialysis population and identifies those research areas that need to be addressed before use of this promising biomarker can be incorporated into routine clinical practice.

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Chapter 3

B-type Natriuretic Peptides for Monitoring Cardiac Risk in Dialysis: Current Role and Future Directions

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ABSTRACT

Dialysis patients have a 50-100 fold increased risk of sudden cardiac death compared to the general population. Fluid overload-induced cardiomyopathy is a key factor underpinning this risk, and progress in mitigating it is hampered by our inability to assess fluid and cardiac risk status in an accurate and dynamic way. There is an urgent need for a biomarker-monitoring strategy that accurately detects the early stages of cardiac injury to enable and guide timely intervention. The B-type natriuretic peptides (BNP / NT-proBNP) play a critical role in circulatory volume and pressure homeostasis in both health and disease, and are secreted from the myocardium during volume/pressure overload, ischaemia, inflammation and sympathetic overactivity. Preliminary studies in dialysis patients have shown that these peptides predict mortality and correlate with cardiomyopathy and fluid state. In this review we discuss the burden and impact of cardiomyopathy in the renal dialysis population and the evidence supporting the role of the B-type natriuretic peptides as biomarkers of volume state, cardiomyopathy and cardiac risk. We also highlight those areas that need to be addressed in order to advance this promising biomarker into clinical practice.

CARDIOVASCULAR DISEASE IN RENAL POPULATIONS – BURDEN AND AVENUES FOR IMPROVEMENT

Cardiovascular disease is highly prevalent in the end-stage kidney disease population treated with dialysis with over 80% of both incident and prevalent patients having at least one cardiovascular diagnosis(1, 2). Cardiovascular disease-related mortality rates are reported to be between 6.6 - 9.6 per 100 patient years and account for 43% of all deaths in this group, making cardiovascular disease

the leading category of death(3, 4). These rates represent a 10 - 30 fold increased risk of cardiovascular death compared with age, gender and diabetic-status matched cohorts from the general population and holds true across geographic regions(5, 6). Indeed, it is likely that these figures underestimate the true mortal burden of cardiovascular disease as over a third of all withdrawals from dialysis therapy are also attributed to this entity(3). Cardiovascular disease is also a major source of morbidity; with a hospitalization rate of 34.7 - 56 per 100 patient years(1, 7) it is the leading cause of hospitalization among patients on haemodialysis irrespective of age.

The excess burden of cardiovascular disease in populations with renal disease is explained by the fact that cardiovascular disease is both an important cause *and* consequence of chronic kidney disease. Individuals with end-stage kidney disease have a higher prevalence of traditional cardiovascular risk factors especially diabetes and hypertension(8), as well as established end-organ disease(1, 2, 5) compared with the general population. Furthermore, end-stage kidney disease is itself an independent risk factor for cardiovascular disease(9), mediated through a variety of pathophysiological mechanisms including volume overload(10), sympathetic overactivity(11), hyperparathyroidism(12) and oxidative stress(13).

Among the various causes of cardiovascular mortality, sudden cardiac death accounts for 15-30% of all deaths in the dialysis population making it the single leading cause of death(1, 3, 4, 14). In contrast to the general population(15), cardiomyopathy and not coronary artery disease is reported to be the primary pathology underlying sudden cardiac death in the dialysis population(2-4, 16). This fact, combined with the high prevalence of cardiomyopathy in the dialysis population (61.8 – 84.5%)(2, 17), have made it a focus of efforts aimed at improving current poor outcomes in dialysis. A number of groups are currently investigating pharmacological(18-20) and dialytic therapies(21) for cardiomyopathy. However, there remains a paucity of validated cardiac-risk monitoring strategies in dialysis. Monitoring is defined as a repeated testing strategy used to assess current health status, detect change in risk/health status over time and evaluate the efficacy of therapy and titrate it accordingly(22). The only guideline on the monitoring of cardiomyopathy in dialysis recommends performing an echocardiogram at 3-yearly intervals(23). The testing modality and interval recommended, however, are entirely opinion-based and the guideline does not detail which echocardiographic parameters to monitor nor the magnitude of change in parameters that should prompt action, making it inadequate as a monitoring strategy. Echocardiography monitoring is also limited pragmatically by the need for specialised training and equipment, and their associated expenses.

This review discusses the emerging role of the B-type natriuretic peptides in monitoring volume state, cardiomyopathy and cardiac risk in the renal dialysis population and identifies those research areas that need to be addressed before use of this promising biomarker can be incorporated into routine clinical practice.

BIOCHEMISTRY AND ESTABLISHED CLINICAL APPLICATIONS OF THE B-TYPE NATRIURETIC PEPTIDES

B-type natriuretic peptide (BNP) is one member of the natriuretic peptide system which additionally consists of the A-, C-, D- and V- type natriuretic peptides and urodilatin(24), although D- and V-natriuretic peptides are not endogenous to humans. These peptides exert endocrine, autocrine and paracrine actions that collectively play a crucial role in maintaining cardiovascular functional and structural homeostasis. BNP is primarily expressed and secreted by the heart, particularly the ventricular myocardium(25, 26). However, expression has also been localized to a number of extra-cardiac sites, including the brain, adrenal gland, kidney, and lungs(27). Transcription of the BNP gene is induced by a number of pathophysiological stimuli, including mechanical myocardial stretch(28, 29) (volume overload) or strain (pressure overload)(30, 31), ischaemia(32, 33), pro-inflammatory cytokines, such as interleukin-1β and TNFα(34, 35), α- and β- adrenergic agonists (sympathetic overactivity)(36, 37), and vasoactive factors, such as endothelin-1(29) and angiotensin-II(38). It is noteworthy that these stimuli overlap significantly with the pathological milieu implicated in the genesis and progression of cardiomyopathy in the dialysis population, positioning BNP as a potentially important biomarker of cardiac risk in this group.

Cardiac myocytes synthesize and secrete relatively low concentrations of BNP at a basal rate under normal physiological conditions. However, in response to the aforementioned stimuli, BNP gene transcription is upregulated leading to the synthesis of the intracellular 134 amino-acid precursor, pre-proBNP. This peptide undergoes cleavage of a 26 amino-acid signal peptide to form the pro-hormone, $proBNP_{1\text{-}108}$, which is in turn cleaved by the convertases furin and corin yielding equimolar amounts of $BNP_{1\text{-}32}$ and its biologically inactive amino-terminal fragment, $NT\text{-}proBNP_{1\text{-}76}$, which are secreted into the circulation(24, 39). The secretory pathway of the B-type natriuretic peptides is illustrated in Figure 1.

BNP exerts most of its physiological actions by binding to natriuretic peptide receptor-A (NPR-A), a guanylyl cyclase-coupled receptor located in the kidneys, adrenal glands, vascular smooth muscle, myocardium, adipose tissue, brain and lungs(40). In concert with the other natriuretic peptides,

BNP counteracts haemodynamic stress caused by volume, vasoactive and neurohormonal related mechanisms by inducing vaso- and veno- dilation of the systemic and pulmonary vasculature, natriuresis, diuresis, lowering the activation threshold of vagal efferents and inhibiting the secretion of renin and aldosterone(41). Circulating BNP is cleared by neutral endopeptidases, receptor mediated binding to NPR-A and by endocytosis following binding to natriuretic peptide receptor-C (NPR-C), while NT-proBNP undergoes glomerular filtration followed by tubular degradation and reabsorption(42). In addition, both peptides are partially excreted unchanged renally with a fractional renal excretion of 15-20%(43).

BNP and NT-proBNP have a number of proven clinical applications in the general population, including improving the accuracy of clinical assessment for diagnosing cardiac failure in patients presenting with dyspnoea(44, 45) and estimating prognosis in patients with an established diagnosis of cardiac failure(46). Over the last decade, 9 randomised controlled trials have examined the role of BNP / NT-proBNP monitoring in guiding heart failure therapy compared to specialist care alone. Two recent meta-analyses of these randomised controlled trials reported that BNP/NT-proBNP-guided heart failure pharmacotherapy resulted in significant reductions in mortality in patients under 75 years of age compared to specialist care alone (adjusted hazard ratio [HR] 0.69, 95% confidence interval [CI] 0.55-0.86(47) and HR 0.76, 95% CI 0.63-0.91(48)). These findings have led to the recommendation that hormonal monitoring be incorporated into specialist care of heart failure patients(49). Both BNP and NT-proBNP have established analytic methods with sufficiently low imprecision(50, 51). However, NT-proBNP has the significant advantage of being less susceptible to pre-analytic variation, including postural, diurnal, and storage effects(52), making it a more robust biomarker for use in daily clinical situations.

CHARACTERISTICS AND CORRELATES OF B-TYPE NATRIURETIC PEPTIDES IN THE DIALYSIS POPULATION

Concentrations of the B-type natriuretic peptides are markedly elevated in virtually all incident and prevalent patients on dialysis including those without a prior diagnosis of cardiac failure. Using a cut-off value for NT-proBNP of 300 pg/ml (the concentration used to exclude heart failure with 98% certainty in non-dialysis patients presenting with dyspnoea(53)), NT-proBNP concentrations in the dialysis population are elevated in the order of 3 – 55 times this diagnostic cut-off(54-60). These concentrations far exceed the increment expected due to reduced renal clearance alone and reflect the high burden of cardiomyopathy in the renal dialysis population.

Concentrations of the B-type natriuretic peptides in the general population are known to be influenced by a number of demographic and non-cardiac physiological variables, including age(61), gender(62), adiposity(63, 64) and glomerular filtration rate. Concordant with observed correlations in the general population, BNP/NT-proBNP concentrations in the dialysis population vary directly with age(54, 65), and indirectly with residual renal function(55, 57) and increasing body mass index(54, 56).

The dialysis procedure has also been shown to affect concentrations of BNP / NT-proBNP, an important consideration when interpreting changes in serial results in both clinical and research practice. The effect of a single haemodialysis session on circulating concentrations of the B-type natriuretic peptides varies according to the ultrafiltration coefficient (flux) of the dialyser membrane and the peptide in question. With low-flux membranes, there is a reduction of 2 – 16% in BNP concentrations and an increase in NT-proBNP concentrations of 0 – 12%(68-71) measured before and after the session. The differential effect of low-flux dialysis on BNP / NT-proBNP is explained by the fact that BNP has a lower molecular weight of 3.5 kDa as compared to 8.5 kDa for NT-proBNP, leading to preferential clearance of BNP. The increase in NT-proBNP concentration likely reflects the effect of haemoconcentration following haemodialysis and ultrafiltration, since BNP and NT-proBNP are secreted in equimolar amounts from the myocardium. In contrast, haemodialysis using a high-flux membrane reduces the concentration of both BNP and NT-proBNP by 9 – 40% and 9.4 – 35%, respectively(68-72). Haemodiafiltration results in even greater clearance of natriuretic peptides due to its even larger dialyser membrane pore size and increased convective clearances(73). Measurement of pre-/post- haemodialysis B-type natriuretic peptide concentrations is of limited clinical value as observed changes reflect dialysis-related clearance rather than short term changes in volume state or cardiac function. This fact has been demonstrated by several investigators who found no correlation between changes in BNP / NT-proBNP concentration across a single haemodialysis session and ultrafiltration volume or changes in blood pressure or weight(73-75). These observations are potentially explained by the extended half-life of B-type natriuretic peptides in the dialysis population.

The ability of peritoneal dialysis to clear plasma BNP / NT-proBNP remains unclear. A recent study of eight peritoneal dialysis patients demonstrated that BNP is present in peritoneal dialysis fluid at mean concentration of 19% ± 4% that of circulating BNP concentrations, suggesting that BNP is cleared by peritoneal dialysis(76). Whether the same holds true for the larger molecular weight peptide, NT-proBNP, was not investigated, nor was the effect of transporter status and/or ultrafiltration volume on clearance kinetics. In contrast, a study of 11 patients on nocturnal

intermittent peritoneal dialysis (NIPD) investigating the effect of a dialysis session on plasma concentrations of BNP / NT-proBNP found no change in circulating concentrations of either peptide across the procedure. Indeed, there was also no difference in concentrations during or between dialysis procedures(77). The discordance between these studies may reflect the small sample sizes used. The clearance of BNP / NT-proBNP by peritoneal dialysis warrants further investigation to aid the interpretation of serial natriuretic peptide measurements.

B-TYPE NATRIURETIC PEPTIDES AS MARKERS OF VOLUME STATE IN DIALYSIS

While BNP / NT-proBNP concentrations have not been shown to correlate with short term changes in volume state(74, 75), their role as volume markers in the medium to long-term remains an important, unresolved issue. Given the crucial role of volume overload in the genesis and progression of cardiomyopathy in end-stage renal disease(18, 78-80) and the independent mortality risk conveyed by volume excess(81-83), a reliable biomarker of volume state would prove to be an invaluable monitoring tool in dialysis.

A minority of cross-sectional studies have found no association between circulating levels of BNP / NT-proBNP and volume state assessed by clinical examination(84) or bioimpedance(85). However, these studies are limited by their very small sample sizes and potential measurement error introduced by the inaccuracy of clinical examination for assessing volume state(86, 87), both of which bias towards a null association. In contrast, seven larger cross-sectional studies have reported significant direct correlations between BNP / NT-proBNP and volume state assessed objectively by bioimpedance analysis(58, 88-91), or a combination of clinical assessment, echocardiography and/or chest X-ray findings(92, 93). The correlation co-efficients for the association between volume state assessed by bioimpedance analysis and NT-proBNP reported in these studies ranged between 0.57 and 0.66 ($P < 0.05$)(88, 89). These findings are also supported by two longitudinal studies, which demonstrated a significant association between change in BNP and change in clinical and surrogate measures of volume state(94, 95).

Thus, the bulk of available evidence suggests that circulating B-type natriuretic peptide concentrations reflect, at least in part, volume state in end-stage renal disease over the medium-long term. Answering this question definitively will require a longitudinal study with an adequate sample size in which natriuretic peptides, volume state and other covariates affecting B-type natriuretic peptide concentrations (inflammation, medication, etc.) are assessed accurately and repeatedly to

delineate the dynamic relationship between volume state and natriuretic peptide concentrations after controlling for other confounders.

B-TYPE NATRIURETIC PEPTIDES AS BIOMARKERS OF CARDIOMYOPATHY IN END-STAGE RENAL DISEASE

Several cross-sectional and cohort studies in dialysis have demonstrated that BNP / NT-proBNP have an independent and powerful direct correlation with left ventricular (LV) mass(65, 96-98), and cardiac diastolic(99, 100) and systolic dysfunction(55, 60, 65, 97).

A multivariate analysis of the predictors of cardiomyopathy in a cohort of 246 prevalent haemo- and peritoneal- dialysis patients found that log-transformed BNP concentration was a significant predictor of LV ejection fraction (β = -0.48, P < 0.0001) and LV mass indexed to height (β = 0.36, P < 0.0001) after adjusting for blood pressure, age, diabetes, albumin, haemoglobin, treatment modality, small molecule clearance, and time on renal replacement therapy(65). These results were echoed by a cross-sectional study of 62 prevalent haemodialysis patients in which NT-proBNP concentration was found to be highly correlated with LV ejection fraction (ρ = -0.77, P < 0.0001), and to be the only independent predictor of LV ejection fraction < 45% (β = 0.69, P < 0.0001) after adjusting for age, haemoglobin, blood pressure, left ventricular hypertrophy, diastolic dysfunction, volume state, haemodialysis vintage, C-reactive protein concentration and erythropoiesis stimulating agent dose(60).

Similar relationships have been demonstrated between BNP and cardiac diastolic dysfunction. In a cross-sectional study of 99 haemodialysis patients with preserved systolic function, investigators reported significantly higher BNP concentrations in individuals with diastolic dysfunction compared to those without cardiac dysfunction (314 ± 162 ng/l vs. 131 ± 77 ng/l, P = 0.01). In a multivariate analysis, gender, interdialytic weight gain, left atrial diameter and diastolic dysfunction were found to be independent predictors of BNP concentration after adjusting for age, BMI, hypertension, diabetes mellitus, dyslipidaemia, haemoglobin concentration, albumin, C-reactive protein and left ventricular mass index(99).

The role of BNP as a biomarker for cardiomyopathy is further highlighted by the findings of a cohort study of 199 dialysis patients investigating the relationship between baseline BNP as the exposure and increase in left atrial volume as the outcome. The study demonstrated that baseline

BNP concentration was a powerful, independent predictor of increase in left atrial volume(101). Finally, a longitudinal study of 21 haemodialysis patients undergoing serial measurement of NT-proBNP and LV mass at baseline, 6- and 12- months demonstrated a powerful direct correlation between change in NT-proBNP concentration and change in LV mass ($r = 0.78$, $P < 0.001$)(98).

The majority of current research into the clinical applications of BNP in the dialysis population has focused on deriving an absolute cut-off value for excluding LV systolic dysfunction in a manner analogous to its use in the general population. This approach has yielded conflicting results and is unlikely to be clinically useful given the multitude of factors affecting natriuretic peptide concentrations, the relatively low prevalence of systolic dysfunction in the dialysis population, and the high prevalence and poor prognosis of diastolic dysfunction. A more meaningful use of this potentially important marker is as a dynamic marker of clinical risk mediated by evolving cardiomyopathy – namely, a monitoring tool of cardiac risk.

B-TYPE NATRIURETIC PEPTIDES AS PROGNOSTIC BIOMARKERS IN END-STAGE RENAL DISEASE

In addition to the important cardiac and physiological correlations discussed previously, cohort studies using a *single baseline* concentration of BNP / NT-proBNP as the exposure have demonstrated that it is a powerful, independent predictor of heart failure(55, 102), sudden cardiac death(103), and cardiovascular(54-57, 59, 65) and all-cause mortality(54-57, 65) in end-stage renal disease.

Wang et al conducted a cohort study of 230 prevalent peritoneal dialysis patients followed for 3-years examining the association between baseline NT-proBNP concentrations, non-fatal cardiovascular congestion and all-cause and cardiovascular mortality. Patients were stratified according to quartiles of baseline NT-proBNP concentrations. Compared to the referent group in the lowest quartile, the group in the highest quartile demonstrated a significant increase in the relative risk (RR) of all-cause mortality (RR = 4.97, 95% CI 1.35 – 18.28), cardiovascular mortality (RR = 7.5, 95% CI = 1.36 – 41.39), and non-fatal cardiovascular congestion (4.25, 95% CI = 1.56 – 11.62) after adjusting for age, gender, dialysis vintage, haemoglobin, high sensitivity C-reactive protein, vascular co-morbidity, LV mass, LV ejection fraction, diabetes, valvular calcification, albumin concentration, systolic blood pressure, history of non-fatal cardiovascular congestion and residual renal function(55).

Similar findings were reported in a cohort study of 150 prevalent haemodialysis patients followed for a median duration of 24-months. Compared to referent group with the lowest quartile of NT-proBNP concentrations at baseline, groups in the 3rd and 4th quartiles had a significantly higher relative risk of both all-cause (3rd quartile HR = 4.78, 95% CI 1.58 – 14.51, 4th quartile HR = 4.03 95% CI 1.31 – 12.40) and cardiovascular death (3rd quartile HR = 10.95, 95% CI 1.38 – 86.73, 4th quartile HR = 8.54 95% CI 1.04 – 69.98)(56).

The strongest support for the potential role of the B-type natriuretic peptides as biomarkers of cardiac risk in the renal dialysis population comes from longitudinal studies employing limited numbers of repeated natriuretic peptide measurements to investigate the relationship between change in peptide concentration and change in risk of mortal events. These studies demonstrate that change in B-type natriuretic peptide concentration correlates directly with change in risk of cardiovascular and all-cause mortality in both incident and prevalent haemodialysis patients(54, 59, 95, 104). While these studies are an important first step in delineating the clinical application of this biomarker in dialysis they are limited by their paucity of repeated measures, and lack of time to event and accuracy measures (sensitivity and specificity); all of which are essential components to advance this promising marker into clinical practice(105).

CONCLUSION

Dialysis patients have a 50-100 fold increased risk of cardiac death compared to the general population. This heightened risk has remained unchanged over the last decade, whilst patient numbers on dialysis have grown exponentially. A key factor underpinning this mortality risk is fluid overload-induced cardiomyopathy. Progress in improving outcomes has been hampered by our current inability to assess fluid state and cardiac risk status in an accurate and dynamic manner. NT-proBNP is a promising biomarker that has been shown in preliminary studies to predict mortality and correlate with cardiomyopathy and fluid state. However, further research with longitudinal studies is needed to derive the essential parameters required to advance this promising biomarker into a clinically useful monitoring strategy.

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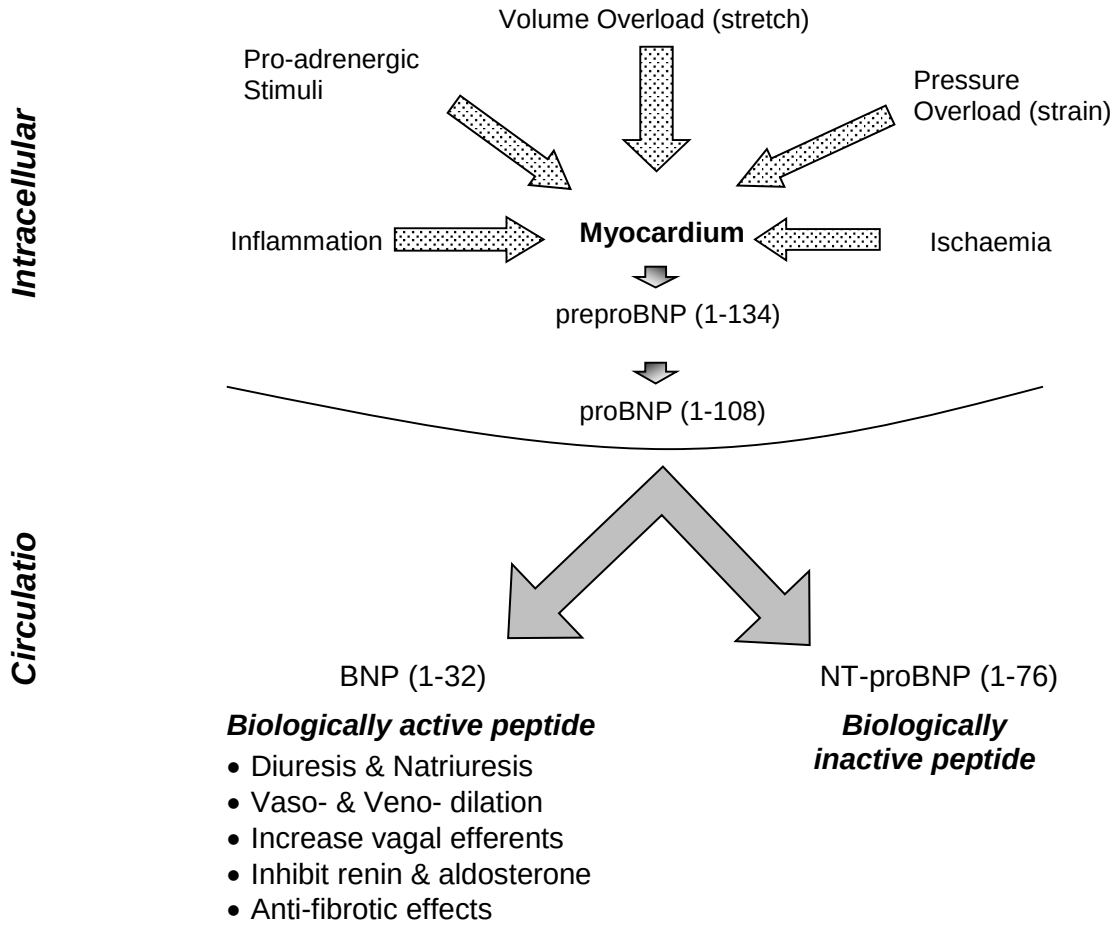
Figure 1. Stimuli, processing pathway and physiological actions of the B-type natriuretic peptides.



Chapter 4

Cardiac Troponins in the Dialysis Population: Current Concepts and Unanswered Questions

The following chapter discusses the biochemistry, and the established and emerging clinical applications of cardiac troponins in the diagnosis and monitoring of cardiac disease in dialysis patients. In addition, it identifies those research areas that need to be addressed in order to advance the use of this biomarker in the acute and chronic care clinical settings.

Chapter 4

Cardiac Troponins in the Dialysis Population: Current Concepts and Unanswered Questions

Introduction

Patients with end-stage kidney disease on dialysis have high rates of emergency presentations with cardiac symptoms and cardiac hospitalizations (34.7 admissions per 100 patient years)(1, 2) and as such, cardiac troponins are amongst the most frequently performed investigations in this group. Recent studies have also demonstrated an association between chronically elevated cardiac troponin concentrations and adverse outcomes in dialysis patients in the absence of acute symptoms(3), implying that cardiac troponins may have a role as prognostic and/or monitoring biomarkers(4). However, several issues continue to confound the interpretation of cardiac troponins in both the acute and chronic settings in this population, including a lack of clarity regarding what constitutes a significant change in serial measurements, disagreement regarding the pathophysiological associations of troponins and hence potential therapeutic targets, and whether interpretation in chronic settings is best undertaken using single or serial measurements. The aims of this review are to discuss the biochemistry of cardiac troponins, their characteristics in the dialysis population and their use in acute and chronic settings. The review will highlight questions that remain unanswered and further research that needs to be undertaken to advance the understanding of cardiac troponins in dialysis and their use in clinical practice.

Biochemistry, release kinetics, detection, and clearance.

The troponin complex is composed of three subunits – Troponin C (TnC), Troponin I (TnI), and Troponin T (TnT) – which work in concert to regulate the interaction between actin and myosin required for myofibrillary contraction. In response to a membrane excitation potential, calcium ions are released from the sarcoplasmic reticulum into the cytoplasm and bind to TnC inducing the release of TnI from actin and TnT from tropomyosin. This in turn allows for the formation of cross-bridges between actin and myosin in a ratchet-like manner to generate a contraction(5). The constituent proteins of the troponin complex are present in both cardiac and skeletal muscle, and while the gene encoding the TnC protein is identical in both muscle types(6), the genes encoding TnI and TnT differ between the two tissues resulting in cardiac- and skeletal-muscle specific TnT and TnI isoforms(7, 8).

Approximately 6% of cTnT and 4% of cTnI are present unbound in the cytoplasm of cardiac myofibrils(9) and can be released rapidly into the circulation following ischemia (with or without necrosis)(10) or in response to myofibrillarly stretch/strain, which result in an increase in cell

membrane permeability(11). The remainders of cTnI and cTnT in cardiac myocytes are present in the form of the ternary troponin complex bound to actin and tropomysosin and are released into the circulation when cardiac myofibrils undergo necrosis following ischaemia, inflammation, infiltration or trauma.

Cardiac troponins take various forms in the circulation depending on the type of troponin, the process inciting its release and the time elapsed since its release from the cardiomyocyte into the circulation. Following myocardial necrosis, cTnT is released in two forms; as part of the ternary troponin complex of TnC-TnI-TnT, as well as in the form of free intact cTnT detectable for up to 12 hours following myocardial necrosis. Thereafter, cTnT undergoes proteolytic degradation to form peptide fragments of varying molecular weights which are detectable by commercial cTnT assays(12). However, in the absence of irreversible myocardial injury, circulating cTnT is only detectable as the intact molecule with a molecular weight of 37 kDa suggesting that degradation associated with irreversible myocardial damage may be mediated by enzymes released at the time of myocardial necrosis(13). In contrast to cTnT, cTnI undergoes rapid proteolytic degradation in the circulation regardless of the process inciting its release(14), and is present in the circulation principally in the form of peptide fragments and to a lesser extent as a binary complex bound TnC(15).

The precise mechanism by which cardiac troponins are cleared from the circulation has not been elucidated, although accumulating evidence suggests that renal clearance plays little, if any, role in the elimination of troponins. Firstly, several cohort studies have demonstrated that a large proportion of renal transplant recipients with elevated pre-transplant cardiac troponin concentrations continue to have elevated concentrations in the first year post-transplant despite a substantial improvement in their glomerular filtration rate(16-19). Secondly, the half-life of cardiac troponins is not significantly different between patients with and those without chronic kidney disease(20). Finally, the only circulating form of cTnT measurable in asymptomatic dialysis patients with elevated cTnT concentrations is the intact peptide, which has a molecular weight of 37 kDa thereby making it too large for glomerular filtration(13). Smaller cTnT peptide fragments resulting from proteolysis have been measured in plasma only after myocardial infarction, and while these are small enough to be filtered through the glomerulus, they have are not detectable in the urine of patients with high plasma cTnT concentrations and normal renal function post-myocardial infarction(21).

Cardiac troponins in the dialysis population: frequency, distribution and effects of the haemodialysis procedure.

A large proportion of dialysis patients have cardiac troponin concentrations which persistently exceed the 99th centile upper reference limit of troponin assays despite being asymptomatic(22-26). The prevalence of this finding varies according to the type of cardiac troponin measured and the sensitivity / generation of the assay employed.

For cTnT, between 29 – 43% of patients have been reported to have elevated concentrations using first generation TnT assays(27, 28), as compared to 99% of dialysis patients when the latest, fifth generation high-sensitivity assays are employed(26). These differences are principally explained by advances in assay technology through generations, which have allowed for the detection of lower concentrations of circulating cTnT. cTnT assays utilise a twin antibody system composed of a capture antibody to isolate circulating TnT and a detection antibody for quantification. First generation TnT assays employed a detection antibody that cross-reacted with skeletal TnT; a limitation that was addressed in second generation assays through the use of capture and detection antibodies that only recognised cardiac specific epitopes thereby improving assay specificity(29). Subsequent generations of the cTnT assay have been characterised by improvements in both specificity and sensitivity through modifications of the capture antibody, and changes in sample volume and buffer optimization, respectively(30).

As with cTnT, prevalence estimates of chronically elevated cTnI concentrations in the dialysis population have increased from 7% using first generation assays(31) to 33% when the latest high generation assays(32), which has been attributed to the use of increased sample plasma volumes and incubation times, and improvements in the microparticle capture bead(33). The frequency distributions of both cardiac troponins demonstrate a right skewed distribution in the dialysis population(32) and thus require appropriate mathematical transformation prior to statistical analysis.

Observational studies have also consistently demonstrated that a higher proportion of dialysis patients have an abnormal cTnT concentration compared with cTnI when both cardiac troponins are measured simultaneously within the same dialysis cohort(31, 32, 34). The precise reasons for the discordance are unknown but may be related to uraemic associated modifications of circulating cTnI, which interfere with measurement, adsorption of cTnI to haemodialysis dialyser membranes(35), and/or anti-cTnI antibodies which interfere with assay performance(36). cTnT and cTnI are partially cleared during haemodialysis with high flux dialysis membranes, but not with low flux membranes(26, 34). This has important implications for the interpretation of serial troponin

measurements, whose measurement should be standardised with respect to the dialysis cycle, especially in the research setting.

Interpretation of troponin measurements from dialysis patients in acute care settings.
Patients on maintenance dialysis have a high incidence of acute coronary syndromes (9.8-16 per 100-patient years), which are a leading cause of emergency presentations, hospitalisation and death in this population(1, 2, 37-40). Accurately diagnosing acute coronary syndromes in dialysis patients is challenging due to the high proportion of patients who present with atypical symptoms and electrocardiographic changes(41, 42). This has led to greater reliance on biomarkers of myocardial injury in this population. However, the interpretation of cardiac troponins from dialysis patients in acute settings is not straightforward given that the majority have cardiac troponin concentrations that persistently exceed the 99th centile upper reference limit of the assay used even when they are well; reflecting established rather than acute cardiovascular pathology (22-26).

In an effort to address this conundrum, the current consensus guideline on the diagnosis of acute myocardial injury requires demonstration of a *change in serial troponin concentrations* with at least one concentration exceeding the 99th centile upper reference limit, occurring in an appropriate clinical context, in order to confirm a diagnosis of acute myocardial injury in dialysis patients (43). Unfortunately, this recommendation is not accompanied by a recommendation on precisely how much change in serial measurements constitutes a significant change. Guidelines have either provided no guidance on the magnitude of change in serial troponins(43, 44) or have based their guidance on the analytic performance of troponin assays rather than clinical studies, wherein a 20% magnitude of change has been recommended based on the fact that such a delta equates to 3 standard deviations of the troponin assay's analytic variation(45).

Guidelines for interpreting serial troponin measurements should incorporate data on both the biological variation of cardiac troponins in stable dialysis patients and serial changes in troponins associated with adverse outcomes in dialysis patients. Biological or within-person variation describes the random fluctuation of biomarker levels around a homeostatic set-point in healthy individuals or those with stable disease; such fluctuations are of no clinical significance and must be distinguished from pathological changes(46).

In an effort to address this important question with relevant patient-level data, several investigators have sought to estimate the within-person coefficient of variation of cardiac troponins from cohorts of dialysis patients. Pianta and colleagues measured pre-dialysis cTnI and hs-cTnT in 103

haemodialysis patients on two occasions at 3-weekly intervals; administering a cardiovascular symptom questionnaire concurrently and excluding two patients with active symptoms. The study reported a within-person coefficient of variation of 9.7%, which can be used to calculate a reference change value - a magnitude of change between two measurements needed to exclude change due to biological variation alone for a pre-specified degree of statistical confidence(47). In a similar study, Jacobs and colleagues(22) measured hs-cTnT and TnI pre-dialysis in 32 haemodialysis patients on 3 occasions at 2-monthly intervals, excluding hospitalized patients from the final analysis. The reported within-person coefficients of variation of this longer two-monthly interval were 15% and 13% for hs-cTnT and cTnI, respectively. These studies lend support to the premise that troponin concentrations can fluctuate in stable dialysis patients. However, they are limited by exclusion of peritoneal dialysis patients, a paucity of repeated measures, and, most importantly, a lack of rigour in ensuring patient stability insofar as they relied either on patient report or a history of hospitalization alone to determine stability. The latter introduces the possibility that unstable patients may have been included in the analyses of these studies and adversely impacted the accuracy of their findings. Thus, there remains a need for well-performed biological variation studies of cardiac troponin, which enrol both haemo- and peritoneal-dialysis patients and ensure the stability of important covariates, including dialysis and pharmacological prescriptions, cardiac rhythm, and patient volume status, while concurrently excluding patients who experience an adverse cardiovascular outcome from the study's analysis.

Reference change values, even when estimated robustly, cannot be used in isolation to set diagnostic and therapeutic decision limits. This is due to the fact that reference change values are principally aimed at excluding biological variation and one cannot assume that changes exceeding the reference change value are always associated with clinically important consequences. This critical question has not been studied in the end-stage kidney disease population, and should be addressed in a large, longitudinal cohort study examining the outcomes of patients with end-stage kidney disease who present emergently with a change in serial cardiac troponins exceeding reference change values. Such a study would then allow formulation of a guideline on both biological variation and clinically important changes(48).

Cardiac troponins in the care of ambulatory dialysis patients.

As previously noted, the majority of prevalent dialysis patients have cardiac troponin concentrations which persistently exceed the upper reference limit of troponin assays despite being systemically well and asymptomatic(26-28, 31, 32). This finding was initially ascribed to impaired renal clearance of troponins and thought to be spurious(49). However, several lines of evidence, which

have been alluded to in the section on the biochemistry of troponins, have largely discredited this explanation. Chief among the latter is the consistent finding by cohort studies that elevated troponin concentrations in asymptomatic, ambulatory dialysis patients are associated with adverse outcomes(3). A recent systematic review and meta-analysis of 98 cohort studies found that cTnT and cTnI concentrations exceeding the assay's upper reference limit were associated with increased risks of all-cause mortality (HR = 3.00 [95% CI 2.36 – 4.26] and HR = 2.70 [95% CI 1.90 – 4.57] respectively), and cardiovascular mortality (HR = 3.31 [95% CI 1.81 – 5.53] and HR = 4.20 [95% CI 2.01 – 9.20] respectively) among asymptomatic dialysis patients even after adjustment for age and presence of cardiovascular co-morbidity(3).

The pathophysiological mechanisms leading to the release of cardiac troponins in asymptomatic dialysis patients remain unclear, although they may include chronic, low level ischaemia caused by a mismatch between cardiac myocytes and perfusing capillaries in scarred, hypertrophied myocardium(50) or integrin-mediated release associated with myocardial stretch/strain in the context of left ventricular hypertrophy and volume overload(11). These putative mechanisms are supported by the findings of cross-sectional studies, which have demonstrated an independent association between troponin concentrations and structural cardiac pathologies in dialysis patients, although the precise
association has varied among studies, including left ventricular hypertrophy(23, 51-55), systolic dysfunction(52, 56, 57) or hydration status(58). These conflicting findings can be attributed, at least in part, to inadequate adjustment for confounding variables, small sample sizes, use of surrogate measures of volume, and insensitive measures of myocardial contractility in these cross-sectional studies.

The association between troponin concentrations and mortality risk in dialysis patients(3) led to the licensing of cardiac troponins by the United States Food and Drug Administration (FDA) for prognostication in this population(4). However, despite this regulatory approval, troponins have failed to enter routine clinical practice due to ambiguity regarding the ideal strategy for testing and interpreting cardiac troponins, and the resultant action that should be taken based on testing(59). In order to advance troponin testing in clinical dialysis practice, longitudinal cohort studies are needed to determine the ideal approach to interpreting significant elevations (absolute cut-off or relative change between serial measurements), the frequency of measurements, the time to event between an abnormal test and an adverse outcome, and the targets of therapy.

Conclusion

The two cardiac troponins, cTnI and cTnT, represent potentially valuable biomarkers for the diagnosis and management of cardiovascular disease in dialysis patients. However, several challenges remain before these biomarkers can be confidently used in both acute and chronic care dialysis practice. In the acute setting, good quality studies are needed to estimate both the biological variation of cardiac troponins, and the magnitude of change in serial measurements associated with adverse outcomes in dialysis patients which should prompt intervention. In the ambulatory setting, longitudinal studies are needed to identify the ideal strategy for interpreting measurements, frequency of measurements, time to event, and targets of therapy. When these indices are characterised, management based on troponin needs to compared to current practice in a randomised controlled trial to establish whether it improves patient level outcomes.

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Chapter 5

Part A - Monitoring cardiovascular risk in the dialysis population: rationale and design of the Monitoring Oscillations of NT-proBNP In guiding Therapy and predicting Outcomes in Renal disease (MONITOR) longitudinal cohort study.

Part B - Week-Week and Month-Month Biological Variation and Reference Change Values of NT-proBNP and hs-cTnT in the Stable Dialysis Population (Study Protocol).

The following chapter details the rationale and design of the two prospective cohort studies conducted as part of this thesis whose aims are to:

Part A - estimate a magnitude of change in serial NT-proBNP measurements that accurately predicts non-fatal and fatal cardiovascular events in prevalent dialysis patients, determine the optimal NT-proBNP monitoring interval and its associated time to event, and to define the pathophysiologic processes that underlie changes in NT-proBNP concentrations which may serve as potential interventional targets

Part B – estimate the within- and between-person variation of NT-proBNP and hs-cTnT measured at weekly and monthly intervals in stable dialysis patients and to use these estimates to calculate the percentage change between serial biomarker measurements needed to exclude change due to biological and analytic variation alone.

Both studies were conceived, designed, and conducted by the candidate. Part A of this chapter is in the process of being submitted for publication.

Chapter 5 – Part A

Monitoring cardiovascular risk in the dialysis population: rationale and design of the Monitoring Oscillations of NT-proBNP In guiding Therapy and predicting Outcomes in Renal disease (MONITOR) longitudinal cohort study.

BACKGROUND

The past decade has seen steady growth in the number of individuals with end-stage renal disease receiving maintenance dialysis therapy, who now number over two million worldwide(1, 2). Unfortunately, patient outcomes on dialysis have not improved substantively over the same period, with cardiovascular disease continuing to be a major cause of morbidity and the leading cause of mortality in this group(2-4). Over 80% of dialysis patients have at least one form of cardiovascular disease and as compared to their non-dialysis counterparts, dialysis patients have a 10-60 times increased risk of cardiovascular mortality (6 – 11 deaths vs. 0.2 deaths per 100 patient years)(2, 3, 5, 6) which has remained largely static over the past decade while cardiovascular mortality in the non-dialysis population has fallen by 27-33%(5, 6).

One of the key reasons for these poor outcomes is our inability to reliably monitor cardiac risk in the dialysis population(7). Monitoring is defined as a repeated testing strategy used to detect changes in risk / health status over time and to evaluate the efficacy of therapy and titrate it accordingly(8, 9). In essence, it provides early warning of escalating risk or ineffective treatment and enables timely intervention to improve outcomes. At the present time there are no validated strategies for monitoring cardiac risk in the dialysis population, thus patients at increased risk of adverse cardiac events are often identified and managed late, and the efficacy of any therapies instituted cannot be reliably monitored.

Cardiomyopathy as a monitoring target

Cardiomyopathy describes abnormalities of cardiac structure and/or function including left ventricular hypertrophy, dilatation, systolic and/or diastolic dysfunction. It is highly prevalent in the dialysis population (61.8 - 84.5%)(10-13), and has been shown in national registries(14, 15), randomised controlled trials(16, 17) and prospective cohort studies(10, 18, 19) to be the strongest

predictor of cardiac-related hospitalization and mortality in the dialysis population. Thus, cardiomyopathy and the pathophysiological processes that underlie its genesis and progression emerge as potentially important targets for monitoring and intervention. Currently, the only guideline on monitoring cardiomyopathy in patients on dialysis recommends performing an echocardiogram at 3-yearly intervals(20). The testing modality and interval recommended, however, are entirely *opinion based* and the guideline does not detail which echocardiographic parameters to monitor nor the magnitude of change in parameters that should prompt action, making it inadequate as a monitoring strategy. Echocardiography is also limited by the fact that the most commonly performed two dimensional echocardiographic measurements have a high within-person coefficient of variation and large inter-observer variability, such that only large changes in cardiac structure and function can be reliably detected(21, 22). Finally, timely access to echocardiography is frequently limited due to the need for specialised operators and equipment, and their associated expenses.

NT-proBNP – a promising biomarker of cardiomyopathy and cardiac risk in dialysis patients

The N-terminal fragment of pro-B-type natriuretic peptide (NT-proBNP) is a biologically inactive peptide secreted primarily from the left ventricle in equimolar amounts to the active hormone B-type natriuretic peptide (BNP). The principal stimuli for NT-proBNP secretion are increasing ventricular wall tension (cardiac hypertrophy) and/or stretch (systolic dysfunction and volume overload). Other stimuli include sympathetic overactivity, myocardial ischaemia and inflammation; a constellation of stimuli that overlap significantly with the pathological milieu implicated in the genesis of cardiomyopathy (23, 24).

Several cross-sectional studies have demonstrated direct associations between NT-proBNP concentrations and the severity of cardiomyopathy and volume overload in dialysis patients, and cohort studies have demonstrated a direct association between baseline NT-proBNP concentrations and subsequent risk of fatal and non-fatal cardiovascular events in the dialysis population(25-27). Of even greater relevance, are longitudinal cohort studies which have demonstrated a direct association between changes in serial NT-proBNP concentrations and changes in volume state(28-30), left ventricular mass(31), and the risk of cardiovascular and all-cause mortality(28, 29, 32, 33) in dialysis patients (Table 1). Taken together, these findings suggest that NT-proBNP monitoring may be useful for assessing and following cardiac risk in the dialysis population.

Moving beyond associations

However, these promising findings alone do not provide the necessary detail needed to develop a NT-proBNP monitoring strategy for use in clinical practice. A clinically useful strategy (Figure 1) requires precise guidance on a magnitude of relative change in serial NT-proBNP concentrations that can safely be ignored (biological variation) versus that should prompt action, the associated time to event of such a change threshold, the ideal monitoring interval, clear targets for intervention, and evidence that such intervention improves patient level outcomes. Published longitudinal studies are unable to define these crucial indices due to their use of infrequent NT-proBNP measurements and arbitrary, predefined categories of change in NT-proBNP (tertiles(29) or deciles(32)), their sole focus on fatal clinical outcomes occurring long after the change in NT-proBNP concentrations or surrogate measures of volume, and their exclusion of peritoneal dialysis patients(34) (Table 1).

METHODS/DESIGN

The **M**onitoring **O**scillations of **N**T-proBNP **I**n guiding **T**herapy and predicting **O**utcomes in **R**enal disease (MONITOR) study was a prospective, longitudinal cohort study which sought to address the aforementioned limitations and advance the development of a NT-proBNP testing strategy for monitoring cardiovascular risk in the dialysis population.

The study protocol complied with the Declaration of Helsinki and the National Statement on Ethical Conduct in Human Research; it received institutional ethics approval from the Metro-South Human Research Ethics Committee (HREC/10/QPAH/303), the University of Queensland Medical Research Ethics Committee (2011000484), and the Greenslopes Research and Ethics Committee (12/39). It was registered with the Australia and New Zealand Clinical Trials Registry (ACTRN12612000836831).

Aims

The aims of the MONITOR study were to estimate a magnitude of change in serial NT-proBNP measurements that accurately predicted non-fatal and fatal cardiovascular events in prevalent dialysis patients. In addition, the study sought to determine the optimal NT-proBNP monitoring interval and its associated time to event, and to define the pathophysiologic processes that underlie changes in NT-proBNP concentrations which may serve as potential interventional targets(9, 35).

Study Setting and Participant Recruitment

Participants were recruited from the in-centre and self-care hemodialysis, and the peritoneal dialysis units of a public, tertiary-care teaching hospital and a private, secondary-care hospital in

metropolitan Brisbane, Australia. Eligible participants were identified from an electronic database of all patients receiving dialysis therapy and were aged over 18 years, able and willing to provide informed consent, established on dialysis therapy for at least 90 days and maintained on the same dialysis prescription for at least 30 days.

In addition, potential participants did not meet any of the exclusion criteria including planned living donor renal transplantation within 3-months of enrolment, one or more contraindications to whole body bioimpedance spectroscopy measurement (permanent pacemaker, implantable cardiac defibrillator, joint replacements, orthopaedic pins, mechanical heart valves and/or limb amputations), congenital heart disease (excluding haemodynamically insignificant patent foramen ovale), advanced malignancy, or pregnancy.

Patient Assessment

Patients were assessed at baseline and thereafter at monthly intervals for up to 24 months. Haemodialysis patients were assessed prior to the mid-week dialysis session, while peritoneal dialysis patients were assessed on a consistent weekday throughout the study. Patients were instructed to avoid strenuous exercise prior to assessment, and were assessed at the same time of day throughout the study to avoid fluctuations in NT-proBNP and hs-cTnT concentrations related to diurnal variation. The study schema is shown in Figure 2.

Patient assessments entailed a structured clinical interview, physical examination, and review of the medical record to ascertain medical history, comorbidities, current treatments and dialysis prescription. The Canadian Cardiovascular Society Angina Grading Scale(36) was used to assess change in cardiac ischaemic symptoms and the Truncated Framingham Heart Failure Score(37) was used to assess for clinical evidence of pulmonary oedema at time of review. In addition, patients underwent a standard 12-lead electrocardiogram, whole body bioimpedance spectroscopy and blood sampling for measurement for NT-proBNP and hs-cTnT measurement on the same occasion.

For haemodialysis patients, the blood pressure recorded was the median of 12 post-dialysis measurements taken at the end of the 12 haemodialysis treatments preceding the study assessment, while for peritoneal dialysis patients it was the median of 12 home blood pressure recordings measured thrice weekly over the 4 weeks prior to the study assessment. The median of these blood

pressure recordings has been shown to have the greatest agreement with 24-hour ambulatory measurements and therefore to be most representative of the patient's blood pressure(38).

Whole body multi-frequency bioimpedance spectroscopy

Hydration status was assessed using whole-body, multi-frequency bioimpedance analysis with the Body Composition Monitor BCM® (Fresenius Medical Care, Asia-Pacific). This instrument has been validated in dialysis patients against radioisotope dilution methods with reported agreement limits (mean ± standard deviation [SD]) of -0.2±2.3L, -0.4±1.4L and 0.2±2L for total body water, extra- and intra-cellular water volumes, respectively(39). In addition, this instrument has been shown to have a detection limit for change in extra cellular volume of (mean ± SD) 0.87±0.64L(40). Peritoneal dialysis patients were assessed with peritoneal dialysis fluid in situ, as this has been previously shown to have no significant or clinically important effect on volume measurements compared with an empty peritoneal cavity(41).

Echocardiography, pulse wave velocity and pulse wave analysis measurement

All of the participants in the cohort study were invited to participate in a sub-study entailing annual echocardiography, and pulse wave analysis and velocity measurements. These measurements were performed annually on a maximum of three occasions, and for haemodialysis patients they were performed on the non-dialysis day immediately following the mid-week dialysis treatment to ensure that cardiac loading conditions are representative of the patient's usual volume state. Echocardiograms were performed and measured by one of two expert operators blinded to the patient's NT-proBNP and hs-cTnT concentrations, hydration status and medical history.

Echocardiograms were performed in the left lateral decubitus position using a 3.5MHz phased array transducer with harmonic imaging (Vivid7, General Electric-Vingmed Medical Systems, Horten, Norway). Grayscale images from the parasternal long-axis and short axis as well as the apical 4-chamber, 2-chamber and apical long axis views were acquired at moderate frame rate (50-70 fps) to allow for processing of 2D strain; apical images in the 4-chamber, 2-chamber and apical long axis were acquired at high frame rate (>120 fps) with a colour tissue Doppler overlay to measure colour tissue Doppler velocity, strain and strain rate. Doppler measurement of left ventricular (LV) inflow, tricuspid valve (TV) inflow and aortic and pulmonary outflow were performed using pulsed and continuous wave Doppler. Colour tissue Doppler measurements were performed using pulsed wave

Doppler in the medial and lateral LV annulus, tricuspid annulus and for all LV segmental measurements.

Cine loops of three cardiac cycles were acquired and the measurements were averaged. If patients are in atrial fibrillation, 5-7 measurements were averaged. Left atrial and ventricular dimensions will be obtained by M-mode according to the American Society of Echocardiography Recommendations(42); endocardial borders were traced at end systole and end diastole in the apical 4—chamber and 2-chamber views to obtain Simpson's Rule ejection fraction; M-mode LV mass were calculated using the formula : LV mass =0.8 × (1.04[(LV internal dimension + septal wall thickness + posterior wall thickness)3 – LV internal dimension3]) + 0.6g, and these were indexed to the patient's body surface area calculated using the Haycock formula (0.024265 x Weight(kg)0.5378 x height(cm)0.3964).

For 2D strain analysis, the endocardial borders were traced at end-systole in the 4-chamber, 2-chamber and apical long axis views; the software (EchoPac PC BTO 11, General Electric-Vingmed Medical Systems, Horten, Norway) divided the LV into basal, mid and apical segments and identifies unique "speckles" within the myocardium and tracked them frame-by-frame throughout the cardiac cycle to obtain strain and strain rate curves. Segments that do not track properly were manually adjusted or eliminated from the analysis. Global longitudinal strain (GLS) was expressed as a mean of all 18 segments.

The SphygmoCor PX system (AtCor Medical, Australia) was used for measurement of pulse wave analysis (augmentation index) and pulse wave velocity (PWV). Carotid-femoral PWV was the only method measured. The distances between the common carotid artery and the suprasternal notch, the suprasternal notch and the umbilicus, and the umbilicus and the femoral artery were measured. Tonometery was performed in the common carotid artery as close to the aortic arch as possible and at the femoral artery. The foot-to-foot times to reach the common carotid artery and the femoral artery were then subtracted and pulse wave velocity was expressed as metres per second (m/s). A total of three measurements were taken and averaged for analysis.

NT-proBNP and hs-cTnT sample collection, storage and analysis

Blood for measurement of plasma NT-proBNP concentrations was collected at each visit while blood for plasma hs-cTnT measurement was only collected at 3-monthly intervals. Blood samples for both biomarkers were collected in lithium-heparin tubes, prior to the commencement of the dialysis treatment for haemodialysis patients. Blood samples were centrifuged and plasma separated within 1 hour of collection and then stored at -80°C until assayed(43, 44).

Plasma NT-proBNP concentration (pg/mL) was measured using a twin-antibody electrochemiluminescence assay on the Elecsys 2010 instrument (Roche Diagnostics, Australia) which has a reported analytical detection range of 5–35,000 pg/mL. Analytic coefficients of variation reported by the analysing laboratory were 2.9% and 1.8% at concentrations of 134 pg/mL and 4534 pg/mL respectively in keeping with desired performance of the assay(45, 46).

Plasma hs-cTnT concentration (ng/L) was measured using the Cobas e170 instrument with the troponin-T hs kit (Roche Diagnostics, Australia); a monoclonal antibody electrochemiluminescence assay which has a reported detection range of 5 – 10,000 ng/L(47). Analytic coefficients of variation reported by the analysing laboratory were 2.8% and 1.4% at concentrations of 26.2 ng/L and 2210 ng/L respectively in keeping with desired performance of the assay(47).

Target Conditions

The primary target condition of interest was a composite of major adverse cardiac events including fatal and non-fatal heart failure, sudden cardiac death, resuscitated cardiac arrest, new onset angina pectoris, unstable angina pectoris, fatal and non-fatal myocardial infarction, and supraventricular tachycardia associated with a rapid ventricular response and compromised cardiac output. Secondary target conditions included all-cause mortality, all-cause hospitalisation, change in extracellular body water of at least one litre assessed using whole body bioimpedance spectroscopy, and changes in indices of left ventricular systolic function and/or diastolic function.

Definitions

Heart failure was defined as new onset or worsening dyspnoea at rest and/or on exertion, paroxysmal nocturnal dyspnoea or orthopnoea with concurrent clinical examination findings or a

chest X-ray consistent with pulmonary congestion and echocardiographic evidence of left ventricular systolic and/or diastolic dysfunction. A diagnosis of heart failure also required an improvement in symptoms following diuresis, ultrafiltration, and/or systemic vasodilator therapy(48).

Sudden cardiac death was defined as death within one hour of new cardiac symptoms or an unexpected, unwitnessed death in a person without any known non-cardiac illness that could be expected to become rapidly fatal and/or no alternative cause of death on autopsy if autopsy is performed(49). *Resuscitated cardiac arrest* was defined as sudden collapse that was witnessed, with either documented absent circulation or ventricular tachy- or brady-arrhythmia and where the patient responded to standard cardiopulmonary resuscitation procedures.

New onset stable angina pectoris was defined as new onset of symptoms of angina pectoris occurring on moderate to severe exert ion together with either a positive non-invasive cardiac stress test demonstrating reversible ischaemia or coronary arteriography demonstrating at least one 50% stenosis in one or more epicardial coronary arteries(50).

Myocardial infarction was defined as a rise and/or fall of cardiac troponin from a previous or baseline measurement by ≥20%, with at least one troponin concentration above the upper reference limit (defined as a level exceeding the 99th percentile of a reference population at which the assay has a coefficient of variation ≤10%) accompanied by symptoms of myocardial ischaemia and/or new significant ECG changes (comprising ST-T wave changes and/or new left bundle branch block and/or pathological Q-waves) and/or imaging evidence of new loss of viable myocardium or new regional wall abnormality and/or identification of intracoronary thrombus on coronary angiography or at autopsy(51).

Sample size and statistical analysis

It is accepted that longitudinal studies employing frequent repeated measures require a modest sample size to establish the presence/absence of a relationship between the biomarker and outcome(52, 53). Based on a similar study investigating cardiac allograft rejection monitoring using BNP(53), we derived a sample size calculation for a desired range of accuracy of a single baseline NT-proBNP level for predicting survival: assuming that the area under the curve (AUC) for baseline NT-proBNP discriminating between patients who live or die will be 0.7 – 0.8 and assuming there are 42 deaths in 150 patients over 2-years (based on an expected annual mortality

rate of 15 deaths per 100 person years), we estimated that we would have a more than sufficient sample size so that the half-width of the 95% confidence interval for the AUC is < 0.1(54). We therefore planned for a sample size of 150 participants comprising 60% haemo- and 40% peritoneal- dialysis (reflecting the proportions of dialysis modalities in current practice), knowing that this sample would give adequate power as the planned analysis using frequent repeated measures methods will require a smaller sample size than a single baseline measure. Estimates of event rates for morbid events were not available, but were expected to exceed fatal event rates. This sample size should also allow findings to be externally generalisable to the wider haemo- and peritoneal- dialysis populations.

Time to cardiovascular event/outcome was analysed using survival analysis, with NT-proBNP being included as a time-varying covariate. To determine the magnitude of change in NT-proBNP predictive of the outcomes, its accuracy, and the optimum monitoring interval we also analysed the relationship between NT-proBNP and outcomes using recently developed statistical methods that allow the joint modelling of time-to-event and longitudinal data(52, 55, 56). To determine whether NT-proBNP monitoring provides an incremental benefit over symptom monitoring, we also included both NT-proBNP and symptoms in our models. Other relevant clinical, biochemical and demographic variables were included in the model as covariates, and will also be used to characterise differences between groups with and without serial increments in NT-proBNP levels. We determined the best combination of monitoring tests (NT-proBNP, bioimpedance and/or symptoms) by checking the ability of our models to discriminate between patients who did or did not manifest the outcomes of interest. To examine the relationship between longitudinal change in NT-proBNP concentrations and longitudinal change in (i) bioimpedance measurements and (ii) echocardiographic outcomes we fitted a longitudinal model with NT-proBNP as the time-varying covariate outcome and the other variables (fluid state and cardiac structural changes) as outcomes.

DISCUSSION

The disproportionately high mortality of the dialysis population has remained largely unchanged for the last decade while the number of patients starting dialysis has grown, and is expected to continue growing exponentially(15, 57). Cardiomyopathy induced by fluid overload and hypertension is the leading cause of death in dialysis(3, 58). At the present time, we do not have a validated monitoring strategy to identify patients at increasing risk of cardiac death in order to intervene in a timely manner nor do we have a tool to tailor fluid targets and cardiac medication dosing for individuals. Our research proposal intends to fill these gaps in dialysis practice by advancing the role of NT-

proBNP monitoring. Our study proposal is structured to deliver the key components needed to build a clinically useful monitoring strategy – a precise magnitude of change that accurately predicts adverse events, its associated time to event, and its incremental value to current practice.

The results of our study will have far reaching clinical and research implications. If NT-proBNP monitoring is proven to be accurate in predicting adverse physiological changes and cardiac events; it will represent the first non-invasive, clinically validated monitoring tool that is able to accurately predict over-hydration, evolving cardiac dysfunction and adverse cardiac/clinical events in dialysis patients. This invaluable tool will be used to alert physicians to the 'at risk' dialysis patient prior to current best practice, enabling timely intervention. Furthermore, once this monitoring strategy is developed it will provide the framework for a treatment guidance tool that will be evaluated to determine if adjusting dry body weight, the dialysis prescription and cardiac medication according to NT-proBNP concentrations produces superior outcomes to current management. A validated biomarker monitoring and treatment strategy will also provide an invaluable surrogate outcome in many future research studies of cardiac interventions in dialysis.

Some international units are already advocating monitoring of NT-proBNP(27). As detailed in this proposal, such adoption is premature and unproven. If this research proposal shows that NT-proBNP monitoring is not superior to current practice then it will help avert the widespread implementation of an inaccurate strategy and in doing so avoid unnecessary costs and/or morbidity.

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Table Legend

Table 1. Longitudinal studies of B-type natriuretic peptides in the dialysis population. The table details the design, findings and limitations of published longitudinal studies measuring either B-type natriuretic peptide (BNP) or the amino terminal fragment of pro-B-type natriuretic peptide (NT-proBNP) in the dialysis population. * , $P < 0.05$; LVMI, left ventricular mass indexed to body surface area; RR, relative risk.

Table 1

Author	Study population characteristics	Number of measurements per subject (frequency)	Outcome and results	Limitations
Gutierrez et al(29)	585 incident haemodialysis patients	2 (3-monthly)	Cardiovascular and all-cause mortality Largest tertile of increase in NT-proBNP concentrations associated with a RR of all cause death of 2.4* & RR of cardiovascular death of 2.9*. NT-proBNP increase predicted by weight gain, systolic blood pressure, fall in albumin & fall in Hb*.	Two NT-proBNP measurements only Haemodialysis only Surrogate measures of fluid state Fatal outcomes only
Winkler et al(32)	1203 prevalent haemodialysis patients	2 (6-monthly)	Cardiovascular and all-cause mortality RR for every unit increase in log NT-proBNP for (i) All-cause death = 1.23* (ii) Sudden death = 1.45* (iii) Cardiac death, stroke or myocardial infarction = 1.19*. Patients with NT-proBNP above median at baseline and >10% fall at 6-months had RR of sudden death of 0.42* compared to patients with stable concentrations.	Post-hoc analysis Two NT-proBNP measurements only Haemodialysis only Surrogate measures of fluid state Fatal outcomes only

Table 1

Choi et al(31)	21 prevalent haemodialysis patients	3 (6-monthly)	Change in LVMI on echocardiography Change in NT-proBNP concentrations during first (r=0.78)* and second (r=0.73)* 6-months of study directly correlated with change in LVMI over corresponding periods.	Two NT-proBNP measurements only Haemodialysis only Small sample size No measure of fluid state No clinical outcomes
Breidthardt et al(33)	113 prevalent haemodialysis patients	5 (6-monthly)	Cardiac related mortality Non-survivors demonstrated a median increase in annual BNP concentrations of 175% versus survivors who had median fall of 14%*	Five NT-proBNP measurements only Haemodialysis only No measure of fluid state
Chazot et al(28)	46 incident haemodialysis patients	2 (3-months)	Percentage decline in BNP correlated significantly with fall in systolic and diastolic blood pressure. Percentage BNP decline independently predicted risk of overall mortality in patients with a cardiac history.	Two BNP measurements only Haemodialysis only Small sample size Fatal outcomes and surrogate measure of fluid state

Figure Legends

Figure 1. The essential components of a biomarker monitoring strategy. The figure depicts serial measurements of a hypothetical biomarker used to predict the occurrence of an adverse outcome (red cross). Patient symptom status and the status of the underlying pathology are depicted above the graph, while potential biomarker sampling time points are labelled as T*x* on the x-axis. During asymptomatic periods when the underlying pathology is stable, biomarker measurements continue to fluctuate randomly around a homeostatic set-point due to biological variation. Thus the fluctuation between sampling points T1 and T2 is of no clinical significance. Deterioration in the underlying pathological process is initially subclinical with the patient remaining asymptomatic. This window is the ideal period during which a biomarker should alert clinicians to a change in pathology. Sampling at time point T3 provides a longer lead time for intervention but has the disadvantage of having a high false positive test detection rate as the magnitude of change in the biomarker level overlaps significantly with biological variation. The T4 sampling time point affords the same lead time advantages as sampling at T3 but with a lower false positive rate. Sampling at time point T5 has low utility as the patient is symptomatic and the lead time is short.

Figure 2. Study schema. Target population, study schedule and target outcomes of the Monitoring Oscillations of NT-proBNP In guiding Therapy and predicting Outcomes in Renal disease (MONITOR) study. NT-proBNP, amino terminal fragment of pro-B-type natriuretic peptide; hs-cTnT, high sensitivity cardiac troponin-T

Figure 1

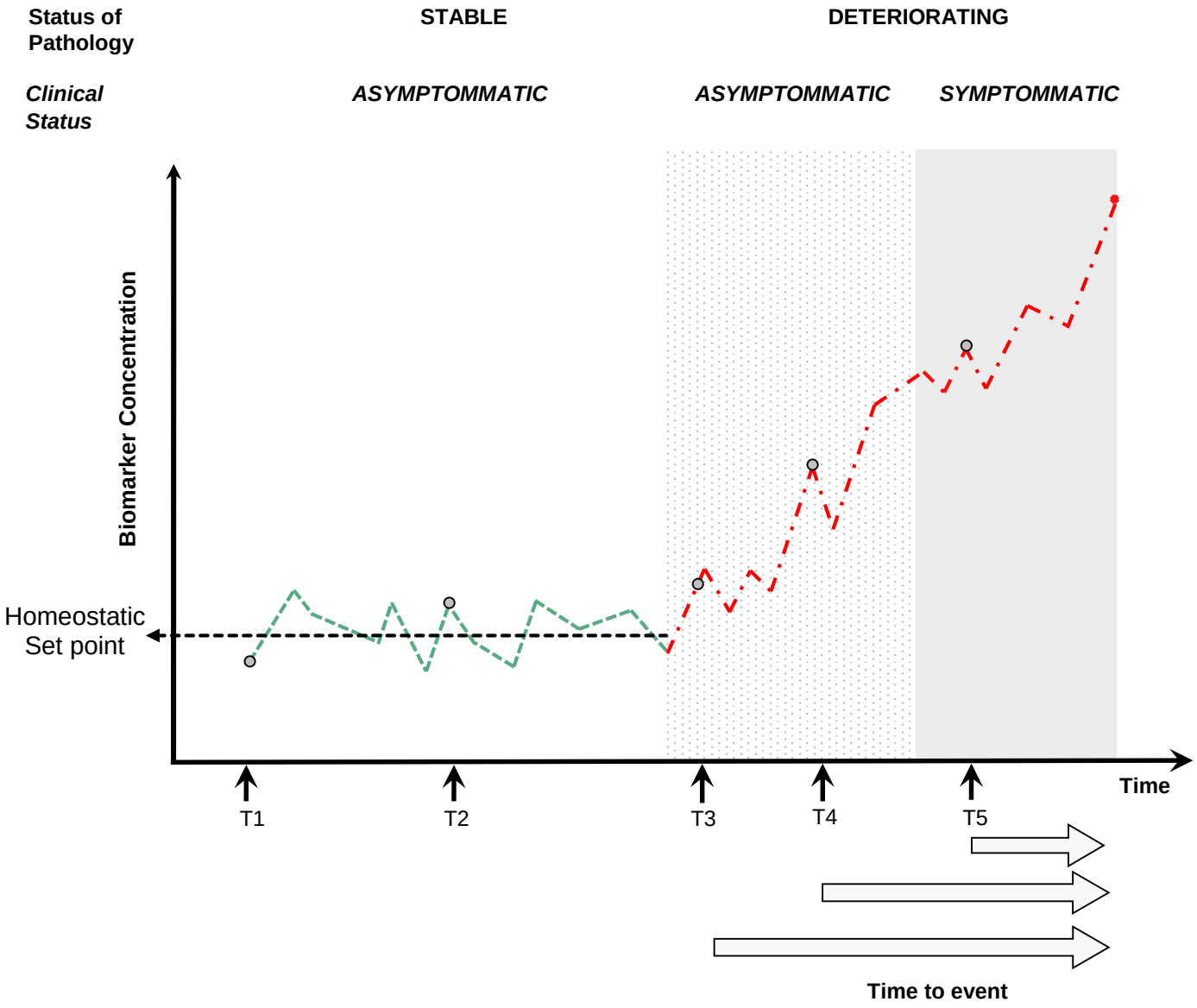
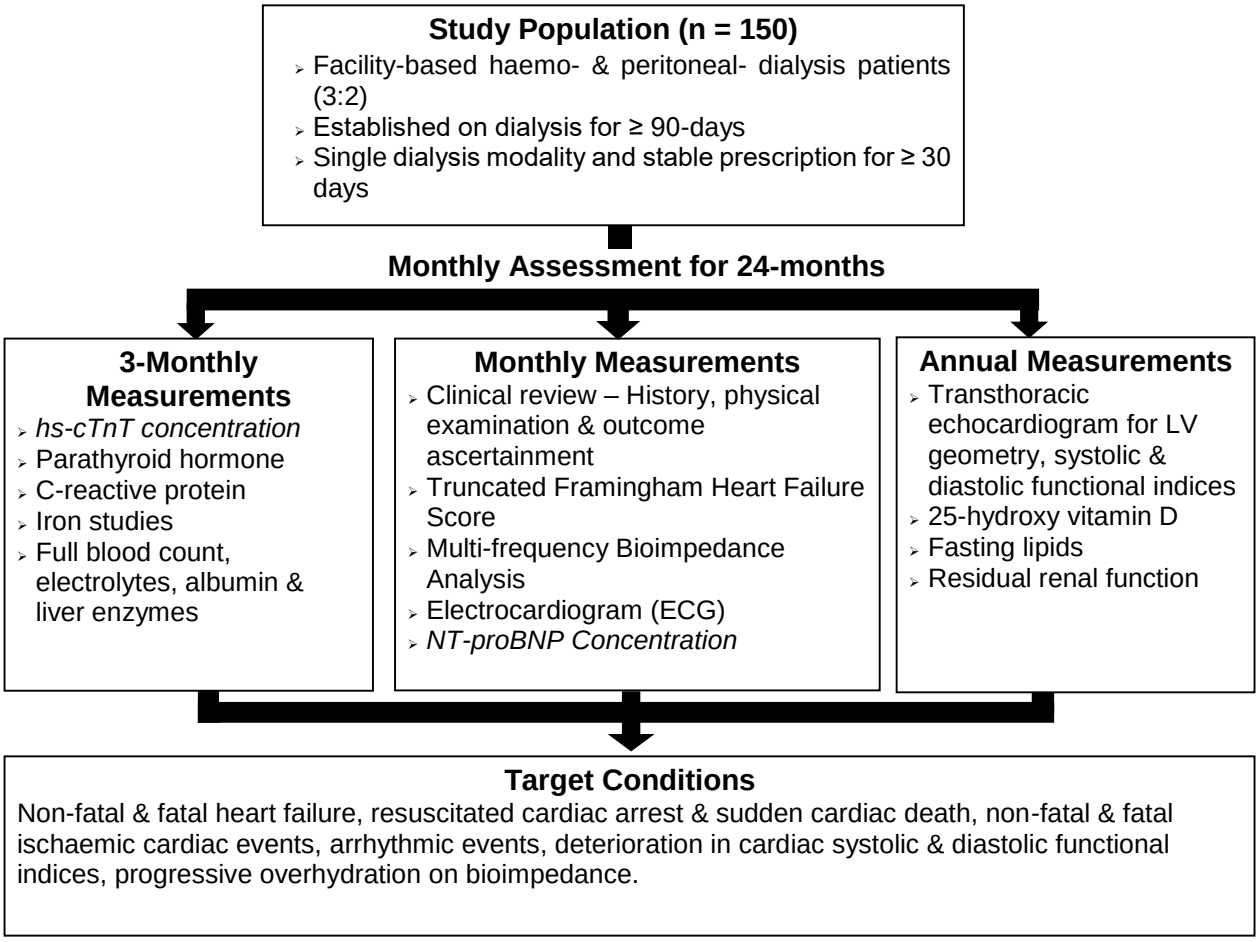

Status of Pathology
STABLE
DETERIORATING
Clinical Status
ASYMPTOMMATIC
ASYMPTOMMATIC
SYMPTOMMATIC
Biomarker Concentration
Homeostatic Set point
T1
T2
T3
T4
T5
Time
Time to event

Figure 2


Study Population (n = 150)
Facility-based haemo- & peritoneal- dialysis patients (3:2)
Established on dialysis for ≥ 90-days
Single dialysis modality and stable prescription for ≥ 30 days
Monthly Assessment for 24-months
3-Monthly Measurements
hs-cTnT concentration
Parathyroid hormone
C-reactive protein
Iron studies
Full blood count, electrolytes, albumin & liver enzymes
Monthly Measurements
Clinical review – History, physical examination & outcome ascertainment
Truncated Framingham Heart Failure Score
Multi-frequency Bioimpedance Analysis
Electrocardiogram (ECG)
NT-proBNP Concentration
Annual Measurements
Transthoracic echocardiogram for LV geometry, systolic & diastolic functional indices
25-hydroxy vitamin D
Fasting lipids
Residual renal function
Target Conditions
Non-fatal & fatal heart failure, resuscitated cardiac arrest & sudden cardiac death, non-fatal & fatal ischaemic cardiac events, arrhythmic events, deterioration in cardiac systolic & diastolic functional indices, progressive overhydration on bioimpedance.

Chapter 5 – Part B

Protocol Title: Week-Week and Month-Month Biological Variation and Reference Change Values of NT-proBNP and hs-cTnT in the Stable Dialysis Population

Rationale:

Cardiac disease is the leading cause of death among incident and prevalent haemo- and peritoneal-dialysis patients. Cohort and cross-sectional studies in the dialysis population have demonstrated that the amino terminal fragment of B-type natriuretic peptide (NT-proBNP) is a powerful predictor of cardiovascular and all cause mortality and varies directly with left ventricular mass and extracellular fluid excess, and inversely with left ventricular systolic function. The next step in the evolution of NT-proBNP as a clinically useful tool is to develop a serial monitoring model with both absolute value cut-offs and a magnitude of serial change that accurately predict adverse events.

Acute myocardial injury is a leading cause of hospitalisation and death in the dialysis population (9.8-16 per 100-patient years), and cardiac troponins are among the most frequently requested tests in this group(1-5). A diagnosis of acute myocardial injury in dialysis patients is contingent on demonstrating a change in serial troponin concentrations in an appropriate clinical context(6, 7). However, there is currently no evidence-based guidance on how much change in serial measurements discriminates between biological variation and acute myocardial injury leading to considerable diagnostic confusion. These concerns are particularly relevant following the recent introduction of high sensitivity cardiac troponin T (hs-cTnT) assays which are able to detect small changes in circulating concentrations

The use of any biological variable for serial monitoring needs to take into account variation of the variable within an individual over time that is not associated with an adverse event. Such variation is attributed to pre-analytic variation, analytic variation (precision and bias) and fluctuations of the variable around a homeostatic set point (intra-individual variation). By minimising pre-analytic variation it is possible to quantify analytic and intra-individual variation over monitoring intervals of interest in a stable cohort of patients. These values can then be used to calculate a bidirectional percentage change range – the reference change value – beyond which a value must change to ensure that the observed change exceeds that expected from biological variation alone.

Reference change values can be calculated for any given level of statistical confidence according to the clinical goal of serial monitoring. If the aim is to avoid missing serious adverse events and the action (evaluation and treatment) prompted by the test poses little risk to the patient, then it may appropriate to use a lower level of statistical confidence in the calculation of the reference change value to minimise false negatives.

Study Design: Prospective observational analytic study.

Hypothesis

Fluctuations in serial NT-proBNP and hs-cTnT concentrations at weekly and monthly intervals among prevalent dialysis patients who are clinically stable and maintain a stable hydration state are explained by quantifiable analytic and intra-individual variation.

Aims:

1. Estimate the within run analytic variation of NT-proBNP and hs-cTnT measurements from a stable cohort of peritoneal- and haemo- dialysis patients assayed using a laboratory based immunoassay.
2. Estimate the intra-individual (within-subject) variation of NT-proBNP and hs-cTnT in a stable cohort of peritoneal- and haemo- dialysis patients at week-week and month-month intervals.
3. Estimate the inter-individual (between-subject) variation of NT-proBNP and hs-cTnT in a stable cohort of peritoneal- and haemo- dialysis patients.
4. Calculate the bidirectional week-week and month-month reference change values of NT-proBNP and hs-cTnT at the 95%, 90%, and 80% levels of statistical significance using estimates of within run and between run analytic and intra-individual variation from a stable cohort of peritoneal- and haemo- dialysis patients.

Sample Size

25 prevalent haemodialysis patients (1:1 M:F)

25 prevalent peritoneal dialysis patients (1:1 M:F)

Selection

Eligible participants will be selected consecutively from the in-centre haemodialysis and peritoneal dialysis units of a single tertiary centre (Princess Alexandra Hospital, Brisbane, Australia).

Inclusion Criteria:

1. Prevalent in-centre haemo- or peritoneal dialysis patients – established on a single dialysis modality for ≥ 90-days
2. Stable dialysis prescription for at least one month
3. Aged 18-years or older
4. Able to provide informed consent
5. Echocardiogram within 6-months of screening

Exclusion Criteria

1. Living renal transplantation planned within 5-months of enrolment.
2. Home haemodialysis
3. Patients on peritoneal dialysis not receiving continuous therapy (e.g. NIPD).
4. Permanent pacemaker and/or implantable cardiac defibrillator.
5. Joint replacements, orthopaedic pins, mechanical heart valves and amputations.
6. Any of the following events / conditions in the month prior to screening:
7. Admission to hospital for any cause.
8. Change in dry body weight target of > 1kg.
9. Unscheduled haemodialysis for treatment of hypertension, dyspnoea or congestive cardiac failure.
10. Change in dialysis prescription.
11. Initiation or dose alteration of angiotensin converting enzyme inhibitor, angiotensin receptor blocker, beta-blocker, or aldosterone receptor antagonist.
12. If has ischaemic heart disease – change in severity of angina – increased frequency and/or occurrence with lesser degrees of exertion and/or alteration in dose of anti-anginal agent(s).
13. If has arrhythmia – change in dose of anti-arrhythmic agent or initiation of new anti-arrhythmic agent.

14. Systemic infection.
15. Congenital heart disease (excluding haemodynamically insignificant patent foramen ovale).
16. Documented severe left ventricular systolic dysfunction defined as an ejection fraction less than 30% by Simpson's rule.
17. Severe valvular heart disease including:
    a. Severe aortic stenosis or regurgitation.
    b. Severe mitral stenosis or regurgitation.
18. Severe pulmonary hypertension (RVSP > [60 mmHg > RA]) due to any cause.
19. ST or non-ST segment myocardial infarction (defined as a combination of serial elevation in Troponin T and any one of new ST/T wave changes on ECG and/or symptoms of cardiac ischaemia) in the 6-months prior to screening.
20. Cardiac surgery and/or coronary angioplasty in the 6-months prior to screening.
21. Pulmonary embolism in the preceding 6-months.
22. Advanced malignancy.
23. Pregnancy.
24. Current immunosuppressive pharmacotherapy for any indication.
25. Patients will also be eliminated from the analysis if during the study or in the interval (7- or 30-days) following completion of the study, the patient:
    a. Undergoes an unscheduled haemodialysis for treatment of hypertension, dyspnoea or congestive cardiac failure.
    b. Has an alteration in their extracellular body volume of ≥ 1l as assessed by bioimpedance analysis or a change in their dry body weight target ≥ 1kg.
    c. Has an alteration of their dialysis prescription.
    d. Has clinical evidence of congestive cardiac failure on the heart failure assessment scale (score ≥ 2).
    e. Undergoes initiation or dose alteration of angiotensin converting enzyme inhibitor, angiotensin receptor blocker, beta-blocker, or aldosterone receptor antagonist.
    f. Is admitted to hospital for any cause.
    g. Develops systemic infection.

h. If has ischaemic heart disease – change in severity of angina – increased frequency and/or occurrence with lesser degrees of exertion and/or alteration in dose of anti-anginal agent(s).
i. If has arrhythmia – change in cardiac rate or rhythm on ECG and/or change in dose of anti-arrhythmic agent or initiation of new anti-arrhythmic agent.
j. Dies.
k. Has a renal transplant

Intervals to be Assessed

Week – week – 4 intervals
Month – month – 4 intervals

Method

- Informed consent.
- Baseline data collection (see appendix 1).
- Patients will be instructed to avoid strenuous exercise prior to attending the assessment and baseline visits.
- All assessment visits will be performed on the same day of the week between 0800 and 1000:
- Mid-week, pre-dialysis for haemodialysis patients.
- Mid-week for peritoneal dialysis patients.
- Clinical assessments, bioimpedance analysis, and blood draws will be conducted at:
    - Day 0 (Baseline)
    - Day 7 (Week 1)
    - Day 14 (Week 2)
    - Day 21 (Week 3)
    - Day 28 (Week 4 **and** Month 1)
    - Day 60 (Month 2)
    - Day 90 (Month 3)
    - Day 120 (Month 4)

Clinical assessments and bioimpedance analysis will also be performed on Day 35 and Day 150 to confirm clinical and physiological stability.

Assessment visit procedure

- Medication review – type and doses.
- Enquiry regarding hospital admission for any cause and/or unscheduled dialysis and/or systemic infection since previous assessment.
- Enquiry about change in dry body weight target and/or change in dialysis regimen since previous assessment.
- Enquiry about change in angina frequency and/or severity if applicable.
- Manual blood pressure.
- Weight using same scales as baseline weight.
- Patient to lie supine for 15-minutes.
- Heart failure assessment scale.
- ECG
- Bioimpedance analysis.
- NT-proBNP and hs-cTnT blood draw and analysis
- Patient supine for 15-minutes
- Haemodialysis - Blood drawn via AV-fistula / graft / permacath following cannulation, and prior to heparinization
- Peritoneal dialysis – Blood drawn from antecubital vein with tourniquet time not exceeding 1-minute
- 2 blood samples (10 ml) will be drawn into Lithium-heparin tubes from the same lot number for all patients across the entire study.
- Collected specimens will be labelled with a unique patient and interval number identifier and stored at 4°C for no more than 1-hour prior to centrifugation.

Samples will be centrifuged at 20°C for 10 minutes at 3000g (5600 rpm) consistently for the entire study. Plasma then will be separated in aliquots of 1 ml and stored at -80°C in a monitored freezer.

Samples from all time points belonging to one individual will be analysed in random duplicate by a single operator in a single run using a single batch of reagent on calibrated Elecsys 2010® and Cobas e170 analysers (Roche Diagnostics).

Remaining plasma will be stored to allow for re-analysis in the future. This information will be included in the consent form.

Assessment schedule

Task	**D 0**	**D 7**	**D 14**	**D 21**	**D 28 M 1**	**D 35**	**D 60 M 2**	**Day 90 M 3**	**D 120 M 4**	**D 150 M 5**
Baseline Data	X	-	-	-	-	-	-	-	-	-
Medication Review	X	X	X	X	X	X	X	X	X	X
Unscheduled dialysis review	X	X	X	X	X	X	X	X	X	X
Any admissions or systemic infections since last review?	X	X	X	X	X	X	X	X	X	X
Change in angina severity	X	X	X	X	X	X	X	X	X	X
Dry weight or dialysis prescription change review	X	X	X	X	X	X	X	X	X	X
Manual BP	X	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X	X
ECG	X	X	X	X	X	X	X	X	X	X
Heart Failure Assessment Scale	X	X	X	X	X	X	X	X	X	X
Bioimpedance Analysis	X	X	X	X	X	X	X	X	X	X
NT-proBNP and hs-cTnT Blood Draw	X	X	X	X	X	-	X	X	X	-

Analysis

We will transform NT-proBNP and hs-cTnT values using an appropriate transformation if required (eg log transformation) so that the data are approximately normally distributed. Variance homogeneity will be analysed at the within run, within person, and between person levels and outliers excluded. We will estimate the within and between run analytic variation, within-person biological variability and between person-variability using a mixed model (Rabe-Hesketh, S. and Skrondal, A. (2008). analysed Multilevel and Longitudinal Modelling Using Stata. (Second Edition). College Station, TX: Stata Press). From this, we will be able to calculate reference change values using standard methods (Changes in serial results; Fraser C. (2009). Biological Variation: From Principles to Practice. AACC Press.) .

Adverse Event Reporting

An adverse event is classified as SERIOUS (SAE) if it meets any one of the following criteria:
DEATH
LIFE-THREATENING: The subject was at substantial risk of dying at the time of the adverse event or it is suspected that the use or continued use of the product would result in the subject's death.
HOSPITALISATION (initial or prolonged): Required admission to the hospital or prolongation of a hospital stay.
DISABILITY: Resulted in a significant, persistent, or permanent change, impairment, damage or disruption in the subject's body function/structure, physical activities or quality of life.
CONGENITAL ANOMALY/BIRTH DEFECT
IMPORTANT MEDICAL EVENT: Other medically important events that, in the opinion of the investigator, may jeopardise the subject or may require intervention to prevent on of the other outcomes listed above.

This study is purely observational with no investigational drugs or interventions and no deviations from current standard practice. Thus we will seek an exemption from reporting Serious Adverse Events unless they are '**unexpected**' or deemed '**clinically significant**' by the Principal Investigator/s.

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Appendix 1

Baseline Data Items

Age

Gender

Ethnicity

Renal diagnosis

Date of dialysis commencement

History of transplantation

Current dialysis prescription

Haemodialysis – frequency, duration of session, dialysis membrane ultrafiltration coefficient, blood flow rate, dialysate flow rate, dialysate composition, last single-pool Kt/V, average inter-dialytic weight gain as a percentage of estimated dry body weight in the week prior to assessment ([Total weight gain across entire week / number of haemodialysis sessions] / estimated dry body weight) x 100.

Peritoneal dialysis – CAPD vs CCPD, total volume of exchanges per 24-hours, transporter status, last recorded weekly Kt/V

Current vascular access

AV fistula / graft flow rate if applicable

Residual renal function (measured within 3-months of enrolment)

Medications - current and changes within last month (commencement / cessation / dose change)

ESA dose – type and dose as mcg or mg/kg per g/l Hb

Last recorded haemoglobin, CRP, calcium, phosphate, PTH, albumin

Co-morbidities

Cardiac

Ischaemic heart disease

Angina - severity, and therapy (medical, PTCA, and/or coronary surgery)

Myocardial infarction – ST-elevation MI, or Non-ST elevation MI (defined as a serial increase in troponin concentration associated with either ischaemic symptoms and/or serial change in ECG)

Document results of previous / most recent:

Coronary angiogram

Non-invasive cardiac stress test

History of left ventricular systolic dysfunction and/or dilated cardiomyopathy, and/or hypertrophic obstructive cardiomyopathy

Document measures of most recent Echocardiogram

Valvular heart disease – document valve involved, type of dysfunction, and severity from most recent echocardiogram. Valve surgery.

Arrhythmia – type, rate, and therapy

Peripheral vascular disease and therapy

Cerebrovascular disease – thrombotic or haemorrhagic stroke, subarachnoid haemorrhage, or transient ischaemic attack

Pulmonary disease – restrictive or obstructive

Dyslipidaemia

Diabetes

Hypertension

Smoking history

Other

Hepatic – viral hepatitis, alcoholic or non-alcoholic steatohepatitis, cirrhosis

Peptic ulcer disease

Endocrine

Neurological

Weight

Height

Waist circumference

BMI

Body temperature (tympanic reading)

Heart failure assessment scale

ECG

Bioimpedance analysis

NT-proBNP and hs-cTnT blood draw

Appendix 2

Heart Failure Assessment Scale – Truncated Framingham Heart Failure Assessment Score

Symptom / Sign	Value	Patient Score
Orthopnoea	0.5	
Paroxysmal nocturnal dyspnoea	1.0	
Reduction in exercise tolerance	0.5	
Resting sinus tachycardia (> 100 / min)	0.5	
Jugular venous pressure > 4 cm	0.5	
Hepatojugular reflex positive	1.0	
Third heart sound	1.0	
Bibasal crackles	1.0	
Hepatomegaly	0.5	
Peripheral Oedema	0.5	
Total		

From Ho KK, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure: the Framingham Study. *J Am Coll Cardiol* 1993; 22: 6A-13A

Chpater 6

N-Terminal pro-B-type Natriuretic Peptide (NT-proBNP) Variability in Stable Dialysis Patients

The following chapter presents the findings of the biological variation study performed to estimate the within- and between-person coefficients of variation of plasma NT-proBNP in a cohort of stable dialysis patients. The within-person coefficients of variation estimated in this study will be key to the interpretation of serial measurements in any future clinical monitoring strategy, as well as for adjusting for any regression-to-mean effects in research practice. In addition, they provide important insights into the ideal strategy for applying NT-proBNP testing in the dialysis population.

The following chapter was published as peer reviewed original research:

Fahim MA, Hayen A, Horvath AR, Dimeski G, Coburn A, Johnson DW, Hawley CM, Campbell SB, Craig JC: N-Terminal Pro-B-Type Natriuretic Peptide Variability in Stable Dialysis Patients. *Clin J Am Soc Nephrol*, 2015 DOI:10.2215/CJN.09060914

The following chapter was also presented by the candidate at the finalist session of Young Investigator Award, Australia and New Zealand Society of Nephrology Annual Scientific Meeting, Brisbane, Australia (2013). *Short and Long Term Biological Variation of high sensitivity Troponin T (hs-cTnT) and N-Terminal B-type Natriuretic Peptide (NT-proBNP) in The Stable Dialysis Population.* ***Fahim M****, Hayen A, Coburn A, Dimeski G, Johnson D, Craig J, Horvath A, Campbell S, Hawley C.*

Chapter 6

N-Terminal pro-B-type Natriuretic Peptide (NT-proBNP) Variability in Stable Dialysis Patients

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ABSTRACT

BACKGROUND AND OBJECTIVES: Monitoring N-terminal pro-B-type natriuretic peptide (NT-proBNP) may be useful for assessing cardiovascular risk in dialysis patients. However its biological variation is unknown, hindering the accurate interpretation of serial concentrations. The aims of this prospective cohort study were to estimate the within- and between-person coefficients of variation of NT-proBNP in stable dialysis patients, and derive the critical difference between measurements needed to exclude biological and analytic variation.

DESIGN, SETTING, PARTICIPANTS AND MEASUREMENTS: 55 prevalent hemo- and peritoneal-dialysis patients attending two hospitals were assessed weekly for 5-weeks then monthly for 4-months between October 2010 and April 2012. Assessments were conducted at the same time in the dialysis cycle and entailed NT-proBNP testing, clinical review, electrocardiography, and bioimpedance spectroscopy. Patients were excluded if they became unstable.

RESULTS: 136 weekly and 113 monthly NT-proBNP measurements from 40 and 41 stable patients respectively were analysed. 22% had ischemic heart disease; 9% and 87% had left ventricular systolic and diastolic dysfunction respectively. Median NT-proBNP concentration was Respective between- and within-person coefficients of variation were 153% and 27% for weekly measurements, and 148% and 35% for monthly measurements. Within-person variation was unaffected by dialysis modality, hydration status, inflammation or cardiac co-morbidity. NT-proBNP concentrations measured at weekly intervals needed to increase by at least 46% or fall by 84% to exclude change due to biological and analytic variation alone with 90% certainty, while monthly measurements need to increase by at least 119% or fall by 54%.

CONCLUSIONS: The between-person variation of NT-proBNP was large and markedly greater than within-person variation indicating that NT-proBNP testing might better be applied in the dialysis population using a relative change strategy. Serial NT-proBNP concentrations need to double or halve to confidently exclude change due to analytic and biological variation alone.

KEYWORDS

Renal Dialysis, N-terminal pro-B-type natriuretic peptide, B-type natriuretic peptide, Variability

INTRODUCTION

The N-terminal fragment of the pro-B-type natriuretic peptide (NT-proBNP) is an inactive peptide secreted from the myocardium in response to stretch, strain, ischemia, inflammation and sympathetic overactivity(1). Cohort studies in the dialysis population have demonstrated a direct association between NT-proBNP concentrations and the risk of cardiovascular and all-cause mortality(2-5); prompting calls to incorporate NT-proBNP testing into dialysis practice as a means of monitoring individual patient's cardiovascular risk(6-8).

However, before these findings can be translated into clinical practice, the biological variation of NT-proBNP in dialysis patients needs to be determined in order to avoid misinterpreting serial measurements. Biological or within-person variation is the random fluctuation of a biomarker around a homeostatic set-point in healthy individuals or those with stable disease(9), resulting in potentially large numerical changes in serial biomarker concentrations which are of no clinical significance. Failure to account for biological variation can result in false reassurance or alarm, and unnecessary changes to therapy with their associated morbidity and costs(10).

The aims of this study were to estimate the within- and between-person variation of NT-proBNP measured at weekly and monthly intervals in stable dialysis patients and to use these estimates to calculate the percentage change between serial NT-proBNP measurements needed to exclude change due to biological and analytic variation alone. We also sought to determine if the within-person variation of NT-proBNP differed according to cardiac co-morbidity, hydration or inflammatory status or between dialysis modalities.

MATERIALS AND METHODS

Study Design and Patient Recruitment

A prospective cohort study was conducted between October 2010 and April 2012 according to methods described by Fraser and Harris(11). The study complied with the declaration of Helsinki and received ethics approval from the Metro-South Human Research Ethics Committee (HREC/10/QPAH/131).

Participants were recruited from the in-centre hemodialysis and peritoneal dialysis units of a tertiary-care teaching hospital in Brisbane, and a secondary-care hospital in Logan, Australia. Eligible participants identified from an electronic database of all patients receiving dialysis therapy were adults (aged ≥18-years) on maintenance dialysis for ≥90-days who had a stable dialysis prescription for ≥30-days and a transthoracic echocardiogram ≤12-months prior to screening.

Eligibility criteria were chosen to ensure that the study cohort were physiologically and clinically stable at enrolment, and likely to remain stable for the duration of the study while still being representative of the dialysis population. Patients were excluded if they had undergone coronary and/or valvular intervention or suffered a myocardial infarction or pulmonary embolism in the 6-months prior to screening; had echocardiographic evidence of severe pulmonary hypertension, severe functional aortic and/or mitral valvular disease, or a left ventricular ejection fraction <30%; had been hospitalised for any indication or undergone an unscheduled dialysis for the treatment of hypertension, heart failure or dyspnoea in the 30-days prior to screening; had been commenced on or undergone a dose change of a diuretic, beta-blocker, aldosterone receptor antagonist, angiotensin converting enzyme inhibitor or angiotensin receptor type-1 blocker in the 30-days prior to screening; had experienced worsening angina, a new cardiac arrhythmia or undergone change in associated therapies in the 30-days prior to screening; had a contraindication to bioimpedance measurement including a pacemaker, joint replacements, or mechanical heart valve; were pregnant; had advanced malignancy; or were unable to provide informed consent.

Patient Assessment

Patients were assessed on 10 consecutive occasions - weekly for 5 weeks then monthly for another 4 months. All assessments were conducted between 6–8 AM, prior to the mid-week dialysis session for hemodialysis patients, and between 8–10 AM on the same weekday for peritoneal dialysis patients. Patients avoided strenuous exercise prior to assessment.

Several factors affect NT-proBNP concentrations including extracellular volume(12), cardiac rhythm(13), myocardial ischemia(14, 15), the dialysis prescription(16) and cardiac pharmacotherapy(17-19). These influences were assessed at every visit using a structured clinical interview, physical examination and medical records review to ascertain interim hospitalisation, changes to medication and/or the dialysis prescription. The Canadian Cardiovascular Society Angina Grading Scale(20) was used to assess change in cardiac ischemic symptoms and the Truncated Framingham Heart Failure Score(21) was used to assess for pulmonary edema. Patients

underwent a standard 12-lead electrocardiogram and whole-body, multi-frequency bioimpedance analysis using the Body Composition Monitor BCM® (Fresenius Medical Care, Asia-Pacific) at each visit to measure extracellular volume. This instrument has a detection limit for change in extracellular volume of 0.87L ±0.64L(22, 23).

Specimen Collection, Storage and Analysis

NT-proBNP concentrations were measured at 8 of 10 visits - baseline then at weeks 1-4 and months 2-4, providing data for 4 weekly and monthly intervals. To ensure that changes in NT-proBNP concentrations during the final measurement interval did not reflect changes in subclinical risk, patients were assessed for stability at week 5 and month 5 without measurement of NT-proBNP. Blood collected in lithium-heparin tubes was centrifuged and plasma separated within 1-hour of collection. Plasma was stored at -80°C until assayed (24).

Samples were batched and analysed together in a single analytic-run in random duplicate by a single expert operator using a single instrument and a single batch of reagent, control, and calibrators. Plasma NT-proBNP concentration (pg/mL) was measured using a twin-antibody electrochemiluminescence assay on the Elecsys 2010 instrument (Roche Diagnostics, Australia) which has a reported analytical detection range of 5–35,000 pg/mL(25). NT-proBNP was chosen in preference to BNP due to its superior stability which minimises preanalytic variation(26), and due to the greater agreement between NT-proBNP assays from different manufacturers(27), allowing the study's findings to be more widely generalizable.

C-reactive protein (mg/L) was measured pre-dialysis at the baseline visit and analysed using a turbidimetric method on the Beckman DxC800 analyser (Beckman Coulter, CA, USA). This method has a lower limit of detection of 2.0 mg/L, and analytic coefficients of variation of 6.3% and 3.1% at concentrations of 6.0 and 85.0 mg/L respectively.

Statistical Analysis

Based on a ratio of analytic to within-person variation of <0.5 for NT-proBNP, we estimated that a study sample of 40 patients undergoing NT- proBNP testing on 8 occasions over 4 weekly and monthly intervals would have power >0.99 to estimate the within-person coefficient of variation with a 95% confidence interval of ±3.6%(28). A sample size of 55 patients was chosen to allow for dropouts as a result of instability.

The principal assumption of biological variation studies is that the cohort is stable with respect to physiological, pathological and extrinsic factors that influence the concentration of the biomarker of interest. In the study presented here, patients were deemed to be unstable if they experienced a change in dose of diuretic, beta-blocker, aldosterone receptor antagonist, angiotensin converting enzyme inhibitor or angiotensin receptor blocker; a change in severity of cardiac ischemic symptoms, dose of anti-anginal agents, or cardiac intervention; a change in anti-arrhythmic agent or new cardiac arrhythmia; a change in extracellular volume >1L on bioimpedance analysis; a change in dialysis modality or prescription; hospitalisation for any reason or exhibited pulmonary edema defined as a score ≥2 on the Truncated Framingham Heart Failure Score. If a study participant was deemed to be unstable, the NT-proBNP concentrations from the intervals preceding and following the event were excluded from the statistical analysis.

Normally distributed variables are presented as mean ± standard deviation, and non-normally distributed variables as median and interquartile range. NT-proBNP concentrations were logarithmically transformed for the variation analyses. We fitted mixed effects models with random intercepts to calculate the between-person coefficient of variation across the cohort (CV_G), the within-person coefficient of variation at weekly and monthly intervals (CV_I) and the within-run analytic co-efficient variation (CV_A). Outlying variances were excluded using the Reed and Cochran tests. Linear regression was used to identify and exclude participants who demonstrated a consistent increase or decrease in log NT-proBNP concentrations throughout the study as such a trend may represent a change in future risk that may not have manifest clinically during the study.

The cohort was also divided into eight subgroups according to dialysis modality, hydration status, ischemic heart disease status, severity of left ventricular diastolic dysfunction, presence or absence of left ventricular systolic dysfunction and left ventricular hypertrophy, tertiles of C-reactive protein concentrations, and quartiles of NT-proBNP concentrations at enrolment. CV_I was estimated for each subgroup and compared using Bartlett's test. Overhydration was assessed using the ratio of absolute overhydration volume to total extracellular volume measured using bioimpedance and categorised as absent (<6.8%), moderate (6.8–15%), or severe (>15%)(29). Ischemic heart disease was defined as any of inducible ischaemia on non-invasive cardiac stress testing and/or ≥50% stenosis in ≥1 epicardial coronary artery on coronary angiography and/or a history of myocardial infarction. Left ventricular diastolic dysfunction(30) and left ventricular hypertrophy(31) were graded as absent, mild, moderate or severe according to established algorithms using

echocardiographic measurements. Left ventricular systolic dysfunction was defined as a left ventricular ejection fraction ≤50% using Simpson's rule(30).

The index of individuality (IOI) was calculated as CV_I/CV_G. This ratio gives an indication of whether a biomarker is best used within a relative-change monitoring strategy (IOI <0.6) or a reference interval strategy (IOI >0.6). The bidirectional reference change value (RCV) was calculated according to the method described by Fokkema et al(32) for logarithmically transformed data as = $\exp(-Z\times\sqrt{2}\times\sigma)$ to $\exp(+Z\times\sqrt{2}\times\sigma)$; where $\sigma = \sqrt{\ln(CV_i^2+1)}$ and Z is the Z-score of a standard normal distribution corresponding to a given probability.

RESULTS

Patient Characteristics

Details of the number of patients assessed, enrolled and included in the final analysis are shown in Figure 1. Fifty five patients were recruited from the hemodialysis (n=28) and peritoneal dialysis units (n=27) of the participating institutions and their baseline characteristics are summarised in Table 1. Cardiovascular risk factors, including hypertension (100%), diabetes mellitus (40%), and current or former smoking (51%) were highly prevalent. A substantial proportion of the cohort also had evidence of established cardiovascular disease including ischemic heart disease (22%), left ventricular hypertrophy (44%), diastolic (87%) and/or systolic (9%) dysfunction, and peripheral- and/or cerebro-vascular disease (9%). Baseline NT-proBNP concentrations demonstrated a right skewed frequency distribution with a median of 1698 (interquartile range 718–3742) pg/mL. 93% of the study cohort had a NT-proBNP concentration above 300 pg/mL; the threshold used to exclude acute decompensated heart failure in the general population(33).

Weekly and Monthly Variation of NT-proBNP

Seven patients and their corresponding NT-proBNP measurements were excluded due to hospital admission (n=3), paroxysmal atrial fibrillation (n=3), and escalating anginal symptoms (n=1). In addition, 36-weekly and 51-monthly NT-proBNP sample pairs were excluded due to a change in extracellular volume of >1L between consecutive visits. None of the participants were excluded on the basis of outlying variances or the linear regression analysis. NT-proBNP measurements made over 136-weekly intervals from 40 patients and 113-monthly intervals from 41 patients were included in the final analysis. These data are shown in Figure 2 with excluded measurements represented by gaps.

The respective analytic, within-person, and between-person coefficients of variation of NT-proBNP were 1.9%, 27%, and 153% for weekly intervals, and 1.6%, 35%, and 148% for monthly intervals (Table 2). Between person variation was much greater than within-person variation (Figure 3), yielding low indices of individuality of 0.18 and 0.24 for the weekly and monthly intervals respectively (Table 2).

Weekly and monthly reference change values for the 70%, 80%, and 90% degrees of statistical confidence are shown in Table 2. Thus, NT-proBNP concentrations measured at weekly intervals needed to increase by 84% or fall by 46% to ensure with 90% confidence that the observed change exceeded analytic and biological variation alone. Monthly reference change values were slightly larger than weekly values for a given degree of statistical confidence.
The within-person co-efficient of variation did not differ significantly between dialysis modalities, by ischemic heart disease, hydration or inflammatory status, by severity of diastolic dysfunction, by presence or absence of left ventricular hypertrophy or across quartiles of NT-proBNP concentration (Table 3). The effect of left ventricular systolic dysfunction on within-person variation was unable to be meaningfully analysed as only one such patient was retained in the final analysis after the exclusion of unstable patients.

DISCUSSION

This study demonstrated that NT-proBNP concentrations vary considerably across the dialysis population with a between-person coefficient of variation of 158%; markedly greater than that reported for healthy individuals (36-70%)(34, 35). This marked variability likely reflects the broad range of hydration status, residual renal function, dialysis regimens, pharmacotherapies, and the numerous types and severities of cardiac pathologies present in the dialysis population. In contrast, the within-person coefficient of variation of NT-proBNP among stable dialysis patients in this study was markedly smaller, equating to 27% and 35% for the weekly and monthly measurement intervals respectively.

The large discrepancy between the within- and between-person coefficients of variation has important implications for how NT-proBNP is interpreted in the dialysis population. The ratio of within- to between-person variation is termed the index of individuality, and in this study equated to 0.18 and 0.24 for the weekly and monthly measurement intervals respectively. Ratios less than 0.6

indicate a high degree of individuality and imply that NT-proBNP testing is better applied in the dialysis population using a relative change strategy wherein serial measurements from the same patient are compared to each other rather than comparing single values to a reference interval or a threshold value(36). This finding represents an important departure from the majority of contemporary studies in this area have sought to derive an absolute NT-proBNP concentration below which cardiac dysfunction and/or over-hydration can be confidently excluded in a manner analogous to which a NT-proBNP concentration less than 300 pg/mL is used to exclude acute decompensated heart failure in the non-dialysis population(33, 37-41). Our findings suggest that such a strategy would be inaccurate for predicting cardiovascular risk in the dialysis population, resulting in unacceptably high false negative or false positive rates depending on the threshold value chosen while also missing the majority of important changes in serial NT-proBNP concentrations measured from an individual dialysis patient(36). Instead, we suggest that a relative change strategy would be of greater clinical value by accurately detecting significant changes in serial NT-proBNP concentrations irrespective of the absolute values. Significant changes in NT-proBNP concentrations would likely reflect important changes in composite cardiovascular risk caused by one or more pathophysiological processes including volume overload, ventricular dysfunction, and/or cardiac ischemia. This hypothesis is supported by longitudinal studies that have demonstrated a direct correlation between change in NT-proBNP concentrations and the risk of fatal cardiovascular events(2-5), change in left ventricular mass(42), and change in surrogate measures of volume status(4, 7).

However, not all changes in serial NT-proBNP concentrations are of clinical significance and may instead be attributed to analytic and/or biological variation. The within-person coefficients of variation estimated in this study will play a crucial role in making this distinction in both the clinical and research settings(10, 43, 44). Using these estimates we calculated a bidirectional magnitude of change between serial NT-proBNP measurements which excludes change due to biological and analytic variation alone for a pre-specified degree of statistical confidence – the reference change value(32, 43). The 90% reference change value for NT-proBNP measured at weekly intervals was -46% and +84%, implying that serial NT-proBNP concentrations must increase by at least 84% or fall by at least 46% to exclude change due to biological and analytic variation alone with 90% certainty. Reducing the degree of statistical confidence reduces the magnitude of change required in either direction and increases the sensitivity but reduces the specificity for detecting a clinically important change. The degree of statistical confidence used is dictated by whether a change in biomarker levels is intended to rule in a given medical condition

(high specificity required) or rule it out (high sensitivity required). While the precise role of NT-proBNP monitoring in the dialysis population has yet to be absolutely defined, we hypothesize that it will primarily be used to identify patients at risk of major adverse cardiac events who require further evaluation to determine which pathophysiologic process(s) is/are responsible. A lower degree of statistical confidence would be suitable for such a strategy as neither diagnosis nor therapy would be solely predicated on changing NT-proBNP concentrations alone. Finally, we found that the within-person coefficient of variation of NT-proBNP was not significantly different between dialysis modalities, by ischemic heart disease, inflammatory or hydration status, by severity of diastolic dysfunction, by presence or absence of left ventricular hypertrophy or across quartiles of baseline NT-proBNP concentrations implying that a single decision limit can be applied across the entire dialysis population.

Our estimates of within-person variation are supported by the findings of Aakre et al(35), who recently reported a within-person coefficient of variation of 26% for NT-proBNP measured at weekly intervals from a cohort of 17 hemodialysis patients. This study was limited by its small sample size, the exclusion of peritoneal dialysis patients and a lack of rigour in ensuring patient stability, wherein the investigators relied either on patient report or a history of hospitalization alone to determine stability and did not measure volume status. As previously described, NT-proBNP concentrations may be affected by volume status, cardiac arrhythmias, the dialysis prescription and changes in angina class that may not necessarily warrant admission. Ensuring stability of all of these parameters is essential to deriving accurate estimates of within-person variation.

Our study has a number of strengths that address these limitations including the use of a rigorous approach to ensure the stability of all factors that influence NT-proBNP concentrations, the enrolment of both peritoneal- and hemo-dialysis patients, measurement of NT-proBNP over both weekly and monthly intervals, and a larger sample size. Nevertheless, the present study has several potential limitations. Firstly, patients with the most severe cardiovascular co-morbidities were excluded to maximise the likelihood that patients would remain stable for the duration of follow-up. Although this potentially limits the generalizability of the study's findings, the baseline characteristics of our study cohort were similar to those reported for prevalent Australasian dialysis patients in the Australian and New Zealand Dialysis and Transplant Registry(45). Secondly, mean hemodialysis session duration in this study was considerably longer than that reported by most North American centres(46), whether hemodialysis session length affects the within-person variation of NT-proBNP could not be investigated in this study due to the homogeneity of dialysis

prescriptions and should be investigated by future studies before these results are applied to populations receiving shorter hemodialysis sessions. Thirdly, it was unable to be determined if the within-person coefficient of variation of NT-proBNP was affected by left ventricular systolic impairment as only one such patient remained stable during the study. Finally, reference change values only exclude biological and analytic variation, and changes in biomarker concentrations exceeding the reference change value are not automatically of clinical significance.

NT-proBNP holds much promise as a biomarker of cardiovascular risk in the dialysis population, however much remains to be established before it can be adopted into clinical practice. This study represents an important step in that direction. Based on its findings we suggest that NT-proBNP testing may be better applied in the dialysis population using a relative change strategy rather than by comparing absolute values to a reference interval or threshold value, and that large changes in NT-proBNP concentrations are needed to confidently exclude change due to biological variation alone. Moving forward, longitudinal cohort studies are needed to determine the accuracy of serial NT-proBNP testing for predicting adverse cardiovascular events in the dialysis population and potential targets for therapeutic intervention.

STATEMENT OF COMPETING FINANCIAL INTERESTS

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FIGURE LEGENDS

Figure 1. Flow diagram of patients assessed for eligibility, enrolled and analysed in the study.

Figure 2. Variation of N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentrations measured from each of the stable participants over the (A) weekly and (B) monthly follow-up phases. Gaps represent measurements excluded due to a change in extracellular volume > 1L between consecutive visits.

Figure 3. Box-plot of NT-proBNP concentrations for stable participants during the weekly follow-up phase plotted in ascending order of median NT-proBNP concentrations. Each box plot depicts five logarithmically transformed NT-proBNP concentrations taken from a single stable participant over the first 5 weeks of the study. The whiskers delimit the range and the middle, lower and upper lines of the box represent the median, upper and lower quartiles respectively. Between-person variation is reflected in the variation between median NT-proBNP concentrations across the entire cohort and was large. By comparison, within-person variation depicted by the box plots was much smaller and largely uniform between patients.

Figure 1

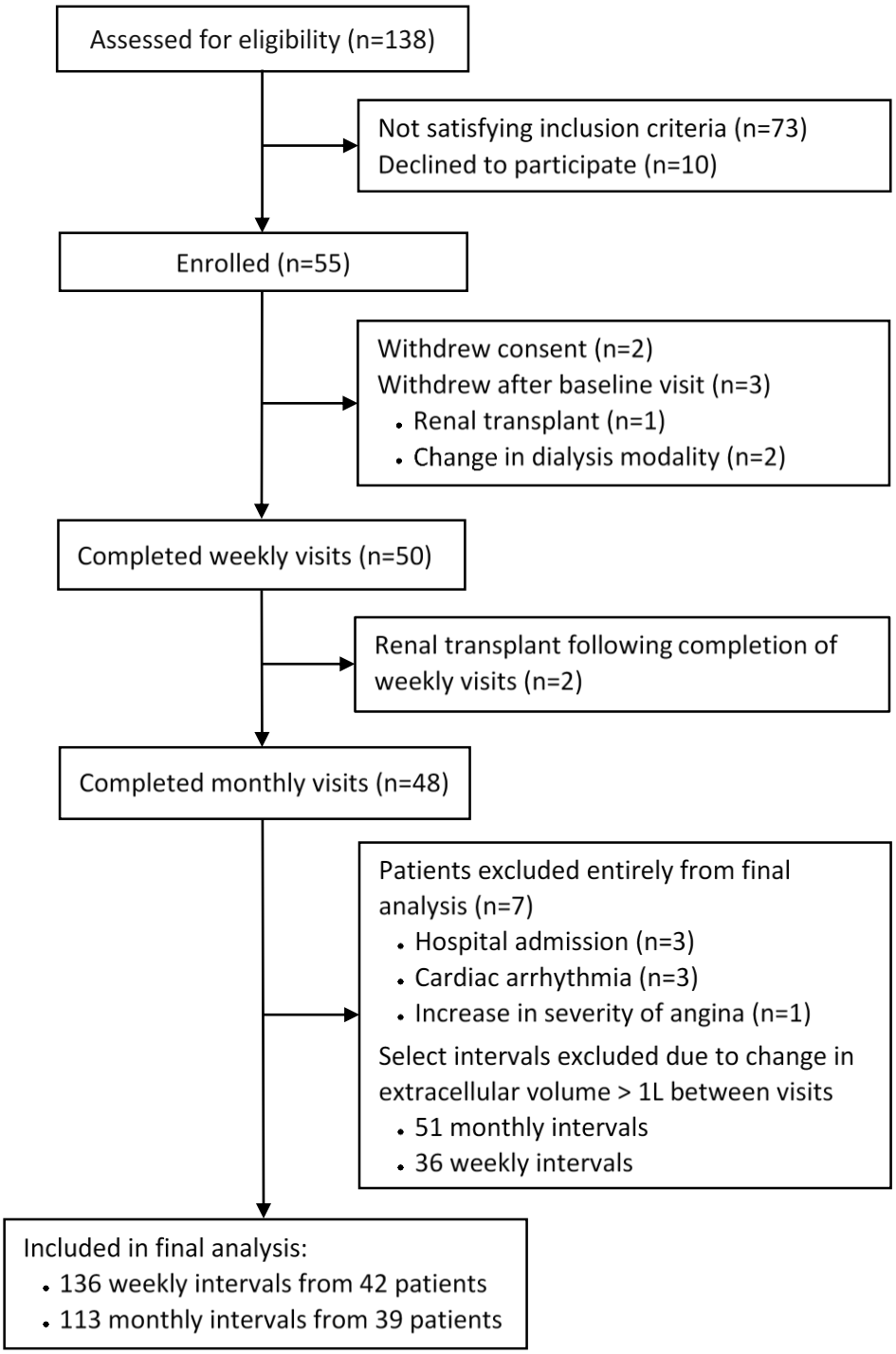
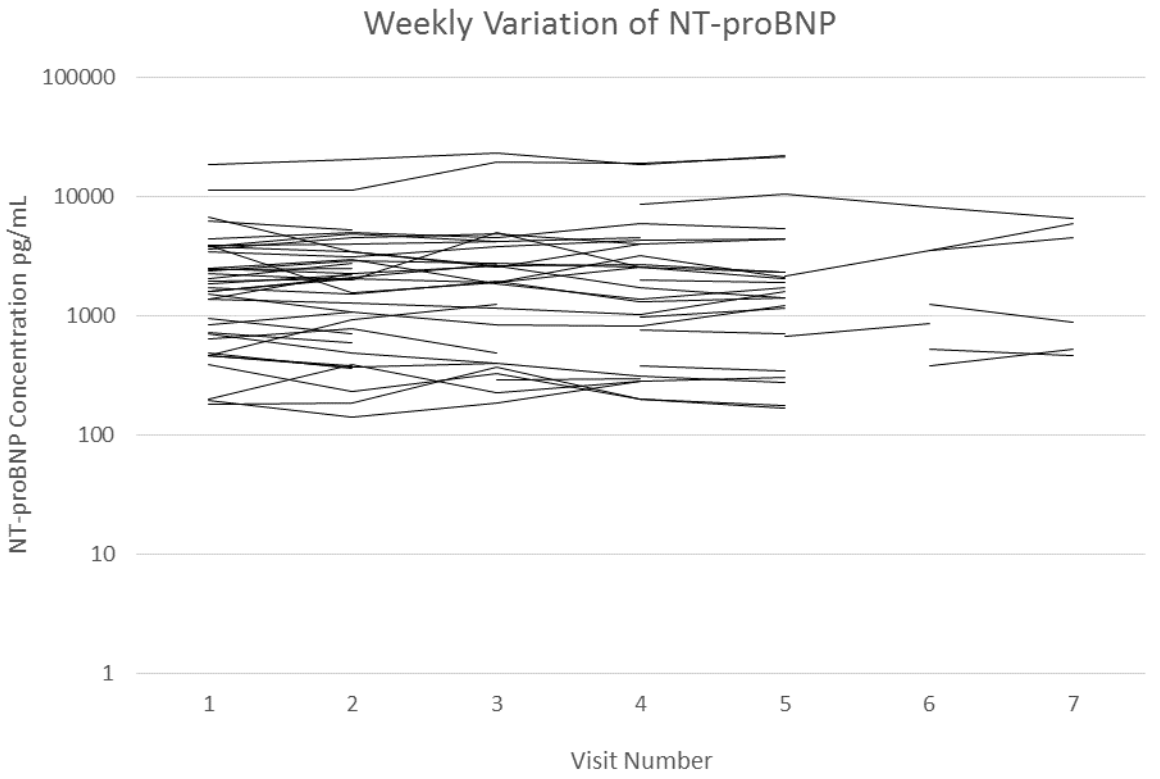

Assessed for eligibility (n=138)
Not satisfying inclusion criteria (n=73)
Declined to participate (n=10)
Enrolled (n=55)
Withdrew consent (n=2)
Withdrew after baseline visit (n=3)
Renal transplant (n=1)
Change in dialysis modality (n=2)
Completed weekly visits (n=50)
Renal transplant following completion of weekly visits (n=2)
Completed monthly visits (n=48)
Patients excluded entirely from final analysis (n=7)
Hospital admission (n=3)
Cardiac arrhythmia (n=3)
Increase in severity of angina (n=1)
Select intervals excluded due to change in extracellular volume > 1L between visits
51 monthly intervals
36 weekly intervals
Included in final analysis:
136 weekly intervals from 42 patients
113 monthly intervals from 39 patients

Figure 2


Weekly Variation of NT-proBNP
100000
10000
1000
100
10
1
NT-proBNP Concentration pg/mL
1
2
3
4
5
6
7
Visit Number

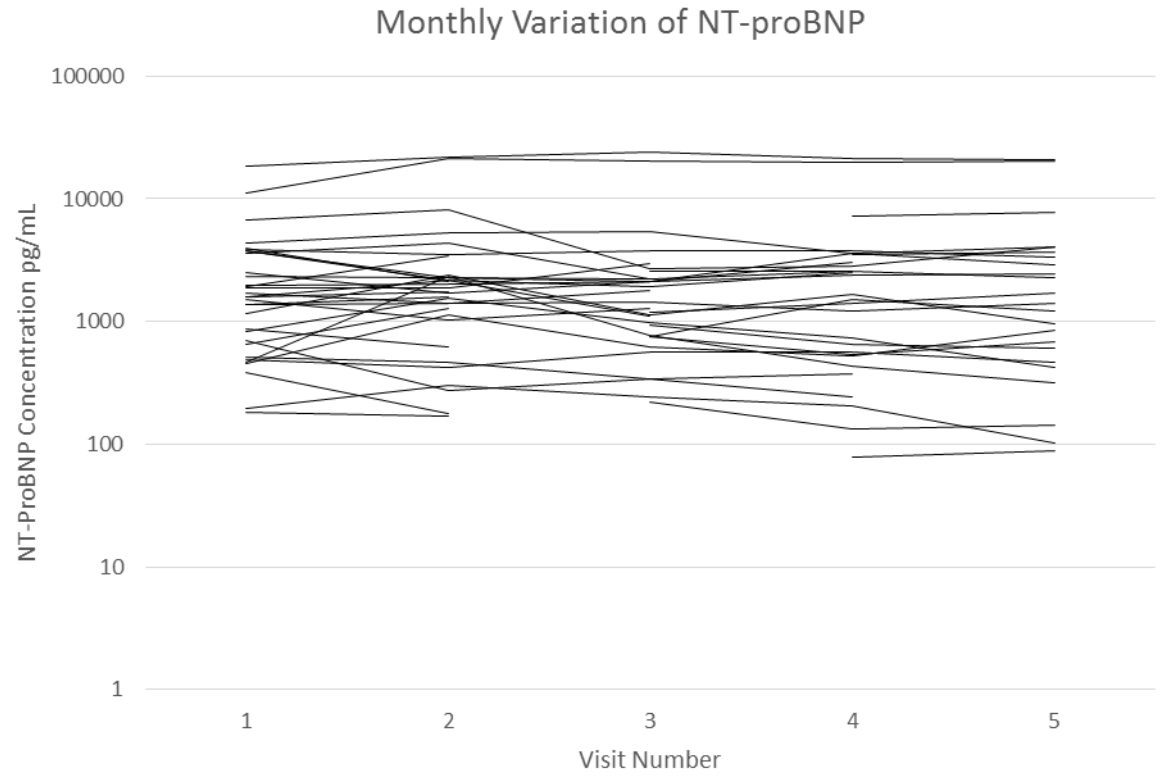

Monthly Variation of NT-proBNP
100000
10000
1000
100
10
1
NT-ProBNP Concentration pg/mL
1
2
3
4
5
Visit Number

Figure 3

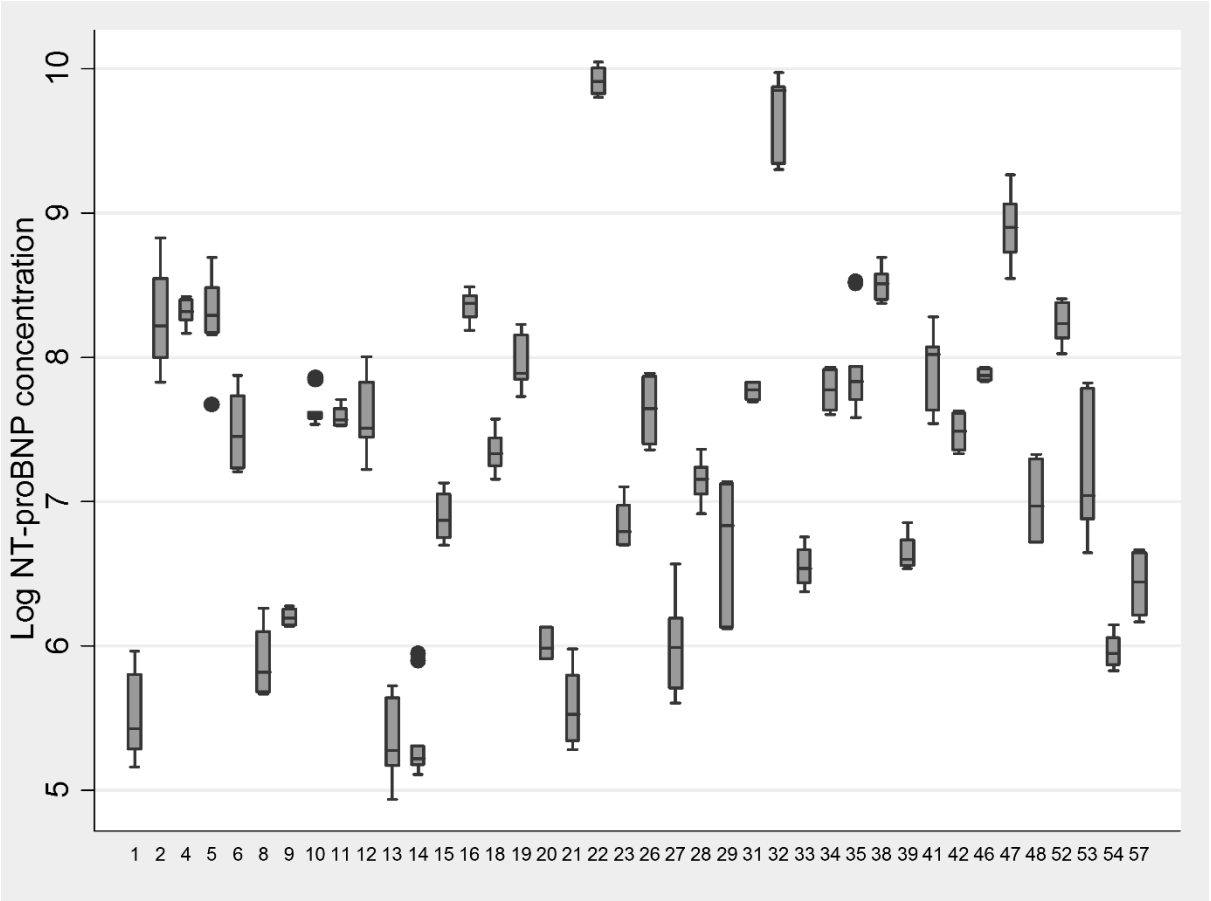

Log NT-proBNP concentration
5
6
7
8
9
10
1 2 4 5 6 8 9 10 11 12 13 14 15 16 18 19 20 21 22 23 26 27 28 29 31 32 33 34 35 38 39 41 42 46 47 48 52 53 54 57

Table 1. Baseline characteristics of the study cohort.

Characteristic	**Value (n=55)**
Male gender (%)	45
Age	
Mean ± SD (years)	59±15
Age distribution (%)	
18 – 49	33
50 – 69	40
70 – 79	22
80 – 90	5
Hemodialysis (%)	51
Dialysis sessions per week (n)	3
Duration of dialysis session (hours)	5.1±0.7
Single pool Kt/V	1.73±0.38
Interdialytic weight gain (Kg)	1.7 (1.5 – 2.3)
Interdialytic weight gain relative to estimated dry weight (%)	2.3±1
Peritoneal dialysis (%)	49
Volume of peritoneal dialysis solution exchanged per 24 hours (L)	8 (8 – 10)
Four hour D/P creatinine	0.72±0.52
Weekly Kt/V	2.21 (1.95 – 2.49)
Time on dialysis (months)	35 (16 – 58)
Body Mass Index (Kg/m^2)	30.2 (28.5 – 34.6)
Systolic blood pressure (mmHg)	130±15

Diastolic blood pressure (mmHg)	74±12
Diabetes mellitus (%)	40
Current or former smoker (%)	51
Ischemic heart disease (%)	22
Peripheral- and/or cerebro-vascular disease (%)	9
Hydration status	
Ratio of extracellular to total body water	0.48±0.04
Overhydration volume relative to extracellular volume (%)	3±9
Normohydrated (%)	69
Moderately overhydrated (%)	21
Severely overhydrated (%)	10
Left ventricular structure and function	
Left ventricular hypertrophy (%)	
Nil	56
Mild	33
Moderate	11
Ejection fraction (%)	60±7
Left ventricular systolic dysfunction (%)	9
Diastolic dysfunction (%)	
Nil	13
Mild	24
Moderate	45
Severe	18
Antihypertensive agents	
Median number of anti-hypertensive agents	1(1-2)

Proportion on beta-blocker (%)	42
Proportion on ACE-I or ARB (%)	38
C-reactive protein (mg/L)	5.0 (2.1 – 12)
NT-proBNP (pg/mL)	
Median (IQR)	1698 (718 – 3742)
Distribution (%)	
< 300 pg/mL	7%
300 – 899 pg /mL	20%
900 – 4999 pg/mL	51%
5000 – 19,999 pg/mL	11%
≥ 20,000 pg/mL	11%

Abbreviations: D/P creatinine, ratio of creatinine concentration in dialysate to plasma; Kt/V, dialysis urea clearance; ACE-I, Angiotensin converting enzyme inhibitor; ARB, Angiotensin type-1 receptor blocker; NT-proBNP, N-terminal pro-B-type natriuretic peptide.

Table 2. Estimates of variance components of NT-proBNP, bidirectional reference change values for stated degrees of statistical confidence and index of individuality over weekly and monthly intervals for stable study participants.

	Coefficient of variation (%)			Reference change value			Index of individuality
	Analytic (CV_A)	**Between-person (CV_G)**	**Within-person (CV_I)**	**90%**	**80%**	**70%**	**CV_I:CV_G**
Weekly	1.9	153	27	-46% and +84%	-38% and +61%	-32% and +47%	0.18
Monthly	1.6	148	35	-54% and +119%	-46% and +84%	-39% and +64%	0.24

Sub-group	Weekly Within-person Coefficient of Variation CV_I % (95% CI)	p-value
Dialysis modality		
Peritoneal dialysis	24.5 (20.5 – 29.2)	0.45
Hemodialysis	29.3 (24.5 – 35.2)	
Coronary artery disease		
Absent	29.3 (25.3 – 34.0)	0.15
Present	18.8 (14.7 – 24.1)	
Left ventricular hypertrophy		
Absent	28.6 (24.3 – 33.7)	0.52
Present	24.3 (19,9 – 29.7)	
Diastolic dysfunction		
Nil / Mild	27.3 (22.8 – 32.8)	0.98
Moderate / Severe	27.1 (22.7 – 32.5)	
Hydration status		
Normohydration	28.4 (24.7 – 32.7)	0.28
Moderate / Severe overhydration	22.3 (15.9 – 31.3)	
CRP concentration (mg/L)		
1st tertile (2 - 4)	29.6 (23.9 – 36.7)	0.43
2nd tertile (5 - 13)	28.8 (23.3 – 35.7)	
3rd tertile (14 - 59)	20.6 (16.3 – 26.0)	
NT-proBNP concentration (pg/mL)		
1st quartile (180 - 718)	29.4 (22.6 – 38.5)	0.89
2nd quartile (719 - 1698)	29.7 (22.6 – 39.0)	

3rd quartile (1699 - 3742)	25.1 (19.6 – 32.1)
4th quartile (3743 - 44091)	23.9 (19.0 – 30.2)

Table 3. Within-person coefficients of variation of N-terminal pro-B-type natriuretic peptide (mean and 95% confidence intervals) by subgroups of dialysis modality, ischemic heart disease status, left ventricular hypertrophy, cardiac diastolic function, hydration status, tertiles of C-reactive protein (CRP) concentration, and quartiles of N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentration.

Chapter 7

Biological Variation of High Sensitivity Cardiac Troponin-T in Stable Dialysis Patients: Implications For Clinical Practice

The following chapter presents the findings of the biological variation study performed to estimate the within- and between-person coefficients of variation of plasma hs-cTnT in stable dialysis patients. The week-week estimates derived in this study aid the interpretation of serial hs-cTnT concentrations measured clinically for the diagnosis or exclusion of acute myocardial injury. The month-month estimates derived in this study will be key to the interpretation of serial measurements in any future clinical monitoring strategy, as well as for adjusting for any regression-to-mean effects in research practice.

The following chapter was published as peer reviewed original research:

Fahim MA, Hayen AD, Horvath AR, Dimeski G, Coburn A, Tan KS, Johnson DW, Craig JC, Campbell SB, Hawley CM: Biological variation of high sensitivity cardiac troponin-T in stable dialysis patients: implications for clinical practice. *Clin Chem Lab Med*, 2014 DOI:10.1515/cclm-2014-0838

The following chapter was also presented by the candidate at the finalist session of Young Investigator Award, Australia and New Zealand Society of Nephrology Annual Scientific Meeting, Brisbane, Australia (2013). Short and Long Term Biological Variation of high sensitivity Troponin T (hs-cTnT) and N-Terminal B-type Natriuretic Peptide (NT-proBNP) in The Stable Dialysis Population. Fahim M, Hayen A, Coburn A, Dimeski G, Johnson D, Craig J, Horvath A, Campbell S, Hawley C.

Chapter 7

Biological Variation of High Sensitivity Cardiac Troponin-T in Stable Dialysis Patients: Implications For Clinical Practice

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Running Title: Biological Variation of hs-cTnT in Dialysis

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ABSTRACT

BACKGROUND Changes in high sensitivity cardiac troponin-T (hs-cTnT) concentrations may reflect either acute myocardial injury or biological variation. Distinguishing between these entities is essential to accurate diagnosis, however the biological variation of hs-cTnT in dialysis population is currently unknown. We sought to estimate the within- and between-person coefficients of variation of hs-cTnT in stable dialysis patients, and derive the critical difference between measurements needed to exclude biological variation with 99% confidence.

METHODS Fifty five prevalent haemo- and peritoneal-dialysis patients attending two metropolitan hospitals were assessed on 10 consecutive occasions; weekly for 5-weeks then monthly for 4-months. Assessments were conducted at the same dialysis cycle time-point and entailed hs-cTnT testing, clinical review, electrocardiography, and bioimpedance spectroscopy. Patients were excluded if they developed clinical or physiological instability.

RESULTS 137 weekly and 114 monthly hs-cTnT measurements from 42 stable patients were analysed. Respective between- and within-person coefficients of variation were 83% and 7.9% for weekly measurements, and 79% and 12.6% for monthly measurements. Within-person variation was unaffected by dialysis modality or cardiac co-morbidity. The bidirectional 99% reference change value was -25% and +33% for weekly measurements, and -37% and +58% for monthly measurements.

CONCLUSIONS The between-person variation of hs-cTnT in the dialysis population is markedly greater than within-person variation indicating that hs-cTnT testing is best applied in this population using a relative change strategy. An increase of 33% or a reduction of 25% in serial hs-cTnT concentrations measured at weekly intervals excludes change due to analytical and biological variation alone with 99% confidence.

KEYWORDS

Renal Dialysis

Troponin T

Variability

INTRODUCTION

Acute myocardial injury is a leading cause of hospitalisation and death in the dialysis population (9.8-16 per 100-patient years), and cardiac troponins are among the most frequently requested tests in this group(1-5). A diagnosis of acute myocardial injury in dialysis patients is contingent on demonstrating a change in serial troponin concentrations in an appropriate clinical context(6, 7). However, there is currently no evidence-based guidance on how much change in serial measurements discriminates between biological variation and acute myocardial injury leading to considerable diagnostic confusion(8).

Biological or within-person variation describes the random fluctuation of biomarker levels around a homeostatic set-point in healthy individuals or those with stable disease, and is of no clinical significance(9). Failure to accurately discriminate between changes in troponin concentrations caused by biological variation versus acute myocardial injury can result in unnecessary alarm, inappropriate management, and patient harm(10). These concerns are particularly relevant following the recent introduction of high sensitivity cardiac troponin T (hs-cTnT) assays which are able to detect small changes in circulating concentrations(11). Current guidelines either do not specify a magnitude of change in hs-cTnT concentrations that excludes biological variation in dialysis patients(6, 8) or have developed theoretical recommendations based on the analytic performance of assays(7).

The aims of our study were to estimate the within- and between-person variation of hs-cTnT measured at weekly and monthly intervals from a cohort of stable dialysis patients and to use these estimates to calculate the percentage change between serial hs-cTnT measurements needed to exclude biological and analytic variation. We also sought to determine if the within-person variation of hs-cTnT differed according to cardiac co-morbidity or between dialysis modalities.

SUBJECTS AND METHODS

Study Design and Patient Recruitment

A prospective cohort study was conducted between October 2010 and April 2012 according to methods described by Fraser and Harris(12). The study complied with the declaration of Helsinki

and received ethics approval from the Metro-South Human Research Ethics Committee (HREC/10/QPAH/131).

Participants were recruited from the in-centre haemodialysis and peritoneal dialysis units of a tertiary-care teaching hospital in Brisbane, and a secondary-care hospital in Logan, Australia. Eligible participants identified from an electronic database of all patients receiving dialysis therapy were adults (aged ≥ 18-years) on maintenance dialysis for ≥ 90-days who had a stable dialysis prescription for ≥ 30-days and a transthoracic echocardiogram ≤ 12-months prior to screening. Eligibility criteria were chosen to ensure that the study cohort were physiologically and clinically stable at enrolment, and likely to remain stable for the duration of the study while still being representative of the dialysis population.

Patients were excluded if they had undergone coronary and/or valvular intervention or suffered a myocardial infarction or pulmonary embolism in the 6-months prior to screening; had echocardiographic evidence of severe pulmonary hypertension, severe functional aortic and/or mitral valvular disease, or a left ventricular ejection fraction < 30%; had been hospitalised for any indication in the 30-days prior to screening; had been commenced on or undergone a dose change of a diuretic, beta-blocker, aldosterone receptor antagonist, angiotensin converting enzyme inhibitor or angiotensin type-1 receptor blocker in the 30-days prior to screening; had experienced worsening angina, a new cardiac arrhythmia or undergone a dose change of associated therapies in the 30-days prior to screening; had a contraindication to bioimpedance measurement including a pacemaker, implantable cardiac defibrillator, joint replacements, or mechanical heart valve; were pregnant; had advanced malignancy; or were unable to provide informed consent.

Patient Assessment

Patients were assessed on 10 consecutive occasions - weekly for 5 weeks then monthly for another 4 months. All assessments were conducted between 6–8 AM, prior to the mid-week dialysis session for haemodialysis patients, and between 8–10 AM on the same weekday for peritoneal dialysis patients. Patients avoided strenuous exercise prior to assessment.

Several factors affect hs-cTnT concentrations including extracellular volume(13), cardiac rhythm(14), myocardial ischaemia(15), and the dialysis prescription(16, 17). These influences were assessed using a structured clinical interview, physical examination and medical records review to ascertain interim hospitalisation, changes to medication and/or the dialysis prescription. The

Canadian Cardiovascular Society Angina Grading Scale(18) was used to assess change in cardiac ischaemic symptoms and the Truncated Framingham Heart Failure Score(19) was used to assess for evidence of pulmonary oedema at time of review. Patients also underwent a standard 12-lead electrocardiogram and whole body, multi-frequency bioimpedance analysis using the Body Composition Monitor BCM® (Fresenius Medical Care, Asia-Pacific) at each visit to measure intra- and extra-cellular volume. This instrument has a detection limit for change in extracellular volume of 0.87L ± 0.64L(20, 21).

Specimen Collection, Storage and Analysis

hs-cTnT concentrations were measured at baseline then at weeks 1-4 and months 2-4, providing data for 4 consecutive weekly and monthly intervals. To ensure that changes in hs-cTnT concentrations during the final measurement interval did not reflect changes in subclinical risk, patients were also assessed for stability at week 5 and month 5.

Blood collected in lithium-heparin tubes was centrifuged and plasma separated within 1-hour of collection. Plasma was stored at -80°C until assayed. hs-cTnT has been shown to be stable under these conditions(22).

All samples were batched and analysed together in a single analytic-run in random duplicate by a single expert operator using a single instrument, and a single batch of reagent, control, and calibrators. Plasma hs-cTnT concentrations (nanograms per litre) were measured on the Cobas e170 instrument using the troponin-T hs kit (Roche Diagnostics, Australia); a monoclonal antibody electrochemiluminescence assay which has a reported detection range of 5 – 10,000 ng/L. The population 99th centile reference limit for the assay is 14 ng/L(11).

Statistical Analysis

Based on a ratio of analytic to within-person variation of < 0.5 for hs-cTnT, we estimated that a study sample of 40 patients undergoing hs-cTnT testing on 8 occasions over 4 weekly and monthly intervals would have power = 1.0 to estimate the within-person coefficient of variation with a 95% confidence interval of ± 1.9%(23). A sample size of 55 was chosen to allow for dropouts and exclusions due to instability.

Patients were deemed to be unstable if between study visits they underwent a change in dose of diuretic, beta-blocker, aldosterone receptor antagonist, angiotensin converting enzyme inhibitor or angiotensin type-1 receptor blocker; a change in severity of cardiac ischaemic symptoms, dose of

anti-anginal agents, or cardiac intervention; a change in anti-arrhythmic agent or new cardiac arrhythmia; a change in extracellular volume > 1L on bioimpedance analysis; a change in dialysis modality or prescription; hospitalisation for any reason or exhibited pulmonary oedema defined as a score ≥ 2 on the Truncated Framingham Heart Failure Score(19). If a study participant was deemed to be unstable, the hs-cTnT concentrations from the intervals preceding and following the event were excluded from the statistical analysis.

Normally distributed variables are summarised as mean ± standard deviation, and non-normally distributed variables as median and interquartile range. hs-cTnT concentrations were logarithmically transformed for the variation analyses. We fitted mixed-effects models with random intercepts to calculate the between-person coefficient of variation (CV_G), the within-person coefficient of variation at weekly and monthly intervals (CV_I) and the within-run analytic coefficient variation (CV_A). Outlying variances were excluded using the Reed and Cochran tests, and linear regression analysis was used to exclude participants who demonstrated a consistent trend in hs-cTnT concentrations in either direction which may reflect a change in subclinical risk.
The cohort was also divided into subgroups according to ischaemic heart disease status, severity of left ventricular diastolic dysfunction, presence or absence of left ventricular systolic dysfunction, quartiles of hs-cTnT concentrations at enrolment, and by dialysis modality. CV_I was estimated for each subgroup and compared using Bartlett's test. Ischaemic heart disease was defined as one or more of inducible ischaemia on non-invasive cardiac testing and/or ≥ 50% stenosis in ≥ 1 epicardial coronary artery on coronary angiography and/or a history of myocardial infarction. Left ventricular diastolic dysfunction was graded as absent, mild, moderate or severe according to an established algorithm using echocardiographic measurements(24). Left ventricular systolic dysfunction was defined as a left ventricular ejection fraction ≤ 50% using Simpson's rule(24).

The index of individuality (IOI) was calculated as CV_I/CV_G. This ratio gives an indication of whether a biomarker is best used within a relative-change monitoring strategy (IOI < 0.6) or a reference interval strategy (IOI > 0.6). The bidirectional reference change value (RCV) was calculated according to the method described by Fokkema et al(25) for logarithmically transformed data as = $\exp(-Z \times \sqrt{2} \times \sigma)$ and $\exp(+Z \times \sqrt{2} \times \sigma)$; where $\sigma = \sqrt{\ln(CV_I^2 + 1)}$ and Z is the Z-score of a standard normal distribution corresponding to a given probability.

RESULTS

Patient Characteristics

Details of the number of patients assessed, enrolled and included in the final analysis are shown in Figure 1. Fifty five patients were recruited from the haemodialysis (n=28) and peritoneal dialysis units (n=27) of the participating institutions and their baseline characteristics are summarised in Table 1. Cardiovascular risk factors, including hypertension (100%), diabetes mellitus (40%), and current or former smoking (51%) were highly prevalent. A substantial proportion of the cohort also had evidence of established cardiovascular disease including ischaemic heart disease (22%), left ventricular diastolic (87%) and/or systolic (9%) dysfunction, and peripheral- and/or cerebro-vascular disease (9%). Baseline hs-cTnT concentrations demonstrated a right skewed frequency distribution with a median of 34 (interquartile range 24 – 54) ng/L. 90% of the study cohort had a hs-cTnT concentration exceeding the 99th centile upper reference limit of the hs-cTnT assay(11).

Weekly and Monthly Variation of hs-cTnT

Seven patients and their corresponding hs-cTnT measurements were excluded due to hospital admission (n=3), paroxysmal atrial fibrillation (n=3), and escalating anginal symptoms (n=1). In addition, 36 weekly and 51 monthly hs-cTnT sample-pairs were excluded due to a change in extracellular volume of > 1L between consecutive visits. hs-cTnT measurements performed over 136 weekly intervals from 42 patients and 113 monthly intervals from 39 patients were included in the final analysis. These data are shown in Figure 2 with excluded measurements represented by gaps.

The respective analytic, within-person, and between-person coefficients of variation of hs-cTnT were 3.1%, 7.9%, and 83% for weekly intervals, and 2.4%, 12.6%, 79% for monthly intervals (Table 2). Between person variation was much greater than within-person variation (Figure 3), yielding low indices of individuality of 0.10 and 0.16 for the weekly and monthly intervals respectively (Table 2).

Weekly and monthly reference change values for the 80%, 90%, and 99% degrees of statistical confidence are shown in Table 2. Thus, hs-cTnT concentrations measured at weekly intervals needed to increase by 33% or fall by 25% to ensure with 99% confidence that the observed change exceeded analytic and biological variation alone. Monthly reference change values were larger than weekly values for a given degree of statistical confidence.

The within-person co-efficient of variation did not differ significantly between dialysis modalities, by ischaemic heart disease status, by severity of diastolic dysfunction or across quartiles of hs-cTnT concentration (Table 3). The effect of left ventricular systolic dysfunction on within-person variation was unable to be meaningfully analysed as only one such patient was retained in the final analysis after the exclusion of unstable patients.

DISCUSSION

This study demonstrated that the between-person variation of hs-cTnT across the dialysis population was large and markedly greater than within-person variation for both the weekly and monthly measurement intervals (83% vs. 7.9%, and 79% vs. 12.6% respectively). This was reflected in the low index of individuality and indicates that hs-cTnT measurements from dialysis patients are best interpreted by comparing serial measurements to each other rather than by comparing single values to a reference interval or a threshold value(26). These findings support current recommendations on interpreting troponin concentrations measured from dialysis patients(6, 7).

Interpreting serial hs-cTnT concentrations in dialysis patients

We found that the within-person coefficients of variation of hs-cTnT in stable dialysis patients were 7.9% and 12.6% for the weekly and monthly measurement intervals respectively. Using these values we calculated bidirectional reference change values for pre-specified degrees of statistical confidence (table 2)(25). The weekly 99% reference change value was +33% and -25%, indicating that hs-cTnT concentrations must increase by at least 33% or fall by 25% in the short-term to exclude change due to biological and analytic variation alone with 99% certainty. We suggest that this short term reference change value has the greatest relevance to clinical practice where troponin is measured over hours or days to diagnose acute myocardial injury. In addition, we suggest using the 99% level of statistical confidence as therapies that may be instituted after excluding biological variation carry risks of serious adverse events necessitating a high degree of certainty. It is also important to highlight that acute myocardial injury may result from either coronary or non-coronary aetiologies(6) and that careful clinical assessment remains imperative to determining the underlying cause of acute myocardial injury and to guide its management. We recommend using a relative (e.g. 33%) rather than absolute (e.g. 5-7 ng/L) change criterion as the former has been shown to be diagnostically superior in populations where the majority of individuals have a baseline hs-cTnT

concentration exceeding the upper reference limit of the assay used and where the between-person variation of hs-cTnT is large, both of which have been demonstrated in this study(27).

Explicit guidance on the interpretation of serial troponins measured from dialysis patients is necessary as the distinction between troponin fluctuations resulting from biological variation versus those occurring during acute coronary syndromes, particularly non-ST elevation myocardial infarction, is frequently unclear(28, 29). To date, consensus guidelines on the interpretation of serial troponin measurements in dialysis patients have either provided no guidance on this issue(6, 8) or have based their guidance on the analytic performance of troponin assays, recommending the use of a 20% magnitude of change on the basis that such a delta equates to 3 standard deviations of the troponin assay's analytic variation(7). Based on the within-person coefficient of variation estimated in our study, a 20% change in serial troponin concentrations would only have a 90% degree of statistical confidence for excluding biological and analytic variation, and would therefore not be sufficiently specific for excluding biological variation.

Impact of dialysis therapy and cardiac co-morbidity on biological variation

The weekly and monthly within-person variation of hs-cTnT estimated from dialysis patients in this study was markedly smaller than corresponding values reported for healthy individuals (15% and 31% respectively)(30), but similar to those reported for non-dialysis dependent individuals with coronary artery disease (7.3% at 4-hourly intervals and 10.7% at 3-weekly intervals)(31). These findings are likely explained by the fact that both dialysis patients and non-dialysis dependent patients with coronary artery disease have baseline troponin-T concentrations around or exceeding the 99th centile upper reference limit of the hs-cTnT assay(16, 31) whereas healthy individuals have much lower baseline values(11). Accordingly, any fluctuations in troponin concentrations caused by biological variation in the former groups represent a smaller proportion of the baseline concentration resulting in a smaller within-person coefficient of variation.

In our study the within-person coefficient of variation of hs-cTnT was not significantly different between dialysis modalities, by ischaemic heart disease status, by severity of diastolic dysfunction, or across quartiles of baseline hs-cTnT concentrations implying that the reference change values described previously can be applied to dialysis patients regardless of their cardiac comorbidity status or dialysis modality. Furthermore, the analytic variation in our study was consistent with that reported from a multi-centre evaluation of the same analytic method using standard laboratory protocols(11). It is therefore unlikely that the reference change values reported in this study would

be appreciably affected by analytic differences related to 'real world' performance of the hs-cTnT assay.

Strengths and limitations

Our estimates of the within-person variation of hs-cTnT in the dialysis population are supported by the findings of Aakre et al(32), Pianta et al(16) and Jacobs et al(33) who reported a within-person coefficient of variation of 8.3%, 9.7% and 13% for hs-cTnT measured weekly, fortnightly and monthly respectively in haemodialysis patients. However, these studies were limited by the exclusion of peritoneal dialysis patients(16, 32, 33), a paucity of repeated measures(16, 33) and most importantly by a lack of rigour in ensuring patient stability, wherein they relied either on patient report(16) or a history of hospitalization(32, 33) alone to determine stability. The latter introduces the possibility that unstable patients may have been included in the analyses of these studies and impacted the accuracy of their findings. Indeed, Aakre et al reported that hs-cTnT concentrations measured over 90-minute intervals in their study demonstrated a consistent decreasing trend; this may reflect recovering acute myocardial injury due to haemodialysis induced cardiac stunning(34) which was not considered by the authors(32).

Our study has a number of important strengths that address these limitations, including the enrolment of both peritoneal- and haemo-dialysis patients, multiple measurements of hs-cTnT, and a rigorous approach towards ensuring stability of all factors affecting hs-cTnT concentrations. Nevertheless, the present study has several limitations. Firstly, patients with the most severe cardiovascular co-morbidities were excluded to maximise the likelihood that enrolled patients would remain stable for the duration of follow-up. Although this potentially limits the generalizability of the study's findings, it is noteworthy that the baseline characteristics of our study cohort were similar to those reported for prevalent Australasian dialysis patients in the Australian and New Zealand Dialysis and Transplant Registry(35). Secondly, the biological variation of hs-cTnT over 3-hourly intervals was not investigated. While this would have most closely approximated the interval over which hs-cTnT is measured in clinical practice, such a study design would have imposed substantial inconvenience on participants, necessitating a 15 hour visit and 5 venepunctures on a non-dialysis day and would likely have limited participation. Finally, our study could not determine if the within-person coefficient of variation of hs-cTnT was affected by left ventricular systolic impairment as only one such patient remained stable during the study.

Conclusions

Based on the findings of this study, it is recommended that hs-cTnT concentrations measured in dialysis patients for the diagnosis of acute myocardial injury be interpreted using a relative change strategy rather than by comparing absolute concentrations to a reference interval. An increase of over 33% or a reduction of 25% in serial hs-cTnT concentrations measured at weekly intervals from dialysis patients excludes change due to biological and analytic variation alone with 99% certainty and should prompt investigation for coronary and/or non-coronary causes of acute myocardial injury; concurrent clinical assessment remains essential to establishing the underlying cause of acute myocardial injury and to guide therapy accordingly. Using the change limits identified in this study, future randomised studies should investigate if early intervention for minor, but significant, changes in cardiac troponin concentrations improves patient outcomes in the dialysis population as has been demonstrated in the non-dialysis population(36).

STATEMENT OF COMPETING FINANCIAL INTERESTS

None of the authors have a relationship with industry or a conflict of interest to declare.

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FIGURE LEGENDS

Figure 1. Flow diagram of patients assessed for eligibility, enrolled and analysed in the study.

Figure 2. Variation of high sensitivity cardiac troponin T (hs-cTnT) concentrations measured from each of the stable participants over the weekly and monthly follow-up phases.

Figure 3. Box-plot of median (horizontal line), upper and lower quartiles (large rectangle), and range (whiskers) of high sensitivity cardiac Troponin T (hs-cTnT) concentrations for each of the stable participants during the weekly follow-up phase.

Figure 1

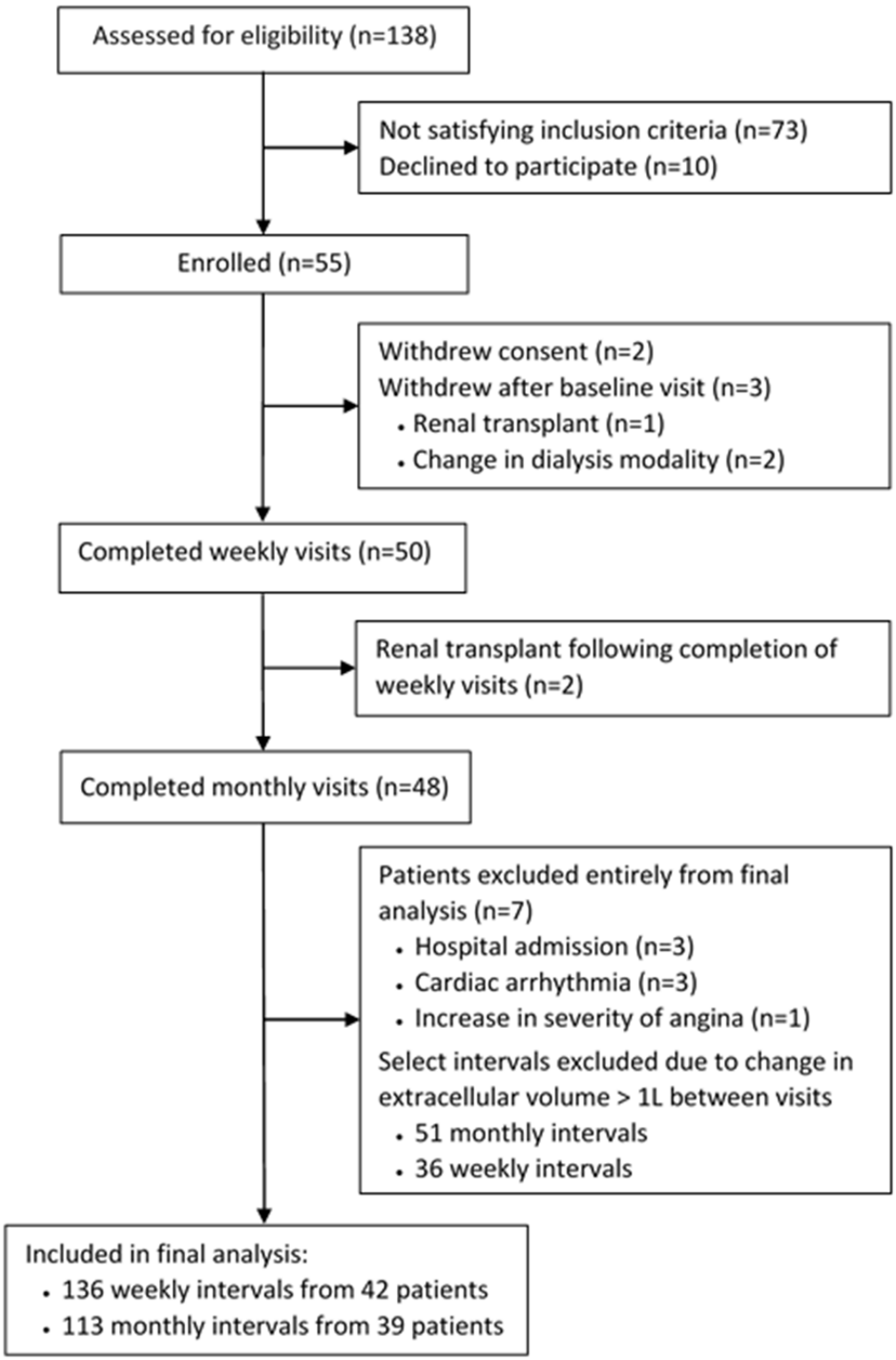
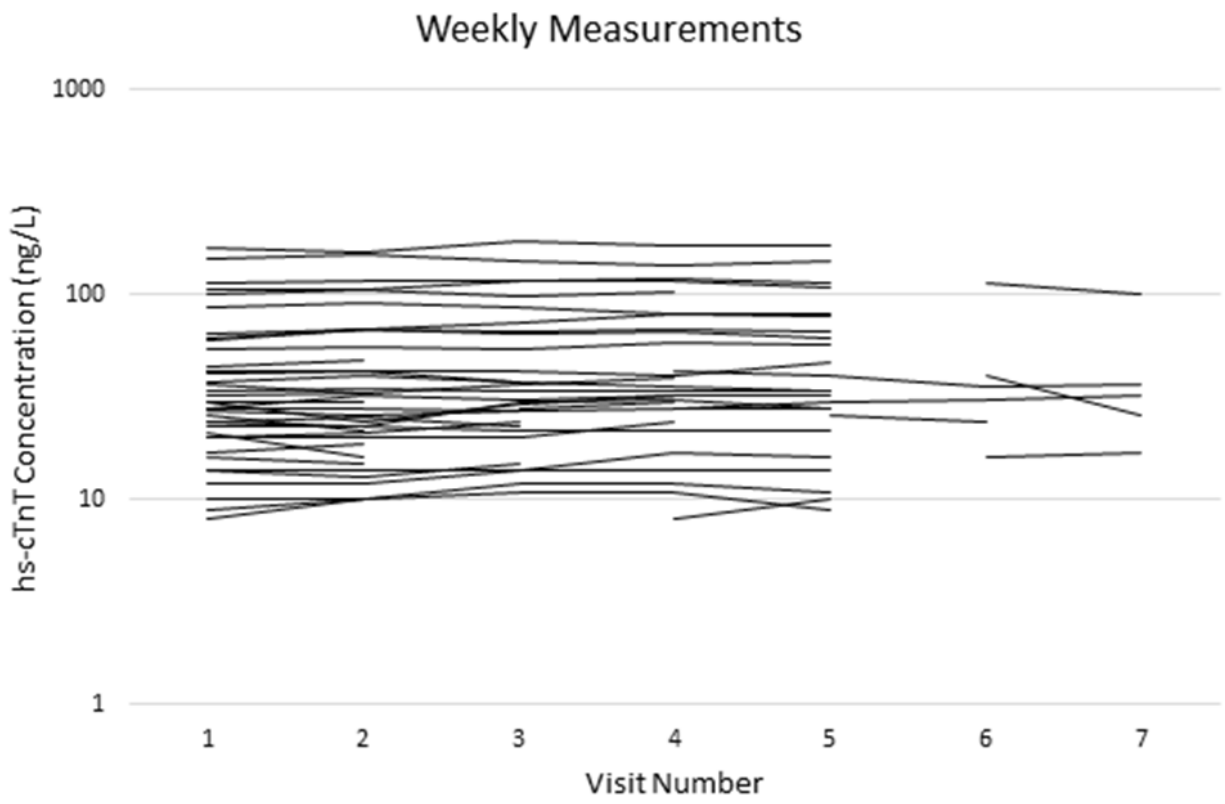

Assessed for eligibility (n=138)
Not satisfying inclusion criteria (n=73)
Declined to participate (n=10)
Enrolled (n=55)
Withdrew consent (n=2)
Withdrew after baseline visit (n=3)
• Renal transplant (n=1)
• Change in dialysis modality (n=2)
Completed weekly visits (n=50)
Renal transplant following completion of weekly visits (n=2)
Completed monthly visits (n=48)
Patients excluded entirely from final analysis (n=7)
• Hospital admission (n=3)
• Cardiac arrhythmia (n=3)
• Increase in severity of angina (n=1)
Select intervals excluded due to change in extracellular volume > 1L between visits
• 51 monthly intervals
• 36 weekly intervals
Included in final analysis:
• 136 weekly intervals from 42 patients
• 113 monthly intervals from 39 patients

Figure 2


Weekly Measurements
1000
100
10
1
hs-cTnT Concentration (ng/L)
1
2
3
4
5
6
7
Visit Number

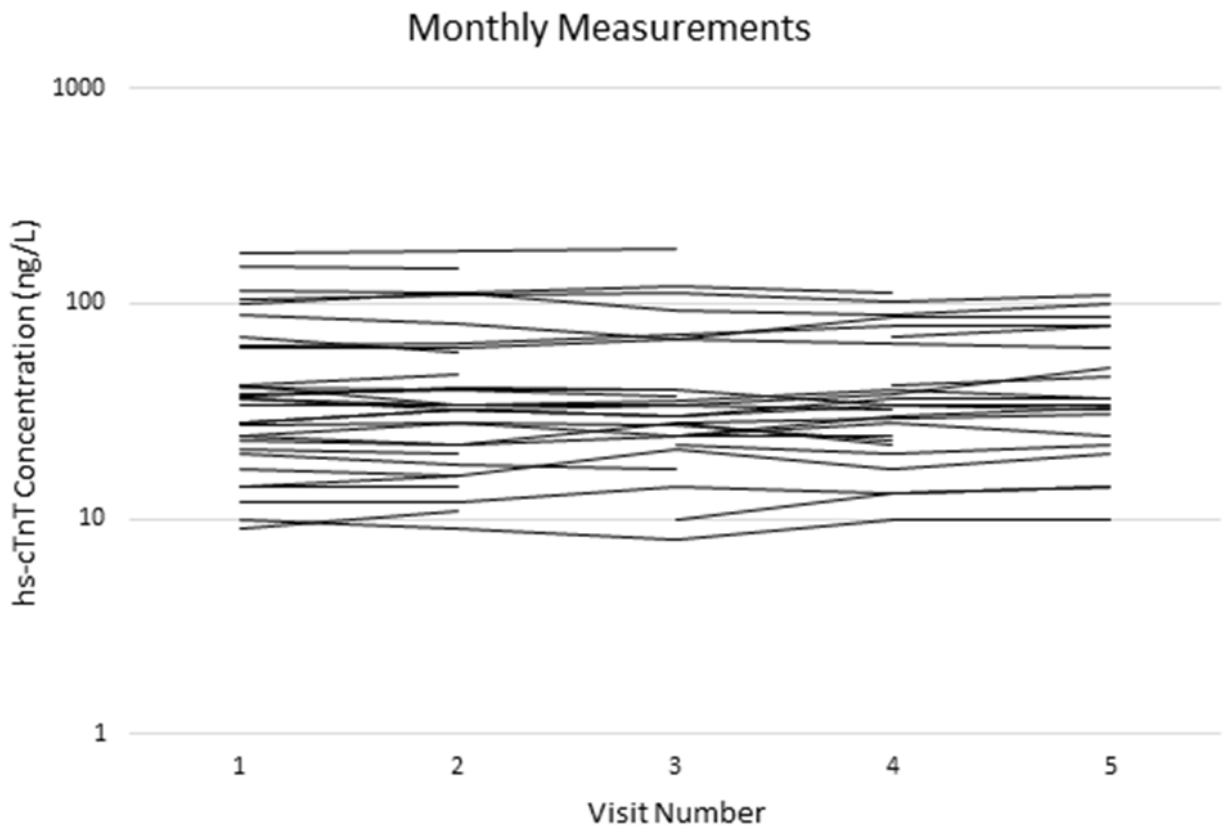

Monthly Measurements
1000
100
10
1
hs-cTnT Concentration (ng/L)
1
2
3
4
5
Visit Number

Figure 3

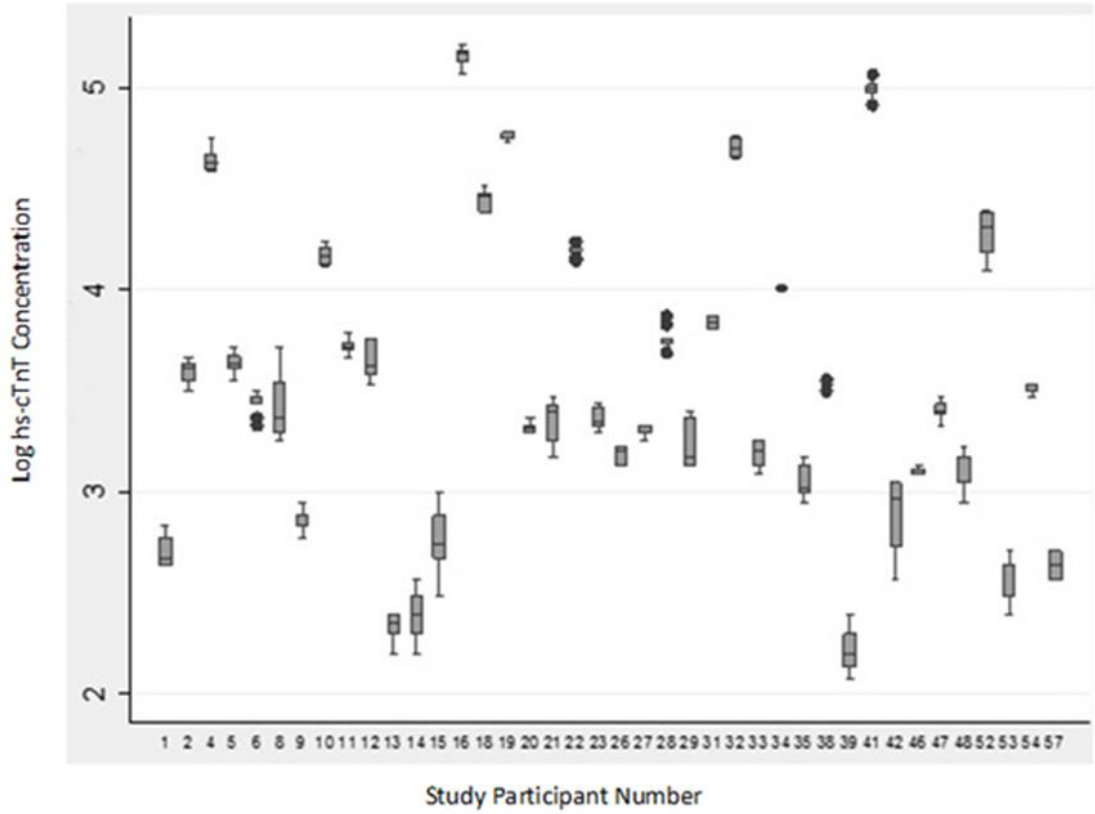

Log hs-cTnT Concentration
2
3
4
5
1 2 4 5 6 8 9 10 11 12 13 14 15 16 18 19 20 21 22 23 26 27 28 29 31 32 33 34 35 38 39 41 42 46 47 48 52 53 54 57
Study Participant Number

Table 1. Baseline characteristics of the study cohort.

Characteristic	**Value (n=55)**
Male gender (%)	45
Age	
Mean ± sd (years)	59±15
Age distribution (%)	
18 – 49 years	33
50 – 69 years	40
70 – 79 years	22
80 – 90 years	5
Haemodialysis (%)	51
Time on dialysis (months)	35 (16 – 58)
Body mass index (Kg/m^2)	30.2 (28.5 – 34.6)
Ratio of extracellular to total body water	0.48±0.04
Systolic blood pressure (mmHg)	130±15
Diastolic blood pressure (mmHg)	74±12
Diabetes mellitus (%)	40
Current or former smoker (%)	51
Ischaemic heart disease (%)	22
Peripheral- and/or cerebro-vascular disease (%)	9
Left ventricular ejection fraction	
Mean±sd (%)	60±7
Left ventricular systolic dysfunction (%)	9
Diastolic dysfunction (%)	
Nil	13

Mild	24
Moderate	45
Severe	18
Antihypertensive agents	
Median number of anti-hypertensive agents	1(1-2)
Proportion on beta-blocker (%)	42
Proportion on ACE-I or ARB (%)	38
hs-cTnT (ng/L)	
Median (IQR)	34 (24 – 150)
Minimum	8
Maximum	241
Proportion of sample (%) with baseline hs-cTnT concentration > 14 ng/L	90%

Abbreviations: ACE-I, Angiotensin converting enzyme inhibitor; ARB, Angiotensin type-1 receptor blocker

Table 2. Estimates of variance components of high sensitivity troponin-T, bidirectional reference change values for stated degrees of statistical confidence and index of individuality over weekly and monthly intervals for stable study participants.

	Coefficient of variation (%)			Reference change value			Index of Individuality
	Analytic (CV_A)	**Between-person (CV_G)**	**Within-person (CV_I)**	**99%**	**90%**	**80%**	**CV_I:CV_G**
Weekly	3.1	83	7.9	-25% and +33%	-17% and +20%	-13% and +15%	0.10
Monthly	2.4	79	12.6	-37% and +58%	-25% and +34%	-20% and +25%	0.16

Table 3. Within-person coefficients of variation of high sensitivity cardiac troponin-T (mean and 95% confidence intervals) by subgroups of dialysis modality, ischaemic heart disease status, cardiac diastolic function and quartiles of high sensitivity cardiac troponin-T concentration (hs-cTnT).

Sub-group	Weekly Within-person Coefficient of Variation CV_I % (95% CI)	p-value
Dialysis modality		
Peritoneal dialysis	7.5 (6.2 – 9.0)	0.68
Haemodialysis	8.2 (6.8 – 10.0)	
Ischaemic heart disease		
Absent	5.7 (4.5 – 7.3)	0.90
Present	8.5 (7.3 – 9.9)	
Diastolic dysfunction		
Nil / Mild	8.2 (6.8 – 9.9)	0.78
Moderate / Severe	7.7 (6.4 – 9.3)	
hs-cTnT concentration (ng/L)		
1st quartile (8 - 24)	10.3 (7.8 – 13.4)	
2nd quartile (25 - 34)	8.3 (6.5 – 10.7)	0.31
3rd quartile (35 - 54)	6.3 (4.6 – 8.8)	
4th quartile (55 - 241)	5.6 (4.5 – 7.1)	

Chapter 8

Pathophysiologic Associations of the N-terminal Fragment of Pro-B-type Natriuretic Peptide (NT-proBNP) in the Dialysis Population.

The following chapter investigates the associations between plasma NT-proBNP concentrations and objective measures of hydration state, vascular function, left ventricular mass and function, and comorbid conditions in a cohort of prevalent haemodialysis and peritoneal dialysis patients. It provides insights into the pathophysiological factors mediating abnormal NT-proBNP concentrations in the dialysis population, and consequently how levels should be interpreted and what interventions might be considered in any future monitoring strategies.

This chapter is in the process of being submitted for publication.

Chapter 8

Pathophysiologic Associations of the N-terminal Fragment of Pro-B-type Natriuretic Peptide (NT-proBNP) in the Dialysis Population

INTRODUCTION

Patients on maintenance dialysis have a 10-60 times increased risk of cardiovascular mortality compared to their non-dialysis counterparts, and this risk has not improved substantively over the past decade(1-4). A key factor underpinning this poor progress is our inability to identify dialysis patients at an increased risk of cardiac morbidity and/or mortality in a timely manner or to monitor the efficacy of cardiac interventions(5).

The N-terminal fragment of pro-B-type natriuretic peptide (NT-proBNP) is an inactive peptide secreted principally from the ventricular myocardium in response to stretch, strain, ischemia, inflammation and sympathetic overactivity(6). NT-proBNP concentrations have been shown in cohort studies to be independently associated with the risk of cardiovascular and all-cause mortality among the dialysis population(7-9) positioning it as a candidate biomarker for monitoring cardiac risk.

However, the translation of NT-proBNP testing into clinical dialysis practice has been hampered by uncertainty regarding the pathophysiological factors mediating abnormal NT-proBNP concentrations in this population, and consequently how levels should be interpreted and what interventions might be used to modify this heightened risk(10, 11). Studies examining the association between NT-proBNP concentrations and volume status, cardiomyopathy, and vascular stiffness in the dialysis population have yielded conflicting conclusions; suggesting it is either a marker of cardiomyopathy alone(12-22) or hydration status alone(23-28). These contradictory findings can be attributed, at least in part, to inadequate adjustment for confounding variables(17-19, 24-26, 28), small sample sizes(22, 24, 25), use of surrogate measures of volume(16-21), and insensitive measures of myocardial contractility(20, 22-26, 28).

The aim of this study was to determine the associations between NT-proBNP concentrations and objective measures of hydration state, vascular function, left ventricular mass and function, and comorbid conditions in a cohort of prevalent haemodialysis and peritoneal dialysis patients.

METHODS

Study Design and Patient Recruitment

A cross-sectional study was conducted between June 2011 and August 2013. The study complied with the declaration of Helsinki and received ethics approval from the Metro-South Human Research Ethics Committee (HREC/10/QPAH/131), the Greenslopes Research and Ethics Committee (Protocol 12/39), and the University of Queensland Medical Research Ethics Committee (2011000484).

Participants were recruited from the in-centre haemodialysis and peritoneal dialysis units of a public, tertiary-care teaching hospital and a private, secondary-care hospital in metropolitan Brisbane, Australia. Eligible participants identified from an electronic database of all patients receiving dialysis were adults (aged ≥18-years) established on maintenance dialysis for ≥90-days who had a stable dialysis prescription for ≥30-days, and were able and willing to provide informed consent.

Patients were excluded if they were planned for living donor renal transplantation within 3-months of enrolment, had advanced malignancy, were pregnant, were unable to provide informed consent or had a contraindication to whole body bioimpedance spectroscopy measurement (including a permanent pacemaker, implantable cardiac defibrillator, joint replacements, orthopaedic pins, mechanical heart valves and/or limb amputations).

Patient Assessment

Haemodialysis patients were assessed between 6–8 AM, prior to the mid-week dialysis session, and peritoneal dialysis patients were assessed between 8–10 AM on a consistent weekday. Patients were instructed to avoid strenuous exercise prior to assessment. Assessments entailed a structured clinical interview, physical examination, and review of the medical record to ascertain medical history, comorbidities, current treatments and dialysis prescription. In addition, patients underwent a standard 12-lead electrocardiogram, whole

body bioimpedance spectroscopy and blood sampling for measurement of NT-proBNP on the same occasion.

For haemodialysis patients, the blood pressure recorded was the median of 12 post-dialysis measurements taken at the end of the 12 haemodialysis treatments that preceded the study assessment, while for peritoneal dialysis patients it was the median of 12 home blood pressure recordings measured thrice weekly over the 4 weeks prior to the study assessment. The median of these blood pressure recordings has been shown to have the greatest agreement with 24-hour ambulatory measurements and therefore to be most representative of the patient's blood pressure(29).

Hydration status was assessed using whole-body, multi-frequency bioimpedance analysis with the Body Composition Monitor BCM® (Fresenius Medical Care, Asia-Pacific). This instrument has been validated in dialysis patients against radioisotope dilution methods with reported agreement limits (mean ± standard deviation [SD]) of -0.2±2.3L, -0.4±1.4L and 0.2±2L for total body water, extra- and intra-cellular water volumes, respectively(30). Peritoneal dialysis patients were assessed with peritoneal dialysis fluid in situ, which has been previously shown to have no significant or clinically important effect on volume measurements compared with an empty peritoneal cavity(31).

Echocardiography and pulse wave velocity measurement

Echocardiograms and pulse wave velocity measurements were performed within one week of NT-proBNP sampling, and for haemodialysis patients they were performed on the non-dialysis day immediately following the mid-week dialysis treatment to ensure that cardiac loading conditions were representative of the patient's usual volume state. Echocardiograms were performed and measured by one of two expert operators blinded to patient's NT-proBNP concentrations and medical history.

Echocardiograms were performed in the left lateral decubitus position using a 3.5MHz phased array transducer with harmonic imaging (Vivid7, General Electric-Vingmed Medical Systems, Horten, Norway). Grayscale images from the parasternal long-axis and short axis as well as the apical 4-chamber, 2-chamber and apical long axis views were acquired at moderate frame rate (50-70 fps) to allow for processing of 2D strain; apical images in the 4-chamber, 2-chamber and apical long axis were acquired at high frame rate (>120 fps) with a

colour tissue Doppler overlay to measure colour tissue Doppler velocity, strain and strain rate. Doppler measurement of LV inflow, TV inflow and aortic and pulmonary outflow were performed using pulsed and continuous wave Doppler. Colour tissue Doppler measurements were performed using pulsed wave Doppler in the medial and lateral LV annulus, tricuspid annulus and for all LV segmental measurements.

Cine loops of three cardiac cycles were acquired and the measurements averaged. If patients were in atrial fibrillation, 5-7 measurements were averaged. Left atrial and ventricular dimensions were obtained by M-mode according to the American Society of Echocardiography Recommendations(32); endocardial borders were traced at end systole and end diastole in the apical 4—chamber and 2-chamber views to obtain Simpson's Rule ejection fraction; M-mode LV mass was calculated using the formula : LV mass =0.8 × (1.04[(LV internal dimension + septal wall thickness + posterior wall thickness)3 – LV internal dimension3]) + 0.6g, and these were indexed to the patient's body surface area calculated using the Haycock formula (0.024265 x Weight(kg)0.5378 x height(cm)0.3964).

For 2D strain analysis, the endocardial borders were traced at end-systole in the 4-chamber, 2-chamber and apical long axis views; the software (EchoPac PC BTO 11, General Electric-Vingmed Medical Systems, Horten, Norway) divided the LV into basal, mid and apical segments, and identified unique "speckles" within the myocardium and tracked them frame-by-frame throughout the cardiac cycle to obtain strain and strain rate curves. Segments that did not track properly were manually adjusted or eliminated from the analysis. Global longitudinal strain (GLS) was expressed as a mean of all 18 segments.

The SphygmoCor PX system (AtCor Medical, Australia) was used for measurement of pulse wave analysis (augmentation index) and pulse wave velocity (PWV). Carotid-femoral PWV was the only method measured. The distances between the common carotid artery and the suprasternal notch, the suprasternal notch and the umbilicus, and the umbilicus and the femoral artery were measured. Tonometery was performed in the common carotid artery as close to the aortic arch as possible and at the femoral artery. The foot-to-foot times to reach the common carotid artery and the femoral artery were then subtracted and pulse wave velocity was expressed as metres per second (m/s). A total of three measurements were taken and averaged for analysis.

NT-proBNP sample collection, storage and analysis

Blood for measurement of NT-proBNP concentrations was collected in lithium-heparin tubes, prior to the commencement of the dialysis treatment for haemodialysis patients. Blood samples were centrifuged and plasma separated within 1 hour of collection and then stored at -80°C until assayed(33).

Plasma NT-proBNP concentration (pg/mL) was measured using a twin-antibody electrochemiluminescence assay on the Elecsys 2010 instrument (Roche Diagnostics, Australia) which has a reported analytical detection range of 5–35,000 pg/mL. Analytic coefficients of variation reported by the analysing laboratory were 2.9% and 1.8% at concentrations of 134 pg/mL and 4534 pg/mL respectively in keeping with desired performance of the assay(34, 35).

Definitions

Ischemic heart disease was defined as any of inducible ischaemia on non-invasive cardiac stress testing and/or ≥50% stenosis in ≥1 epicardial coronary artery on coronary angiography and/or a history of myocardial infarction and/or a history of coronary artery angioplasty, stenting or coronary artery bypass grafting.

Systolic dysfunction was classified either as an ejection fraction ≤ 50% measured using Simpson's rule(36) or a global longitudinal strain > -15% which has been associated with increased risk of mortality in both the general and dialysis populations(37-39). Diastolic dysfunction was graded as absent (normal diastolic function) or assigned a grade from one to three reflecting worsening diastolic dysfunction according to a consensus guidelines(40). Gender specific limits from consensus guidelines were used to classify left ventricular hypertrophy as absent, mild, moderate or severe according to left ventricular mass indexed to body surface area(32).

Hydration status was expressed as the ratio of extracellular to total body water volumes, and overhydration defined as a ratio two standard deviations above the mean age and gender matched ratio in the normal population(11, 31).

Statistical analysis

Categorical variables are presented as frequencies and percentages, and continuous variables as mean ± standard deviation if normally distributed and as median and interquartile range if

non-normally distributed. NT-proBNP concentrations were logarithmically transformed for correlation, univariable and multivariable linear regression analyses. Bivariate correlation between log transformed NT-proBNP concentrations and independent variables were analysed using pairwise correlation.

Thirteen participants had at least one missing measurement for either global longitudinal strain (n=1), left ventricular mass (n=7), and/or pulse wave velocity (n=12) which could not be measured due to body habitus or arrhythmia. Examination of the missing data demonstrated an arbitrary pattern and missing values were assumed to be missing at random. Multiple imputation was used to impute missing values using a multivariable normal model with 20 imputations(41). Univariable and multivariable linear regression analyses were performed using imputed data as the primary analyses, and repeated using non-imputed data as a sensitivity analysis (supplement). The latter did not alter the results in a clinically meaningful manner.

Independent variables for inclusion in the linear regression models were chosen based on published associations with NT-proBNP. Independent variables identified as being significant at the 5% level ($P < 0.05$) from the univariable analyses were analysed in the multivariable linear regression model. Variable selection was not performed. Global longitudinal strain and ejection fraction are both measures of left ventricular contractility with differing sensitivity, thus they were used individually as independent variables in two separate multivariable linear regression analyses. Regression diagnostics were performed for both multivariable models and were satisfactory. Semi-partial correlations were calculated for independent variables to determine increment to R^2. All analyses were performed using Stata/MP 12.1 (College Station, Tx, USA).

RESULTS

Participant characteristics

Seventy eight prevalent patients were recruited between June 2011 and January 2014 from the haemodialysis (n=47) and peritoneal dialysis (n=31) units of the participating institutions and their baseline characteristics are shown in Table 1. Cardiovascular risk factors and established cardiovascular disease were highly prevalent among the study cohort, including

diabetes mellitus (45%), hypertension (100%), current or former smoking (61%), ischaemic heart disease (41%) and peripheral and/or cerebrovascular disease (16%). In addition, a substantial proportion of participants had evidence of cardiomyopathy on echocardiography including left ventricular hypertrophy (67%) and/or diastolic dysfunction (84%). The prevalence of systolic dysfunction differed according to the measure of left ventricular systolic function used with 32% of the cohort having an ejection fraction under 50%, while 56% of the cohort had a global longitudinal strain measurement > -15%. Fifty seven percent of males and seventy one percent of females in the study cohort were overhydrated on bioimpedance analysis. Baseline NT-proBNP concentrations demonstrated a right skewed frequency distribution with a median of 3344 (interquartile range 1080-9786) pg/mL. Ninety five percent of the study cohort had a NT-proBNP concentration above 300 pg/mL, the threshold used to exclude acute decompensated heart failure in the general population.

Bivariate correlation and univariable linear regression analyses are shown in Table 2 and Figure 1. These analyses demonstrated significant direct correlations between NT-proBNP concentrations and age, female gender, hydration status, global longitudinal strain, increasing grade of diastolic dysfunction, ischaemic heart disease and left ventricular mass indexed to body surface area. In addition, significant inverse correlations were demonstrated between NT-proBNP concentrations and left ventricular ejection fraction and residual renal function.

Multivariable linear regression

Two multivariable linear regression models were used to analyse associations between NT-proBNP concentrations and significant independent variables identified from the univariable analyses. The models differed in terms of the measure of left ventricular systolic function used, with model 1 using global longitudinal strain and model 2 using left ventricular ejection fraction (table 3). Both models demonstrated significant associations between NT-proBNP concentrations and hydration status and left ventricular mass. However a significant association with left ventricular systolic function was only demonstrated in model 1 using global longitudinal strain, which also resulted in a significant increment to R^2 of 0.06 ($p=0.003$) as shown in Figure 2. In the multivariable model, hydration status explained the greatest proportion of the variation in NT-proBNP concentrations (standardised $\beta = 0.313$, $p<0.01$) followed by left ventricular mass (standardised $\beta = 0.238$, $p<0.01$), global longitudinal strain (standardised $\beta = 0.233$, $p<0.01$) and residual renal function (standardised $\beta = -0.182$, $p=0.05$).

DISCUSSION

This study demonstrated an independent association between NT-proBNP concentrations and hydration status, left ventricular systolic function and left ventricular mass, and furthers understanding of the pathophysiological mechanisms underlying the association between NT-proBNP concentrations and the heightened risk of mortality in dialysis patients.

Overhydration, impaired myocardial contractility and left ventricular hypertrophy each result in cardiac volume and/or pressure overload and stretch/strain of cardiac myocytes, which in turn secrete BNP and NT-proBNP in equimolar amounts(42). It is noteworthy that ventricular pressure overload can occur in overhydrated dialysis patients, even in the absence of structural or functional cardiomyopathy, accounting for the independent association of plasma NT-proBNP concentrations with hydration status observed in this study(43).

Cross-sectional studies have previously reported a significant association between NT-proBNP concentrations and either hydration status(23-28) or left ventricular systolic function(12-22) on multivariable analysis, but not with both independent variables concurrently. The discrepancy between these findings and those of the present study might be explained the limitations of the previous studies, including small sample sizes(22, 24, 25), inadequate control for confounding variables(17-19, 22, 24-26, 28) and the use of surrogate measures of hydration(16-21) and/or ejection fraction as the measure of systolic function(20, 22, 23, 25, 26, 28) and the measurement error introduced as a consequence. In the present study, whole body, multi-frequency bioimpedance spectroscopy was used as an objective, validated measure of hydration status(11, 30) and global longitudinal strain as a sensitive measure of ventricular contractility(37, 39). The importance of the latter is highlighted by the finding in this study that NT-proBNP concentrations were significantly associated with global longitudinal strain but not ejection fraction. Global longitudinal strain measures deformation of the ventricular myocardium by frame-by-frame tracking of natural acoustic markers generated from the interaction between ultrasound and the myocardium, and is less prone to errors caused by variation in heart rate and cardiac loading(44). It has been independently associated with mortality in dialysis patients and compared with ejection fraction, has been shown to be a more sensitive and objective measure of myocardial contractility(37).

Overhydration(45, 46), systolic dysfunction and left ventricular hypertrophy(47, 48) have all been independently associated with the risk of cardiovascular morbidity and mortality in the dialysis population, although strategies for monitoring them remain suboptimal. Clinical assessment of hydration status is inaccurate and subject to high interobserver variability(49), while bioimpedance, lung ultrasound(50), and echocardiography are likely to entail substantial financial and personnel costs, which may prohibit their universal and/or on a regular application(51). Echocardiography and technological volume assessment are also limited by the fact that they each measure a single risk factor without quantifying its effect on composite cardiac risk in the individual being evaluated. NT-proBNP has the potential to address these limitations, however the ideal strategy for implementing this biomarker in practice remains unclear.

Efforts at translating NT-proBNP testing into clinical usage have so far focused on establishing a threshold concentration below which cardiomyopathy or overhydration(13, 16, 21, 52) can be confidently excluded in a manner akin to which a NT-proBNP concentration less than 300 pg/mL is used to exclude acute decompensated heart failure in the non-dialysis population(53). The finding in this study that NT-proBNP concentrations are independently associated with three important pathophysiologies makes it unlikely that any single threshold concentration will have the sensitivity or specificity required to exclude systolic dysfunction, ventricular hypertrophy and overhydration concurrently. In addition, recent studies have demonstrated that the between-person variation of NT-proBNP in the dialysis population is large and much greater than its within-person variation implying that a relative change strategy may be more appropriate(54, 55) to monitor cardiovascular risk related to hydration status and cardiomyopathy.

This study also demonstrated a significant, inverse correlation between NT-proBNP concentrations and residual renal function, which can be attributed to the fact that NT-proBNP has a fractional renal extraction of approximately 20%(56). However, as demonstrated in this study, impaired renal clearance only accounts for a small proportion of the elevation and variation in NT-proBNP concentrations among dialysis patients which is principally explained by hydration state and cardiomyopathy. Significant associations demonstrated between NT-proBNP and age, gender, diastolic dysfunction and ischaemic heart disease on univariable linear regression analyses were not borne out on multivariable

linear regression analyses suggesting that univariable associations were confounded by other independent variables.

This study has several strengths including the standardisation of NT-proBNP sampling, handling and storage to minimise pre-analytic variation, its use of sensitive and objective measures of myocardial contractility (global longitudinal strain) and volume (whole body bioimpedance spectroscopy), and its inclusion of both haemodialysis and peritoneal-dialysis patients thereby broadening the generalizability of its findings. Nevertheless, this study had two principal limitations including its sample size, which restricted the number of independent variables that could be explored in the multivariable linear regression models, and the lack of measurement of inflammatory markers which have been associated with NT-proBNP concentrations in the dialysis population in previous reports(23).

Conclusion

This study demonstrated that NT-proBNP concentrations in prevalent haemodialysis and peritoneal dialysis patients were independently associated with hydration status, left ventricular mass, left ventricular systolic function (assessed using global longitudinal strain) and residual renal function. Coupled with the established association between NT-proBNP concentrations and mortality, these findings suggest that NT-proBNP testing may have a role in monitoring the risk of adverse cardiac events related to hydration state and cardiomyopathy in the dialysis population. Longitudinal studies are needed to determine the accuracy of such a strategy before it can be adopted into clinical practice.

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Figure Legends

Figure 1. Bivariate correlation between NT-proBNP concentrations and hydration status, left ventricular mass and systolic function. Scatter plots, regression line and 95% confidence interval (grey area). a, P = < 0.001; BSA = body surface area; ECW = extracellular water volume; LV = left ventricular; r = correlation coefficient; TBW = total body water.

Figure 2. Proportion of variation in NT-proBNP concentrations explained by hydration status, residual renal function, and left ventricular mass and systolic function. Increment to the coefficient of determination (R^2) due to residual renal function (semi-partial correlation = 0.025, P = 0.049), hydration status (semi-partial correlation 0.067, P = 0.001), left ventricular mass indexed to body surface area (semi-partial correlation 0.087, P < 0.001) and global longitudinal strain (semi-partial correlation 0.060, P = 0.003). IHD = ischaemic heart disease; GLS = global longitudinal strain; LVMI = left ventricular mass indexed to body surface area; RRF = residual renal function.

Figure 1

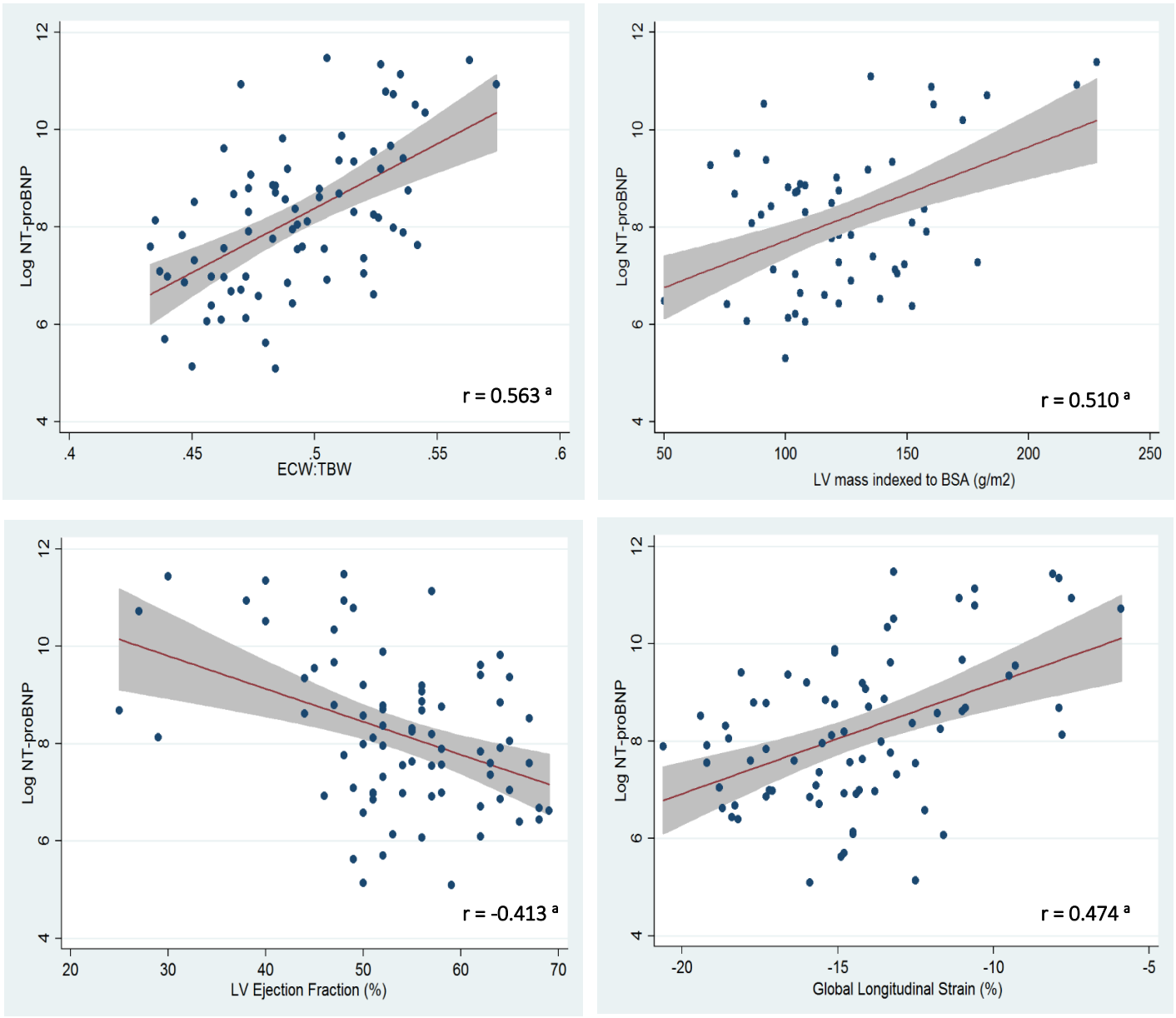
Log NT-proBNP
ECW:TBW
r = 0.563 a
Log NT-proBNP
LV mass indexed to BSA (g/m2)
r = 0.510 a
Log NT-proBNP
LV Ejection Fraction (%)
r = -0.413 a
Log NT-proBNP
Global Longitudinal Strain (%)
r = 0.474 a

Figure 2

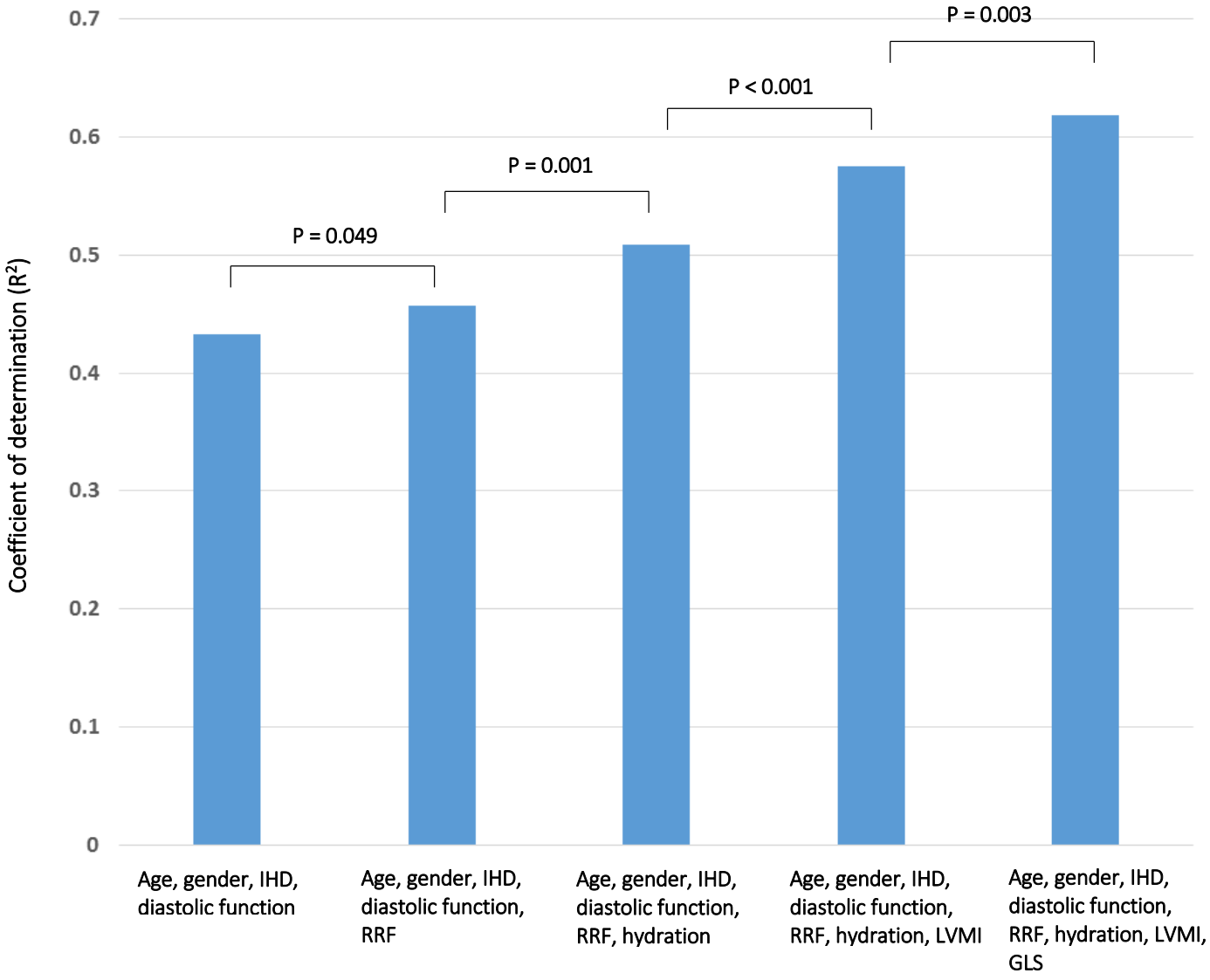
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Coefficient of determination (R^2)
P = 0.049
P = 0.001
P < 0.001
P = 0.003
Age, gender, IHD, diastolic function
Age, gender, IHD, diastolic function, RRF
Age, gender, IHD, diastolic function, RRF, hydration
Age, gender, IHD, diastolic function, RRF, hydration, LVMI
Age, gender, IHD, diastolic function, RRF, hydration, LVMI, GLS

Table 1. Baseline characteristics of the study cohort. Abbreviations: ACE-I = Angiotensin converting enzyme inhibitor; ARB = Angiotensin type-1 receptor blocker; D/P creatinine = ratio of creatinine concentration in dialysate to plasma; Kt/V = dialysis urea clearance; NT-proBNP = N-terminal pro-B-type natriuretic peptide.

Characteristic	**Value (n=78)**
Male gender n (%)	50 (64)
Age	
Mean ± SD (years)	64±13
Age distribution n (%)	
18 – 49	12 (15)
50 – 69	35 (45)
70 – 79	20 (26)
80 – 90	11 (14)
Hemodialysis n (%)	48 (61)
Dialysis sessions per week (n)	3
Duration of dialysis session (hours)	4.7±0.7
Single pool Kt/V	1.58±0.3
Interdialytic weight gain (kg)	1.83 (1.40 – 2.23)
Interdialytic weight gain relative to estimated dry weight (%)	2.4±1.1
Peritoneal dialysis n (%)	30 (39)
Volume of peritoneal dialysis solution exchanged per 24 hours (L)	8 (8 – 8.9)
Four hour D/P creatinine	0.72±0.1
Weekly Kt/V	1.93 (1.76 – 2.25)
Time on dialysis (years)	6 (4-9.5)

Body Mass Index (kg/m^2)	29.3±6.4
Systolic blood pressure (mmHg)	128±20
Diastolic blood pressure (mmHg)	70±13
Diabetes mellitus n (%)	35 (45)
Current or former smoker n (%)	48 (61)
Ischemic heart disease n (%)	32 (41)
Peripheral- and/or cerebro-vascular disease n (%)	13 (16)
Hydration status	
Ratio of extracellular to total body water	0.49±0.03
Proportion overhydrated n (%)	
Male	29 (57)
Female	20 (71)
Residual renal function (mL/min)	0 (0-3)
Left ventricular structure and function	
Left ventricular mass indexed to BSA (g/m^2)	119 (97-146)
Left ventricular hypertrophy n (%)	
Nil	26 (33)
Mild	10 (13)
Moderate	15 (19)
Moderate	27 (35)
Ejection fraction (%)	54 (49-62)
Global longitudinal strain (%)	-14±3.2
Left ventricular systolic dysfunction n (%)	
Ejection fraction	25 (32)
Global longitudinal strain	44 (56)
Diastolic dysfunction n (%)	

Nil	12 (16)
Grade 1	33 (42)
Grade 2	26 (33)
Grade 3	7 (9)
Pulse wave velocity (m/s)	9.4 (8.2-11.8)
Antihypertensive agents	
Median number of anti-hypertensive agents	1(1-2)
Proportion on beta-blocker n (%)	45 (58)
Proportion on ACE-I or ARB n (%)	27 (34)
NT-proBNP (pg/mL)	
Median (IQR)	3344 (1080-9786)
Distribution n (%)	
< 300 pg/mL	4 (5)
300 – 899 pg /mL	9 (12)
900 – 4999 pg/mL	34 (44)
5000 – 19,999 pg/mL	20 (27)
≥ 20,000 pg/mL	11 (12)

Table 2. Univariable associations of NT-proBNP. Linear regression coefficients (β) and bivariate correlation coefficients (r) of NT-proBNP and dependent variables. ACE-I = Angiotensin converting enzyme inhibitor; ARB = Angiotensin type-1 receptor blocker; BSA = body surface area; ECW = extracellular water volume; LV = left ventricular; TBW = total body water.

	r	***β***	**(95% CI)**	**P value**
Age (years)	0.328	0.039	(0.013 – 0.065)	0.004
Gender (female)	0.237	0.754	(0.044 – 1.465)	0.038
Hydration status (ECW:TBW)	0.563	26.476	(17.537 – 35.414)	<0.001
Diabetes	-0.080	-0.246	(-0.950 – 0.458)	0.489
Ischaemic heart disease (absence)	-0.367	-1.139	(-1.803 – -0.475)	0.001
Left ventricular ejection fraction (%)	-0.413	-0.067	(-0.102 – -0.033)	<0.001
Global longitudinal strain (%)	0.474	0.220	(0.128 – 0.320)	< 0.001
Diastolic Dysfunction	0.492	0.883	(0.523 – 1.243)	<0.001
Grade 1		0.682	(-0.197 – 1.560)	0.126
Grade 2		1.092	(0.187 – 1.998)	0.019
Grade 3		3.419	(2.185 – 4.653)	<0.001
Left ventricular mass indexed to BSA (g/m^2)	0.510	0.020	(0.012 – 0.028)	<0.001
Pulse wave velocity (m/s)	0.205	0.085	(-0.013 – 0.183)	0.09
Mean arterial pressure (mmHg)	0.019	0.002	(-0.025 – 0.029)	0.864
Residual renal function (mL/min)	-0.268	-0.141	(-0.260 – -0.021)	0.022
Beta-blocker use	0.056	0.173	(-0.539 – 0.886)	0.629
ACE-I or ARB use	0.084	0.270	(-0.471 – 1.011)	0.470

Table 3. Multivariable associations of NT-proBNP. Multivariable linear regression models with NT-proBNP as the dependent variable. Model 1 uses global longitudinal strain as the measure of left ventricular systolic function while model 2 uses left ventricular ejection fraction as the measure of left ventricular systolic function. Unstandardized (β) and standardised (Std β) linear regression coefficients; 95% CI = 95% confidence intervals. BSA = body surface area; ECW = extracellular water volume; TBW = total body water.

	Model 1 – Global longitudinal strain as the measure of systolic function					**Model 2 – Left ventricular ejection fraction as the measure of systolic function**				
	β	**(95% CI)**	**Std β**	**P value**	**R^2**	**β**	**(95% CI)**	**Std β**	**P value**	**R^2**
Age (years)	0.019	(-0.002 – 0.040)	0.160	0.08	0.631	0.020	(-0.002 – 0.042)	0.170	0.08	0.597
Gender (female)	0.430	(-0.092 – 0.957)	0.136	0.10		0.406	(-0.138 – 0.951)	0.128	0.14	
Hydration status (ECW:TBW)	14.726	(5.132 – 24.319)	0.313	<0.01		15.514	(5.476 – 25.552)	0.330	<0.01	
Ischaemic heart disease	-0.081	(-0.647 – 0.485)	-0.026	0.78		-0.088	(-0.693 – 0.515)	-0.028	0.77	
Global longitudinal strain (%)	0.111	(0.028 – 0.193)	0.233	<0.01		--------	----------	--------	-------	
Left ventricular ejection fraction (%)	-------	----------	--------	--------		-0.006	(-0.039 – 0.027)	-0.038	0.71	
Diastolic dysfunction	0.073	(-0.291 – 0.436)	0.040	0.69		0.052	(-0.290 – 0.477)	0.05	0.62	
Grade 1	-0.295	(-1.068 – 0.479)	-0.095	0.45		-0.268	(-1.089 – 0.553)	-0.086	0.51	
Grade 2	-0.251	(-1.094 – 0.591)	-0.078	0.55		-0.289	(-1.181 – 0.604)	-0.089	0.52	
Grade 3	-0.558	(-0.737 – 1.853)	0.105	0.39		0.819	(-0.540 – 2.178)	0.154	0.23	
Left ventricular mass indexed to BSA (g/m^2)	0.011	(0.003 – 0.019)	0.238	<0.01		0.013	(0.005 – 0.021)	0.339	<0.01	
Residual renal function (mL/min)	-0.086	(-0.173 – 0.000)	-0.182	0.05		-0.084	(-0.175 – 0.006)	-0.159	0.07	

Supplement

Table 1. Multivariable associations of NT-proBNP using non-imputed data. Multivariable linear regression models with NT-proBNP as the dependent variable. Model 1 uses global longitudinal strain as the measure of left ventricular systolic function while model 2 uses left ventricular ejection fraction as the measure of left ventricular systolic function. Unstandardized (β) and standardised (Std β) linear regression coefficients; 95% CI = 95% confidence intervals. BSA = body surface area; ECW = extracellular water volume; TBW = total body water.

	Model 1 – Global longitudinal strain as the measure of systolic function					**Model 2 – Left ventricular ejection fraction as the measure of systolic function**				
	β	**(95% CI)**	**Std β**	**P value**	**R^2**	**β**	**(95% CI)**	**Std β**	**P value**	**R^2**
Age (years)	0.018	(-0.005 - 0.041)	0.146	0.13	0.653	0.017	(-0.007 - 0.041)	0.141	0.17	0.609
Gender (female)	0.564	(0.013 - 1.115)	0.172	0.045		0.512	(-0.072 - 1.097)	0.157	0.09	
Hydration status (ECW/TBW)	15.605	(5.431 - 25.779)	0.328	<0.01		16.573	(5.811 - 27.335)	0.349	<0.01	
Ischaemic heart disease	-0.053	(-0.659 - 0.551)	-0.017	0.86		-0.064	(-0.713 - 0.585)	-0.020	0.84	
Global longitudinal strain (%)	0.119	(0.031 - 0.208)	0.244	<0.01		-------	----------	--------	--------	
Left ventricular ejection fraction (%)	-------	----------	--------	--------		-0.008	(-0.046 - 0.030)	-0.046	0.68	
Diastolic dysfunction	0.028	(-0.358 - 0.414)	0.014	0.89		0.055	(-0.362 - 0.473)	0.029	0.79	
Grade 1	-0.492	(-1.393 - 0.410)	-0.152	0.28		-0.329	(-1.276 - 0.618)	-0.102	0.49	
Grade 2	-0.458	(-1.401 - 0.485)	-0. 39	0.34		-0.392	(-1.390 - 0.607)	-0.119	0.44	
Grade 3	0.279	(-1.111 - 1.668)	0.053	0.69		0.669	(-0.818 - 2.156)	0.128	0.37	
Left ventricular mass indexed to BSA (g/m^2)	0.012	(0.004 - 0.020)	0.294	<0.01		0.014	(0.005 - 0.022)	0.331	<0.01	
Residual renal function (mL/min)	-0.099	(-0.197 - -0.001)	-0.167	0.045		-0.098	(-0.201 - 0.005)	-0.165	0.06	

Chapter 9

Pathophysiologic Associations of High Sensitivity Cardiac Troponin-T (hs-cTnT) in the Dialysis Population

The following chapter investigates the associations between plasma hs-cTnT concentrations and objective measures of hydration state, vascular function, left ventricular mass and function, and comorbid conditions in a cohort of prevalent haemodialysis and peritoneal dialysis patients. It provides insights into the pathophysiological factors mediating abnormal hs-cTnT concentrations in the dialysis population, and consequently how levels should be interpreted and what interventions might be considered in any future monitoring strategies.

This chapter is in the process of being submitted for publication.

Chapter 9

Pathophysiologic Associations of High Sensitivity Cardiac Troponin-T (hs-cTnT) in the Dialysis Population

INTRODUCTION

Cardiac troponin measurements have an established role in the diagnosis of myocardial injury in acute care settings(1), however they may also have a role in risk stratifying dialysis patients in the chronic care setting. Up to 94% of physiologically stable and asymptomatic dialysis patients have a persistently elevated cardiac troponin concentration(2-4), which has been independently associated with the risk of morbidity and mortality in this group(4, 5). These findings have spurred calls to use cardiac troponin testing as means of risk stratifying dialysis patients(6, 7), and has led to the licensing of cardiac troponins by the United States Food and Drug Administration (FDA) for this indication(8).

However, there remains considerable uncertainty regarding the pathophysiological factors mediating abnormal troponin concentrations in this population, and consequently what interventions might be used to modify this heightened risk. The majority of studies examining the associations of cardiac troponins in the dialysis population have reported associations with left ventricular hypertrophy(3, 9-13) and/or systolic dysfunction(10, 14, 15), while others have reported associations with hydration status(16) and surrogate measures of vascular stiffness(15). These contradictory findings can be attributed, at least in part, to inadequate adjustment for confounding variables, small sample sizes, use of surrogate measures of volume, and insensitive measures of myocardial contractility.

The aim of this study was to determine the associations between high sensitivity cardiac troponin-T (plasma hs-cTnT) concentrations and objective measures of hydration state, vascular function, left ventricular mass and function, and comorbid conditions in a cohort of prevalent haemodialysis and peritoneal dialysis patients.

METHODS

Study Design and Patient Recruitment

A cross-sectional study was conducted between June 2011 and August 2013. The study complied with the declaration of Helsinki and received ethics approval from the Metro-South Human Research Ethics Committee (HREC/10/QPAH/131), the Greenslopes Research and Ethics Committee (Protocol 12/39), and the University of Queensland Medical Research Ethics Committee (2011000484).

Participants were recruited from the in-centre haemodialysis and peritoneal dialysis units of a public, tertiary care teaching hospital and a private, secondary care hospital in metropolitan Brisbane, Australia. Eligible participants identified from an electronic database of all patients receiving dialysis were adults (aged ≥18-years) established on maintenance dialysis for ≥90-days who had a stable dialysis prescription for ≥30-days, and were able and willing to provide informed consent.

Patients were excluded if they were planned for living donor renal transplantation within 3-months of enrolment, had advanced malignancy, were pregnant, were unable to provide informed consent or had a contraindication to whole body bioimpedance spectroscopy measurement (including a permanent pacemaker, implantable cardiac defibrillator, joint replacements, orthopaedic pins, mechanical heart valves and/or limb amputations).

Patient Assessment

Haemodialysis patients were assessed between 6–8 AM, prior to the mid-week dialysis session, and peritoneal dialysis patients were assessed between 8–10 AM on a consistent weekday. Patients were instructed to avoid strenuous exercise prior to assessment. Assessments entailed a structured clinical interview, physical examination, and review of the medical record to ascertain medical history, comorbidities, current treatments and dialysis prescription. In addition, patients underwent a standard 12-lead electrocardiogram, whole body bioimpedance spectroscopy and blood sampling for measurement of plasma hs-cTnT on the same occasion.

For haemodialysis patients, the blood pressure recorded was the median of 12 post-dialysis measurements taken at the end of the 12 haemodialysis treatments that preceded the study

assessment, while for peritoneal dialysis patients it was the median of 12 home blood pressure recordings measured thrice weekly over the 4 weeks prior to the study assessment. The median of these blood pressure recordings has been shown to have the greatest agreement with 24-hour ambulatory measurements and therefore to be most representative of the patient's blood pressure(17).

Hydration status was assessed using whole-body, multi-frequency bioimpedance analysis with the Body Composition Monitor BCM® (Fresenius Medical Care, Asia-Pacific). This instrument has been validated in dialysis patients against radioisotope dilution methods with reported agreement limits (mean ± standard deviation [SD]) of -0.2±2.3L, -0.4±1.4L and 0.2±2L for total body water, extra- and intra-cellular water volumes, respectively(18). Peritoneal dialysis patients were assessed with peritoneal dialysis fluid in situ, which has been previously shown to have no significant or clinically important effect on volume measurements compared with an empty peritoneal cavity(19).

Echocardiography and pulse wave velocity measurement

Echocardiograms and pulse wave velocity measurements were performed within one week of plasma hs-cTnT sampling, and for haemodialysis patients they were performed on the non-dialysis day immediately following the mid-week dialysis treatment to ensure that cardiac loading conditions were representative of the patient's usual volume state. All echocardiograms were performed and measured by one of two expert operators blinded to patient's plasma hs-cTnT concentrations and medical history.

Echocardiograms were performed in the left lateral decubitus position using a 3.5MHz phased array transducer with harmonic imaging (Vivid7, General Electric-Vingmed Medical Systems, Horten, Norway). Grayscale images from the parasternal long-axis and short axis as well as the apical 4-chamber, 2-chamber and apical long axis views were acquired at moderate frame rate (50-70 fps) to allow for processing of 2D strain; apical images in the 4-chamber, 2-chamber and apical long axis were acquired at high frame rate (>120 fps) with a colour tissue Doppler overlay to measure colour tissue Doppler velocity, strain and strain rate. Doppler measurement of LV inflow, TV inflow and aortic and pulmonary outflow were performed using pulsed and continuous wave Doppler. Colour tissue Doppler measurements were performed using pulsed wave Doppler in the medial and lateral LV annulus, tricuspid annulus and for all LV segmental measurements.

Cine loops of three cardiac cycles were acquired and the measurements averaged. If patients were in atrial fibrillation, 5-7 measurements were averaged. Left atrial and ventricular dimensions were obtained by M-mode according to the American Society of Echocardiography Recommendations(20); endocardial borders were traced at end systole and end diastole in the apical 4—chamber and 2-chamber views to obtain Simpson's Rule ejection fraction; M-mode LV mass was calculated using the formula : LV mass =0.8 × (1.04[(LV internal dimension + septal wall thickness + posterior wall thickness)^3 – LV internal dimension^3]) + 0.6g, and these were indexed to the patient's body surface area calculated using the Haycock formula (0.024265 x Weight(kg)0.5378 x height(cm)0.3964).

For 2D strain analysis, the endocardial borders were traced at end-systole in the 4-chamber, 2-chamber and apical long axis views; the software (EchoPac PC BTO 11, General Electric-Vingmed Medical Systems, Horten, Norway) divided the LV into basal, mid and apical segments, and identified unique "speckles" within the myocardium and tracked them frame-by-frame throughout the cardiac cycle to obtain strain and strain rate curves. Segments that did not track properly were manually adjusted or eliminated from the analysis. Global longitudinal strain (GLS) was expressed as a mean of all 18 segments.

The SphygmoCor PX system (AtCor Medical, Australia) was used for measurement of pulse wave analysis (augmentation index) and pulse wave velocity (PWV). Carotid-femoral PWV was the only method measured. The distances between the common carotid artery and the suprasternal notch, the suprasternal notch and the umbilicus, and the umbilicus and the femoral artery were measured. Tonometery was performed in the common carotid artery as close to the aortic arch as possible and at the femoral artery. The foot-to-foot times to reach the common carotid artery and the femoral artery were then subtracted and pulse wave velocity was expressed as metres per second (m/s). A total of three measurements were taken and averaged for analysis.

Plasma hs-cTnT sample collection, storage and analysis

Blood for measurement of plasma hs-cTnT concentrations was collected in lithium-heparin tubes, prior to the commencement of the dialysis treatment for haemodialysis patients. Blood samples were centrifuged and plasma separated within 1 hour of collection and then stored at -80°C until assayed(21).

Plasma hs-cTnT concentration (ng/L) was measured using the Cobas e170 instrument with the troponin-T hs kit (Roche Diagnostics, Australia); a monoclonal antibody electrochemiluminescence assay which has a reported detection range of 5 – 10,000 ng/L(22). Analytic coefficients of variation reported by the analysing laboratory were 2.8% and 1.4% at concentrations of 26.2 ng/L and 2210 ng/L respectively in keeping with desired performance of the assay(22).

Definitions

Ischemic heart disease was defined as any of inducible ischaemia on non-invasive cardiac stress testing and/or ≥50% stenosis in ≥1 epicardial coronary artery on coronary angiography and/or a history of myocardial infarction and/or a history of coronary artery angioplasty, stenting or coronary artery bypass grafting.

Systolic dysfunction was classified either as an ejection fraction ≤ 50% measured using Simpson's rule(23) or a global longitudinal strain > -15% which has been associated with increased risk of mortality in both the general and dialysis populations(24-26). Diastolic dysfunction was graded as absent (normal diastolic function) or assigned a grade from one to three reflecting worsening diastolic dysfunction according to a consensus guideline(27). Gender specific limits from consensus guidelines were used to classify left ventricular hypertrophy as absent, mild, moderate or severe according to left ventricular mass indexed to body surface area(20).

Hydration status was expressed as the ratio of extracellular to total body water volumes, and overhydration defined as a ratio two standard deviations above the mean age- and gender-matched ratio in the normal population(19, 28).

Statistical analysis

Categorical variables are presented as frequencies and percentages, and continuous variables as either mean ± standard deviation if normally distributed or as median and interquartile range if non-normally distributed. Plasma hs-cTnT concentrations were logarithmically transformed for correlation, univariable and multivariable linear regression analyses.

Bivariate correlation between log transformed plasma hs-cTnT concentrations and independent variables were analysed using pairwise correlation.

Thirteen participants had at least one missing measurement for either global longitudinal strain (n=1), left ventricular mass (n=7), and/or pulse wave velocity (n=12) which could not be measured due to body habitus or arrhythmia. Examination of the missing data demonstrated an arbitrary pattern and missing values were assumed to be missing at random. Multiple imputation was used to impute missing values using a multivariable normal model with 20 imputations(29). Univariable and multivariable linear regression analyses were performed using imputed data as the primary analyses, and repeated using non-imputed data as a sensitivity analysis (supplement). Differences between the results of the two analyses are presented and discussed below.

Independent variables for inclusion in the linear regression models were chosen based on published associations with plasma hs-cTnT. Independent variables identified as being significant at the 5% level ($P < 0.05$) from the univariable analyses were analysed in the multivariable linear regression model. Global longitudinal strain and ejection fraction are both measures of left ventricular contractility with differing sensitivity, thus they were used individually as independent variables in two separate multivariable linear regression analyses. Regression diagnostics were performed for both multivariable models and were satisfactory. Semi-partial correlations were calculated for independent variables to determine increment to coefficient of determination (R^2). All analyses were performed using Stata/MP 12.1 (College Station, TX, USA).

RESULTS

Participant characteristics

Seventy eight prevalent patients were recruited between June 2011 and January 2014 from the haemodialysis (n=47) and peritoneal dialysis (n=31) units of the participating institutions. Their baseline characteristics are shown in Table 1. Cardiovascular risk factors and established cardiovascular disease were highly prevalent among the study cohort, including diabetes mellitus (45%), hypertension (100%), current or former smoking (61%), ischaemic heart disease (41%) and peripheral and/or cerebrovascular disease (16%). In addition, a substantial proportion of participants had evidence of cardiomyopathy on echocardiography

including left ventricular hypertrophy (67%) and/or diastolic dysfunction (84%). The prevalence of systolic dysfunction differed according to the measure of left ventricular systolic function used with 32% of the cohort having an ejection fraction under 50%, while 56% of the cohort had an abnormal global longitudinal strain measurement of > -15%. Fifty seven percent of males and 71% of females in the study cohort were overhydrated on bioimpedance analysis. Baseline plasma hs-cTnT concentrations demonstrated a right skewed frequency distribution with a median of 51 ng/L (interquartile range 32-91 ng/L). Ninety six percent of the study cohort had a plasma hs-cTnT concentration above the currently accepted assay upper reference limit of 14 ng/L(22), and 84% of males and 100% of women (91% of the cohort) had a plasma hs-cTnT exceeding recently proposed age and gender based upper reference limits(30).

Bivariate correlation and univariable linear regression analyses are shown in Table 2 and Figure 1. These analyses demonstrated significant direct correlations between plasma hs-cTnT concentrations and age, hydration status, a history of either diabetes mellitus or ischaemic heart disease, global longitudinal strain and pulse wave velocity. In addition, a significant inverse correlation was demonstrated between plasma hs-cTnT concentrations and left ventricular ejection fraction. There were no significant associations between plasma hs-cTnT concentrations and either left ventricular mass indexed to body surface area nor with central or peripheral augmentation indices.

Multivariable linear regression

Two multivariable linear regression models were used to analyse associations between plasma hs-cTnT concentrations, significant independent variables identified from the univariable analyses, and left ventricular mass indexed to body surface area which was included in the multivariable models *a priori* as numerous published studies have suggested it is significantly associated with plasma hs-cTnT concentrations. The two multivariable models differed in terms of the measure of left ventricular systolic function used, with model 1 using global longitudinal strain and model 2 using left ventricular ejection fraction (table 3). Plasma hs-cTnT concentrations were significantly associated with both hydration status and pulse wave velocity in both models, although a significant association with pulse wave velocity was only demonstrated in the analysis using imputed data. Neither multivariable model demonstrated a significant association with left ventricular systolic function. Hydration status explained the greatest proportion of the variation in plasma hs-cTnT

concentrations (standardised $\beta = 0.379$, $p<0.01$), and led to a significant improvement in the coefficient of determination of the multivariable model with an increment to R^2 of 0.109, $P < 0.01$ (Figure 2).

DISCUSSION

This study demonstrated an independent association between plasma hs-cTnT concentrations and hydration status, as well as a possible independent association with vascular stiffness assessed using pulse wave velocity in prevalent dialysis patients. These findings improve our understanding of potential pathophysiological mechanisms underpinning chronically elevated plasma hs-cTnT concentrations and their associated adverse outcomes in the dialysis population.

Several mechanisms have been posited to explain the release of cardiac troponins in overhydrated states including the stimulation of stretch sensitive integrins in viable myocardial fibrils(31), stretch- and/or strain-induced cardiomyocyte apoptosis(32), and volume overload-induced sub-endocardial ischaemia(33). Overhydration in dialysis patients has been shown to result in cardiac pressure overload even in the absence of established cardiomyopathy(34), and may stimulate cardiac troponin release by some or all of the aforementioned mechanisms.

An independent association between plasma troponin T and hydration status in dialysis patients has only been reported in one previous investigation. In their study of 74 haemodialysis patients, Park and colleagues reported an independent association between troponin T measured using a third generation assay and hydration status assessed using bioimpedance spectroscopy ($\beta = 0.37$, $P < 0.01$)(16). However, this study was significantly limited by the fact that participants did not undergo echocardiography, with the only independent cardiac variable included in the multivariable analysis being left ventricular hypertrophy assessed using electrocardiography. The insensitivity of the latter measure, and the lack of inclusion of echocardiographic measures of systolic and/or diastolic function cast doubt on the significance of this study's findings. The study presented here addresses these limitations through its use of standardised echocardiography with measurement of both established and novel cardiac structural and functional indices.

Overhydration is as an independent predictor of mortality in the dialysis population(35) and provides a partial explanation for the adverse prognosis portended by elevated plasma hs-cTnT concentrations in this group. Hydration status in dialysis patients is currently assessed using either clinical examination or devices, such as bioimpedance or lung ultrasound, which are variously limited by their inaccuracy, subjectivity and/or cost(36). The association between plasma hs-cTnT and hydration status reported in this study raises the prospect that plasma hs-cTnT testing could be used to monitor hydration status in dialysis populations, although the accuracy of such a strategy and its impact on patient outcomes need to be established before it can be routinely adopted in clinical practice(37).

This study also demonstrated an independent association between plasma hs-cTnT concentrations and pulse wave velocity in analyses using imputed data. This is a novel and plausible association. Pulse wave velocity measures the velocity of the propagated cardiac pulse wave which is a function of vascular compliance(38). Vascular calcification in the dialysis population increases vascular stiffness(39) resulting in an increase in pulse wave velocity which has in turn been independently associated with the risk of mortality in this group(40). This heightened mortality risk is thought to be related to an increase in cardiac afterload leading to ventricular hypertrophy, and from an earlier reflection of the cardiac pulse wave which impairs coronary diastolic flow(41, 42). Both of these physiological alterations may result in myocardial ischaemia and release of cardiac troponins. The association between cardiac troponin and vascular stiffness in the dialysis population has not been previously reported, but is lent support the findings of Artunc et al(15) who reported an independent association between high sensitivity troponin I and pulse pressure in dialysis patients.

However, it is important to view the association with pulse wave velocity reported in this study with caution as a significant association could not be demonstrated between plasma hs-cTnT and other measures of vascular dysfunction, including central and peripheral augmentation indices, and because the association with pulse wave velocity was only demonstrated on multivariable analysis using imputed data. As such, the association between plasma hs-cTnT and vascular stiffness should be considered preliminary until it is either confirmed or refuted in future studies.

The majority of published studies investigating the pathophysiologic associations of cardiac troponin in the dialysis population have reported an independent association with left ventricular hypertrophy(3, 9-13) and/or left ventricular systolic dysfunction(10, 14, 15). However, these studies were limited by their lack of control for confounding variables, particularly hydration status which was not measured in most of these studies. In this study, significant associations between plasma hs-cTnT and cardiac structural and functional indices were only demonstrated in univariable linear regression analyses and multivariable analyses that did not include hydration status. The inclusion of hydration status as independent variable in the multivariable models resulted in cardiomyopathic indices losing their significance and led to a significant improvement in the coefficient of determination of the multivariable model.

This study has several strengths including the its use of the latest generation high sensitivity troponin assays to minimise measurement error, standardisation of plasma hs-cTnT sampling, handling and storage to minimise pre-analytic variation, its use of sensitive and objective measures of myocardial contractility (global longitudinal strain) and volume (whole body bioimpedance spectroscopy), and its inclusion of both haemodialysis and peritoneal-dialysis patients thereby broadening the generalizability of its findings. Nevertheless, this study had two principal limitations including its sample size, which restricted the number of independent variables that could be explored in the multivariable linear regression models, and the lack of measurement of inflammatory markers which have been associated with plasma hs-cTnT concentrations in previous dialysis population studies.

Conclusion

This study demonstrated that plasma hs-cTnT concentrations in prevalent haemodialysis and peritoneal dialysis patients were independently associated with hydration status, and possibly pulse wave velocity. These findings raise the possibility that plasma hs-cTnT testing could be used to monitor hydration status in dialysis patients, however longitudinal studies are needed to determine the accuracy of such a strategy and its impact on patient outcomes before it can be routinely implemented in clinical practice. In addition, further studies are needed to either confirm or refute an association between plasma hs-cTnT and vascular stiffness in the dialysis population.

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Figure Legends

Figure 1. Bivariate correlation between plasma hs-cTnT concentrations and hydration status, pulse wave velocity, and left ventricular mass and systolic function. Scatter plots, regression line and 95% confidence interval (grey area). BSA = body surface area; ECW = extracellular water volume; LV = left ventricular; r = correlation coefficient; TBW = total body water.

Figure 2. Proportion of variation in plasma hs-cTnT concentrations explained by hydration status, and pulse wave velocity. Increment to the coefficient of determination (R^2) due to pulse wave velocity (semi-partial correlation = 0.032, $P < 0.01$) and hydration status (semi-partial correlation = 0.109, $P < 0.01$). DM = diabetes mellitus, IHD = ischaemic heart disease; GLS = global longitudinal strain; LVMI = left ventricular mass indexed to body surface area.

Figure 1

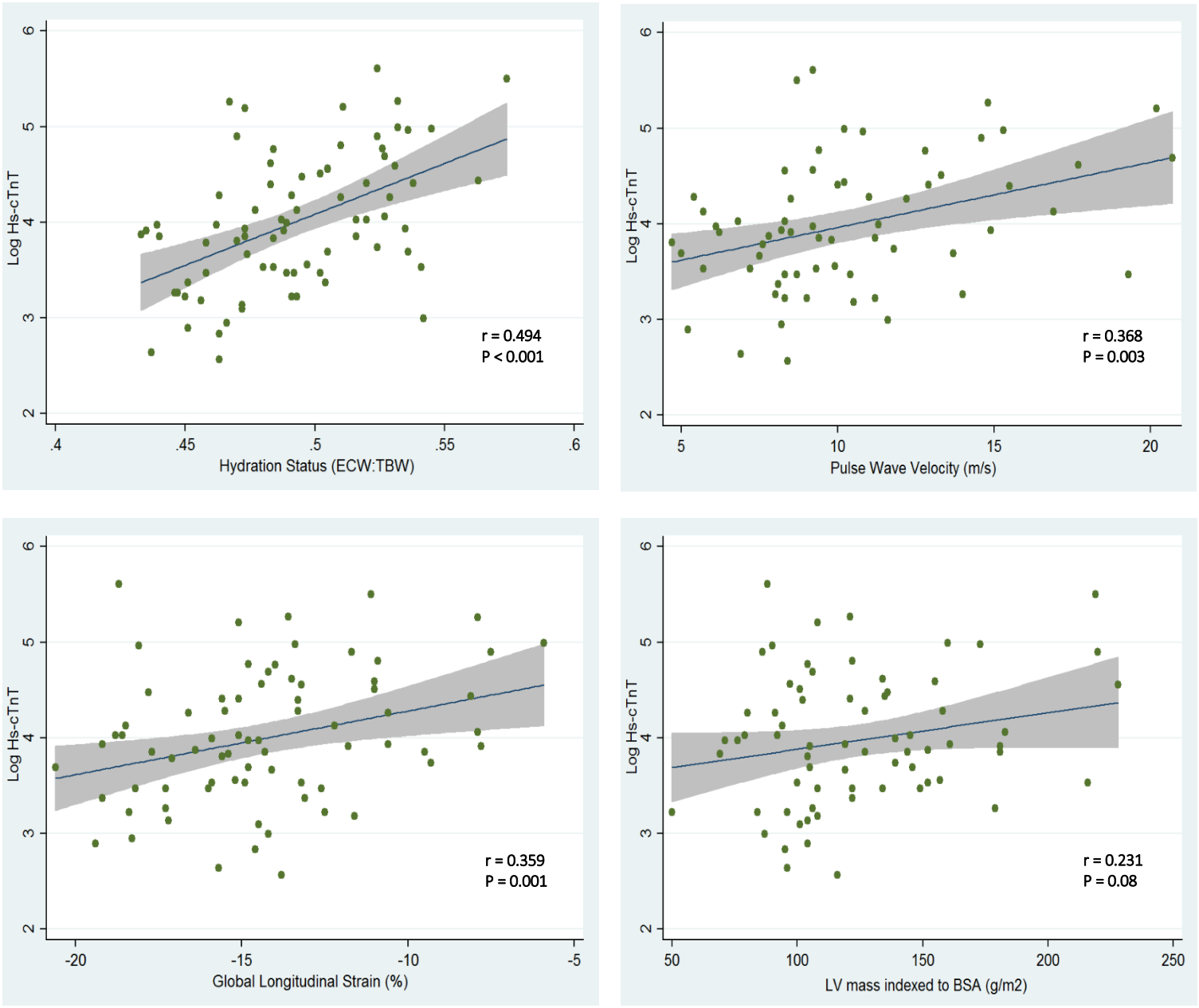
Log Hs-cTnT
Hydration Status (ECW:TBW)
r = 0.494
P < 0.001
Log Hs-cTnT
Pulse Wave Velocity (m/s)
r = 0.368
P = 0.003
Log Hs-cTnT
Global Longitudinal Strain (%)
r = 0.359
P = 0.001
Log Hs-cTnT
LV mass indexed to BSA (g/m2)
r = 0.231
P = 0.08

Figure 2

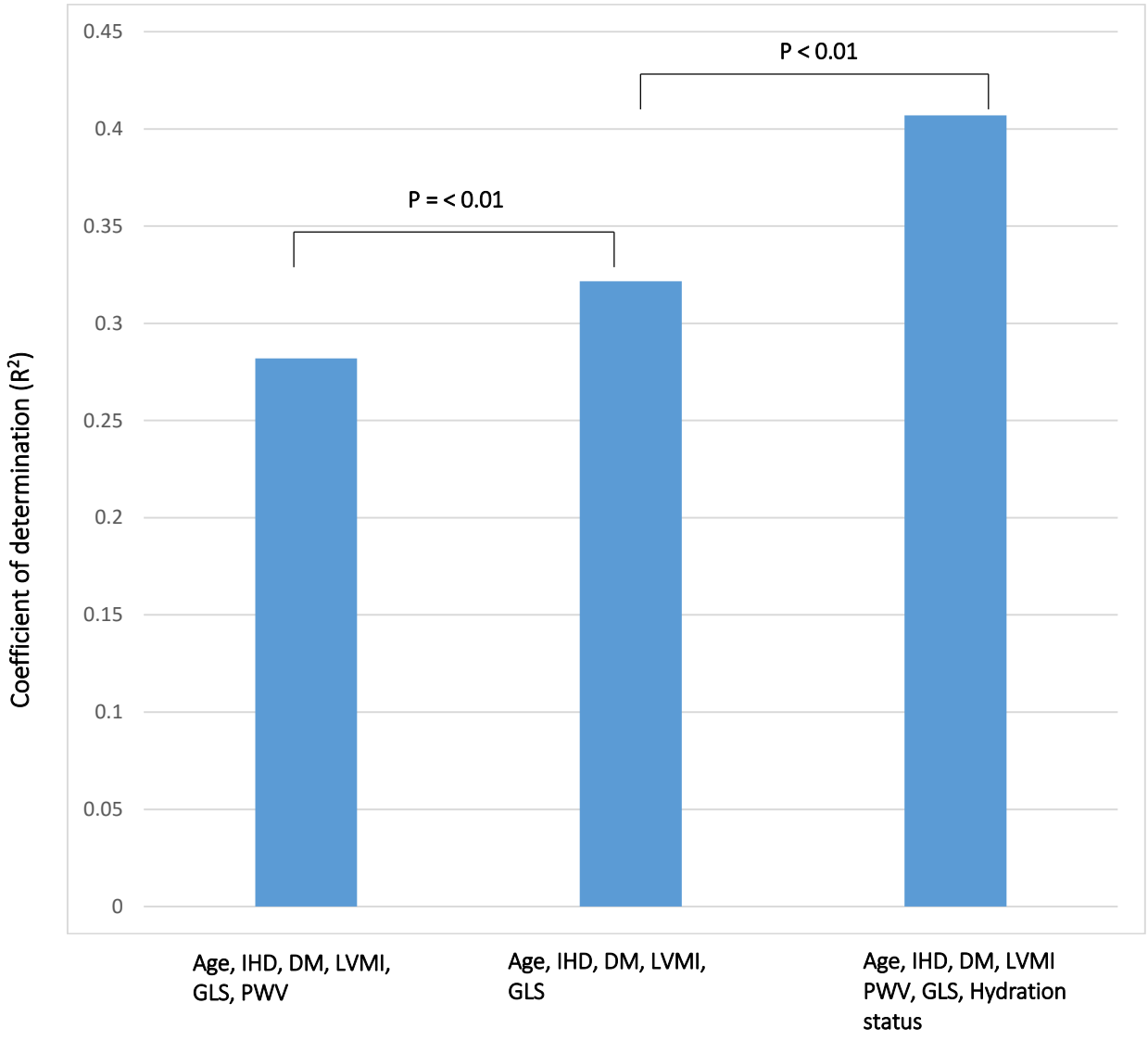
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Coefficient of determination (R^2)
P = < 0.01
P < 0.01
Age, IHD, DM, LVMI, GLS, PWV
Age, IHD, DM, LVMI, GLS
Age, IHD, DM, LVMI PWV, GLS, Hydration status

Table 1. Baseline characteristics of the study cohort. Abbreviations: ACE-I = Angiotensin converting enzyme inhibitor; ARB = Angiotensin type-1 receptor blocker; D/P creatinine = ratio of creatinine concentration in dialysate to plasma; Kt/V = dialysis urea clearance; plasma hs-cTnT = high sensitivity cardiac troponin-T; URL = upper reference limit.

Characteristic	**Value (n=78)**
Male gender n (%)	50 (64)
Age	
Mean ± SD (years)	64±13
Age distribution n (%)	
18 – 49	12 (15)
50 – 69	35 (45)
70 – 79	20 (26)
80 – 90	11 (14)
Haemodialysis n (%)	48 (61)
Dialysis sessions per week (n)	3
Duration of dialysis session (hours)	4.7±0.7
Single pool Kt/V	1.58±0.3
Interdialytic weight gain (kg)	1.83 (1.40 – 2.23)
Interdialytic weight gain relative to estimated dry weight (%)	2.4±1.1
Peritoneal dialysis n (%)	30 (39)
Volume of peritoneal dialysis solution exchanged per 24 hours (L)	8 (8 – 8.9)
Four hour D/P creatinine	0.72±0.1
Weekly Kt/V	1.93 (1.76 – 2.25)
Time on dialysis (years)	6 (4-9.5)

Body Mass Index (kg/m^2)	29.3±6.4
Systolic blood pressure (mmHg)	128±20
Diastolic blood pressure (mmHg)	70±13
Diabetes mellitus n (%)	35 (45)
Current or former smoker n (%)	48 (61)
Ischemic heart disease n (%)	32 (41)
Peripheral- and/or cerebro-vascular disease n (%)	13 (16)
Hydration status	
Ratio of extracellular to total body water	0.49±0.03
Proportion overhydrated n (%)	
Male	29 (57)
Female	20 (71)
Residual renal function (mL/min)	0 (0-3)
Left ventricular structure and function	
Left ventricular mass indexed to BSA (g/m^2)	119 (97-146)
Left ventricular hypertrophy n (%)	
Nil	26 (33)
Mild	10 (13)
Moderate	15 (19)
Moderate	27 (35)
Ejection fraction (%)	54 (49-62)
Global longitudinal strain (%)	-14±3.2
Left ventricular systolic dysfunction n (%)	
Ejection fraction	25 (32)
Global longitudinal strain	44 (56)
Diastolic dysfunction (%)	

Nil	12 (16)
Grade 1	33 (42)
Grade 2	26 (33)
Grade 3	7 (9)
Pulse wave velocity (m/s)	9.4 (8.2-11.8)
Antihypertensive agents	
Median number of anti-hypertensive agents	1(1-2)
Proportion on beta-blocker (%)	45 (58)
Proportion on ACE-I or ARB (%)	27 (34)
Plasma hs-cTnT (ng/L)	
Median (IQR)	51 (32-91)
Distribution by age and gender specific URL n (%)	
Plasma hs-cTnT > 14 ng/L	
Males < 50 years	7 (78%)
Females < 65 years	14 (100%)
Plasma hs-cTnT > 17 ng/L	
Males 50 – 64 years	13 (100%)
Females ≥ 65 years	14 (100%)
Plasma hs-cTnT > 31 ng/L	
Males ≥ 65 years	22 (79%)

Table 2. Univariable associations of plasma hs-cTnT. Linear regression coefficients (β) and bivariate correlation coefficients (r) of plasma hs-cTnT and dependent variables. ACE-I = Angiotensin converting enzyme inhibitor; ARB = Angiotensin type-1 receptor blocker; BSA = body surface area; ECW = extracellular water volume; TBW = total body water.

Variable	*r*	*β*	(95% CI)	P value
Age (years)	0.293	0.015	(0.004 – 0.028)	0.01
Gender (female)	-0.081	-0.119	(-0.453 – 0.215)	0.48
Hydration status (ECW/TBW)	0.494	10.643	(6.341 – 14.945)	<0.001
Diabetes mellitus (absence)	-0.334	-0.470	(-0.775 – -0.166)	0.003
Ischaemic heart disease status (absence)	-0.290	-0.411	(-0.725 – -0.099)	0.01
Left ventricular ejection fraction (%)	-0.233	-0.017	(-0.034 – -0.001)	0.04
Global longitudinal strain (%)	0.359	0.070	(0.028 – 0.111)	0.001
Diastolic Dysfunction	0.191	0.157	(-0.029 – 0.342)	0.10
Grade 1		0.310	(-0.163 – 0.783)	0.20
Grade 2		0.327	(-0.161 – 0.815)	0.19
Grade 3		0.608	(-0.057 – 1.273)	0.07
Left ventricular mass indexed to BSA (g/m^2)	0.231	0.004	(-0.004 – 0.008)	0.08
Pulse wave velocity (m/s)	0.368	0.068	(0.025 – 0.111)	0.003
Central augmentation index	0.034	0.001	(-0.006 – -0.008)	0.78
Peripheral augmentation index	0.140	0.006	(-0.004 – 0.016)	0.27
Mean arterial pressure (mmHg)	-0.061	-0.003	(-0.015 – 0.009)	0.59
Residual renal function (mL/min)	-0.086	-0.021	(-0.077 – 0.035)	0.46
Beta-blocker use	0.143	0.204	(-0.119 – 0.527)	0.52
ACEI / ARB use	0.012	0.017	(-0.323 – 0.357)	0.92

Table 3. Multivariable associations of plasma hs-cTnT. Multivariable linear regression models with plasma hs-cTnT as the dependent variable. Model 1 uses global longitudinal strain as the measure of left ventricular systolic function while model 2 uses left ventricular ejection fraction as the measure of left ventricular systolic function. Unstandardized (β) and standardised (Std β) linear regression coefficients; 95% CI = 95% confidence intervals. BSA = body surface area; ECW = extracellular water volume; TBW = total body water.

	Model 1 – Global longitudinal strain as the measure of systolic function					**Model 2 – Left ventricular ejection fraction as the measure of systolic function**				
	β	**(95% CI)**	**Std β**	**P value**	**R^2**	**β**	**(95% CI)**	**Std β**	**P value**	**R^2**
Age (years)	-0.001	(-0.013 - 0.012)	-0.010	0.93	0.407	-0.001	(-0.013 - 0.013)	-0.001	0.99	0.379
Hydration status (ECW/TBW)	8.156	(2.930 - 13.381)	0.379	<0.01		8.045	(2.680 - 13.409)	0.374	<0.01	
Ischaemic heart disease	0.048	(-0.268 - 0.363)	0.034	0.77		0.069	(-0.263 - 0.400)	0.048	0.68	
Global longitudinal strain (%)	0.038	(-0.005 - 0.080)	0.192	0.09		-------	----------	--------	-------	
Left ventricular ejection fraction (%)	-------	----------	---------	--------		-0.005	(-0.023 - 0.012)	-0.068	0.56	
Diabetes mellitus	-0.173	(-0.466 - 0.120)	-0.123	0.24		-0.199	(-0.500 - 0.100)	-0.142	0.19	
Pulse wave velocity (m/s)	0.045	(0.002 - 0.087)	0.250	0.04		0.052	(0.009 - 0.095)	0.290	0.02	
Left ventricular mass indexed to BSA (g/m^2)	0.002	(-0.002 - 0.006)	0.099	0.37		0.002	(-0.002 - 0.007)	0.130	0.75	

SUPPLEMENT

Table 1. Multivariable associations of plasma hs-cTnT using non-imputed data. Multivariable linear regression models with plasma hs-cTnT as the dependent variable. Model 1 uses global longitudinal strain as the measure of left ventricular systolic function while model 2 uses left ventricular ejection fraction as the measure of left ventricular systolic function. Unstandardized (β) and standardised (Std β) linear regression coefficients; 95% CI = 95% confidence intervals. BSA = body surface area; ECW = extracellular water volume; TBW = total body water.

	Model 1 – Global longitudinal strain as the measure of systolic function					**Model 2 – Left ventricular ejection fraction as the measure of systolic function**				
	β	**(95% CI)**	**Std β**	**P value**	**R^2**	**β**	**(95% CI)**	**Std β**	**P value**	**R^2**
Age (years)	0.002	(-0.011 - 0.016)	0.049	0.72	0.368	0.002	(-0.011 - 0.015)	0.048	0.73	0.366
Hydration status (ECW/TBW)	8.835	(2.925 - 14.747)	0.445	<0.01		8.998	(3.048 - 14.948)	0.453	<0.01	
Ischaemic heart disease	0.038	(-0.325 - 0.402)	0.028	0.83		0.034	(-0.333 - 0.400)	0.025	0.85	
Global longitudinal strain (%)	0.012	(-0.041 - 0.064)	0.053	0.66		-------	----------	--------	-------	
Left ventricular ejection fraction (%)	-------	----------	---------	--------		0.001	(-0.018 - 0.020)	0.015	0.91	
Diabetes mellitus	-0.079	(-0.426 - 0.267)	-0.058	0.65		-0.083	(-0.429 - 0.263)	-0.061	0.63	
Pulse wave velocity (m/s)	0.038	(-0.009 - 0.086)	0.208	0.11		0.040	(-0.006 - 0.086)	0.218	0.09	
Left ventricular mass indexed to BSA (g/m^2)	0.001	(-0.003 - 0.006)	0.63	0.63		0.001	(-0.003 - 0.006)	0.080	0.55	

Chapter 10

Longitudinal correlation of the amino terminal fragment of pro-B-type natriuretic peptide (NT-proBNP) and hydration status in dialysis patients.

This chapter investigates whether NT-proBNP and hydration status assessed using bioimpedance analysis are independently correlated over time and consequently whether NT-proBNP testing can be used to monitor hydration status in dialysis patients. This chapter provides important insights into potential interventional targets for future monitoring strategies.

The following chapter is in the process of being finalised for publication.

Chapter 10 - Longitudinal correlation of the amino terminal fragment of the pro-B-type natriuretic peptide (NT-proBNP) and hydration status in dialysis patients.

Word count: 2909

INTRODUCTION

Overhydration is common among patients on maintenance dialysis therapy(1), contributes to the genesis and progression of cardiomyopathy(2) and has been independently associated with the risks of morbidity and mortality in this group(1, 3). However, current methods for the assessment of hydration status are suboptimal and rely principally on clinical examination which is inaccurate and has high interobserver variability(4). The introduction of technologies such as bioimpedance(5) and lung ultrasound(6), have improved the accuracy of hydration assessment, although these devices incur significant personnel and equipment costs thereby limiting their universal and/or regular application.

The amino terminal fragment of the pro-B-type natriuretic peptide (NT-proBNP) is an inactive peptide secreted from the myocardium in response to cardiac stretch and/or strain(7) such as occur during volume overload(8). Furthermore, plasma NT-proBNP has been independently associated with risks of incident heart failure(9) and cardiovascular death in dialysis patients(10), raising the possibility that NT-proBNP testing may have a role in the monitoring of hydration status of dialysis patients. However, the studies investigating the correlation of NT-proBNP and volume status in dialysis patients have so far yielded conflicting results(11). The majority of these studies have been cross-sectional in design(12-17) with limited applicability to clinical longitudinal monitoring, whereas preliminary longitudinal studies have been limited by their use of surrogate measures of volume status(18-24), small sample sizes(19-23), limited numbers of repeated measures(22-25) and short-term follow-up(22-24).

The aim of this study was to investigate the longitudinal correlation of plasma NT-proBNP and hydration status assessed using bioimpedance spectroscopy in a cohort of prevalent haemodialysis and peritoneal dialysis patients and to determine whether this correlation was affected by dialysis modality and/or cardiac functional or structural abnormalities.

METHODS

Study Design and Patient Recruitment

A prospective, longitudinal cohort study was conducted between June 2011 and January 2015. The study complied with the declaration of Helsinki and received ethics approval from the Metro-South Human Research Ethics Committee (HREC/10/QPAH/131), the Greenslopes Research and Ethics Committee (Protocol 12/39), and the University of Queensland Medical Research Ethics Committee (2011000484).

Participants were recruited from the in-centre haemodialysis and peritoneal dialysis units of a public, tertiary-care teaching hospital and a private, secondary-care hospital in metropolitan Brisbane, Australia. Eligible participants identified from an electronic database of all patients receiving dialysis were adults (aged ≥18 years) established on maintenance dialysis for ≥90 days who had a stable dialysis prescription for ≥30 days, and were able and willing to provide informed consent.

Patients were excluded if they were planned for living donor renal transplantation within 3 months of enrolment, had advanced malignancy, were pregnant, were unable to provide informed consent or had a contraindication to whole-body bioimpedance spectroscopy measurement (including a permanent pacemaker, implantable cardiac defibrillator, joint replacements, orthopaedic pins, mechanical heart valves and/or limb amputations).

Patient Assessment

Patients were assessed at baseline and thereafter at monthly intervals for up to 24 months. Haemodialysis patients were assessed prior to the mid-week dialysis session, while peritoneal dialysis patients were assessed on a consistent weekday throughout the study. Patients were instructed to avoid strenuous exercise prior to assessment, and were assessed at the same time of day throughout the study to avoid fluctuations in NT-proBNP concentrations related to diurnal variation. Patient assessments entailed a structured clinical interview, physical examination, and review of the medical record to ascertain medical history, comorbidities, current treatments and dialysis prescription. In addition, patients underwent a standard 12-lead electrocardiogram, whole body bioimpedance spectroscopy and blood sampling for measurement of NT-proBNP on the same occasion.

For haemodialysis patients, the blood pressure recorded was the median of 12 post-dialysis measurements taken at the end of the 12 haemodialysis treatments that preceded the study assessment, while for peritoneal dialysis patients it was the median of 12 home blood pressure recordings measured thrice weekly over the 4 weeks prior to the study assessment. The median values of these blood pressure recordings have been shown to have the greatest agreement with 24-hour ambulatory measurements and therefore to be most representative of the patients' blood pressures(26).

Whole body multi-frequency bioimpedance spectroscopy

Hydration status was assessed using whole-body, multi-frequency bioimpedance analysis with the Body Composition Monitor BCM® (Fresenius Medical Care, Asia-Pacific). This instrument has been validated in dialysis patients against radioisotope dilution methods with reported agreement limits (mean ± standard deviation [SD]) of -0.2±2.3L, -0.4±1.4L and 0.2±2L for total body water, extra- and intra-cellular water volumes, respectively(5). In addition, this instrument has been shown to have a detection limit for change in extracellular volume (mean ± SD) of 0.87±0.64L(27). Peritoneal dialysis patients were assessed with peritoneal dialysis fluid in situ, as this has been previously shown to have no significant or clinically important effect on volume measurements compared with an empty peritoneal cavity(28).

NT-proBNP sample collection, storage and analysis

Blood for measurement of NT-proBNP concentrations was collected in lithium-heparin tubes prior to the commencement of the dialysis treatment for haemodialysis patients. Blood samples were centrifuged and plasma separated within 1 hour of collection and then stored at -80°C until assayed(29). Plasma NT-proBNP concentration (pg/mL) was measured using a twin-antibody electrochemiluminescence assay on the Elecsys 2010 instrument (Roche Diagnostics, Australia), which has a reported analytical detection range of 5–35,000 pg/mL. Analytic coefficients of variation reported by the analysing laboratory were 2.9% and 1.8% at concentrations of 134 pg/mL and 4534 pg/mL, respectively in keeping with desired performance of the assay(30, 31).

Echocardiography

All of the participants in the cohort study were invited to participate in an echocardiographic sub-study. Echocardiograms were performed within one week of baseline NT-proBNP sampling, and for haemodialysis patients they were performed on the non-dialysis day immediately following the mid-week dialysis treatment to ensure that cardiac loading conditions were representative of the patient's usual volume state. Echocardiograms were performed and measured by one of two expert operators blinded to the patient's NT-proBNP concentrations, hydration status and medical history.

Echocardiograms were performed in the left lateral decubitus position using a 3.5MHz phased array transducer with harmonic imaging (Vivid7, General Electric-Vingmed Medical Systems, Horten, Norway). Grayscale images from the parasternal long-axis and short axis as well as the apical 4-chamber, 2-chamber and apical long axis views were acquired at moderate frame rate (50-70 fps) to allow for processing of 2D strain. Apical images in the 4-chamber, 2-chamber and apical long axis were acquired at high frame rate (>120 fps) with a colour tissue Doppler overlay to measure colour tissue Doppler velocity, strain and strain rate. Doppler measurement of left ventricular (LV) inflow, tricuspid valve (TV) inflow and aortic and pulmonary outflow were performed using pulsed and continuous wave Doppler. Colour tissue Doppler measurements were performed using pulsed wave Doppler in the medial and lateral LV annulus, tricuspid annulus and for all LV segmental measurements.

Cine loops of three cardiac cycles were acquired and the measurements averaged. If patients were in atrial fibrillation, 5-7 measurements were averaged. Left atrial and ventricular dimensions were obtained by M-mode according to the American Society of Echocardiography Recommendations(32). Endocardial boarders were traced at end systole and end diastole in the apical 4-chamber and 2-chamber views to obtain Simpson's Rule ejection fraction. M-mode LV mass was calculated using the formula : LV mass =0.8 × (1.04[(LV internal dimension + septal wall thickness + posterior wall thickness)3 – LV internal dimension3]) + 0.6g, and was indexed to the patient's body surface area calculated using the Haycock formula (0.024265 x Weight(kg)0.5378 x height(cm)0.3964).

For 2D strain analysis, the endocardial boarders were traced at end-systole in the 4-chamber, 2-chamber and apical long axis views; the software (EchoPac PC BTO 11, General Electric-Vingmed Medical Systems, Horten, Norway) divided the LV into basal, mid and apical

segments, and identified unique "speckles" within the myocardium and tracked them frame-by-frame throughout the cardiac cycle to obtain strain and strain rate curves. Segments that did not track properly were manually adjusted or eliminated from the analysis. Global longitudinal strain (GLS) was expressed as a mean of all 18 segments.

Definitions

Ischemic heart disease was defined as any of inducible ischaemia on non-invasive cardiac stress testing and/or ≥50% stenosis in ≥1 epicardial coronary artery on coronary angiography and/or a history of myocardial infarction and/or a history of coronary artery angioplasty, stenting or coronary artery bypass grafting.

Systolic dysfunction was classified either as a global longitudinal strain > -15% which has been associated with increased risk of mortality in both the general and dialysis populations(33-35). Diastolic dysfunction was graded as absent (normal diastolic function) or assigned a grade from one to three reflecting worsening diastolic dysfunction according to a consensus guidelines(36). Gender-specific limits from consensus guidelines were used to classify left ventricular hypertrophy as absent, mild, moderate or severe according to left ventricular mass indexed to body surface area(32).

Hydration status was expressed as the ratio of extracellular to total body water volumes, and overhydration defined as a ratio two standard deviations above the mean age- and gender-matched ratio in the normal population(28, 37).

Statistical analysis

Categorical variables were presented as frequencies and percentages, and continuous variables as mean ± standard deviation if normally distributed and as median and interquartile range if non-normally distributed. NT-proBNP concentrations demonstrated a right skewed distribution and were logarithmically transformed.

Participants with less than three paired NT-proBNP and hydration status measurements were excluded from the correlation analysis as they skewed the correlation. The correlation between the logarithm of NT-proBNP and hydration status (expressed as the ratio of extracellular water to total body water [ECW:TBW]) for each participant was calculated using Pearson's correlation at each time point. The correlation coefficient and associated standard error for each participant were then transformed using the Fisher's transformation. A

random effects meta-analysis was then conducted on the Fisher's transformed correlation coefficients across all participants, and the resulting combined estimate was then back-transformed to determine the combined correlation coefficient and its 95% confidence interval(38, 39).

To investigate if the correlation between NT-proBNP and hydration status differed according to dialysis modality, left ventricular hypertrophy, systolic and/or diastolic function, the entire cohort was divided according to dialysis modality and the echocardiographic study subgroup was divided according to the presence or absence of systolic dysfunction measured using global longitudinal strain, grade of diastolic dysfunction and severity of left ventricular hypertrophy. The correlation coefficients in the subgroups were compared to the referent subgroup category using meta-regression.

RESULTS

Participant and echocardiographic characteristics

One hundred and three patients comprising 68 haemodialysis and 35 peritoneal dialysis patients were enrolled in the study and 78 of these patients also participated in the echocardiographic sub-study. The baseline characteristics of the whole cohort and the echocardiographic sub-study cohort were comparable and are shown in Table 1. Cardiovascular risk factors and established cardiovascular disease were highly prevalent among the study cohort, including diabetes mellitus (43%), hypertension (96%), current or former smoking (57%), ischaemic heart disease (39%) and peripheral and/or cerebrovascular disease (15%). A substantial proportion of the cohort was prescribed a beta-blocker (54%) and/or a angiotensin converting enzyme inhibitor / angiotensin type-1 receptor blocker (31%).

Echocardiographic abnormalities were common among the sub-study cohort including left ventricular hypertrophy (67%) and diastolic dysfunction (84%). The prevalence of systolic dysfunction differed according to the measure of left ventricular systolic function used with 32% of the cohort having an ejection fraction ≤ 50%, while 56% of the cohort had a global longitudinal strain measurement > -15%.

Correlation between plasma NT-proBNP and hydration status

At baseline, the median plasma NT-proBNP concentration was 2299 pg/mL (IQR 956-11697), the mean (±SD) ratio of extracellular water volume to total body water volume was 0.49±0.04, and 48% of males and 53% of females were overhydrated. Participants were each reviewed for a median of 14 months (IQR 9-22) providing a total of 1431 paired bioimpedance and plasma NT-proBNP measurements. Ten participants with 14 corresponding paired measurements were excluded from the analysis as these participants each had less than 3 measurements performed. Meta-analysis of the correlation coefficients calculated for each analysed participant yielded a significant correlation coefficient of 0.273 (95% CI 0.200-0.342, I^2 = 48.3%) between plasma NT-proBNP concentration and ECW:TBW. Time series plots of plasma NT-proBNP concentration and ECW:TBW ratio for three study participants are shown in Figure 1.

Meta-regression was used to compare the correlation coefficients of plasma NT-proBNP and hydration status between subgroups of dialysis modality, left ventricular function, grade of diastolic dysfunction and severity of left ventricular hypertrophy. The correlation coefficients remained significant for all subgroups and did not differ between categories within a subgroup.

DISCUSSION

The present study is the largest and most comprehensive examination to date of the longitudinal correlation between plasma NT-proBNP and hydration status in prevalent haemodialysis and peritoneal dialysis patients. This study demonstrated that NT-proBNP concentrations measured at monthly intervals were significantly and directly correlated with changes in hydration status assessed using bioimpedance spectroscopy and were independent of dialysis modality, and cardiac structural and functional abnormalities.

The physiological basis for this correlation lies in the fact that overhydration in dialysis patients is able to cause ventricular pressure overload which in turn results in stretch and/or strain of myocardial fibrils thereby stimulating the secretion of B-type natriuretic peptide (BNP) and NT-proBNP in equimolar amounts(8, 40). Of note, ventricular pressure overload can occur in the absence of established cardiomyopathy and improves with ultrafiltration during the dialysis procedure(8). The findings of this study suggest that monthly NT-proBNP

testing may have roles in identifying and risk stratifying dialysis patients with worsening overhydration who might benefit from further specific assessment and management, and in monitoring their response to optimization treatment. Compared to a bioimpedance / lung ultrasound monitoring strategy alone, NT-proBNP testing would have the added advantage of not only identifying overhydration but also quantifying its impact on the risk of adverse cardiac events(10, 41, 42).

To date, the relationship between plasma NT-proBNP and hydration status in dialysis patients has been controversial(11) with studies yielding conflicting findings(12-16, 19, 20, 25, 43, 44). However, the vast majority of these investigations have been cross-sectional in design(12-14, 16, 43, 44) and therefore of limited direct relevance to clinical monitoring which requires demonstration of a longitudinal correlation between NT-proBNP and hydration status. A small number of longitudinal studies have also examined the association between NT-proBNP concentrations and surrogate measures of volume over the short- and medium- terms. Cohort studies examining the correlation between changes in NT-proBNP concentrations and either ultrafiltration volume or weight over a single haemodialysis session(22, 45) or across three haemodialysis treatments(22, 23) did not demonstrate a significant association between the two variables suggesting that it is not a short-term marker of hydration status. These findings are not surprising given that B-type natriuretic peptides are not stored in intracellular secretory granules, but are instead are regulated through changes in gene expression and are thus unlikely to change significantly over a short time frame(7). Conversely, cohort studies(19, 20) examining the associations between plasma B-type natriuretic peptides and surrogate measures of hydration such as serum albumin concentration, weight, and blood pressure over monthly or quarterly intervals have demonstrated significant correlations between these two variables. The findings of these studies lend support to an association between volume status and plasma B-type natriuretic peptide concentrations in dialysis patients, although they have significant limitations attributable to their use of surrogate measures of volume, infrequent repeated measures and small sample sizes. Finally, a recent cohort study by Davenport et al demonstrated a significant correlation between change in plasma NT-proBNP and hydration status assessed using bioimpedance measured on two occasions over 6-months(25). However, the paucity of repeated measures in this study introduced the risk that the correlation between the two variables may have been overestimated. The study presented here confirmed a significant correlation between NT-proBNP and hydrations status in the dialysis population while

addressing the limitations of previous studies through its use of a large inclusive sample, objective measures of volume status, and frequent repeated measurements.

A cross sectional study by David et al(46) has previously reported that plasma NT-proBNP concentration and hydration status were only correlated in dialysis patients with systolic dysfunction. To investigate whether the correlation between plasma NT-proBNP concentration and hydration status differed according to cardiomyopathy and/or dialysis modality, patients were divided into four subgroups based on dialysis modality, and left ventricular hypertrophy, and systolic and diastolic function. Correlation coefficients were then compared within subgroups using meta-regression. This analysis found that the correlation coefficients of plasma NT-proBNP and hydration status were of similar magnitudes, and remained statistically significant irrespective of subgroup implying that if NT-proBNP testing was to be used for monitoring hydration status in the dialysis population, then the same decision limits could be used regardless of dialysis modality or cardiomyopathy.

This study has several strengths including its large sample size and the large number of repeated measures per participant, its use of multi-frequency bioimpedance spectroscopy as an objective measure of hydration status, its standardisation of plasma NT-proBNP sampling, handling and storage to minimise pre-analytic variation, and its inclusion of both haemodialysis and peritoneal dialysis patients to extend the generalizability of its findings. Nevertheless, the study was limited by the fact that not all participants underwent echocardiography potentially impacting the findings of the subgroup analysis.

Conclusion

This study demonstrated that plasma NT-proBNP concentrations were significantly and directly correlated with hydration status measured longitudinally in both haemodialysis and peritoneal dialysis patients irrespective of dialysis modality. These findings raise the possibility that NT-proBNP could be used to identify patients with worsening overhydration and to monitor their response to adjustment of the dialysis prescription. However, further longitudinal studies are needed to determine the accuracy and impact of such a monitoring strategy on patient outcomes before it can be routinely adopted into clinical practice.

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Figure Legends

Figure 1. Time series plot of plasma NT-proBNP concentrations and hydration status. Time series plot of plasma NT-proBNP concentrations (pg/mL, red line) and ratio of extracellular water (ECW) volume to total body water (TBW) volume (blue line) for three study participants.

Figure 1

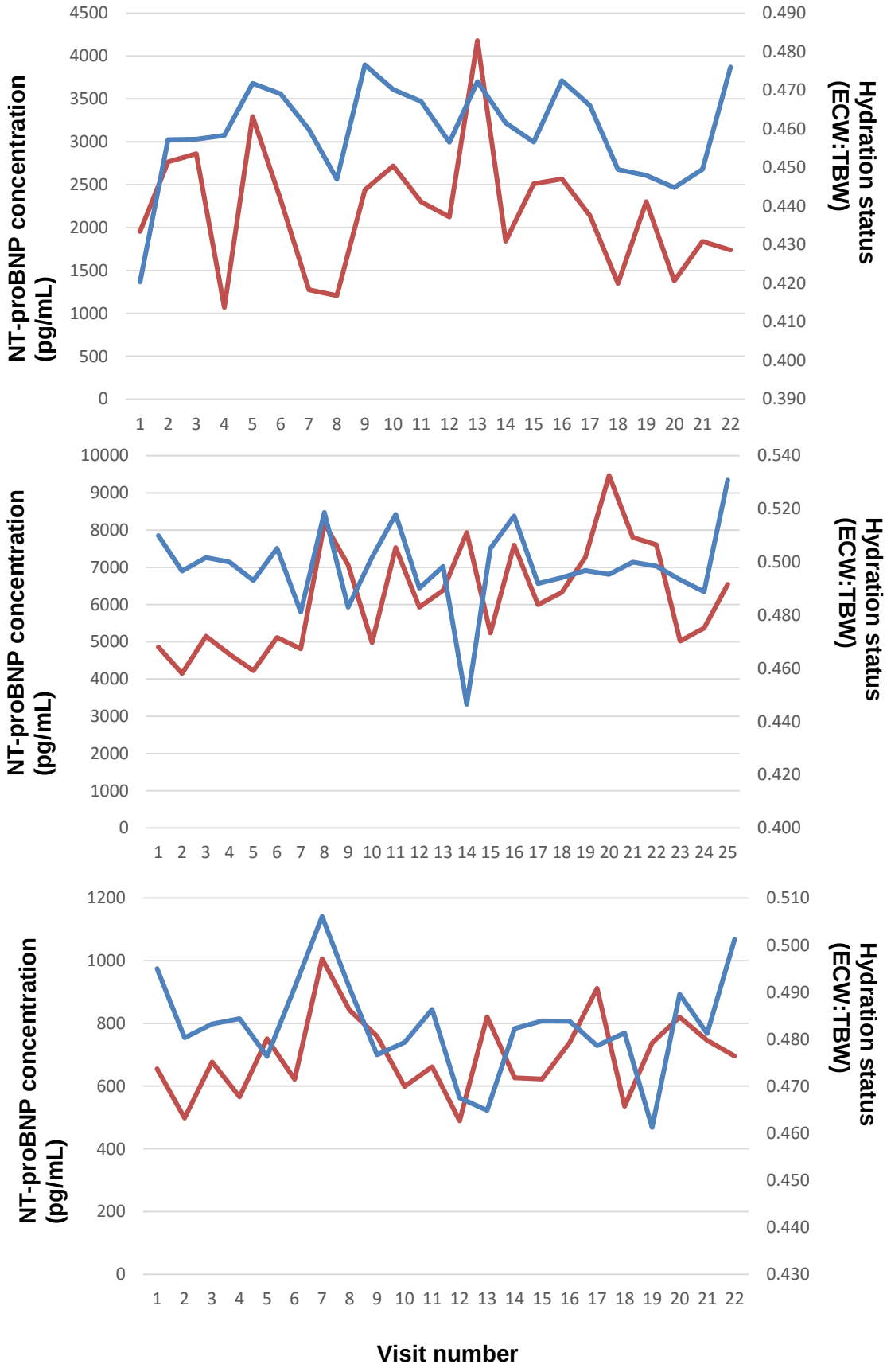

NT-proBNP concentration (pg/mL)
Hydration status (ECW:TBW)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0.490
0.480
0.470
0.460
0.450
0.440
0.430
0.420
0.410
0.400
0.390
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
NT-proBNP concentration (pg/mL)
Hydration status (ECW:TBW)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0.540
0.520
0.500
0.480
0.460
0.440
0.420
0.400
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
NT-proBNP concentration (pg/mL)
Hydration status (ECW:TBW)
1200
1000
800
600
400
200
0
0.510
0.500
0.490
0.480
0.470
0.460
0.450
0.440
0.430
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Visit number

Table 1. Baseline characteristics of the entire study and echocardiographic sub-study cohorts.

Abbreviations: ACE-I = Angiotensin converting enzyme inhibitor; ARB = Angiotensin type-1 receptor blocker; D/P creatinine = ratio of creatinine concentration in dialysate to plasma; Kt/V = dialysis urea clearance; NT-proBNP = N-terminal pro-B-type natriuretic peptide.

Characteristic	**Entire study cohort (n=103)**	**Echocardiographic sub-study (n=78)**
Male gender n (%)	64 (62)	50 (64)
Age		
Mean ± SD (years)	63±13	64±13
Age distribution n (%)		
18 – 49	19 (18)	12 (15)
50 – 69	46 (44)	35 (45)
70 – 79	26 (25)	20 (26)
80 – 90	12 (13)	11 (14)
Hemodialysis n (%)	68 (66)	48 (61)
Dialysis sessions per week (n)	3	3
Duration of dialysis session (hours)	4.6±0.7	4.7±0.7
Single pool Kt/V	1.57±0.3	1.58±0.3
Interdialytic weight gain relative to estimated dry weight (%)	1.9±1.0	2.4±1.1
Peritoneal dialysis n (%)	35 (34)	30 (39)
Volume of peritoneal dialysis solution exchanged per 24 hours (L)	8 (8-9.5)	8 (8 – 8.9)
Four hour D/P creatinine	0.72±0.1	0.72±0.1
Weekly Kt/V	2.02 (1.76-2.52)	1.93 (1.76 – 2.25)
Time on dialysis (years)	4 (2-6.3)	6 (4-9.5)

Body Mass Index (kg/m^2)	28.7±6.4	29.3±6.4
Systolic blood pressure (mmHg)	129±18	128±20
Diastolic blood pressure (mmHg)	69±13	70±13
Diabetes mellitus n (%)	59 (43)	35 (45)
Current or former smoker n (%)	59 (57)	48 (61)
Ischemic heart disease n (%)	41 (39)	32 (41)
Peripheral- and/or cerebro-vascular disease n (%)	16 (15)	13 (16)
Hydration status		
Ratio of extracellular to total body water	0.49±0.04	0.49±0.03
Proportion overhydrated n (%)		
Male	31 (48)	29 (57)
Female	27 (53)	20 (71)
Residual renal function (mL/min)	0 (0-2)	0 (0-3)
Antihypertensive agents		
Median number of anti-hypertensive agents	1 (0-3)	1(1-2)
Proportion on beta-blocker n (%)	56 (54)	45 (58)
Proportion on ACE-I or ARB n (%)	32 (31)	27 (34)
NT-proBNP (pg/mL)		
Median (IQR)	2299 (956-11697)	3344 (1080-9786)
Distribution n (%)		
< 300 pg/mL	4 (4)	4 (5)
300 – 899 pg /mL	14 (13)	9 (12)
900 – 4999 pg/mL	45 (44)	34 (44)
5000 – 19,999 pg/mL	25 (25)	20 (27)
≥ 20,000 pg/mL	15 (14)	11 (12)
Left ventricular structure and function		
Left ventricular mass indexed to BSA (g/m^2)	----------	119 (97-146)

Left ventricular hypertrophy n (%)		
Nil	----------	26 (33)
Mild	----------	10 (13)
Moderate	----------	15 (19)
Moderate	----------	27 (35)
Ejection fraction (%)	----------	54 (49-62)
Global longitudinal strain (%)	----------	-14±3.2
Left ventricular systolic dysfunction n (%)		
Ejection fraction	----------	25 (32)
Global longitudinal strain	----------	44 (56)
Diastolic dysfunction n (%)		
Nil	----------	12 (16)
Grade 1	----------	33 (42)
Grade 2	----------	26 (33)
Grade 3	----------	7 (9)

Table 2. Correlation of plasma NT-proBNP and hydration status by dialysis modality and cardiomyopathic features. Meta-regression of correlation coefficient between plasma NT-proBNP concentration and ratio of extracellular water volume to total body water volume by dialysis modality, left ventricular (LV) systolic function, and severities of left ventricular diastolic dysfunction and hypertrophy. 95% CI = 95% confidence interval

Sub-group	Correlation coefficient (95% CI)	p-value
Dialysis modality		
Haemodialysis	0.303 (0.211 – 0.391)	0.23
Peritoneal dialysis	0.203 (0.059 – 0.337)	
LV systolic dysfunction		
Absent	0.224 (0.125 – 0.320)	0.11
Present	0.350 (0.227 – 0.461)	
LV diastolic dysfunction		
Nil	0.245 (0.086 – 0.392)	0.87
Grade 1	0.316 (0.180 – 0.440)	
Grade 2	0.245 (0.100 – 0.380)	
Grade 3	0.286 (-0.016 – 0.539)	
LV hypertrophy		
Nil	0.252 (0.125 – 0.372)	0.94
Mild	0.253 (0.028 – 0.420)	
Moderate	0.319 (0.118 – 0.497)	
Severe	0.283 (0.135 – 0.419)	

Chapter 11 – Conclusion.

The findings presented in this thesis advance understanding of the pathophysiological factors underpinning NT-proBNP and hs-cTnT in the dialysis population and improve interpretation of serial measurements of these biomarkers in clinical practice. The five studies presented yielded novel and important findings, which are summarized below.

N-terminal B-type natriuretic peptide (NT-proBNP)

There has been considerable debate regarding the ideal strategy for applying NT-proBNP testing in the dialysis population. To date, most studies have pursued an absolute cut-off strategy whereby a single threshold concentration would be used to rule out left ventricular hypertrophy, systolic dysfunction or volume overload. The findings of the biological variation and longitudinal studies presented in this thesis greatly inform this debate and argue in favour of an alternate, relative monitoring strategy.

The biological variation study of plasma NT-proBNP in the dialysis population was the first of its kind to be performed and reported in the dialysis population, and found that plasma NT-proBNP has a low index of individuality in the dialysis population. This finding suggests threshold values to rule out cardiac pathology and/or hypervolaemia would need to be set at the extremes of the frequency distribution of NT-proBNP concentrations in the dialysis population, and would therefore have poor sensitivity and specificity for the target pathology and be of limited clinical utility. Instead, the study's findings strongly argue in favour of a relative monitoring strategy whereby a significant change in serial values may alert physicians to patients at increasing risk of adverse cardiovascular events and who require more detailed evaluation to determine which pathophysiological factor(s) require intervention. The within-person coefficients of variation estimated in the biological variation study will also be invaluable for guiding the interpretation of changes in serial measurements of NT-proBNP in any future monitoring strategy and for making appropriate adjustments for regression-to-the-mean effects in interventional and observational studies using plasma NT-proBNP as an outcome or exposure, respectively.

The findings of the cross-sectional investigation of the pathophysiological associations of plasma NT-proBNP in the dialysis population also support the use of a relative monitoring strategy. The study found that NT-proBNP was independently associated with left ventricular systolic

dysfunction, hydration status, left ventricular hypertrophy and residual renal function whereas previous studies had demonstrated an association with either cardiomyopathy or hydration status but not both. This novel finding can be attributed in part to the use of left ventricular global strain as the measure of left ventricular systolic function in the study. These findings lend support to the conclusion that NT-proBNP testing is likely to be better applied in the dialysis population using a relative change testing strategy as any single threshold value is unlikely to have sufficient sensitivity or specificity to exclude overhydration, left ventricular systolic dysfunction and hypertrophy concurrently. The findings of the study also identify pathophysiologic associations that may serve as targets of intervention in a potential future NT-proBNP monitoring strategy.

The longitudinal study investigating the correlation of serial measurements of plasma NT-proBNP and hydration in dialysis patients is the largest and most comprehensive study of its kind performed to date. It demonstrated, for the first time, that longitudinal changes in plasma NT-proBNP and hydration status were independently correlated thereby providing the most clinically relevant support to date for the role of NT-proBNP testing for monitoring hydration state and its impact on prognosis in the dialysis population. However, the correlation coefficient of 0.273 also indicates that NT-proBNP is not solely a marker of volume status in the dialysis population.

High sensitivity cardiac troponin-T (hs-cTnT)

The biological variation study of plasma hs-cTnT concentrations in the dialysis population was the most comprehensive such investigation to have been performed and reported in a dialysis population to date. It found that serial hs-cTnT concentrations need to increase by at least 33% or fall by at least 25% over one week to exclude changes due to biological variation alone with 99% confidence. These findings help to inform the interpretation of serial hs-cTnT concentrations measured in the acute setting for the diagnosis of myocardial injury. In addition, should hs-cTnT monitoring be adopted in clinical practice, the within-person coefficients of variation estimated in this study will be invaluable for guiding the interpretation of changes in serial measurements of hs-cTnT concentrations and for making appropriate adjustments for regression-to-the-mean effects in interventional and observational studies using plasma hs-cTnT as an outcome or exposure, respectively.

The cross-sectional investigation of the pathophysiological associations of plasma hs-cTnT in the dialysis population yielded the novel findings that plasma hs-cTnT concentration was independently associated with hydration status and possibly also with pulse wave velocity. These findings suggest

that hs-cTnT testing may additionally have a role, either alone or together with plasma NT-proBNP concentration, in identifying patients with increasing overhydration and monitoring their response to therapy. The association of plasma hs-cTnT concentration with pulse wave velocity could be explained by myocardial hypoperfusion during diastole due to vascular calcification, and generates further potential targets for intervention, although it should be noted that the association was only demonstrated using imputed data. Thus, whilst this observed relationship is biologically plausible, it requires further confirmation.

Future directions

The findings of the aforementioned studies suggest the NT-proBNP and hs-cTnT are both associated with important pathophysiological processes in the dialysis population and represent the essential preliminary steps required to devise a cardiac biomarker monitoring strategy in the dialysis population. However, important questions persist regarding the accuracy and lead time of such a monitoring strategy for predicting adverse clinical events, precise decision limits, and potential targets of intervention. Further analyses of the MONITOR study are expected to answer these vital questions.

Accuracy of clinical, biomarker, and bioimpedance based monitoring strategies for predicting adverse clinical events.

The accuracy and lead time of a monitoring strategy for predicting adverse clinical events are among the most critical factors determining its utility. The ideal strategy should identify adverse clinical events accurately and with sufficient lead time to allow intervention to potentially reduce the risk of future adverse events. In addition, such a strategy should be superior in both accuracy and lead time to clinical monitoring so as to justify a change in practice.

As detailed in chapter 5A and Appendix 1, the MONITOR study performed serial measurements of the cardiac biomarkers NT-proBNP and hs-cTnT, bioimpedance spectroscopy, and clinical assessments including weight, blood pressure, and the truncated Framingham heart failure score. The study also collected data on the occurrence of non-fatal cardiovascular events, all cause hospitalisation, fatal cardiovascular events, and all-cause mortality – each of which will be adjudicated by investigators blinded to the patient's biomarker and bioimpedance measurements. The accuracy and time to event of each monitoring strategy will be investigated using mixed models that allow the joint modelling of longitudinal and time to event data. The study will individually examine the Framingham truncated heart failure score, bioimpedance spectroscopy, hs-

cTnT and NT-proBNP monitoring in this manner, in addition to assessing the incremental benefit, or otherwise, of the latter three strategies to clinical monitoring alone. In addition, we will explore whether a combination strategy employing both NT-proBNP and hs-cTnT is superior to a monitoring strategy which only uses a single biomarker.

Longitudinal association of cardiac biomarkers with therapeutic interventions and pathophysiological processes

Should NT-proBNP and/or hs-cTnT be shown to predict adverse clinical events in a timely and accurate manner, then further evaluation of the longitudinal association between the cardiac biomarkers and both therapeutic interventions and pathophysiologic processes will be essential to guiding intervention(s) based on monitoring.

The analyses presented in the thesis have already demonstrated an association between NT-proBNP concentrations and both left ventricular global strain and volume status in cross-sectional studies and a longitudinal correlation between NT-proBNP concentrations and volume status. The latter association will be examined further using both time series analysis and mixed models to explore if a lag exists in the correlation, which may in turn alter the strength of the correlation. Similar methods will also be used to examine the longitudinal association between NT-proBNP concentrations and changes in cardiac structure, systolic and diastolic function, systemic inflammation, vascular stiffness, cardiac pharmacotherapy, and the dialysis prescription. Similar analyses will also be performed with hs-cTnT. By determining the relative contribution of each of these therapeutic and pathophysiological factors to changes in cardiac biomarker concentrations, these analyses will guide the development of a hierarchical intervention strategy to be tested in a pilot randomised study of biomarker guided therapy.

Should a pilot randomised study prove to be safe and feasible, we would endeavour to perform a large randomised study with the goal of assessing if biomarker based monitoring improves patient outcomes and quality of life and is cost-effective which is the definitive pre-requisite for instituting biomarker monitoring in clinical practice.

Appendix 1

SERIAL NT-PROBNP MONITORING IN THE DIALYSIS POPULATION FOR THE PREDICTION OF MAJOR CARDIOVASCULAR EVENTS

STUDY PROTOCOL VERSION 3.0

Title: Serial NT-proBNP Monitoring in the Dialysis Population for The Prediction of Major Cardiovascular Events

Study Rationale

Introduction

The all-cause mortality rate in the dialysis population is 15% per annum with cardiovascular disease being the leading cause of death (34%). Compared with the general population, dialysis patients have a 100-fold increased risk of cardiac arrest which has been attributed to traditional and non-traditional risk factors including extracellular volume expansion leading to cardiomyopathy(1-3). This appallingly high mortality has remained largely unchanged over the last decade. Current tools to identify patients at high risk of adverse cardiac events and guide dry body weight, cardiac pharmacotherapy and the dialysis prescription are inadequate and there is urgent need for novel tools to guide interventions.

In the dialysis population, cardiomyopathy rather than coronary artery disease is the major risk factor for sudden death. Volume overload and hypertension have been identified as critical risk factors for cardiomyopathy, and pilot studies of anti-hypertensive and beta-blockade therapy in dialysis have yielded promising results. Progress has been hampered, however, by the fact that current tools to assess patients' hydration and cardiac risk status are insensitive, and a validated tool to guide dry body weight, cardiac pharmacotherapy, and the dialysis prescription does not exist. The development of a tool that accurately identifies patients at high risk of future adverse events, and may also be used to guide therapy has been labelled a priority.

N-terminal proBNP

In the non-dialysis population, the amino terminal fragment of the cardiac hormone proBNP (NT-proBNP) has demonstrated excellent diagnostic utility for excluding cardiac systolic / diastolic dysfunction and accurately predicts prognosis in individuals with established cardiac failure. There

is also increasing evidence from randomised controlled trials and meta-analyses that among individuals aged under 75-years of age, heart failure pharmacotherapy guided by NT-proBNP concentrations produces superior clinical outcomes compared to best clinical practice alone(4, 5).

The amino terminal fragment of the cardiac hormone B-type natriuretic peptide (NT-proBNP) has emerged as an extremely strong candidate biomarker for monitoring cardiovascular disease in the dialysis population. NT-proBNP is a biologically inactive 76 amino-acid peptide secreted in equimolar amounts to the biologically active hormone BNP. Twenty percent of the peptide is excreted unchanged by the kidneys and the remainder is eliminated by passive endocytosis. The ventricular myocardium is the major site of synthesis of NT-proBNP which occurs primarily in response to ventricular strain or stretch, reflecting underlying hypertrophy or dilatation. Other stimuli include ischaemia/hypoxia, endothelin-I, angiotensin-II, interleukin-Iβ, and adrenergic agonists(6, 7). Thus from a physiological stand point, NT-proBNP reflects left ventricular structural and functional injury in response to a milieu of pathological stimuli known to be prevalent in the dialysis population. Indeed, if the general population cut-off for NT-proBNP is applied to the dialysis population, NT-proBNP concentrations are universally elevated in the order of 2-15 times the upper limit of normal. This marked elevation primarily reflects the widespread prevalence of left ventricular structural and functional abnormalities in the dialysis population and far exceeds the elevation that can be attributed to impaired renal excretion alone.

NT-proBNP in the Dialysis Population

The prognostic value of NT-proBNP in dialysis has been demonstrated in both haemo- and peritoneal- dialysis patients. In a cohort of 965 peritoneal dialysis patients followed for a minimum of 2-years, Paniagua et al demonstrated that NT-proBNP concentrations at baseline were highly predictive of cardiovascular and all-cause mortality at 30-months after controlling for demographic factors, co-morbidity, dialysis treatment dose, inflammatory markers, and residual renal function. In this study, patients with NT-proBNP concentrations below the lowest quintile (3465 pg/ml) had a relative risk of all cause mortality of 0.64 compared to patients with higher concentrations (95% CI 0.45 – 0.92, p = 0.016)(8). Similarly, Madsen et al investigated the prognostic value of NT-proBNP in a cohort of 109 haemodialysis patients followed for a mean duration of 20-months. A multivariate Cox regression analysis using demographic factors, co-morbidities, inflammatory markers, left ventricular ejection fraction, left ventricular mass, and NT-proBNP as independent variables found that only NT-proBNP (HR 1.52 95% CI 1.18-1.92 p

= 0.001) and age (HR 1.04 95% CI 1.01 – 1.06 p = 0.009) were independent predictors of all cause mortality(9).

Cross-sectional studies have also demonstrated a strong inverse correlation between NT-proBNP and left ventricular ejection fraction(9-11) and a strong direct correlation between NT-proBNP and left ventricular mass indexed for body surface area(9, 12) in both haemo- and peritoneal dialysis patients. NT-proBNP concentrations measured pre- and post- single dialysis sessions have not been shown to correlate with ultrafiltration volume(9), however, a strong direct correlation has been demonstrated between NT-proBNP and extracellular fluid volume assessed by multifrequency bioimpedance at 2-monthly intervals over 6-months in a cohort of 44 haemodialysis patients(13), suggesting that NT-proBNP is a marker of medium-term extracellular fluid volume.

Of greatest interest are the findings of a study of 585 incident haemodialysis patients in whom NT-proBNP was measured at dialysis inception and again 3-months later to evaluate the association between longitudinal change in NT-proBNP over the first 90 days of dialysis and mortality at 1-year (14). Kaplan-Meier estimates of survival were significantly worse in subjects with a net increase in NT-proBNP compared with those with a net decrease over the first 90-days of dialysis. When examined in a multivariate Cox regression analysis adjusted for baseline NT-proBNP, age, sex, race, aetiology of renal disease, and albumin, the highest tertile of delta NT-proBNP had a 2.4 fold greater risk of all-cause mortality and a 2.9 fold greater risk of cardiovascular mortality than patients in the lowest tertile.

Moving Forward: Formulating a Serial Monitoring Strategy

Whereas current studies support the hypothesis that changes in NT-proBNP predict changes in extracellular volume, cardiac structure and adverse clinical events their design does not provide the critical information needed to develop a clinically useful serial monitoring model. Specifically, the vast majority of published studies use *single or very small* numbers of repeated measures of NT-proBNP, *subjective* assessments of fluid state, and have focused on *fatal* clinical outcomes only. A clinically useful serial monitoring model needs to:

1. Distinguish between changes in concentration that accurately predict adverse events and should therefore prompt clinical action versus those related to expected biological variation that can be ignored. This can only be defined through serial testing.

2. Predict *non-fatal* clinical outcomes and early physiological derangements to enable timely intervention in order to improve outcomes.
3. Provide an estimate of the time interval between a change in a longitudinal marker and the outcome of interest (time to event).
4. Improve on established monitoring strategies by providing *earlier* and/or *more accurate* detection of the disease of interest
5. The next step in the evolution of NT-proBNP as a clinically useful tool is to develop a serial monitoring model that incorporates absolute value cut-offs and a magnitude of serial change that accurately predict adverse events.

The use of a biomarker in serial monitoring has several similarities to a screening program. The aim is to use the test (biomarker) to identify a pre-clinical stage of the condition of interest before the condition becomes irreversible, allowing earlier intervention and leading to an improved outcome. The major difference between traditional screening tests and serial monitoring are that serial monitoring is often applied to diseased populations, and places more importance on change in the test/biomarker rather than using a fixed cut-off for case identification. Nevertheless, it is worthwhile applying the criteria for a screening program to the serial monitoring model. In order to justify a screening program several criteria need to be satisfied(15):

- **The condition** needs to be 'important' to the population of interest, being either common and/or severe – cardiomyopathy, cardiovascular disease and sudden cardiac death in the dialysis population certainly satisfy this criterion
- **The natural history** of the condition needs to be understood well enough to justify screening. We need to be reasonably confident that early detection will capture a *reversible* stage of the disease and that intervening at this earlier stage will result in superior outcomes compared with usual practice.
- In this regard, there is mounting evidence that sudden cardiac death in the dialysis population is related more closely to cardiomyopathy than coronary artery disease, and that fluid overload, hypertension and cardiac fibrosis represent integral mechanisms in the pathogenesis of cardiomyopathy. Unfortunately there are no large randomized trials of interventions in the dialysis population demonstrating improved cardiovascular outcomes with any intervention, however, a recent meta-analysis of randomized control trials of blood pressure control reported reduced cardiovascular event rates, and reduced cardiovascular and all cause mortality

associated with use of blood pressure lowering agents(16) while a number of other trials of fluid control, and beta-blockade have demonstrated improvement in surrogate cardiovascular measures.

- **The test** needs to acceptable, easily applied, and accurate. This is the focus of this study.
- The screening program needs to formally **evaluated** to ensure that it is able to be implemented effectively and produces superior outcomes compared with established practice.
- **Economic analyses** need to be performed to determine the cost-effectiveness of screening and ensure that adequate resources/facilities exist to deal with the outcomes of screening.

Research Questions

1. In prevalent dialysis patients, is there a magnitude of change in serial NT-proBNP concentrations that predicts future adverse clinical events? Over what time frame and how accurately? Do progressively larger changes predict increasingly severe events (non-fatal vs fatal)?
2. In prevalent dialysis patients, is there a magnitude of change in serial NT-proBNP concentrations that accurately correlates with progressive over-hydration and predicts evolving cardiac dysfunction.
3. In prevalent dialysis patients, is there an absolute concentration(s) of NT-proBNP that diagnoses over-hydration and/or established cardiac dysfunction, and predicts adverse clinical events? Over what time frame and how accurately?
4. How do these derived change/absolute values of NT-proBNP compare with patient symptoms and/or bioimpedance for predicting evolving cardiac dysfunction and adverse clinical events? Does it do it earlier and more accurately? Is its predictive value modified by bioimpedance analysis and/or patient symptom score?
5. What are the clinical, physiological and biochemical characteristics (potential therapeutic targets) of the individuals who have these abnormal absolute NT-proBNP values and/or abnormal change values?

Hypotheses

In the prevalent dialysis population, progressive elevation of serial NT-proBNP concentrations of a magnitude exceeding biological variation will accurately correlate with:

- Overhydration on bioimpedance analysis

- Patient reported symptoms of congestive heart failure

and predict:

- Fatal and non-fatal cardiovascular events
- Evolving adverse cardiac structural and functional changes
- All cause mortality

In the prevalent dialysis population, NT-proBNP concentrations exceeding an absolute value will accurately diagnose:

- Overhydration on bioimpedance analysis
- Established cardiac structural and functional abnormalities

and predict:

- Fatal and non-fatal cardiovascular events
- All cause mortality

Aims

A. Predictive Aims

1. Establish a magnitude(s) of change in serial NT-proBNP concentrations that accurately predicts:
    a. Fatal and non-fatal cardiovascular and clinical adverse events
    b. Evolving adverse cardiac structural and functional changes
2. Establish the time to event (lead time) between the determined change value(s) and the adverse outcome(s).
3. Determine if changes of progressively increasing magnitude predict increasingly severe adverse events (fatal vs non-fatal events)
4. Establish an absolute value(s) of NT-proBNP that accurately diagnoses:
    a. Over-hydration
    b. Established cardiac dysfunction / abnormal cardiac structure

    and predicts:
    c. Adverse clinical events
5. Establish the time to event (lead time) between the determined absolute concentration and the adverse event

B. Comparative Accuracy

1. Compare the accuracy and time to event of the derived NT-proBNP change value with the accuracy and time to event of:
    a. Change in bioimpedance (normal hydration to over-hydration)
    b. Change in symptom score (from < 2 to ≥ 2) on truncated Framingham score

for:

- Adverse cardiovascular and clinical events
- Evolving cardiac dysfunction / adverse structural change
- Determine if the predictive accuracy of the derived NT-proBNP change value for adverse clinical events is modified by:
- concurrent change in patient symptom score on the truncated Framingham heart failure score
- concurrent change in hydration state on bioimpedance analysis

C. Mechanistic Aims

1. Evaluate the correlation between change in NT-proBNP concentrations and change in:
    a. Symptom score on the truncated Framingham heart failure score
    b. Hydration state on bioimpedance
    c. Indices of cardiac systolic and diastolic function
2. Determine the clinical, physiological, biochemical and therapy-related patient associations of the derived NT-proBNP change and absolute values.

Study Design and Sample

Study Design: Prospective Cohort Study

Study Population: Prevalent peritoneal dialysis and in-centre haemodialysis patients

Sample Size: 150 participants in total consisting of 50-70% haemodialysis and 30-50% peritoneal dialysis patients to reflect the proportions of these dialysis modalities in the dialysis centre. Methodology for calculating samples sizes in longitudinal studies investigating the predictive value of biomarkers is not established.

The sample size in this study is chosen based on:

Event rate: The all cause mortality rate of prevalent haemodialysis patients is 19.3 per 100 patient years in the first year following dialysis inception and 7.3 per 100 patient years in the second year following dialysis inception. For prevalent peritoneal dialysis patients, the all cause mortality rate is 12.5 per 100 patient years in the first year following dialysis inception and 15 per 100 patient years in the second year following dialysis inception (Princess Alexandra Hospital ANZDATA Unit Report 2008). Estimates of non-fatal morbid event rates are not available, but are expected to exceed fatal event rates.

Feasibility: It is anticipated that study visits will be conducted by Dr Fahim and a study nurse. 150 participants is the maximum number of participants that can followed monthly by this study staff.

Generalizability: The proposed sample sizes represent approximately 33% of all haemo- and peritoneal- dialysis patients at the Princess Alexandra and Logan Hospitals and should provide a participant case mix that is representative of both the study units' and Australasian dialysis populations.

Setting : In-centre haemo-, Minimal care haemo- and peritoneal- dialysis units of the Princess Alexandra Hospital, Brisbane, and Greenslopes Hospital, Brisbane, QLD, Australia.

Recruitment and Selection : Participants meeting entry criteria will be selected consecutively.

Inclusion Criteria

- In-centre haemo- or peritoneal dialysis patients established on dialysis for ≥ 90-days
- Established on a *single* dialysis modality for ≥ 30 days
- Aged 18-years or older
- Temporary or permanent vascular access
- Able to provide informed consent

Exclusion Criteria

- Advanced malignancy and/or current malignancy undergoing evaluation and/or treatment – excludes skin cancers
- Permanent pacemaker and/or implantable cardiac defibrillator
- Coronary artery stent
- Limb amputee
- Living donor renal transplant planned within 3-months of enrolment

- Home haemodialysis
- Congenital heart disease (excluding haemodynamically insignificant patent foramen ovale)
- Acute renal impairment with the expectation of spontaneous recovery of renal function
- Pregnancy

Target Condition / Outcomes

Primary:

Non-fatal coronary vascular events - New onset stable angina pectoris, or unstable angina pectoris, or non-fatal ST or non-ST elevation myocardial infarction or coronary artery revascularisation - angiographic or surgical.
Non-fatal heart failure (with/out unscheduled dialysis)
Unscheduled dialysis for hypertension (symptomatic or asymptomatic)
Non-fatal peripheral vascular events – Stroke (thrombotic or haemorrhagic), or intervention for peripheral vascular disease – revascularization or amputation including carotid revascularization procedures.
Arrhythmic events:
New onset atrial fibrillation requiring initiation of rate control therapy
Atrial flutter
Ventricular tachycardia
Ventricular fibrillation
2nd or 3rd degree atrioventricular block
Resuscitated cardiac arrest
Death due to coronary artery disease
Death due to heart failure
Sudden cardiac death
Death due to stroke
Death due to peripheral vascular disease
Cardiovascular death (composite of 7-11)

Secondary outcomes:

Death due to any cause
Hospitalisation for any cause

Dialysis modality change from peritoneal to haemo- dialysis attributed to ultrafiltration failure

Deterioration in indices of left ventricular systolic function:

Ejection fraction – relative change within or across diagnostic categories by 10% **OR** change from > 50% to < 50%

Global longitudinal strain by 8%

Left ventricular volumes by 12%

Deterioration in indices of left ventricular diastolic function:

Left atrial volume by 10%

Deceleration time by 10% **OR** from Mild (Stage 1) to moderate or severe diastolic (Stage 2 or 3) dysfunction.

E/e' by 15% **OR** from normal filling pressure (E/e' <8) to elevated filling pressure (E/e' >15)

For each outcome event, a record will be made of whether or not emergency department review and/or hospital admission was required for the event.

NOTE: In situations where more than one event occurs concurrently, only the most severe event will be recorded the outcome for that time point.

Index Tests

Change in serial NT-proBNP concentrations

Absolute NT-proBNP concentration

Bioimpedance analysis

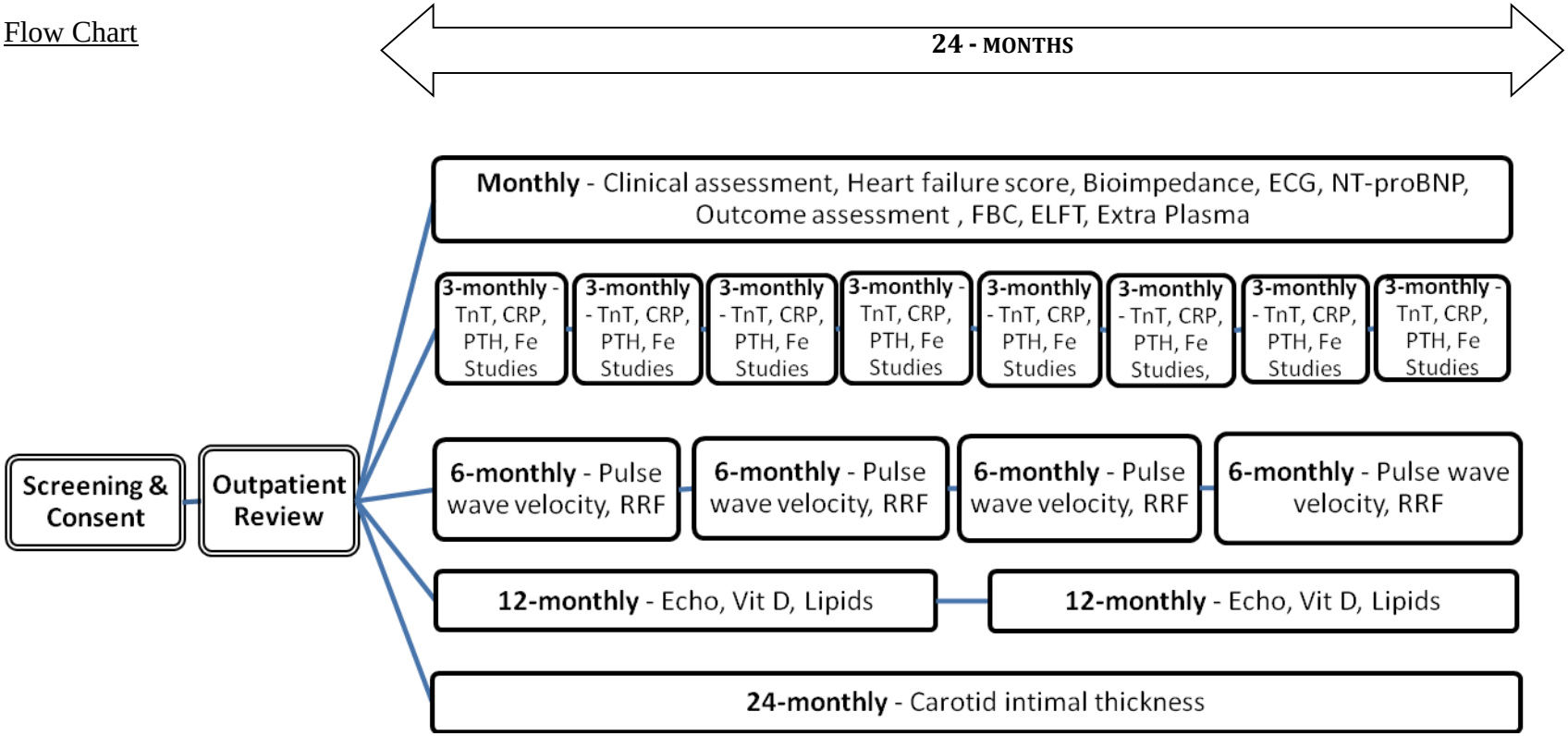
Truncated Framingham Heart Failure Assessment Score

Reference Standard for Target Condition

The reference standard is clinical review as per standard clinical practice undertaken in the dialysis unit and/or nephrology clinic either as a routine or initiated by the patient. *Outcome definitions are detailed in Appendix 6*

Methods

Flow Chart



Method Description

Informed consent.

Information will be collected about eligible participants who refuse to participate for comparison with the study group to determine if they are comparable.

All participants will be reviewed at monthly intervals as outpatients.

Outpatient review

Participants will be instructed to avoid strenuous exercise prior to attending the baseline and assessment visits.

All baseline and assessment visits will be performed on the same day of the week:

Mid-week, pre-dialysis for haemodialysis patients.

Mid-week for peritoneal dialysis patients.

Baseline data collection (*see Appendix 1 for Baseline Visit Procedure*).

Assessment visits and clinical outcome ascertainment will be conducted monthly (*see Appendix 2 for Assessment Visit Procedure*).

Investigational measures will be conducted at varying intervals (*see schedule in Appendix 5*).

Participants will be followed up for 24-months or until transplant, death, withdrawal from dialysis or transfer out of the study centres.

Study Measures

Physical examination and Heart failure assessment score

These assessments will be performed by Dr Magid Fahim throughout the study who will be blinded to the NT-proBNP concentrations throughout the study. In addition, this assessment will **always** be performed prior to bioimpedance analysis, so that Dr Fahim will be blinded to the bioimpedance analysis at the time of clinical assessment.

NT-proBNP and undesignated plasma collection and storage

15 ml of whole blood will be collected into a lithium heparin tube *pre-dialysis* from the participant's AV fistula/graft or central venous catheter (if on haemodialysis) or from his/her arm (if on peritoneal dialysis).

Samples will be stored at 4°C for a maximum of 1-hour before undergoing centrifuge. Plasma will be pipetted into 2ml aliquots and stored at -80°C. Samples for NT-proBNP will be analysed in batches at 6-month intervals on the Elecsys 2010 (Roche Diagnostics) after a quality control specimen analysis.

Undesignated plasma will be stored for up to 15-years and re-analysed in the future for new biomarkers relevant to the area. HREC approval will be sought prior to such analysis and participants will be consented for this at enrolment.

12-lead ECG and Bioimpedance analysis

These procedures will be performed by Dr Magid Fahim and/or a study nurse in accordance with manufacturer's instructions. ECG interpretation will be performed by Dr Magid Fahim and any queries referred to Assoc Prof Richard Troughton.

Echocardiograms

Echocardiograms will be:
Performed mid-week on a non-dialysis day (the day after the second dialysis of the week) throughout the study
Performed by the one of four echocardiographers who will be blinded to all clinical and biochemical information including NT-proBNP concentrations
Assoc Prof Richard Troughton will assess a random sample of serial Echo's (all 3 Echo's from a given patient) in a blinded fashion to ensure measurements are accurate (quality control procedure)

Carotid intimal thickness, and Pulse wave velocity
Carotid intimal thickness and pulse wave velocity will be performed by the same operator throughout the study, mid-week pre-dialysis (after the second dialysis of the week for haemodialysis).

<u>Outcome Ascertainment</u>

Clinical outcomes will be ascertained by:
Monthly participant interview
Monthly review of dialysis notes *(redundancy procedure)*
Monthly review of local hospital computer admission records *(redundancy procedure)*

Monthly review of Princess Alexandra and Logan Hospitals' computerized nephrology database *(redundancy procedure)*
Death certificate review (if applicable)

Any potential clinical outcomes detected by these methods will be confirmed by review of the participant's medical notes and/or death certificate to ensure that the event satisfies the required definitions. Confirmatory reviews will be carried out by Dr Magid Fahim and in cases where there is any ambiguity, the notes/cases will be referred to Assoc Prof Carmel Hawley and Dr Scott Campbell for further review.

Cardiac functional/structural outcomes will be ascertained by comparison of serial echocardiograms. Echocardiograms from *all time points* for a single patient will be measured (interpreted) by a single interpreter blinded to patient clinical and biochemical data at the end of the study.
Serial changes in carotid intimal thickness and pulse wave velocity will be ascertained by comparison of serial recorded images. Images from *all time points* for a single patient will be measured (interpreted) by a single interpreter blinded to patient clinical and biochemical data at the end of the study.

Statistical Analysis

Time to cardiovascular event/outcome will be analysed using survival analysis, with NT-proBNP being included as a time-varying covariate. We will also analyse the data using recently developed statistical methods that allow joint modelling of time-to-event data and longitudinal data (NT-proBNP). To determine whether BNP monitoring provides an incremental benefit over symptom monitoring, we will include both BNP and symptoms in our models. Other relevant clinical and demographic variables will be included in the model as covariates. We will determine the best combinations of monitoring (BNP,bioimpedance and symptoms) by checking the ability of our models to discriminate between patients who do or do not have the primary outcomes of interest.
To examine the relationship between longitudinal change in NT-proBNP concentrations and longitudinal change in (i) bioimpedance measurements and (ii) echocardiographic outcomes we will fit a longitudinal model with NT-proBNP as the time-varying covariate outcome and the other variables (echo + fluid state change) as outcomes.

Losses to follow-up and withdrawal of consent

Patients' data will be censored at time of death, renal transplant, transfer out of study centre, and withdrawal of consent. Any data collected prior to these events will be included in the analysis.

Safety and Monitoring

An adverse event is classified as 'serious' (SAE) if it meets any one of the following criteria:
Death
Life threatening: The subject was at substantial risk of dying at the time of the adverse event or it is suspected that the use or continued use of the product would result in the subject's death.
Hospitalisation (initial or prolonged): Required admission to the hospital or prolongation of a hospital stay.
Disability: Resulted in a significant, persistent, or permanent change, impairment, damage or disruption in the subject's body function/structure, physical activities or quality of life.
Congenital anomaly / birth defect
Important Medical Event: Other medically important events that, in the opinion of the investigator, may jeopardise the subject or may require intervention to prevent one of the other outcomes listed above.

This study is purely observational with no investigational drugs or interventions and no deviations from current standard practice. We will seek an exemption from reporting Serious Adverse Events unless they are 'unexpected' or deemed 'clinically significant' by the Principal Investigator/s.

Clinical findings consistent with life threatening heart failure or other relevant life threatening clinical pathology will be reported to the participant's usual doctor at the time of the study visit for further review and/or intervention.

Bioimpedance spectroscopy readings have been validated against models of total body water but have **not** been shown to be related to adverse clinical outcomes. Thus both bioimpedance measures and NT-proBNP concentrations will NOT be communicated to the clinicians caring for the participants.

Investigators

Principal Investigator

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Associate Investigators

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Consultant Nephrologist, The Children's Hospital at Westmead, Sydney
Professor (Personal Chair), Clinical Epidemiology, School of Public Health, University of Sydney, Australia

Professor David Johnson
Consultant Nephrologist, Clinical Director, Professor of Medicine (University of Queensland),
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Christchurch, New Zealand

Mr Goce Demiski
Supervising scientist, Chemical Pathology Princess Alexandra Hospital, Brisbane, Australia

Collaborators

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MSc, PhD, RDCS, FASE

Echocardiographer and Research Fellow, Cardiovascular Imaging Research Centre of The University of Queensland

Appendix 1 - Baseline Data Items & Investigations

Study participant ID number

ANZDATA number

Age

Gender

Ethnicity

Renal diagnosis

Time from referral to renal service to dialysis commencement (weeks)

Date of dialysis commencement and duration of dialysis (months)

Dialysis access at dialysis commencement – temporary vs permanent

Single dialysis modality since commencement (Y/N) – if 'No' – Record (i) dialysis modalities used, (ii) duration of each modality (weeks), and (iii) reason for change of modality

Duration of dialysis on current modality

Current vascular access – CVC (tunnelled or non-tunnelled), AV fistula, AV graft, PD catheter

If AV fistula / graft - record 2 most recent flow rates and dates

Current dialysis prescription and adequacy

Haemodialysis – frequency, duration of session, dialysis membrane ultrafiltration coefficient, blood flow rate, dialysate flow rate, dialysate sodium concentration, dialysate potassium concentration 1.0 mmol/l (Y/N), last two single-pool Kt/V and dates, average monthly inter-dialytic weight gain as a percentage of estimated dry body weight in the month prior to assessment ([Total inter-dialytic weight gain across entire month / number of haemodialysis sessions] / estimated dry body weight) x 100.

Peritoneal dialysis – CAPD vs CCPD vs NIPD, total volume of exchanges per 24-hours, transporter status, last recorded weekly Kt/V and number of episodes of peritonitis

Residual renal function (measured within 3-months of enrolment)

History of transplantation (Y/N) – if 'Yes' – record (i) number of transplants, (ii) duration of dialysis independent graft function and (iii) reason for graft failure.

Current medications – generic names, dose and frequency (including IV iron)

ESA dose – type and dose as mcg or mg/kg per g/l Hb

Co-morbidities

Cardiovascular

Ischaemic Heart Disease

Angina

Method of diagnosis – Clinical only (*probable*) (Y/N) vs clinical and (i) non-invasive stress test (record date, type of test and result) or (ii) evidence of > 50% stenosis of epicardial coronary artery on coronary angiography (*definite*)

Severity on Canadian Cardiovascular Society grading of angina pectoris (*see Appendix 4*)

Current and past therapy – medical, PTCA, and/or coronary surgery

Myocardial infarction (MI) – type, number and therapy:

ST elevation MI and therapy – thrombolysis / PTCA / conservative

Non-ST elevation MI

Document most recent (i) non-invasive cardiac stress test (type, date, and result) and/or (ii) coronary angiogram if performed *(even if normal)*

Congestive Heart Failure / Cardiomyopathy

Previous admission for congestive heart failure (Y/N)

Current or previous:

Left ventricular systolic dysfunction and/or

Dilated cardiomyopathy

Hypertrophic obstructive cardiomyopathy

Document measures of most recent echocardiogram and date

Valvular Heart Disease

Document valve(s) involved, type of dysfunction, and severity from most recent echocardiogram.

Valve surgery and date.

Arrhythmia

Type, rate (from ECG), and therapy

Peripheral Vascular and Carotid Artery Disease

Diagnosis - clinical only (*probable*) and/or doppler ultrasound or angiography (document date and results) (*definite*)

Therapy - Angioplasty, endarterectomy, stenting, or amputation

Cerebrovascular Disease

Transient ischaemic attack

Thrombotic or haemorrhagic stroke

Subarachnoid haemorrhage

IF NO IMAGING – classify as *probable*

Dyslipidaemia

Record most recent lipid profile – total cholesterol, LDL, HDL, and TAG concentrations – date and fasting or not

Diabetes

Duration and therapy

Hypertension – (Y/N)

Smoking history

Current / former / never – if current/former document pack years

Pulmonary Disease

Restrictive or obstructive (document respiratory function test results and date)

Hepatic

Viral hepatitis, alcoholic or non-alcoholic steatohepatitis, cirrhosis – record investigational evidence of any clinical diagnosis

Peptic Ulcer Disease – record if endoscopically proven (*definite*) or not (*probable)*

Endocrine – type of disorder

Alcohol consumption – standard drinks per week

Neurological (non-cerebrovascular)

Previous Malignancies – excluding non-melanoma skin cancer

Examination

Weight

Height

Waist circumference

BMI

Pulse rate and rhythm

Manual sitting blood pressure

Heart failure assessment scale (*see Appendix 3*)

Investigations

Full blood count and differential (FBC)

Urea, creatinine and electrolytes – Na^{+}, K^{+}, Cl^{-}, $HCO3^{-}$, Corr. Ca^{2+}, PO_4^{3-}, Urea, Creatinine

Albumin and liver enzymes

Ferritin, and transferrin saturation

Troponin T (TnT)

C-reactive protein (CRP)

Parathyroid hormone (PTH)

Fasting lipid profile

25-hydroxy vitamin D

NT-proBNP

Hs-cTnT

5-ml plasma to be stored at -80°C for future analysis of relevant biomarkers (patient will be consented for this)

Residual renal function

ECG

Bioimpedance analysis

Echocardiogram (mid-week on non-dialysis day)

Carotid intimal thickness

Pulse wave velocity

Appendix 2 - Follow-up Assessment Visit and Outcome Ascertainment Redundancy Procedures

Visits will be conducted mid-week pre-dialysis for haemodialysis patients and mid-week for peritoneal dialysis patients – visits will always be performed on the same day of the week throughout the study

Patient Visit

History – *(Monthly)*

Enquiry regarding clinical events since previous assessment:

presentation to dialysis service for unscheduled dialysis

presentation to emergency department - presenting complaint and diagnosis

admission to hospital - presenting complaint and diagnosis

cardiac investigations and/or interventions and indication

vascular investigations and/or interventions and indication

change in dry body weight target and indication

change in dialysis modality or prescription and indication

change in angina severity on Canadian Cardiovascular Society grading of angina pectoris (*see Appendix 4*)

Medication list review – record changes and reasons for changes

Examination – *(Monthly)*

Weight using identical set of scales throughout study for all participants

Pulse rate and rhythm

Manual sitting blood pressure

Heart failure assessment scale (*see Appendix 3*)

Investigations – TO BE PERFORMED PRE-DIALYSIS

Full blood count and differential (FBC) – (*Monthly*)

U & E's – Na^{+}, K^{+}, Cl^{-}, $HCO3^{-}$, Corr. Ca^{2+}, PO_4^{3-}, Urea, Creatinine – (*Monthly*)

Albumin and liver enzymes – (*Monthly*)

Ferritin and transferrin saturation – *(3-monthly)*

High sensitivity cardiac troponin T (hs-cTnT) – *(3-monthly)*

C-reactive protein (CRP) – *(3-monthly)*

Parathyroid hormone (PTH) – *(3-monthly)*

25-hydroxy vitamin D – (Y*early*)

NT-proBNP – (*Monthly*)

5-ml plasma to be stored at -80°C for future analysis of relevant biomarkers (patient will be consented for this) – (*Monthly*)

ECG – (*Monthly*)

Residual renal function (*3-monthly*)

Bioimpedance analysis – (*Monthly*)

Imaging

Echocardiogram (mid-week on non-dialysis day) – *(Yearly)*

Carotid intimal thickness – *(2-yearly)*

Pulse wave velocity – *(6-monthly)*

Redundancy Procedures for Ascertaining Outcomes

Local hospital admission records and the nephrology database will be reviewed monthly to check if the patient has been admitted to hospital. If an admission is found then the patient's hospital record will be retrieved and the following information recorded:

Indication for admission

Diagnosis

Relevant diagnostic procedures

Medication and dialysis prescription changes

Dialysis notes will be reviewed monthly for:

- mean post-dialysis blood pressure across the entire month.
- average interdialytic weight gain over month as percentage of estimated dry body weight
- unscheduled dialyses and indication

If a patient fails to attend a follow-up visit and cannot be contacted directly:

Local hospital records and the nephrology database will be checked to determine if they have been admitted to hospital.

If it transpires that the participant is deceased, a copy of the death certificate will be obtained. (Permission from the Australian Institute of Health and Welfare will be obtained.)

If it transpires that the patient has withdrawn from dialysis, the reason for this shall be ascertained through the patient's medical records and discussion with the physician responsible for their care

If the patient transfers out of the study centre then they shall still be followed up for mortality

Appendix 3 - Heart Failure Assessment Scale

Truncated Framingham Heart Failure Assessment Score

Symptom / Sign	Value	Patient Score
Orthopnoea	0.5	
Paroxysmal nocturnal dyspnoea	1.0	
Reduction in exercise tolerance	0.5	
Resting sinus tachycardia (> 100 / min)	0.5	
Jugular venous pressure > 4 cm	0.5	
Hepatojugular reflex positive	1.0	
Third heart sound	1.0	
Bibasal crackles	1.0	
Hepatomegaly	0.5	
Peripheral Oedema	0.5	
Total		

From: Ho KK, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure: the Framingham Study. *J Am Coll Cardiol* 1993; 22: 6A-13A

Appendix 4 - Canadian Cardiovascular Society grading of angina pectoris

Grade	Description
Grade I	Ordinary physical activity does not cause angina, such as walking and climbing stairs. Angina with strenuous or rapid or prolonged exertion at work or recreation
Grade II	Slight limitation of ordinary activity. Walking or climbing stairs rapidly, walking uphill, walking or stair climbing after meals, or in cold, or in wind, or under emotional stress, or only during the few hours after awakening. Walking more than two blocks on the level and climbing more than one flight of ordinary stairs at a normal pace and in normal conditions
Grade III	Marked limitation of ordinary physical activity. Walking one or two blocks on the level and climbing one flight of stairs in normal conditions and at normal pace
Grade IV	Inability to carry on any physical activity without discomfort, anginal syndrome may be present at rest

From: Campeau Lucien. Grading of angina pectoris. *Circulation* 1976; 54: 522-523

Appendix 5 - Study Procedure Schedule

	Baseline	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13
History	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Examination	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CCS angina severity	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Heart Failure score	X	X	X	X	X	X	X	X	X	X	X	X	X	X
FBC	X	X	X	X	X	X	X	X	X	X	X	X	X	X
U & E	X	X	X	X	X	X	X	X	X	X	X	X	X	X
LFT	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Iron studies	X	-	-	X	-	-	X	-	-	X	-	-	X	-
TnT	X	-	-	X	-	-	X	-	-	X	-	-	X	-
CRP	X	-	-	X	-	-	X	-	-	X	-	-	X	-
PTH	X	-	-	X	-	-	X	-	-	X	-	-	X	-
Lipids	X	-	-	-	-	-	-	-	-	-	-	-	-	X
25-OH Vit D	X	-	-	-	-	-	-	-	-	-	-	-	-	X
NT-proBNP	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5-ml plasma	X	X	X	X	X	X	X	X	X	X	X	X	X	X

ECG	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Bioimpedance	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Residual renal function	X	-	-	-	-	-	X	-	-	-	-	-	X	-
Echocardiogram	X	-	-	-	-	-	-	-	-	-	-	-	X	-
Carotid intimal thickness	X	-	-	-	-	-	-	-	-	-	-	-	-	-
Pulse wave velocity	X	-	-	-	-	-	X	-	-	-	-	-	X	-
Clinical outcome assessment	-	X	X	X	X	X	X	X	X	X	X	X	X	X

	M14	**M15**	**M16**	**M17**	**M18**	**M19**	**M20**	**M21**	**M22**	**M23**	**M24**
History	X	X	X	X	X	X	X	X	X	X	X
Examination	X	X	X	X	X	X	X	X	X	X	X
CCS angina severity	X	X	X	X	X	X	X	X	X	X	X
Heart Failure score	X	X	X	X	X	X	X	X	X	X	X
FBC	X	X	X	X	X	X	X	X	X	X	X
U & E	X	X	X	X	X	X	X	X	X	X	X
LFT	X	X	X	X	X	X	X	X	X	X	X
Iron studies	-	X	-	-	X	-	-	X	-	-	X
TnT	-	X	-	-	X	-	-	X	-	-	X

CRP	-	X	-	-	X	-	-	X	-	-	X
PTH	-	X	-	-	X	-	-	X	-	-	X
Lipids	-	-	-	-	-	-	-	-	-	-	-
25-OH Vit D	-	-	-	-	-	-	-	-	-	-	-
NT-proBNP	X	X	X	X	X	X	X	X	X	X	X
5-ml plasma	X	X	X	X	X	X	X	X	X	X	X
ECG	X	X	X	X	X	X	X	X	X	X	X
Bioimpedance	X	X	X	X	X	X	X	X	X	X	X
Residual renal function	-	-	-	-	X	-	-	-	-	-	X
Echocardiogram	-	-	-	-	-	-	-	-	-	-	X
Carotid intimal thickness	-	-	-	-	-	-	-	-	-	-	X
Pulse wave velocity	-	-	-	-	X	-	-	-	-	-	X
Clinical outcome assessment	X	X	X	X	X	X	X	X	X	X	X

Appendix 6 - Outcome Definitions

Non-fatal Myocardial Infarction (ST elevation or Non-ST elevation myocardial infarction)

A rise and/or fall of cardiac Troponin from a previous or baseline measurement by ≥20%, with at least one level above the upper reference limit (defined as a level exceeding the 99th percentile of a reference population at which the assay has a coefficient of variation ≤10%) ***together with at least one of the following***:

Symptoms of myocardial ischaemia (various combinations of chest, upper limb, jaw, or epigastric discomfort at rest or on exertion with/out accompanying dyspnoea, diaphoresis, nausea and/or syncope lasting ≥ 20 minutes);

ECG changes –

ST elevation in two contiguous leads of ≥0.2mV in men or ≥0.15mV in women in V2 or V3 and/or ≥0.1mV in other leads *OR*

New horizontal/down-sloping ST depression ≥0.05mV in two contiguous leads, *OR*

T-wave inversion ≥0.1mV in two contiguous leads (with prominent R or R/S ratio>1) *OR*

New left bundle branch block *OR*

Any Q-waves in leads V_2-V_3 ≥ 0.02 s or QS complex in leads V_2 and V_3 *OR*

Q-wave ≥ 0.03 s and ≥ 0.1 mV deep or QS complex in leads I, II, aVL, aVF, or V_4-V_6 in any two leads of a contiguous lead grouping.

Imaging evidence of new loss of viable myocardium or new regional wall abnormality.

From: Thygesen K et al. Universal Definition of Myocardial Infarction. *Circulation.* 2007;116: 2634-2653.

New Onset Stable Angina Pectoris

New onset of symptoms of angina pectoris (various combinations of chest, upper limb, jaw, or epigastric discomfort with/out accompanying dyspnoea, diaphoresis, nausea and/or syncope) occurring on moderate to severe exertion (CCS class I and II)

AND one of:

Positive non-invasive cardiac stress test demonstrating reversible ischaemia

OR

Coronary arteriography demonstrating at least one 50% stenosis in one or more epicardial coronary arteries.

From: Hemmingway et al. Incidence and Prognostic Implications of Stable Angina Pectoris Among Women and Men**.** *JAMA.* 2006; 295: 1404-1411

Unstable Angina Pectoris

Symptoms of myocardial ischaemia (various combinations of chest, upper limb, jaw, or epigastric discomfort with/out accompanying dyspnoea, diaphoresis, nausea and/or syncope) meeting *any one* of the following criteria:

Rest angina - Angina occurring at rest and prolonged, usually greater than 20 min

New-onset angina - New-onset angina of at least CCS class III severity

Increasing angina severity - Previously diagnosed angina that has become distinctly more frequent, longer in duration, or lower in threshold (i.e., increased by 1 or more CCS class to at least CCS class III severity)

With or without ECG changes

New horizontal/down-sloping ST depression ≥0.05mV in two contiguous leads, *OR*

New T-wave inversion ≥0.1mV in two contiguous leads

AND

In the absence of ST elevation on the ECG or detectable quantities of a marker of myocardial injury (troponin I (TnI), troponin T (TnT), or CK-MB) based on 2 or more samples collected at least 6 h apart, with a reference limit of the 99th percentile of the normal population.

From: Anderson J et al. ACC/AHA 2007 Guidelines for the Management of Patients With Unstable Angina/Non_ST-Elevation Myocardial Infarction: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non_ST-Elevation Myocardial Infarction) Developed in Collaboration with the American College of Emergency Physicians, the Society for Cardiovascular Angiography and Interventions, and the Society of Thoracic Surgeons Endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation and the Society for Academic Emergency Medicine. *J Am Col Cardiol* 2007; 50: e1 – 157

Heart Failure

New onset or worsening dyspnoea at rest, exertional dyspnoea, paroxysmal nocturnal dyspnoea, or orthopnoea AND
Chest X-ray consistent with pulmonary congestion AND
Echocardiogram performed before or at presentation demonstrating left ventricular systolic and/or diastolic dysfunction AND
Improvement in symptoms following diuresis, ultrafiltration, and/or systemic vasodilator therapy

From: Zannad et al. Heart Failure as an endpoint in heart failure and non-heart failure cardiovascular clinical trials: the need for a consensus definition. *Eur Heart J*. 2008; 29: 413-421

Stroke

New focal neurological dysfunction attributable to brain or retinal ischaemia that lasts more than 24 hours (unless death occurs within 24 hours, in which case it will be classified as a fatal stroke) or where brain imaging demonstrates an acute infarction or haemorrhage consistent with the presentation.

Coronary Revascularisation

Percutaneous transluminal coronary angioplasty with or without stent. NOT diagnostic coronary angiograms. OR
Coronary artery bypass graft surgery.

Evidence in the form of a hospital discharge summary or procedure sheet will be required.

Unscheduled Haemodialysis for Hypertension

Haemodialysis / ultrafiltration procedure performed outside the participant's usual schedule where the primary indication is a blood pressure > 170/100 with/out documented clinical evidence of overhydration with/out symptoms/signs of hypertensive encephalopathy (headache, nausea, confusion, seizure, papilloedema, retinal haemorrhages or exudates)

Unscheduled Haemodialysis for Shortness of Breath

Haemodialysis / ultrafiltration procedure performed outside the participant's usual schedule where the primary indication is a complaint of dyspnoea at rest or exertion, or paroxysmal nocturnal dyspnoea, or worsening orthopnoea *and* clinical evidence of overhydration with or without a chest X-ray demonstrating pulmonary congestion.

Intervention for peripheral vascular disease

Revascularisation of the lower limb by percutaneous or surgical means.
Lower limb amputation that is not directly caused by trauma or infection, but where vascular insufficiency is deemed the primary reason for the amputation.

Death Due to Coronary Artery Disease

Any one of the following:

Death attributed to myocardial infarction (as defined above)

Sudden, unexpected death involving cardiac arrest with symptoms consistent with myocardial ischaemia and accompanied by new ST-elevation, or new LBBB and/or evidence of fresh thrombus by coronary angiography and/or autopsy and/or pathological evidence of acute myocardial infarction but death occurring before blood samples could be obtained or at a time before cardiac biomarkers would be expected to be abnormal.

Death following cessation of dialysis if coronary artery disease is the primary reason for cessation of dialysis.

Death during coronary revascularization procedure.

From: Thygesen K et al. Universal Definition of Myocardial Infarction. *Circulation.* 2007;116: 2634-2653.

Death due to Heart Failure

Death in a patient who presents with heart failure OR
Death following cessation of dialysis if heart failure is the primary reason for cessation of dialysis

Death due to Stroke

Death following presentation with stroke OR

Death following cessation of dialysis if stroke is the primary reason for cessation of dialysis.

Death due to Peripheral Vascular Disease

Death following presentation with a peripheral vascular disease event such as an ischaemic or gangrenous lower limb, where vascular insufficiency and not infection or trauma is the reason for the ischaemia OR Death following cessation of dialysis if peripheral arterial disease is the primary reason for cessation of dialysis

Resuscitated Cardiac Arrest

Sudden collapse of a participant that is witnessed, with either documented absent circulation or ventricular tachy- or brady-arrhythmia and where the patient responds to standard cardiopulmonary resuscitation procedures.

Sudden Cardiac Death

Death within one hour of new cardiac symptoms or an unexpected, unwitnessed death in a person without any known non-cardiac illness that could be expected to become rapidly fatal and/or no alternative cause of death on autopsy if autopsy is performed.

Peritoneal Dialysis Ultrafiltration Failure

Clinical syndrome of overhydration (various combinations of dyspnoea at rest or on exertion, orthopnoea, paroxysmal nocturnal dyspnoea, hypertension, tachycardia, Bibasal crepitations, pleural effusion, and/or oedema.) AND

An ultrafiltration volume of < 400 ml after 2-hour dwell of 2 litres of 4.25% peritoneal dialysis fluid

AND

Exclusion of constipation, peritoneal leak, catheter entrapment, obstruction, or malposition.

From: Mujais et al. Evaluation and management of ultrafiltration failure problems in peritoneal dialysis. Perit Dial Int 2000; 20(S4): S5 - S21

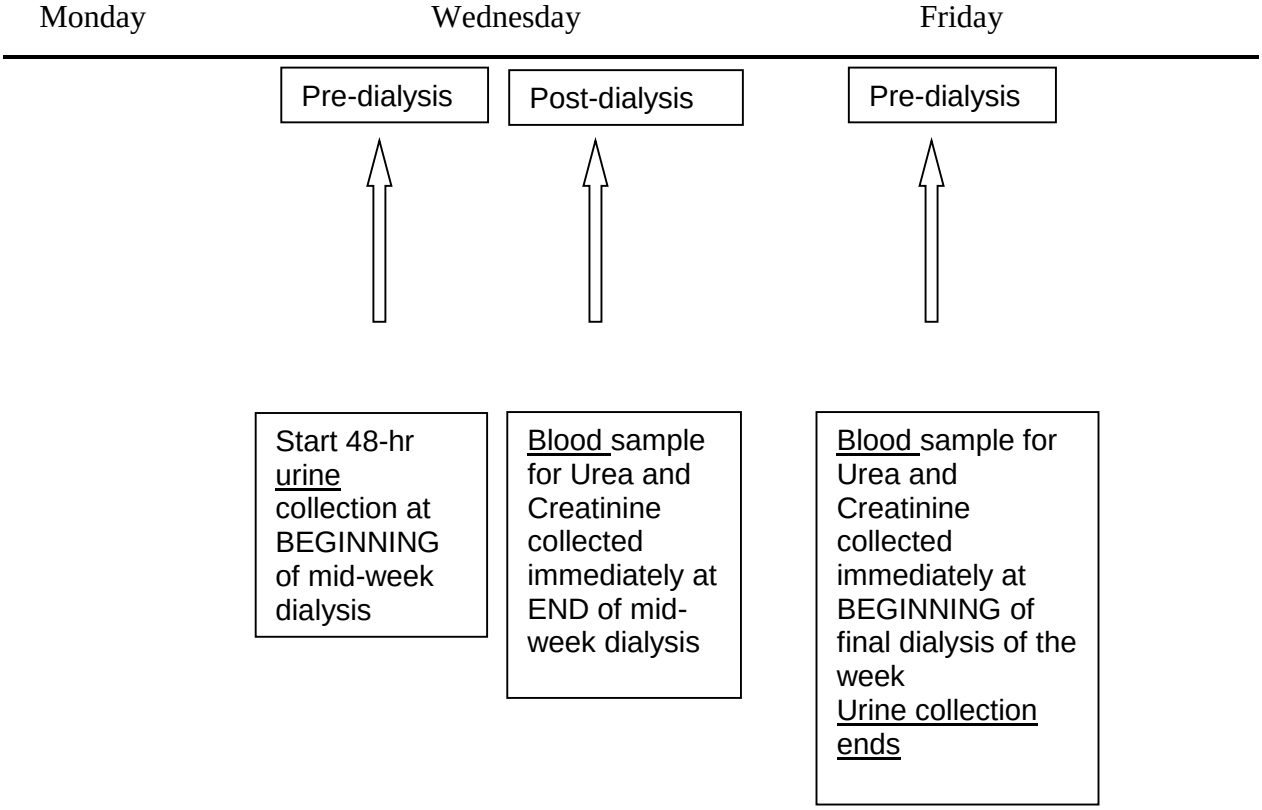
Appendix 7- Measurement of Residual Renal Function

Residual renal function (rGFR adjusted for body surface area) will be estimated as the mean of creatinine and urea clearances using formula:

rGFR (ml/min/1.73m^2) = ½ (Creatinine clearance + Urea clearance) (1.73/BSA)

Haemodialysis

Example for patients on Monday, Wednesday, Friday Schedule



Procedure

Urine collection starts at the beginning of the mid-week dialysis:

Prior to dialysis the patient is asked to empty their bladder

All urine from the start of the mid-week haemodialysis session and for the next 48-hours is to be collected into the supplied container

The patient is asked to empty their bladder into the container just prior to their final dialysis of the week. This concludes the urine collection

Blood sampling

Blood to be collected POST-MID WEEK DIALYSIS ***AND***

PRE-FINAL WEEK DIALYSIS for urea and creatinine concentrations

Pre-Haemodialysis Blood Sampling Procedure

When using an AV fistula or graft,

Obtain the blood specimen from the arterial needle prior to connecting the arterial blood tubing or flushing the needle. Ensure that no saline and/or heparin is present in the arterial needle and tubing prior to drawing the sample for measurement of plasma urea.

Do not draw a sample for use as pre-dialysis bloods if haemodialysis has been initiated.

When using a venous catheter,

Use a 5ml syringe, and withdraw any heparin and saline from the arterial port of the catheter, along with blood, to a total volume of 5ml. Discard the contents of this syringe.

Connect a new syringe and draw the sample for plasma urea measurement.

Do not draw a sample for use as pre-dialysis bloods if haemodialysis has been initiated.

Post-Haemodialysis Blood Sampling Procedure

At the completion of haemodialysis, turn off the dialysate flow and decrease the ultrafiltration rate (UFR) to 50ml/h, (to the lowest TMP/UFR setting, or off).

Decrease the blood flow to 100ml/min for 15s (longer if the bloodline volume to the sampling port exceeds 15ml).

Proceed to obtain the blood sample.

Calculations

Creatinine clearance (ml/min) = $(U_{Cr}\,V_1)/\,[0.5(\text{PreCr} + \text{PostCr})t]$

U_{Cr} is the urinary creatinine concentration (μmol/L) in the interdialytic urine collection

V_1 is the volume (ml) of the interdialytic urine collection

PreCr is the plasma creatinine concentration (μmol/L) at the beginning of the final dialysis of the week

PostCr is the plasma creatinine concentration (μmol/L) immediately post the middle dialysis of the week

t is time (min) of inter-dialytic period

Urea clearance (ml/min) = $(U_{ur} V_1)/ [0.5(PreUr + PostUr)t]$

U_{ur} is the urinary urea concentration (μmol/L) in the interdialytic urine collection

V_1 is the volume (ml) of the interdialytic urine collection

PreUr is the plasma urea concentration (μmol/L) at the beginning of the final dialysis of the week

PostUr is the urea concentration (μmol/L) immediately post the middle dialysis of the week

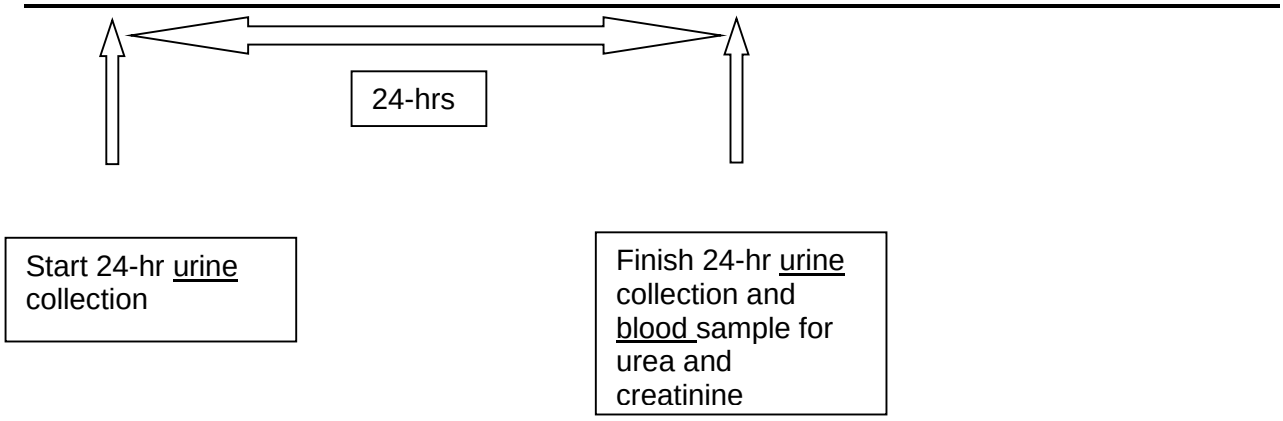
t is time (min) of inter-dialytic period

Residual GFR (residual renal function) =

rGFR (ml/min/1.73m^2) = ½ (Creatinine clearance + Urea clearance) (1.73/BSA)

Peritoneal Dialysis

Any day of the week



Procedure

Can be performed on any day of the week

Empty bladder into toilet prior to start of 24-hour collection

Start 24-hour urine collection

Empty bladder into collection bottle immediately at the end of 24-hour collection and obtain blood sample for urea and creatinine concentrations.

Calculation

rGFR (ml/min/1.73m^2) = ½ (Creatinine clearance + Urea clearance) (1.73/BSA)

Creatinine clearance (ml/min): $(U_{Cr}\ V)/1440P_{Cr}$

Where U_{Cr} is the urinary creatinine concentration in 24-hour urine collection (μmol/L), V is the volume of the 24-hour urine collection (ml), P_{Cr} is the plasma creatinine concentration at the end of the 24-hour urine collection (μmol/L)

Urea clearance (ml/min): $(U_{ur}\ V)/1440P_{Ur}$

Where U_{Ur} is the urinary urea concentration in the 24-hour urine collection (mmol/L), V is the volume of the 24-hour urine collection (ml), P_{Ur} is the plasma urea concentration at the end of the 24-hour urine collection (mmol/L)

Body Surface Area (BSA) (m^2): Dubois formula

Appendix 8 - Echocardiography Protocol

Echocardiograms will be performed mid week on a non-dialysis day throughout the study.
Echocardiograms will be performed by the same sonographers/scientist who will be blinded as to the patient's clinical status throughout the study.
Measurements will be performed at the end of the study with the scientist/sonographer blinded to the sequence of the echocardiograms, and the patient's clinical and biochemical details.
Assoc Prof Richard Troughton will assess a random sample of serial Echo's (all 3 Echo's from a given patient) in a blinded fashion to ensure measurements are accurate (quality control procedure)
Digital cine loops of three (3) cardiac cycles will be acquired and stored for offline analysis.

Parasternal Long Axis view:
2D loop at moderate (50-70/min) frame rate.
2D loop with colour tissue Doppler at high (>120/min) frame rate
M-mode (still frame) of aortic root/left atrium
M-mode (still frame) of left ventricle

Parasternal Short Axis view:
2D loop at aortic valve level
2D loop at basal left ventricular level at moderate frame rate
2D loop with colour tissue Doppler at basal left ventricular level at high frame rate
2D loop at mid left ventricular level
2D loop at apical left ventricular level

Apical Four Chamber View:
2D loop at moderate frame rate focusing on left and right ventricles
2D loop with colour tissue Doppler at high frame rate focusing on left and right ventricles
2D loop at high frame rate to include both atria and pulmonary veins
Pulsed wave Doppler of the right upper pulmonary vein (sweep)
Pulsed Doppler of the mitral inflow (sweep)
Pulsed Doppler of the left ventricular outflow (sweep)
Pulsed Doppler of the tricuspid inflow (sweep)
Continuous wave Doppler of the mitral, aortic and tricuspid valve flows (sweep)

Pulsed tissue Doppler of the right ventricular free wall annulus

Pulsed tissue Doppler of the septal mitral annulus

Pulsed tissue Doppler of the lateral mitral annulus.

Apical Two Chamber view:

2D loop at moderate frame rate focusing on left ventricle

2D loop with colour tissue Doppler at high frame rate focusing on left ventricle

2D loop at high frame rate to include left atrium and pulmonary veins

Apical Long Axis view:

2D loop at moderate frame rate focusing on left ventricle

2D loop with colour tissue Doppler at high frame rate focusing on left ventricle

3D Echocardiography:

Full volume 3D left ventricular acquisition of four (4) cardiac cycles (X2)

Measurements:

M-mode	Aortic root and left atrial AP diameter (Ao/LA)
	Left ventricular end diastolic diameter (LVEDD)
	Left ventricular end systolic diameter (LVESD)
	Septal wall thickness (diastole) (IVS)
	Posterior wall thickness (diastole) (PW)
2D	Left ventricular end diastolic volume (4 chamber and 2 chamber) (LVEDD)
	Left ventricular end systolic volume (4 chamber and 2 chamber) (LVESD)
	Left ventricular mass and mass index (LMV/LVMI)
	Left atrial area (4 chamber and 2 chamber) (LAA)
	Left atrial volume (4 chamber and 2 chamber) (LAV)
	Twist and untwist velocities and rates (Torsion)
	Global longitudinal strain and strain rate (GLS)
Doppler--	Pulmonary vein S, D and a velocities and a duration
	Mitral E, A velocities, E/A ratio, deceleration time
	Isovolumic relaxation and contraction times (IVRT and IVC)
	Aortic and mitral valve opening and closing
	Aortic valve velocity and velocity time integral (VTI)

Tricuspid valve regurgitant velocity (TR)

Tissue Doppler—Left ventricular S', E' and A' velocities

Right ventricular S' velocity

Left ventricular filling pressure (E/E')

3D-- Left ventricular end diastolic volume

Left ventricular end systolic

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Appendix 2

Awards, invited lectures and research grants during candidature

AWARDS

1. National Health and Medical Research Council Postgraduate Scholarship (2010 -2013)
2. Finalist - Young Investigator of The Year. Short and Long Term Variation of High Sensitivity Troponin-T and N-Terminal B-Type Natriuretic Peptide in The Stable Dialysis Population. Australia & New Zealand Society Of Nephrology (ANZSN) Annual Scientific Meeting, Brisbane, Australia (2013)

INVITED LECTURES

1. 'Biological Variation of NT-proBNP in the Stable Dialysis Population' Presented June 20th 2012 at Screening Test Evaluation Program (STEP) seminar series, Sydney University
2. 'Biological Variation in Clinical and Research Practice' Presented on 17th March 2012, Shire Clinical Insights Meeting 2012, Hilton Hotel, Sydney

RESEARCH GRANTS

Dr Magid Fahim (the candidate) was responsible for authoring the successful research grant proposals list below and all associated reviewer rebuttals in their entirety

Year(s) Awarded	Nature of Funding	Funding Body	Peer Reviewed	Role	Value (AUD)
2010 - 13	Postgraduate Medical Research Scholarship	National Health & Medical Research Council, Australia	Y	Principal Investigator	102,750
2010 - 13	In-kind Equipment Support	Fresenius Medical Care, Australia	Y	Chief Investigator	65,000
2011 - 12	Project Grant	Princess Alexandra Research Foundation, Australia	Y	Chief Investigator	75,000
2011 - 12	Project Grant	Kidney Health Australia	Y	Chief Investigator	55,000

Year(s) Awarded	Nature of Funding	Funding Body	Peer Reviewed	Role	Value (AUD)
2012 - 13	Project Grant	National Health & Medical Research Council, Australia	Y	Chief Investigator	281,193
2011	Infrastructure Support Grant	Princess Alexandra Research Foundation, Australia	Y	Chief Investigator	7,500
2012	Equipment Support	Roche Diagnostics, Australia	Y	Chief Investigator	20,000
2012 - 13	Equipment Support	Roche Diagnostics, Switzerland	Y	Chief Investigator	156,723
2012	Infrastructure Support Grant	Princess Alexandra Research Foundation, Australia	Y	Chief Investigator	7,100