

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

“Chronic kidney disease (CKD) is defined as either persistently low glomerular filtration rate (GFR) less than 60ml/min/1.73m², or presence of markers of kidney damage (haematuria, proteinuria or abnormalities in imaging studies) for more than 3 months” (1). Using this definition, about 10-13% of the general population is reportedly affected by CKD (2). More specifically, CKD is at least 3-4 times more frequent in Africa than in developed countries (3). The prevalence of CKD in South Africa was reported as 15-24% by Matsha *et al* (4). Chronic kidney disease patients are more likely to develop cardiovascular disease (CVD) than their age-matched counterparts in the general population, so much so that in CKD patients, the risk of death particularly due to cardiovascular disease is much higher than the risk of eventually requiring dialysis (5). Sarnak *et al* (6) in their study further showed that CKD is a strong predictor of CVD even after correcting for other risk factors.

It is well documented that systemic inflammation plays a major role in the development of atherosclerosis as well as cardiovascular morbidity and mortality among CKD patients (7). The chronic inflammatory state is prevalent in CKD and CVD and represent a connecting factor in the pathogenesis and progression of these two chronic conditions (8). However, the cause of the inflammation and the process by which chronic low grade inflammation leads to vascular disease is yet to be proven.

“Endotoxin (lipopolysaccharide [LPS]) is a glycolipid that comprises most of the outer wall of Gram-negative bacteria (GNB), and is a potential source of inflammation” (9, 10). Gram negative organisms colonize various systems in the human body thereby generating endotoxins not only from overt infections but also in common chronic subclinical bacterial infections leading to endotoxaemia (11). It is now recognized that endotoxaemia

constitutes a strong risk factor for early atherogenesis in patients with chronic bacterial infection (12). These findings were further supported by a previous study that show that endotoxaemia was strongly related to atherosclerosis (13). The study demonstrated that close to half of the newly diagnosed atherosclerosis was attributed to the presence of low grade bacterial infection. In an animal study, weekly exposure to endotoxin lead to progression of atherosclerosis in rabbits on a hypercholesterolaemic diet (14). This further suggests that chronic exposure to endotoxin may be related to subclinical atherosclerosis and represents a reversible CVD risk factor.

Although previous studies have postulated that elevated levels of circulating plasma endotoxin are associated with risk of atherosclerosis and cardiovascular disease (15-18), it is still difficult to firmly establish the mechanism by which endotoxin-related inflammation leads to atherosclerosis. This present study therefore aimed to establish the potential role of endotoxin/toll-like receptor-4 related immune activation, to explain the association between circulating endotoxin and atherosclerosis in CKD patients.

Very few studies have investigated endotoxaemia across the spectrum of CKD patients, and most importantly, there is a paucity of data on the association between immune activation and endotoxin-induced atherosclerosis among African CKD patients. In addition, this study is unique in that a body composition monitor – bio-impedance spectroscopy (BIS) device, a convenient, accurate and potentially safe technique to measure body composition was used to objectively assess fluid overload as a possible stimulus for inflammatory activation in CKD patients. This study therefore, aimed to investigate endotoxaemia across the spectrum of South African CKD patients and also sought to determine the pro-inflammatory effects of endotoxin that may be relevant to the development of atherosclerosis in CKD patients.

This review will explore the link between CKD and CVD as well as the potential source of endotoxaemia in patients with CKD. In addition, it will describe the cellular and molecular mechanisms of inflammatory activation by endotoxaemia while highlighting various consequences of inflammation particularly in CKD. Finally, it will also provide an overview of therapeutic strategies for reduction of the inflammation through endotoxin reduction.

1.1 Epidemiology of CVD in CKD

Numerous studies have demonstrated that there is an inverse relationship between GFR and the incidence of CVD and interestingly, this observation is independent of associated risk factors (19-21). The Chronic Kidney Disease Prognosis Consortium in a meta-analysis of studies in the general population, after adjusting for traditional CVD risk factors reported an association between impaired kidney function and increased risk of death from any cardiovascular cause when compared with the reference group with normal kidney function (20). Similarly, Go *et al* (21) reported an association between lower GFR and development of CVD. On further analysis, the adjusted hazard ratio increased from 1.4 in the group of patients with CKD stage 3 to 3.4 in patients with end stage renal disease (ESRD). More important is the association between the degree of proteinuria and risk of CVD morbidity and mortality. In a meta-analysis of cohorts with cardiovascular events, graded increase in the risk of coronary artery disease (CAD) was observed with elevated proteinuria (22). Microalbuminuria was associated with a 50% increase in the risk of CAD (HR = 1.47; 95% CI = 1.30 to 1.66) while the risk is doubled in patients with macroalbuminuria (HR = 2.17; 95% CI = 1.87 to 2.52). Likewise, Ljungman *et al* (23) in a 10-year follow-up study of 120 middle-aged non-hypertensive and non-diabetic patients with no CVD at baseline reported higher baseline urine albumin excretion in those that developed CVD compared to those who remained free of CVD. This association remained significant even after adjusting

for blood pressure, with proteinuria still more strongly associated with CVD than with smoking and serum cholesterol. Similarly, data from diabetic cohorts as well as a meta-analysis reported a graded increase in CVD risk with elevations in proteinuria (20, 24). Moreover, the association between albuminuria and cardiovascular mortality seems somewhat independent of GFR. Schmieder *et al* (25) showed that elevations in proteinuria predicted mortality among patients with vascular disease but normal GFR.

According to the United States Renal Data System (2009) annual data report, the annual incidence of sudden cardiac death in CKD patients is 2.8%, which is five folds higher than the incidence in the general population (26). The prevalence of CAD ranges between 24% among young non-diabetic dialysis patients and 85% among older diabetic patients on long-standing dialysis (27, 28). Liu *et al* (29) reported a significant association between CAD and CKD among patients who underwent coronary angiography; 18.8% of patients with CAD had CKD compared with 5.4% without CAD ($p < 0.001$). Therefore, strategies that target atherosclerotic disease may be effective in reducing the burden of CVD in populations with advanced CKD.

1.2 Spectrum of Cardiovascular Pathology in Patients with Chronic Kidney Disease

Pathological changes in the cardiovascular system can be broadly divided into those that affect the vessels (specifically arteries) and heart structures.

1.2.1 Arterial Disease

The term “arteriosclerosis” generally used to describe a range of vascular pathology was derived from Greek word meaning “hardening of vessels”. However, the term stringently refers to atherosclerosis, arteriolosclerosis, and Mönckeberg’s medial calcific sclerosis (30). Atherosclerosis is characterized by the development of lipid-enriched plaques in the intima layer of the artery. Occurrence of calcification in a plaque is important to determine the stage of the lesion thereby making calcification an important feature of atherosclerosis (31). In

contrast, arteriolosclerosis, which was first described in patients with Bright's disease in 1868, is characterized by noncalcified, nonatheromatous stiffening of smaller muscular arteries (31). The third distinct form of arterial disease was described in 1903 by Mönckeberg. It is characterized by non-atheromatous thickening and heavy calcification involving the tunica media of the artery (32). Chronic kidney disease patients may be affected by all of the three aforementioned forms of arterial disease. Arteriosclerosis in an individual will be determined by various factors including the patient's age, exposure to risk factors as well as duration of CKD.

1.2.2 Cardiac Disease

Left ventricular remodeling develops well before initiation of dialysis and may even be detectable in patients with CKD stage 2 or 3 (33). Association between CKD and CVD was first observed among young dialysis patients, with increased left ventricular mass reported in 74% of them (34). Left ventricular hypertrophy (LVH) is commonly categorized according to the predominant pattern of abnormality on echocardiogram. Foley *et al* (34) described concentric hypertrophy and eccentric hypertrophy in 44% and 30% of their studied population respectively. Concentric LVH is associated with pressure overload (typically accompanying hypertension, arteriosclerosis or occasionally aortic stenosis) and is characterized by increased wall thickness and normal or decreased left ventricular chamber (34). Eccentric LVH is characterized by an increase in myocyte length that is proportional to the increase in left ventricular diameter (34). Risk factors for eccentric LVH include volume overload secondary to salt and water retention, anaemia and arteriovenous fistula (6, 34).

1.3 Overview of Risk Factors for Atherosclerotic cardiovascular disease in chronic kidney disease

The Framingham Heart Study group in 1961 introduced the concept of CVD risk factors by linking the presence of previously identified clinical conditions (elevated

cholesterol, hypertension, diabetes mellitus, and tobacco use) to development of future CVD (35). These risk factors are presumed to cause the CVD (35). The major CVD risk factors are broadly classified into traditional and non-traditional risk factors (36). The potentially treatable traditional risk factors are hypertension, diabetes, dyslipidaemia, smoking and obesity (2). Potentially modifiable non-traditional risk factors include anaemia, hyperphosphataemia, hypovitaminosis D, hyperhomocysteinaemia and inflammation (2). However, there is an ever expanding list of non-traditional risk factors and these are summarized in Table 1.

Table 1.1: Risk factors for cardiovascular disease in CKD

Traditional	Non-traditional
Hypertension	Inflammation
Diabetes mellitus	Oxidative stress
Smoking	Hyperhomocysteinaemia
Older Age	Anaemia
Gender	Abnormal calcium/phosphate metabolism
Higher LDL Cholesterol	Lipoprotein (a) and apolipoprotein(a) isoforms
Lower HDL Cholesterol	Advanced glycation end products (AGES)
Family History of CVD	Asymmetric dimethylarginine (ADMA)
Menopause	Malnutrition
Physical inactivity	Chronic Kidney Disease
LVH	Proteinuria
	Extracellular fluid volume overload
	Renin angiotensin system activity
	Decrease in glomerular filtration rate (GFR)
	Endothelial dysfunction
	Thrombogenesis
	Fibroblast growth factor 23 (FGF-23)

Notes: LDL, low density lipoprotein; HDL, high density lipoprotein; CVD, cardiovascular disease; LVH, left ventricular hypertrophy. See text for details and references.

Although traditional risk factors have been validated for diagnosis and management of CVD in many populations, identification of these risk factors in CKD patients still does not fully explain the occurrence of CVD (36). Also, the underlying mechanistic pathways linking traditional risk factors and CVD are yet to be elucidated (2). However, it has been

suggested that novel risk factors (non-traditional) may further enhance CVD risk stratification by facilitating detection and evaluation of atherosclerotic disease (37).

1.3.1 Traditional risk factors

1.3.1.1 Hypertension

Association between high blood pressure and kidney damage exemplifies a vicious cycle of rising blood pressure and deteriorating kidney function. Kidney dysfunction may trigger hypertension via various mechanisms including salt and water retention, sympathetic over-activity, activation of the renin-angiotensin system, and accumulation of endogenous vasopressors (38). Conversely, hypertension in turn can further damage the kidneys. A report from a meta-analysis on risk for hypertension in living kidney donors suggests that a fall in GFR of 10ml/min is directly related to an increase of 5-mmHg in systolic blood pressure (39). It follows that there is a strong relationship between hypertension and different types of CVD. Prospective epidemiological studies thus far have revealed a direct association between elevated blood pressure and CAD. A meta-analysis of 61 prospective studies which included a total of 1 million adults without CVD at baseline, showed that a prolonged 20-mmHg rise in usual systolic blood pressure was associated with more than two-times higher risk of stroke-related deaths and two-fold higher risk of death from CAD and death from heart failure (40). Furthermore, the Action to Control Cardiovascular Risk in Diabetes BP trial (ACCORD BP) which randomly assigned 4,733 hypertensive-diabetic patients to either the intensive group (SBP <120 mmHg) or the standard group (SBP <140 mmHg), demonstrated that lowering blood pressure to below the standard level significantly reduce the risk of stroke alone by about 40% in type 2 diabetes cohorts; even though, the researchers found no significant differences in the rates of a combined endpoint including nonfatal heart attack, nonfatal stroke, or cardiovascular death between the intensive group and the standard group (41). Comparable to the general population, hypertension is a risk factor for mortality

in CKD patients before or at initiation of dialysis. The relationship between blood pressure and mortality is U-shaped, implying that isolated systolic blood pressure and wide pulse pressure perhaps represent a long term risk while low mean blood pressure and low diastolic blood pressure predict early mortality (42).

Moreover, optimal blood pressure control in CKD patients is quite challenging. Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines for the management of blood pressure in CKD recommends initiating antihypertensive treatment for all non-diabetic, non-proteinuric adults with CKD whose office blood pressure is regularly greater than 140/90mmHg (43). The authors equally suggested treating individuals with proteinuric CKD whose office blood pressure is persistently higher than 130/90mmHg. Available evidence however suggests that lower targets in adults with hypertension and CKD are not advised. The African-American Study of Kidney Disease and Hypertension (AASK) Trial enrolled and randomized 1094 hypertensive patients to either 1 or 2 basic groups with BP aggressively lowered to a mean arterial pressure (MAP) of ≤ 92 mmHg or the usual BP control of $\text{MAP} \leq 102$ mmHg (44). The findings of the AASK study showed that there were no significant differences in the rate of decline of GFR in either group nor were there significant differences in the secondary composite outcomes of greater than 50% reduction from the baseline GFR, end-stage renal disease or the cardiovascular events (44). Nevertheless, previous studies have suggested that systolic blood pressure in haemodialysis (interdialytic period) and peritoneal dialysis patients should not exceed 160 mmHg (45). On the other hand, excessive lowering of blood pressure in dialysis patients might be harmful and can aggravate myocardial systolic stunning (46-48). Despite the high burden of CVD in CKD patients, there is a relative scarcity of randomized controlled trial evidence on blood pressure control in CKD (49).

1.3.1.2 Dyslipidaemia

The typical lipid abnormality resulting from moderate to severe kidney failure is an accumulation of very low density lipoprotein (LDL) and intermediate density lipoprotein with resultant elevated serum triglycerides and reduced high density lipoprotein (HDL) cholesterol levels (50). Aside from nephrotic syndrome characterized by high levels of LDL cholesterol, LDL cholesterol concentrations in CKD are comparable to or lower than the general population (50). Reduced HDL concentration leads to diminished hepatic clearance of LDL, thus increasing the LDL residence time in plasma, which makes it more prone to oxidation, glycation, and carbamylation. There is continuous cleavage of cholesterol from the LDL particles and subsequent replacement with triacylglycerol, thereby resulting in accumulation of small dense LDL particles which may be more atherogenic (51).

Moreover, albuminuria is related to various lipid abnormalities (52). Kahri *et al* (53) in their comparative study of lipid profiles between patients with microalbuminuria and those without, documented that HDL was eleven-fold lower in microalbuminuric patients compared with normoalbuminuric patients. The ratio of HDL cholesterol to apolipoprotein A was significantly lower in microalbuminuric than normoalbuminuric patients (54). Furthermore, the ratio of anti-atherogenic plasma lipoprotein lipase to the pro-atherogenic hepatic lipase was also significantly lower in microalbuminuric patients compared to normoalbuminuric patients (55, 56). Plasma activity of cholesterol ester transfer protein, which redistributes cholesterol esters from HDL to LDL and VLDL has been shown to be higher in patients with microalbuminuria(57). Nevertheless, it remains unclear whether these types of lipid abnormalities would be expected to result in a large increase in the risk of atherosclerotic events in patients with non-nephrotic CKD.

Although it is clear from epidemiologic studies and large-scale randomized trials that an elevated LDL cholesterol level is a contributing cause of atherosclerotic events, however,

it is uncertain whether the observed incidence of atherosclerotic events in the CKD population is mainly caused by increased LDL cholesterol concentrations because such an abnormality occurs in few patients with nephrotic-range proteinuria (58-60). Similarly, a meta-analysis of prospective studies has shown that hypertriglyceridaemia may not be a major determinant of an increase in the risk of atherosclerotic events. After adjusting for HDL cholesterol and other cofounders, there was no clear association between hypertriglyceridaemia and atherosclerotic CVD events such as myocardial infarction and ischaemic events (58).

Furthermore, elevated lipoprotein (a) [Lp (a)] levels might further contribute to the increased risk of atherosclerotic events in CKD; this abnormality is a direct result of impaired kidney function since levels can be normalized by kidney transplantation (61, 62). Moderate to severe kidney failure is associated with elevated Lp(a) concentrations and existing evidence suggests a causal relationship between elevated Lp(a) concentrations and ischaemic heart disease in the general population (61, 63). In ESRD, plasma concentrations of Lp(a) are determined mainly by the apolipoprotein(a) [apo(a)] phenotype and shows an inverse relationship with the molecular weight of apo(a) (64). There is a complex link between plasma Lp(a) concentrations and atherogenesis in ESRD. Unlike in the general population, where Lp(a) levels are primarily determined by genetic mutation on the apolipoprotein(a) [apo(a)] gene locus, there is a phenotype-associated elevation of Lp(a) concentrations in ESRD. It is difficult to predict the effect of CKD-induced changes in Lp (a) concentrations (62).

1.3.1.3 Diabetes mellitus

Diabetes mellitus (DM) affects about 350 million people worldwide and the prevalence is progressively increasing, owing to the progressive rise in prevalence of obesity and an ageing population (65). About 40% of ESRD patients commencing dialysis in the

United States have diabetic nephropathy and an additional 10% were reported to have diabetes as a comorbid disease (26). In South Africa, the occurrence of DM is also increasing, with the prevalence of diabetic nephropathy between 14-16% (66). Diabetic patients are at an increased risk of mortality and approximately two-thirds of deaths in diabetics could be attributed to cardiovascular causes (67). Data from a meta-analysis of 102 prospective studies demonstrated that the presence of diabetes doubles the risk of CAD (68). More specifically, the risk of ischaemic stroke was increased by about half, the risk of haemorrhagic stroke by around four-fifths and the risk of other vascular death, including heart failure, by about three quarters. Regarding CKD patients, occurrence of DM seems likely to contribute additional risk over and above that arising from other traditional risk factors related to CKD. Consequently, only a few of these patients are offered renal replacement therapy in the South African public health sector facilities because of inadequate resources and associated comorbid conditions including CVD (69).

1.3.1.4 Cigarette smoking

Data from a prospective observational study clearly showed that cigarette smoking is associated with an increased risk of cardiovascular morbidity and mortality (70). The study also provided strong evidence suggesting that the association between smoking and risk of CVD is that of cause and effect by demonstrating the beneficial effects of smoking cessation among cohorts of male smokers followed up for 50yrs (70). There is also evidence that cigarette smoking may also be associated with increased risk of developing CKD. In the analysis of the Framingham Offspring study of 1,916 individuals, cigarette smoking was found to be associated with a two-fold increased risk of new onset albuminuria, even after exclusion of those with reduced estimated glomerular filtration rate (eGFR) and diabetes (71). In another case control study, heavy cigarette smoking increases the risk of CKD overall and the risk is particularly higher among hypertensive and diabetic patients (72).

In addition, current smokers had a two-fold increased risk of CKD while former smokers had no increased risk. The data also suggested there is close association between the numbers of cigarettes smoked daily and the likelihood of having CKD in the smokers. However, an observational study carried out in an outpatient clinic population with background CKD did not show an independent association between smoking and progression of CKD (73). Nonetheless, cessation or complete avoidance of smoking might be an importance goal in preventing as well as stemming risks of CVD among CKD patients.

1.3.1.5 Age

The report of the United State National Health and Nutrition Examination Survey (NHANES) demonstrated a relationship between presence of cardiovascular risk factors, age and degree of renal failure (26). According to the study, patients around 60yrs of age were the most common group initiating renal replacement therapy in the United States; the same age when CVD is most prevalent (26). This further confirms the association between risk of developing CVD and ageing. In addition, the importance of systolic, diastolic and pulse pressure variation in relation to ageing was clearly demonstrated in the Framingham study (74). Diastolic blood pressure was an independent risk factor for coronary heart disease (CHD) among individual less than 50yrs; all three blood pressure indices were comparable predictors in patients between 50 and 59yrs, while isolated systolic pressure or wide pulse pressure was the strongest CHD risk predictor among individuals who were 60yrs and above. In contrast, some risk factors including dyslipidaemia, glucose intolerance and elevated fibrinogen had a reduced effect on the risk of CHD with advancing age (75, 76). It follows that lower relative risk is counteracted by higher absolute risk in elderly patients. Consequently, all major risk factors continue to be critical predictors of CHD risk in the elderly.

1.3.1.6 Gender

Despite the fact that CVD is diagnosed about a decade later in women than in men, it still remain the commonest cause of mortality among women over the age of 65 years (77). According to the recent data from the NHANES, while the prevalence of ischaemic heart disease has risen among women between the ages of 35 to 54 years, it is declining among men of the same age group (78). It has been postulated that exposure to endogenous oestrogen during the child-bearing age probably slows down the process of atherosclerosis in women (77). Life expectancy is two-years lower in women with early menopause compared with women with late or normal menopause (79). In addition, transition to menopause has been associated with a higher CHD risk profile (80).

Even though the majority of the traditional risk factors are common to males and females, the impact of these factors on the development of CVD in men and women are quite different (77). Before the age of 50, the relative risk of smoking is higher in women than in men, with higher risk associated with the total number of cigarettes smoked per day (81). Similarly, diabetic women are at a greater risk of cardiovascular morbidity when compared to their male counterparts. Data from a meta-analysis of 37 prospective cohort studies showed that the risk of fatal cardiovascular events is 50% higher in diabetic women than men with diabetes mellitus (82).

During ageing, systolic blood pressure rises sharply in women compared to men, and this may be as a result of relative oestrogen deficiency during transition to menopause (83, 84). Furthermore, in the elderly (>75 years), isolated systolic hypertension is 14% more common, and is an independent predictor of LVH, diastolic heart failure and stroke in women (84). Blood pressure less than 140/90 mmHg is more associated with endothelial dysfunction and cardiovascular complications in females than in males (85).

In women of child-bearing age, hypercholesterolaemia is less common in women compared with men. However, during menopause, elevated total cholesterol, low density lipoprotein and lipoprotein (a) levels are more prevalent in women than in men (80). Evidence from the Framingham Study suggests that a reduced high density lipoprotein cholesterol level is associated with a higher cardiovascular risk in females than in males (86). The report from the JUPITER trial further highlighted the significance of the age difference in the manifestation of cardiovascular events among men and women, where similar benefits were established in healthy men ≥ 50 years and in women ≥ 60 years, using a statin for primary prevention of coronary heart disease events (87). Currently, many gender differences in the occurrence of atherosclerosis are yet to be elucidated, thus necessitating further future research in this field of cardiovascular medicine.

1.3.1.7 Physical activity

Several studies have consistently associated high levels of physical activity with reduced risk of CHD morbidity and mortality. Data from several population studies have shown that the risk of CHD is almost double in inactive individuals compared to active counterparts (88-90). Available evidence suggests that the relative risk associated with sedentary lifestyle is equivalent to that conferred by hypertension, cigarette smoking and hypercholesterolaemia, thus highlighting physical inactivity as an independent predictor of CVD (91). The level of physical activity is inversely related to the incidence of CVD in both men and women, and this association persists after adjusting for other classic risk factors (92, 93).

Regarding patients with established CHD, mortality rate is significantly lower among those who participate in an exercise program compared to those who do not exercise (94). Furthermore, data from the British Regional Heart Study showed that light to moderate

exercise in men with established CVD is associated with fewer risk of all-cause mortality (95).

Mechanisms through which physical activity reduces risk of CVD may possibly involve changes in lipid and lipoprotein metabolism as well as improve endothelial function (96). Reduced risk of CAD, lower triglycerides levels and higher HDL-cholesterol concentrations have been documented in individuals who regularly exercise compared to untrained individuals (97). In addition, there seems to be a direct association between levels of physical activity and HDL concentrations (98). It has been shown that physical exercise improves endothelium-dependent vasodilation of coronary vessels in patients with chronic heart failure (99). Similarly, a report from a previous study showed that exercise training in patients with chronic cardiac failure restores endothelium-dependent flow-mediated vasodilation, a measure of endothelial nitric oxide synthesis (100).

1.3.1.8 Left ventricular hypertrophy

Stewart *et al* (101) in their study of 296 patients with CKD demonstrated LVH in all stages of CKD, progressing with deteriorating renal function. Similarly, in an earlier report by Levin *et al* (102), prevalence of LVH increases with advancing CKD. Left ventricular hypertrophy was found in 26.7% of patients with creatinine clearance (CrCl) greater than 50 ml/min, 30.8% of those with CrCl between 25 and 49 ml/min, and 45.2% of patients with CrCl of < 25 ml/min. In addition, the mean left ventricular mass index in patients with CrCl greater than 50 ml/min, 25-50 ml/min and less than 25 ml/min were 97.5 g/m², 100.8 g/m² and 114.4 g/m² respectively. In CKD patients, uraemia facilitate abnormal structural remodelling of the heart, resulting in LVH, fibrosis and left ventricular dysfunction (103, 104). This together with electrolyte abnormalities may trigger and facilitates arrhythmias which is commonly seen in ESRD patients (105).

1.3.1.9 Genetic susceptibility for cardiovascular disease

Genome-wide association studies (GWAS) have successfully identified numerous novel genetic variants that have been implicated in CVD. These include reports of the association of genetic variants at the 9p21 locus with myocardial infarction (106, 107). Since it was first reported, this association has been replicated in different ethnic groups. Recently, a meta-analysis of these studies reported an allele relative risk of 1.27 (95% confidence interval 1.23 to 1.31, $P=1.62 \times 10^{-12}$) for CAD (108). To date, the role and the mechanism through which this genetic variant at 9p21 locus increases CVD risk is yet to be clarified (109). Furthermore, GWAS for hypertension (110, 111) and lipid abnormalities have identified other new loci that are associated with hypertension and dyslipidaemia (112, 113).

More recently, APOL1 variants (G1 and G2 alleles) were shown to contribute substantially to atherosclerotic CVD risk among African Americans (114). The APOL1 alleles consist of 2 missense mutations rs73885319 (p.S342G) and rs60910145 (p.I384M), together are denoted G1; and rs71785313 (deletion of p.N388 and p.Y389) are denoted G2 (114). These two alleles are localised to gene encoding apolipoprotein L1 (APOL1), a major component of HDL which confers protection against *Trypanosoma brucei rhodesiense* infection (115) and also increases the risk for CKD (116, 117). In addition, APOL1 is a major component of dense HDL3 particles that play a key role in cholesterol transport and inhibit oxidation of LDL (118). In view of the fact that inflammation and immunity play crucial roles in the process of atherosclerosis, APOL1 alleles may possibly contribute to the severity of atherosclerosis through this pathway (119).

Furthermore, atherosclerosis is a polygenic disease involving a complex interplay of various genes. A number of inflammatory genetic markers have been linked to atherosclerosis. Single-nucleotide polymorphisms (-463G /A and -129G /A) are localized to the myeloperoxidase (MPO) gene on chromosome 17. Associations between MPO gene

variants and CAD have been documented by previous studies (120, 121). A genetic locus localized to the interleukin-6 (IL-6) gene on chromosome 7 has also been identified; this polymorphism (–174G/C) has been associated with atherosclerosis and CAD (122, 123). However, the presence of the TLR4 Gly299 allele on chromosome 9 has been associated with a reduced risk of developing carotid plaques in Caucasian carriers (124). Although it remains to be seen whether the presence of the TLR4 allele in black CKD patients will protect against the development of atherosclerosis.

Moreover, other genetic polymorphisms that have been linked to coronary atherosclerosis have been localized to the genes of the surfactant protein D (SFTPD), Met11Thr (125) and the regulated upon normal T-cell expressed and secreted (RANTES, CCL5) –403G/A polymorphisms (126).

Despite the increase in the number of CAD – associated risk alleles, genetic risk scores have not been found to significantly improve cardiovascular risk prediction when compared to traditional risk factors (127). A positive family history of premature CVD, an established risk factor for incident CVD (128, 129), is a useful surrogate marker for increased genetic cardiovascular risk (109). The addition of family history into the risk prediction models significantly improves CVD risk prediction (128-130). A comprehensive family history will predict 16% of CVD-associated genetic polymorphisms, which is much more better than the CVD predictive rate of any previously studied genetic risk scores, thus further highlighting the clinical usefulness of establishing family history of CVD as a surrogate marker for genetic cardiovascular risk prediction (109).

1.3.2 Non-traditional risk factors

1.3.2.1 Inflammation

“A milieu of chronic subclinical inflammation is common to both CKD and CVD and has been suggested to play a role in the bidirectional relationship between these two systems”

(131). There is strong evidence from both experimental and clinical studies that inflammation plays a central role in the pathogenesis of atherosclerosis (132). Chronic inflammation is directly related to several complications of CKD, including accelerated atherosclerosis and vascular calcification (133). As a result, inflammation is a predictor of mortality in this group of patients (134).

Accumulation of minimally oxidized LDL in the arterial wall is the main trigger factor for the inflammatory response which is characterized by disorders of both the innate and adaptive systems, leading to a complex immune dysfunction (134). Activation of endothelial cells leads to induction and secretion of adhesion molecules which binds to circulating leucocytes, thereby resulting in transmigration of leucocytes and subsequent accumulation of inflammatory macrophages and T-cells within the arterial wall (135). This process is mediated by chemoattractants such as monocyte chemoattractant protein-1 (MCP-1). The activated lymphocytes release a variety of inflammatory cytokines which further activate smooth muscle cells, endothelial cells and macrophages. Oxidized LDL accumulates in the macrophages leading to formation of foam cells and fatty streak. Previous data has shown that CD40 ligands are expressed by macrophages, T and B lymphocytes, endothelial cells, vascular smooth muscle cells and fibroblasts (136). The CD40 receptor proteins play a significant role in leucocyte adhesion, matrix degeneration and cytokine-induced inflammatory responses. The study further demonstrated that interruption of the CD40 signaling pathway in an experimental model reduced progression of atherosclerosis (136).

Moreover, there has been increasing evidence suggesting the role of infectious agents as the triggering factor chronic inflammatory state (132). For instance, laboratory analysis has identified antibodies to chlamydia pneumonia within atherosclerotic plaques (137). Also, antibodies against organisms like chlamydia, *helicobacter pylori*, herpes simplex virus, and cytomegalovirus have been isolated in the serum samples of patients with atherosclerosis

(138, 139). Furthermore, inflammatory markers such as C-reactive protein (CRP) are elevated in patients with acute coronary syndrome and peripheral arterial disease, and the levels are closely associated with future cardiovascular events (140).

Although individuals with CKD have been shown to have elevated levels of CRP, interleukin-2 (IL-2), interleukin-6 (IL-6), interferon gamma (IFN- γ), tumour necrosis factor alpha (TNF- α), sIL2R, ICAM and VCAM-1 (141-144) however, to date, no interventional study has been carried out in humans to control inflammation and assess whether or not this has a positive effects on cardiovascular and renal outcomes.

1.3.2.2 High-sensitivity C-reactive protein (hsCRP)

Inflammation plays a significant role in the process of arterial thrombosis and elucidates the underlying pathophysiological mechanisms linking atherosclerotic plaque formation and acute rupture, leading to vascular occlusion and infarction (145). C-reactive protein (CRP), a simple downstream marker of inflammation is a member of the circulating pentraxin family, is made up of 23-kDa subunits, and plays a major role in the innate immune responses in humans (146). Even though other cells and tissues in the body can elaborate CRP, it is primarily produced in liver. C-reactive protein, an acute phase reactant secreted in the liver in response to expression of IL-6 at the site of inflammation, has now become known as a major CVD risk marker (147). However, it remains unclear whether CRP is simply a marker of inflammation or is direct involved in the atherothrombotic process. Pertaining to the latter, CRP may influence vascular susceptibility to atherosclerosis by stimulating increased production of adhesion molecules and endothelial plasminogen activator inhibitor type 1 (PAI-1), or by blocking lipid uptake by macrophages, and co-localization with complement within atherosclerotic lesions (146).

Moreover, a prospective epidemiological study has demonstrated that hsCRP is a major predictor for, and sudden cardiac death in seemingly healthy populations, even when

LDL-C and PAI-1 levels are low (148). In addition, meta-analyses have further shown that the relative risk associated with hsCRP was larger than that of blood pressure and cholesterol (149). The findings of this study are relevant to women and men across all age groups and have been consistent with data from diverse populations. Similarly, collaborative studies from the United States and Europe showed that hsCRP levels have a superior predictive value for CVD risk compared to LDL-C levels (150). Available data showed that CVD risk is higher in individuals with elevated hsCRP levels and low LDL-C levels than in those with elevated LDL-C levels but low levels of hsCRP (140). Even in studies that have reported fairly low levels of hsCRP, the magnitude of CVD risk was at least as large as that of hypertension and smoking, thus demonstrating the importance of inflammation in the pathogenesis of atherosclerosis (151).

According to the American Heart Association and the Centers for Disease Control and Prevention, hsCRP levels <1, 1-3, and >3 mg/liter should be classified as lower, moderate, and higher cardiovascular risk respectively, when considered along with traditional CVD risks (152). This was further supported by data from the Framingham Heart Study after reclassification of CVD risk using CRP (153). Furthermore, screening for hsCRP should be done as part of global risk evaluation and not as a replacement for LDL and HDL testing. High-sensitivity C-reactive protein levels higher than 8 mg/liter may represent an acute-phase response caused by an underlying acute infection, thus the hsCRP test should be repeated at approximately 2 to 3 weeks after treatment of infection. Persistently elevated hsCRP levels, however, represent very high future CVD risk, since hsCRP is directly associated with vascular risks (154). Moreover, testing for hsCRP can easily be done on an outpatient basis when assessing cholesterol levels because hsCRP levels are stable over long periods, have no circadian variation, and do not depend on prandial state (154).

1.3.2.3 Oxidative stress

The term oxidative stress refers to tissue injury resulting from an excess of oxidant compounds, which are crucial for tissue repair and protection against infection. Oxidative stress results when the anti-oxidant defenses are overwhelmed by pro-oxidants (155, 156), leading to damage of compounds such as lipids, protein and DNA. Oxidized low density lipoprotein (oxLDL) in the blood plays a key role in the pathogenesis of coronary heart disease, hypertension and atherosclerosis (157-159). Massive production of reactive oxygen species (ROS) has been implicated in the pathophysiology of atherosclerosis, through endothelial cell activation and cellular immune activation (159). Increased NADPH enzyme activity as well as catastrophic mitochondrial respiratory enzyme activities may contribute to ROS generation and oxidative burst (160). Moreover, it has been established that elevated NADPH oxidase subunit proteins in humans is associated with increased superoxide production and severity of atherosclerosis (161). Also, in animal models, NADPH oxidase-deficient mice had significantly lower atherosclerosis compared to mice with normal NADPH activity (162). Available evidence also suggests that mononuclear cells as well as endothelial cell play a significant role in the patho-mechanism of atherosclerosis through production of superoxide which is also consistent with oxidative stress (163). Platelets also contribute to ROS production leading to enhanced release of ADP and platelet recruitment (164).

Oxidative stress depletes endogenous nitric oxide thereby causing endothelial dysfunction, which plays a role in the process of left ventricular remodeling and fibrosis, as well as oxidation of lipoproteins (165, 166). Interestingly, an association between CKD and oxidative stress has been shown to contribute to the pathogenesis and progression of renal failure (167-170). It is well established that CKD patients, most specifically haemodialysis patients have elevated levels of markers of oxidative stress compared with the general population (171, 172). This may possibly be caused by loss of anti-oxidants during dialysis

therapy, release of ROS by neutrophils in response to bacteria crossing the dialysis membrane and reduced dietary uptake of anti-oxidants as a result of malnutrition (171-174).

In the general population, dietary intake or plasma concentrations of antioxidant vitamins (vitamins A, C, and E) are inversely related to cardiovascular disease incidence and mortality (175). A systematic review of 56 studies which evaluated anti-oxidant therapy in haemodialysis patients showed that vitamin D, alpha-tocopherol and N-acetylcysteine significantly reduced cardiovascular morbidity and mortality (171). Nevertheless, larger and more adequately powered randomized controlled trials are needed to confirm this observation.

Given that ROS have short half-lives of just a few seconds (176, 177), oxidative stress is usually quantified by the detection of various modified macromolecules that are generated from non-enzymatic binding of lipids and proteins with ROS in the tissues, plasma and other body fluids. These marker molecules usually have longer half-lives and include molecules derived from lipid peroxidation such as malondialdehyde oxidized LDL (oxLDL) and F2 isoprostanes (172, 178). Oxidative stress has been investigated using different methodologies: Mune *et al* (179), after dialyzing patients with antioxidant coated dialyzers for 24 months demonstrated a significant reduction in the levels of markers of oxidative stress as well as a delayed in the progression of aortic calcification, a marker of atherosclerosis. Higher plasma malondialdehyde-LDL levels were also reported in patients with insulin resistance compared with controls (180). Adults with metabolic syndrome had nearly 4 times the level of plasma 8-isoprostanes compared with those with normal lipids and normal weight (181). These reports, in summary, support the hypothesis that oxidative stress is involved in multiple processes that are relevant to the development of CVD.

1.3.2.4 Endothelial dysfunction

Although vascular endothelium was originally regarded as an inert barrier between the blood and blood vessel, it is now well recognized that the endothelium plays an important role in controlling vascular activities. Endothelium play a central in the regulation of inflammatory process, thrombosis, and coagulation through production of autocrine and paracrine hormones such as nitric oxide (NO), prostaglandins, endothelin, and angiotensin II (182). Of all these substances, NO is the most well studied (183, 184). Nitric oxide is constitutively produced by endothelial NO synthase (eNOS), otherwise known as NOS III (183), has vasodilator, antiplatelet, anti-proliferative, anti-adhesive, permeability-decreasing and anti-inflammatory properties (185). Reduced bioavailability of NO with associated increased production of vasoconstrictors facilitates the process of thrombosis and development of atherosclerosis. This implies that any interruption in NO functions or availability will significantly increase the risk of CAD.

In addition to adversely affecting blood flow and nutrient delivery, endothelial dysfunction also induces immune activation and systemic inflammation (182). Endothelial cells play a part in the recruitment and adhesion of leukocytes to the endothelial lining of the vessels through secretion of chemokines and cytokines (182, 183).

Asymmetric dimethylarginine (ADMA), an inhibitor of NOS has been shown to accumulate in patients with CKD and may also contribute to the CVD burden (186). Dimethylarginines, naturally occurring in the human body, are formed from protein catabolism (187). In CKD patients, ADMA was significantly elevated as a result of reduced clearance in the kidney. It contributes to CVD by blocking the endothelial cellular uptake of L-arginine, causing reduced NO synthesis and ultimately resulting in widespread vascular inflammation (187-189), thus providing a the link between CKD and CVD (2).

1.3.2.5 *Hyperhomocysteinaemia*

Chronic kidney disease is closely associated with elevated levels of homocysteine, which has been linked with CVD (190-193). A causal relationship may exist between hyperhomocysteinaemia and renal impairment; available evidence suggests that elevated homocysteine levels have a strong inverse correlation with GFR; in addition, the levels were reduced after kidney transplantation (194). However, the mechanisms underlying hyperhomocysteinaemia in CKD are multifaceted and complex. They are not only attributable to reduced renal clearance, because renal homocysteine excretion accounts for <1% of its elimination, but also due to metabolic disturbances in remethylation and trans-sulfuration pathways (194). Moderate reductions in GFR are associated with an increase in total plasma homocysteine (195). Results from a study showed that reduction in homocysteine concentration was associated with lower risk of CHD and stroke (196). Furthermore, results of genetic studies of mutations in the methylenetetrahydrofolate reductase gene (MTHFR) appeared consistent with the view that this association is causally related (197).

Available evidence suggests that vitamin B12 and folate supplementation is associated with a reduction in the homocysteine levels (193). The study further recommended that CKD patients should be evaluated for elevated levels of homocysteine and subsequently treatment with vitamin supplementation for hyperhomocysteinaemia (193). In spite of this, available evidence did not show any cardiovascular benefit from vitamin supplementation (198, 199). This observation could be explained by persistent hyperhomocysteinaemia despite rigorous vitamin supplementations.

1.3.2.6 *Adiponectin*

Adiponectin (ADPN) belongs to the family of adipokine-related hormones. It has an immuno-modulatory properties and protects against insulin resistance, obesity and other

metabolic disorders (200). It exists in 3 different forms in the blood namely low, medium, and high molecular weight adiponectin, with the high-molecular-weight form appearing to be more pathogenic (201). Secretion of adiponectin is strictly controlled by peroxisomal proliferator-activated receptor gamma (201). Even though, adiponectin receptors are localized to the majority of organs in the human body, including muscle (receptor 1) and brain (201), it is still not clear whether adiponectin only acts through these target organs. In cross-sectional studies in adults, adiponectin was identified as an independent predictor of obesity, hyperinsulinemia and insulin resistance, metabolic syndrome, visceral adiposity, T2DM (202) and atherosclerosis (203).

Adiponectin is a collagen-like protein, has anti-atherogenic and anti-inflammatory properties, hence its protective role for cardiovascular disease (204, 205). Previous studies have shown that ADPN was inversely related to renal function, and significantly elevated in ESRD patients (203, 206, 207). Likewise, several studies have demonstrated higher levels in CKD, haemodialysis, and peritoneal dialysis patients (206, 208). Although, it is thought that impaired clearance of ADPN in ESRD patients may be due to its high molecular weight (207), it is yet to be established whether the increased ADPN levels in kidney failure patients are as a result of impaired clearance or whether it is a compensatory mechanism to offset the increased cardiovascular risks in these patients. Hence, this needs to be established in larger prospective studies.

1.3.2.7 Fibroblast growth factor 23

Recently, fibroblast growth factor 23 (FGF-23) was discovered and has been associated with regulation of the body's calcium-phosphate metabolism, essentially by increasing phosphate excretion in the kidneys (209, 210). Fibroblast growth factor 23 levels steadily increase with worsening renal function, starting at the early stages of kidney failure, increasing by multiple folds in ESRD (211, 212). In studies involving CKD patients, it was

demonstrated that severely elevated levels of FGF-23 were independently associated with myocardial hypertrophy and endothelial dysfunctions (209, 213). Faul *et al* (214) showed that FGF-23 was closely associated with LVH in a large study of ethnically diverse CKD patients. They reported that LVH was mediated through FGF receptor activation pathway, independent of klotho, the co-receptor of FGF-23 which is only expressed in the kidneys and parathyroid glands. The same group reported that treatment with a FGF-23 receptor antagonist reduced LVH. It follows that pharmacological reduction of FGF-23 may be a potential therapeutic strategy to reduce CVD in CKD (209).

1.4 An overview of pathogenesis of atherosclerosis

Increase uptake and accumulation of low density lipoprotein (LDL) in macrophages, has been established as the rate-limiting step in the development of atherosclerosis (215). As shown in Figure 1.1, in the presence of endothelial dysfunction and/or injury LDL moves into the subendothelial space where it become oxidized and get converted to oxidized LDL (OxLDL) (216). Oxidized LDL in turn activate endothelial cells leading to increased production of adhesion molecules as well as MCP-1, which facilitate attachment of lymphocytes and monocytes to endothelial layer (132, 135). Through secretion of chemo-attractant substances, the monocytes and the leucocytes migrate into the sub-endothelial space, where the monocytes differentiate into macrophage scavenger cells (217). Macrophage cells then ingest OxLDL and transform into foam cells (217). This process is continuous and as the foam cells combine with leucocytes, a fatty streak is formed (135). The foam cells in turn contribute to the severity of inflammatory process by releasing more inflammatory cytokines, thereby stimulating proliferation as well as migration of smooth muscle cells into the intima layer of the artery. Thus resulting in the formation of a fibrous plaque, which later progress to advanced atherosclerotic plaque and may eventually lead to acute coronary syndrome (135, 215, 216), (218).

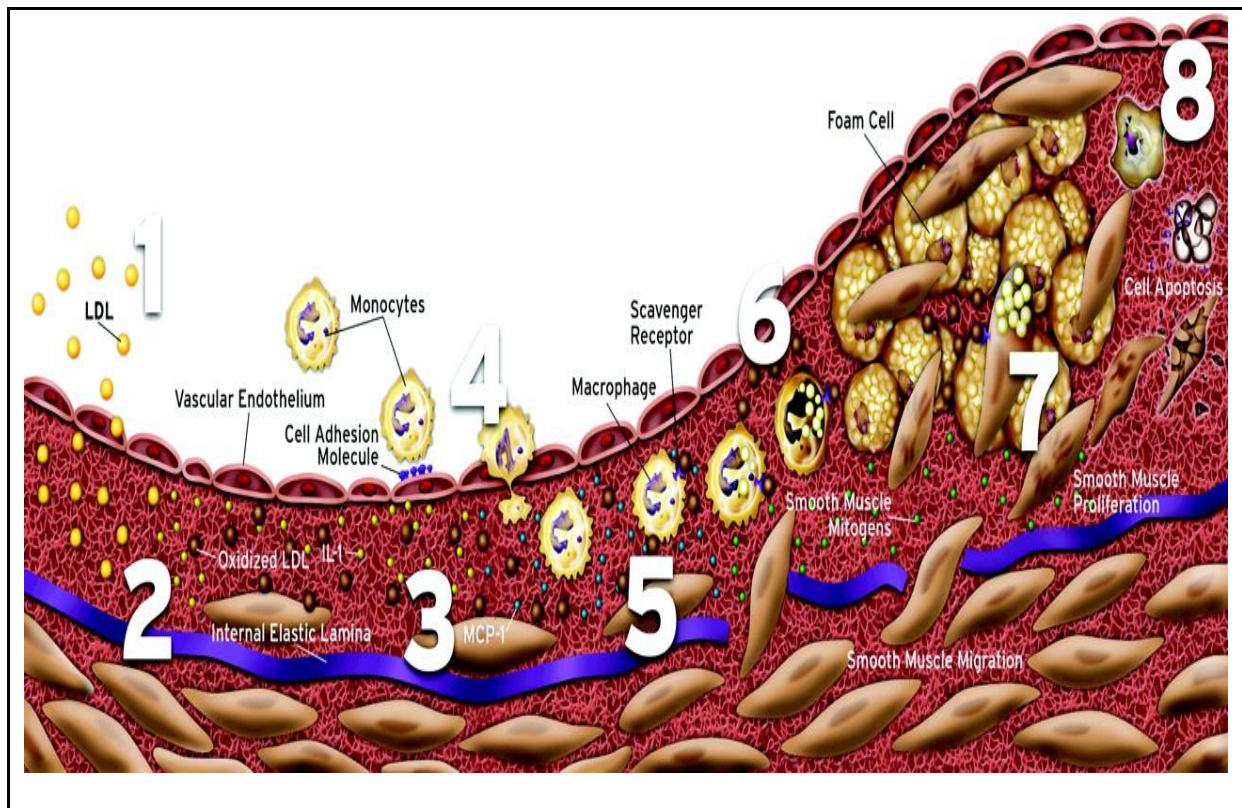


Figure 1.1: Diagram illustrating the development of an atherosclerotic plaque. LDL is converted to oxidized LDL by inflammatory cells. Oxidized LDL subsequently cross the endothelial layer into the subendothelial space. Secretion of inflammatory cytokines leads to recruitment of more monocytes to the subendothelium. Accumulation and proliferation of foam cells as well as smooth muscle cell leads to the formation of atherosclerotic plaque. LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein-1. (Reproduced with permission from Faxon D, Fuster V, Libby P, Beckman J, Hiatt W, Robert W, *et al.* Atherosclerotic Vascular Disease Conference Writing Group III: Pathophysiology. *Circulation*. 2004; 109:2617-25)

1.5 Bacteria Endotoxins

Endotoxin, also known as lipopolysaccharide, is a components of the outer membrane of the cell wall of Gram-negative bacteria (GNB) (133, 219). The outer membrane of GNB functions as a protective permeability barrier, impermeable to large molecules and hydrophobic compounds from the environment and is critical to the function of the outer membrane (220). Structurally, LPS is a 10kDa molecule with different chemical composition. Lipopolysaccharide is made up of three components namely, lipid A, a core oligosaccharide, and an O-side chain (9). The O-side chain and the core polysaccharides are both immunogenic in nature, with the ability to stimulate antibody production.

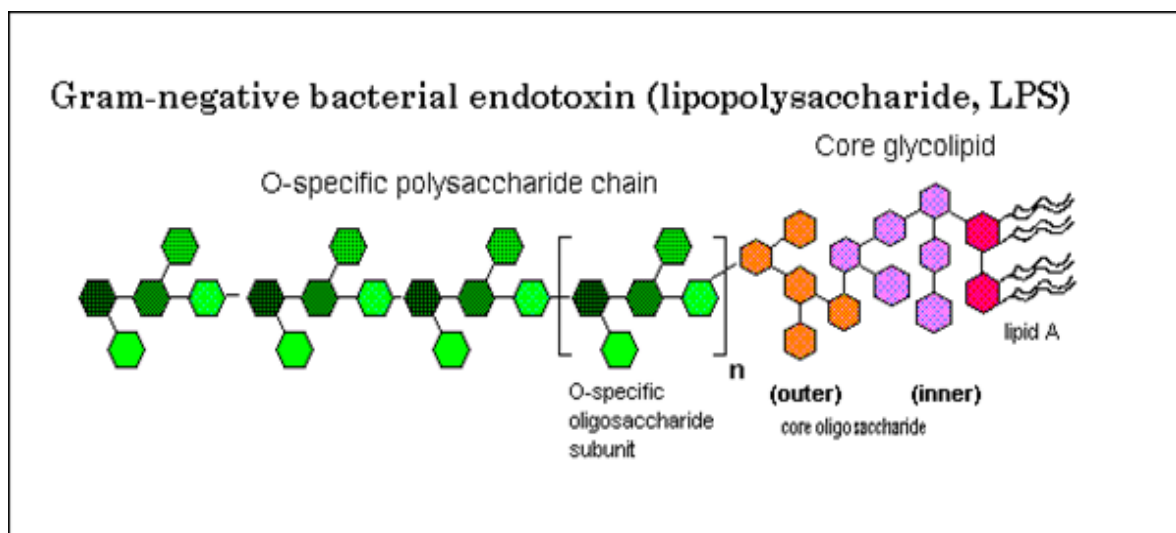


Figure 1.2: Schematic of bacterial endotoxin (lipopolysaccharide). Lipopolysaccharide consists of three covalently linked regions: lipid A, a core oligosaccharide, and an O-side chain.

(Source: <http://temp.biomin.net/solutions/endotoxin-risk-management/>)

In other words, lipopolysaccharide component is associated with biological functions of endotoxin, lipid A is the toxic component while the polysaccharide component has the ability to produce antibodies (immunogenicity) (220, 221).

1.6 Detection and quantitation of endotoxins (LPS)

Levin and colleagues in 1950 reported that gram negative bacterial infection in *Limulus polyphemus* (a horseshoe crab) leads to intravascular coagulation of the crab blood cells (Amoebocyte), and postulated that the coagulation of the amoebocyte resulted from the activation of the clotting factors by endotoxin in the bacterial wall (222) (223). Following this discovery, Limulus Amoebocyte Lysate test (a chromogenic test) was subsequently developed for the detection of endotoxin levels (224). Recently, a novel LPS detection method based on the ability of toll-like receptor-4 (TLR4) to recognize lipid A of LPS, ultimately leading to activation of nuclear factor kappa-B(NF- κ B), was developed (225). The presence of very low concentrations of LPS, as low as 0.03ng/ml, is detected by this method (226).

1.7 Endotoxin and Vascular Dysfunction

During GNB infection, endotoxin cause endothelial dysfunction, ultimately leading to septic shock, disseminated intravascular coagulation, and vascular leak syndromes (227). Endotoxin levels equivalent to those observed during sepsis, induce numerous pro-inflammatory responses in circulating monocytes and macrophages as well as in cultured endothelial and smooth muscle cells, resulting in up regulation of adhesion molecules, increased production of inflammatory cytokines and oxidative stress, ultimately leading to endothelial damage (228). Even though these observations provide evidence that endotoxaemia can elicit vascular inflammation and endothelial dysfunction, endotoxin levels in inflammation-related atherosclerosis is much more lower compared to the levels in sepsis (159). Available evidence from a previous study showed that subclinical endotoxaemia is associated with an increased risk of atherosclerosis (13). This further suggests that low levels of endotoxin may induce chronic low grade inflammation and accelerated atherosclerosis, hence indicating a potential link between circulating endotoxaemia and subclinical atherosclerosis.

1.8 Signaling by endotoxin

1.8.1 Activation of cellular signaling pathways

Several immune mediators have been identified to play a major role in the endotoxin signaling pathway (159). Based on the proposed model of interaction between endotoxin and vascular cells, LPS induced immune activation involves a number of proteins, including LPS binding protein (LBP), CD14, Toll-like receptor-4 (TLR-4), and lymphocyte antigen 96 (also known as MD-2 protein). These modulatory proteins cooperatively activate a receptor activity ultimately leading to inactivation of nuclear factor-kappa B (159). The following

review therefore focuses on the generation of ligands of the innate immune response by circulating endotoxaemia.

1.8.2 *Lipopolysaccharide binding protein (LBP)*

Lipopolysaccharide binding protein, a 60-kDa phospholipid, binds and transfers proteins with moderate specificity in human blood (229). The LBP level has been suggested as a clinical marker of circulating endotoxaemia (230, 231). Lipopolysaccharide binding protein extracts endotoxin monomers from the bacterial cell wall or from aggregates of circulating endotoxin, forming a complex LPS-LBP that interacts with the MD-2 portion of the TLR4/MD-2 receptor ligand attached to CD14, resulting in target cell activation (232, 233). Alternatively, the LPS-LBP complex may bind to high-density lipoprotein (HDL), which facilitate the clearance of circulating endotoxin in the liver (234, 235).

1.8.3 *CD14*

CD14, a pattern-recognition receptor, mediates inflammatory response to endotoxin (236). It stimulates monocytes and neutrophils via NF- κ B, leading to translation and production of inflammatory cytokines (133, 159). Serum CD14 levels is a marker of endotoxin-induced cellular activation (236, 237). Genetic mutation in the promoter gene for a human CD14 has been shown to correlate with reduced incidence of (238). Levels of soluble CD14 (sCD14) were found to be closely associated with aortic stiffness and carotid plaque formation in a population-based study, further supporting the hypothesis that CD14 may contribute to severity of atherosclerosis in humans (239). Notably, a more recent study demonstrated that elevated sCD14 levels, a marker of activation of endotoxin/TLR4 system, is directly associated with inflammatory markers and inversely related to nutritional status, and is the major determinant of mortality in long-term haemodialysis patients (240).

1.8.4 *The TLR signaling pathway*

Toll-like receptors are part of the innate immune system, specifically recognize pathogen-associated molecules, and have been shown to be activated by LPS in numerous cells including mammalian phagocytes (133, 134). They are transmembrane proteins containing both extracellular and cytosolic regions (159). The endotoxin–CD14 complex binds to a toll-like receptor-4 (TLR-4) via MD-2, a TLR-4 co-receptor (241). This secreted accessory protein binds to both TLR-4 and endotoxin in phagocytes and other endotoxin-responsive cells (242). In short, TLR4 forms a large complex with several associated proteins, including CD14 and MD-2, to achieve efficient LPS-induced cell activation (243).

1.9 Pro-inflammatory effects of endotoxin

As a result of the presence of circulating endotoxin, a variety of pro-inflammatory responses may be initiated, ultimately generating a chronic inflammatory state and accelerated atherosclerosis (133, 159). These pro-inflammatory responses are illustrated below in Figure 1.3.

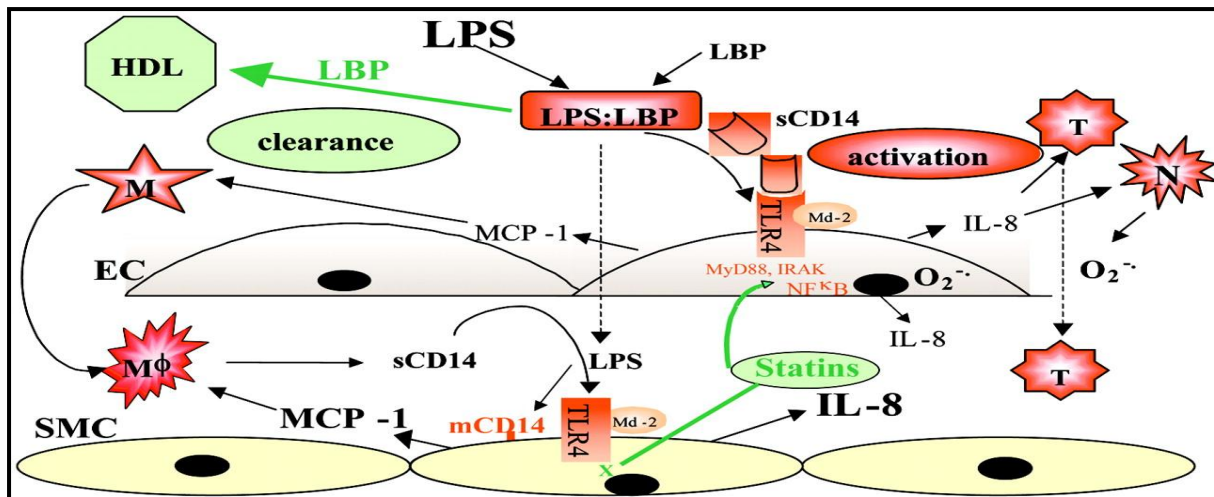


Figure 1.3: Diagram illustrating pro-atherogenic interactions between endotoxin and vascular cells. Circulating endotoxin binds to LBP forming a complex that binds to HDL, ultimately leading to elimination of endotoxin-HDL complex in the liver. Otherwise, endotoxin-LBP complex binds to sCD14 and leading to LPS-induced immune activation via TLR-4/MD2 Pathway, resulting in production of pro-inflammatory cytokines such IL-8, MCP-1 and reactive oxygen species. Statins inhibit endotoxin/TLR-4 signaling pathway. LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; HDL, high density lipoprotein; sCD14, serum CD14; mCD14, membrane-bound CD14; TLR4, toll-like receptor-4; MD-2, lymphocyte antigen 96; NF- κ B, Nuclear factor kappa B; M, monocyte; EC, endothelial cell; MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin-8; T, T lymphocyte; N, neutrophil; SMC, smooth muscle cell; M ϕ , macrophages; O $_2^{\cdot-}$, reactive oxygen species. (Reproduced with permission from Stoll LL, Denning GM, Weintraub NL. Potential role of endotoxin as a proinflammatory mediator of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004 24(12):2227-36).

Oxidative stress plays a key role in the pathophysiology of many chronic inflammatory disorders, including early atherosclerosis. *In vitro* studies have demonstrated that injection of endothelial cells with endotoxin resulted in increased antioxidant enzyme activity, consistent with an upsurge of oxidative stress (244, 245), leading to increased levels of inflammatory cytokines, proliferation of smooth muscle cell and programmed cell death, thus suggesting a link between endotoxin-induced oxidative stress and the mechanisms underlying the development of atherosclerosis (246, 247). More specifically, LPS has been shown to be associated with elevated markers of oxidative stress in human tissue, endotoxin induced dose-dependent production of reactive oxygen species in human tissue culture (248).

Of note, regarding atherosclerosis, is the production of inflammatory cytokines and chemokines, which recruit inflammatory cells to foci of inflammation (249). Endotoxin

stimulates increased production of pro-inflammatory cytokines such as INF- γ , IL-1, IL-6, IL-8, TNF- α (250, 251) leading to activation of chemokines and adhesion molecules (159).

Available evidence suggests that cytokines and adhesion molecules are important regulators of vascular inflammation, and act by initiating and perpetuating the interaction of immune cells with the vascular endothelium, consequently leading to plaque formation and cardiovascular events (133, 159, 252). During vascular inflammation, leucocytes undergo stepwise interaction with vascular endothelium comprising selectin-mediated leucocyte rolling, mild adhesion facilitated by adhesion molecules such as VCAM-1 and ICAM-1, and subsequent firm adhesion mediated by MCP-1 and IL-8 (133, 159). In addition, elevated circulating adhesion molecules are found in CKD patients and are closely associated with systemic inflammation, lipid abnormality and cardiovascular events in haemodialysis patients (253).

1.10 Potential therapeutic strategies for the reduction of inflammation through endotoxin reduction

Endotoxaemia constitutes a potentially important target for interventions directed to reducing cardiovascular morbidity and mortality in CKD patients. Theoretically, endotoxaemia can be prevented either through treating foci of endotoxin including periodontal disease, catheters and vascular access or by reducing translocation of endotoxin from the gut, ultimately resulting in a decrease in inflammatory response (133). One of these strategies would involve the use of sevelamer carbonate, a phosphate binder. Sevelamer binds and forms a complex with anions, bile acids as well as conjugated amino acids, leading to increase in faecal excretion and reduced intestinal absorption, including translocation of endotoxin into the circulation (133). Although, sevelamer has been widely used in CKD patients as a phosphate binder, it is increasingly being recognized to possess anti-atherogenic

and anti-inflammatory properties in haemodialysis patients (254). This suggests the possibility of cholesterol-independent effects of sevelamer, through its regulatory effects on systemic inflammation and oxidative stress, on atheroma formation (255). A clinical study in haemodialysis patients demonstrated that sevelamer treatment leads to a decrease in hsCRP levels which was accompanied by a corresponding decrease in circulating endotoxin levels, thus suggesting that endotoxaemia may contribute to the inflammatory state in haemodialysis, and was partly reduced by the use of sevelamer (256).

Statins, inhibitor of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, widely used clinically for their effective lipid lowering action, have also been shown to possess anti-inflammatory effects (257, 258). Available evidence suggested that lipid lowering statins may have an additional protective role in blocking some of the endotoxin signaling pathways, leading to a decrease in inflammatory response. (248). Possibly, this may be due to reduced synthesis of mevalonate, a cholesterol precursor that play a critical role in the process of lipidation of proteins, thereby resulting in disordered receptor signaling pathway (259). More recently, a clinical trial explored the approach of inhibiting protein lipidation with some degree of success in the treatment of a multisystem disorder manifesting with accelerated atherosclerosis in the affected children (260). These aforementioned studies therefore suggest that anti-inflammatory strategies may possibly reduce cardiovascular complications related to systemic inflammation in patients with CKD.

1.11 Transforming growth factor β levels and atherosclerosis

Transforming growth factor β , a growth factor that is produced by many inflammatory cells including leucocytes, macrophages, smooth muscle cells and platelets (261). There are three isoforms of transforming growth factor β (TGF β) which are designated as TGF β 1, TGF β 2, and TGF β 3. Transforming growth factor- β 1, the most extensively studied of these three

isoforms, exhibits anti-inflammatory and anti-proliferative properties by inhibiting the synthesis of tumour necrosis factor- α (TNF- α) or by down-regulating the pro-inflammatory effects of IL-1 β and interferon- γ (262), ultimately leading to reduction of inflammatory cytokine-induced VCAM-1, chemotaxis and leucocyte adhesion to vascular endothelial lining, and decreased macrophage activity (263, 264). Previous studies have provided evidence that suggests that low serum levels of TGF β 1 are a risk factor for atherosclerosis in non-CKD (265, 266) and haemodialysis patients (267). However, there is paucity of data on whether TGF β 2 and TGF β 3 contribute to the susceptibility and the severity of atherosclerosis in CKD patients.

1.12 Circulating endotoxaemia, renal function, and volume status

Elevated levels of inflammatory markers have been reported in non-dialytic CKD, and worsens with the development of ESRD (167, 268). As GFR progressively worsens, CKD patients develop impaired capacity to excrete salt and water by the kidneys, resulting in fluid retention and hypertension, which is common in dialysis patients, and is also associated with poor clinical outcomes (269). It has been shown that CKD patients develop fluid overload from the early stages of the disease, and this may induce inflammatory response, leading to worsening kidney failure and CVD (270). Patients with circulatory overload, for example those with congestive heart failure, present with signs of systemic inflammation that diminish when the cardiac failure is compensated (271). It has been postulated that inflammatory state in CKD patients is related to gut oedema and increased gut permeability, leading to translocation of endotoxins from the gut lumen into the blood circulation (133). Thus, the presence of endotoxins in the circulation leads to systemic immune activation, and finally generating a chronic inflammatory state in CKD patients (133, 271).

1.13 Carotid intima media thickness (CIMT) as a surrogate marker for CVD

Carotid intima media thickness is an independent risk factor for CVD, and the presence of carotid plaques is a strong predictor of cardiovascular events and mortality (272, 273). Available evidence has validated CIMT as a good surrogate marker for CVD. Previous studies have shown that CIMT is an independent risk factor for future CVD events (274-276). Also, the predictive association between baseline CIMT and increased CVD risk has been demonstrated in healthy young adult populations (277, 278).

Data from intervention studies have also linked CIMT with increased risk of cardiovascular events. A strong association was observed between progression of CIMT and the incidence of cardiovascular death (combined relative risk was 2.2 for each annual increase in CIMT of 0.03 mm) in the CLAS trial, involving patients who received colestipol-niacin or placebo following CABG surgery (279). Recently, a meta-analysis of statin therapy trials also showed a close association between the progression of CIMT and the incidence of cardiovascular events, with a mean annual decrease in CIMT thickness of 0.012 mm associated with a significant reduction in the risk of cardiovascular events (280). In addition, presence of carotid artery plaque in an individual is a strong predictor of future cardiovascular events (281).

In spite of the evidences highlighting the usefulness of CIMT as a predictive marker of atherosclerotic CVD, there are limitations in the assessment of change in CIMT as a suitable marker of atherosclerosis and of increased cardiovascular risk (282). Firstly, CIMT is obtained by combined measure of the intimal and medial layer of the arterial wall, even though in the early phase, atherosclerotic process is only limited to the intima layer. Secondly, despite the fact that majority of the population of the westernized societies die from CAD, several studies have demonstrated a weak correlation between increased CIMT

and atherosclerosis. A third critique deals with scarcity of data showing that progression of CIMT predicts CAD as well as stroke, which further supports criticism of the utility of CIMT measurements in clinical study.

1.14 Measurement of inferior vena cava diameter (IVCD)

Estimation of fluid status in ESRD patients is quite challenging, since many factors including reduced lean body mass, dialysis-induced hypotension, as well as increased peripheral vascular resistance and altered venous capacity, may cause measurement errors in this group of patients (283). Usually, clinicians estimate fluid status by the presence of dyspnoea/orthopnoea, dyspnoea on exertion, jugular venous distension, peripheral oedema, congestive hepatomegaly, pulmonary crackles, weight gain or hypertension, which are valuable but not specific for detecting fluid overload (284, 285). Measurement of inferior vena cava diameter (IVCD) and the calculation of the collapsibility index (CI) are thought to be appropriate estimates of body fluid status in CKD, as cardiac filling pressures in CKD patients are more likely to reflect fluid overload rather than intrinsic cardiac dysfunction. Inferior vena cava diameter measurement is a non-invasive, simple and quick method, correlates well with right atrial pressure and circulating blood volume (286, 287). Although there are issues about intra- and inter-observer variability during IVCD assessment, nevertheless, IVCD measurement is still a viable technique for evaluation of fluid status in ESRD patients (283, 288).

1.15 Bio-impedance spectroscopy

Estimation of body composition is useful in various clinical settings for obtaining information about nutritional state and fluid status of different body compartments. Bio-impedance spectroscopy (BIS) is an attractive technique for this purpose, because it provides a precise and specific method for assessment of nutritional and body fluid status (289). Bio-

impedance spectroscopy is a simple and inexpensive tool for evaluating total body water, extracellular water (ECW) as well as intracellular water (ICW); thus, assessment of hydration status in ESRD patients (290-292). This measurement technique is based on the assumption that direct current is conducted faster in fatty tissue than in lean tissue mass with high water content (289, 293). A bio-impedance meter is attached to the body and a small electrical signal measures the body's ability to conduct the current (294). Bio-impedance spectroscopy has been documented to be useful in the assessment of fluid status in haemodialysis (295) as well as in peritoneal dialysis patients (296). However, there is lack of information on the utility of BIS-derived measurements for evaluation of fluid status in pre-dialytic CKD patients.

Although, BIS is a simple device providing valuable evaluation of patients' fluid status, the method is limited by lack of standards of normality (297). In addition, variations in electrolytes, red cells, protein, body temperature or presence of implantable devices affect BIS measurements, thereby further limiting the use BIS-derived measurements for evaluating fluid status of CKD patients (289, 297). For accuracy, standard conditions are needed such as fasting state, correct posture during measurement as well as timing of measurements after supine position or haemodialysis therapy (298). The patients should be counselled against strenuous exercise, excess alcohol consumption and the use of diuretics prior to examination (289). Accurate measurement of patient's weight and height is essential, since over- or underestimation of weight or height can cause an error in the estimation of total body water (289). Therefore, patient's weight and height should be measured to the nearest 0.1kg and 0.5cm respectively (289).

Nevertheless, assessment of fluid status by BIS has been validated against the gold standard reference method (isotope dilution) in both patients and healthy individuals (299, 300). Most importantly, the bio-impedance spectroscopy measurement technique is highly

reproducible and very specific in the detection of fluid status in CKD patients (301-303). However, this needs to be confirmed by adequately powered studies in South African CKD patients and healthy controls.

1.16 Rationale for the study

As a result of impaired renal function, CKD patients are fluid overloaded, resulting in increased bacterial translocation across the gut and increased endotoxin levels in the blood circulation, activating cellular immune activation, leading to systemic inflammation. Clearly, translocated endotoxin derived from both endogenous and environmental microbes has a wide range of adverse effects on cardiovascular structure and function, driving systemic inflammation, oxidative stress and atherosclerosis. It is therefore hypothesized that endotoxaemia is related to volume overload and represents a potentially reversible risk factor for subclinical atherosclerosis in CKD patients. This study investigated endotoxin in different cohorts of CKD patients and also evaluated the role of endotoxaemia as an inflammatory mediator of atherosclerosis in CKD patients.

1.17 Aim of study

The aim of the study is to investigate endotoxaemia across the spectrum of CKD and to determine the pro-inflammatory effects of endotoxin that may be relevant to the development of atherosclerosis in CKD patients.

1.18 Objectives

1. To determine blood levels of endotoxins in stage 3 CKD and dialysed patients.
2. To evaluate the association between renal function, volume status and endotoxaemia in stage 3 CKD and dialysed patients.

3. To measure carotid intima-media thickness (CIMT) as a surrogate marker of atherosclerosis in stage 3 CKD and dialysed patients.
4. To establish the role of circulating endotoxaemia as a mediator of inflammation in stage 3 CKD and dialysed patients.
5. To determine the relationship between circulating endotoxaemia, markers of systemic inflammation and subclinical atherosclerosis as determined by CIMT in stage 3 CKD and dialysed patients.

CHAPTER 2

2.0 MATERIALS AND METHODS

The study was approved by the University of the Witwatersrand, Human Research Ethics Committee (Protocol M130127) and was in accordance with the Helsinki Declaration of 1975, as revised in 2000.

2.1 Study design and population

This was a cross-sectional descriptive study that involved 120 chronic kidney disease (CKD) patients attending Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa and a comparator cohort of 40 age- and sex-matched controls.

2.1.1 *Sample size*

Sample size was calculated based on the two sample t-test to detect a difference of 0.5 EU/ml in the level of endotoxin using data from a previous study (18). A minimum of 38 patients in each of the comparison groups was needed to detect this difference at 95% confidence interval ($\alpha=0.05$) with a power of 90% ($\beta=0.10$). The final sample size in each of the comparison group was increased to 40 to allow for patient attrition. Total sample size was 160 (40 in each group-peritoneal dialysis [PD], haemodialysis [HD], CKD stage 3 and controls).

2.1.2 *Inclusion criteria*

- Stage 3 CKD patients who attended the renal clinic for ≥ 3 months as well as patients on haemodialysis and peritoneal dialysis for ≥ 3 months at the dialysis unit who agreed to participate in the study were recruited.
- Age between 18 years and 60 years.

2.1.3 Exclusion criteria

Patients with the following conditions were excluded:

- Clinical signs of active or chronic infection.
- Diabetes mellitus.
- Seropositive for hepatitis B, C and the human immunodeficiency virus.
- Autoimmune disease.
- Liver dysfunction.
- Malignancy.
- Heart failure (NYHA III-IV).
- Anti-inflammatory or immunosuppressive therapy at least three months prior to enrolment.
- Amputated limbs.
- Pacemakers and/or implants.
- Incomplete measurements.

2.1.4 Controls

Forty age and gender-matched volunteers who were staff and students of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) were recruited.

2.2 Methods

Enrolment of all patients and controls commenced in April 2013 and was completed in December 2013. Using a structured interview form, information on age, race, gender and tobacco use was documented. Patients were classified as smokers if they were current smokers, former smokers if they stopped smoking for at least six months prior to the study and non-smokers if they had never smoked (203). Blood pressure for HD patients was

recorded non-invasively in the arm without the A-V fistula with an accu-sion mercury sphygmomanometer in the sitting position before a dialysis session commenced. Blood pressure was estimated by averaging all pre-dialysis and post dialysis blood pressure recordings taken during the month before the study (3 measurements per week for a total of 12 measurements; that is 3/wk). Among PD and CKD patients, blood pressure was recorded at the time of the clinic visit. The blood pressure average of four clinic visits was taken as the patient's actual BP. For the controls, blood pressure was taken in the sitting position after resting for 5 minutes. The average of three readings, recorded 5 minutes apart, was taken as the blood pressure. Pulse pressure was calculated as systolic blood pressure (SBP) minus diastolic blood pressure (DBP). Mean arterial blood pressure (MABP) was calculated as diastolic blood pressure plus one third pulse pressure. Height was measured to the nearest 0.5 cm and weight to the nearest 0.1 kg using the Seca 220 telescopic measuring rod (Seca gmbh GmbH & Co, Germany) and body mass index (BMI) was calculated as the ratio weight to height squared. In haemodialysis patients, the dry weight was obtained before dialysis therapy. Waist circumference was assessed using a tape measure. Measurements were taken at the level of belly button with the clothing raised to just below the chest. Estimated glomerular filtration rate (eGFR) was calculated using CKD-EPI formula.

2.2.1 Blood collection and processing

Blood was taken from the study participants for measurements of haemoglobin concentration, creatinine, albumin, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, calcium, phosphate and intact parathormone. The mean of three measurements taken over 3-months from the time of recruitment, including the month of entry into the study, were used for analysis. All tests were analysed in the National Health Laboratory as part of the routine standard of care using ADVIA^R auto-analysers (Siemens Healthcare Diagnostics Inc, USA). Blood for measurements of endotoxin,

lipopolysaccharide binding protein (LBP), C-reactive protein (CRP), interleukin-6 (IL-6), serum CD14 (sCD14), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), oxidized LDL (OxLDL) and transforming growth factor- β (TGF- β) was collected into anticoagulant-free tubes after an overnight fast. Blood samples from patients receiving haemodialysis were drawn before a dialysis session commenced. Samples were kept on ice until the serum was separated within 30 minutes of collection and centrifuged at 3000 rpm for 10 minutes at room temperature (U-320R, Boeco, Germany). Serum was separated and stored in appropriate 1.5ml micro-centrifuge tubes at -70° until ready for assay. Samples were only thawed once, at time of assay. Whole blood was collected into ethylene diamine tetra-acetic acid (EDTA) bottle and stored at -20° for cytokine genotyping.

2.2.2 Bio-impedance spectroscopy analysis – assessment of fluid and nutritional status

Fluid status and markers of nutritional status [lean tissue index (LTI), fat tissue index (FTI), lean tissue mass (LTM), fat tissue mass (FTM), adipose tissue mass (ATM) and body cell mass (BCM)] were measured automatically with the aid of a body composition monitor – BIS device (Fresenius Medical Care, Germany). Measurements were performed in the supine position after the patient had rested for 5 minutes. The electrodes were fixed on the dorsal surfaces of the hand and foot at the distal metacarpals and metatarsals on the non-dominant side, respectively. Anthropometric data including weight, height, age and gender were input into the machine and results calculated by in-built software and displayed on the LCD screen. The measurements were repeated in cases where erroneous measurements were detected by machine-based measurement quality indicator. Three readings were recorded and average values used for the analysis. (304). Fluid overload was defined by absolute overhydration (in litres) or as $OH/ECW\% \geq 15\%$ (305). In addition to the fluid overload measurement by the BCM, patients' fluid status was also assessed by evaluating for the presence of oedema. Malnutrition was defined as lean tissue index (LTI) less than the 10th percentile of a

reference population (306). Percentage body cell mass (%BCM) was computed using the formula: $100 \times \text{BCM} / \text{current weight}$. Male and female patients with %BCM less than 35% and 30% respectively were considered malnourished

2.2.3 *Inferior vena cava diameter measurement*

All echocardiographic/ultrasonographic measurements were performed by a trained cardiologist/sonographer who was blinded to the clinical status of the patients and controls. Inferior vena cava examination with simultaneous electrocardiographic monitoring was performed in a supine position after 5–10 min of rest during normal expiration and inspiration while trying to avoid Valsalva manoeuvres. Inferior vena cava diameter was measured on the same day on which the bio-impedance analysis was done to avoid interference of daily fluctuations of fluid status. The anteroposterior dimension of IVCD was measured using two-dimensional and Doppler recordings 1.5 cm below the diaphragm in the hepatic segment. The IVCD was expressed as IVCD in expiration, adjusted for body surface area (BSA). Fluid overload was defined as $\text{IVCD} > 11.5 \text{ mm/m}^2$ (286). In addition, the collapsibility index (CI) was calculated as the percentage of decrease in IVCD in inspiration versus expiration. Patients with a CI lower than 40% were considered to be fluid overloaded (307).

2.2.4 *Echocardiography*

Echocardiography was performed according to the American Society of Echocardiography recommendations (308, 309) using the Philips iE33 machine equipped with a S5-1 1-5 MHz transducer, allowing for M-mode, two dimensional and colour doppler measurements (Philips Corporation USA). All measurements were done at the time of BIS analysis. The following parameters were determined: left atrium diameter (LAD), left ventricular end-diastolic diameter, left ventricular end-systolic diameters, posterior wall thickness and interventricular septal thickness in diastole. From these measurements, left

ventricular mass (LVM) was calculated according to the equation described by Devereux and Reichek (310). Left ventricular mass index (LVMI) was computed by dividing LVM by body surface area. Left ventricular hypertrophy was diagnosed when LVMI is greater than 131 g/m^2 in males and 100 g/m^2 in females (311). Relative wall thickness was calculated and four pattern of left ventricular geometry were identified (normal, concentric, eccentric and concentric remodelling) based on LVMI and RWT (312). Left ventricular systolic function was assessed by ejection fraction while diastolic function was assessed using E/A ratio, deceleration time and left ventricular filling pressure (E/e').

2.2.5 Carotid intima media thickness (CIMT)

As previously described (281), CIMT was assessed using high resolution B-mode ultrasonography with the aid of L3-11 MHz linear array transducer (Philips Corporation USA). The sonographer visualised and focused on the far walls of the common carotid artery, 1 cm proximal to the dilatation of the carotid bulb in the longitudinal plane (Figure 2.1) and then acquired the CIMT measurements automatically with percentage measurement quality, ranging from less than 50% to 100%. A percentage measurement quality of greater than 95% was used in this study. A similar process was followed for both right and left common carotid artery and the mean of right and left common CIMT was used for analysis. Carotid plaque was defined as the echogenic structure protruding into the lumen with the distance between the media adventitia interface and the internal side of the lesion $\geq 1.2 \text{ mm}$ (313). Carotid intima media thickness was measured in plaque-free areas and all measurements were performed by the same sonographer who was blinded to the clinical details and laboratory data of the participants.

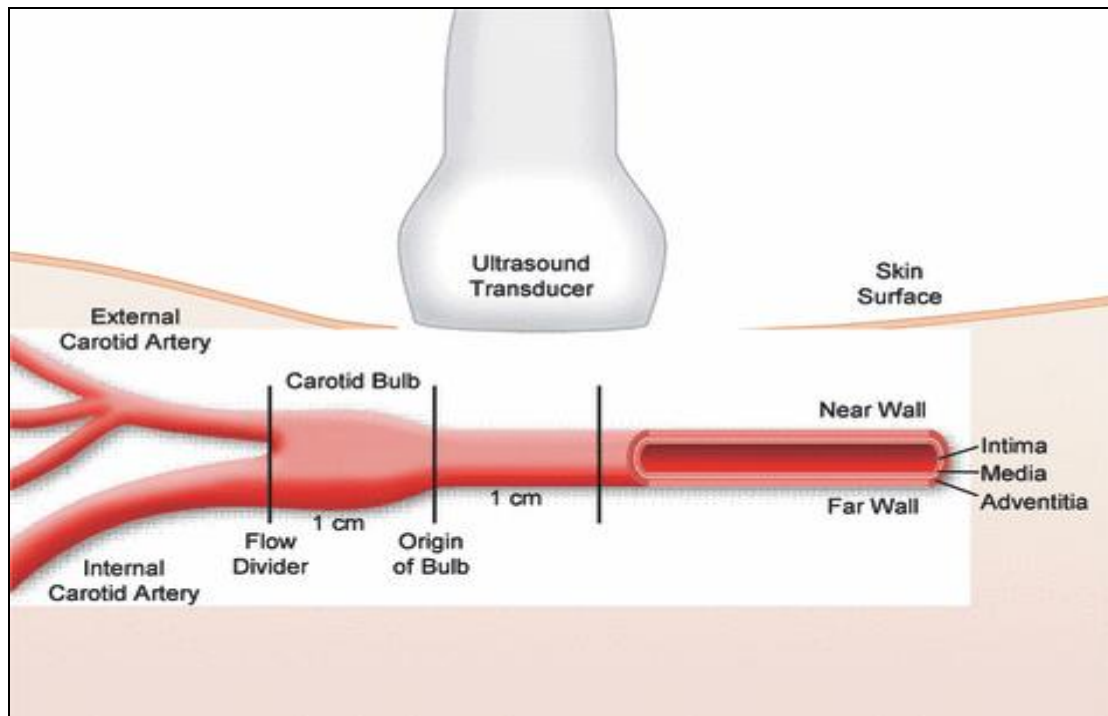


Figure 2.1: Schematic showing the anatomy and sites of carotid intima media thickness measurement. The figure illustrates different segments of the carotid artery; namely common carotid artery, internal carotid artery, and the carotid bifurcation. Using a B-mode ultrasound, CIMT measurements was obtained from the far walls of the common carotid artery, 1 cm proximal to the dilatation of the carotid bulb in the longitudinal plane (Reproduced with permission from Mookadam F, Moustafa S, Lester S, Warsame T. Subclinical Atherosclerosis: Evolving Role of Carotid Intima-Media Thickness. *Prev Cardiol.* 2010; 13:186-97).

2.2.6 Measurement of serum endotoxin levels

All laboratory assays including ELISA and DNA genotyping were done by the investigator under the supervision of a laboratory scientist. Serum endotoxin was quantified using the Limulus amoebocyte lysate QCL-1000TM assay (Lonza Walkersville, USA) according to manufacturer's instructions, using a previously described method (314) (Appendix 5). To further assess the effect of haemodialysis on generation of endotoxin, serum samples for endotoxin quantification were obtained pre and immediately post dialysis. Absorbance was measured using an ELx800 Universal plate reader (BioTek Instruments, Inc, VT, USA). Haemodialysis cohorts underwent more detailed evaluation of factors associated

with endotoxaemia, including intra-dialytic blood pressure and ultrafiltration volume. Patients were diagnosed to have intra-dialytic hypotension when SBP was lower than 100 mmHg or SBP decreased by more than 40 mmHg.

2.2.7 C-reactive protein, IL-6, IL-8, sCD14 and MCP-1 assays

Serum CRP, IL-6, IL-8, sCD14 and MCP-1 assays were analyzed using Luminex® Performance Assay multiplex kits (R&D Systems, Inc. Minneapolis, USA). Assays were carried out in accordance with the manufacturer's instructions (Appendix 7 and 8). For CRP measurements, the sample dilution was 1:1000 (first dilution was 1:100 and second dilution was 1:10) while samples for sCD14 measurements was diluted 1 in 50 times (first dilution was 1:5 and second dilution was 1:10). Samples for IL-6, IL-8 and MCP-1 were not diluted. Absorbance for CRP, IL-6, MCP-1, IL-8 and sCD14 were read in the bead region 8, 19, 53, 54 and 59 respectively, on the Bio-Plex™ 200 system (Bio-Rad, Texas, USA) and concentrations were generated automatically with Bio-Plex manager software, version 5.0 (Bio-Rad Laboratories Inc, Hercules, USA).

2.2.8 Measurement of lipopolysaccharide binding protein (LBP) concentrations

Serum levels of LBP were measured using commercial human LBP ELISA kit, Hycult HK315 (Hycult biotechnology, Uden, the Netherlands), in accordance with manufacturer's instruction (Appendix 6). The sample dilution ratio was 1:3000 (first dilution was 1:1000 and second dilution was 1:3). Absorbance was measured using an ELx800 Universal plate reader (BioTek Instruments, Inc, VT, USA), and concentrations were calculated using an automated spreadsheet for 4- and 5- parameter logistics curve fitting of bioassay calibrations (©A. Swart 2012-2014).

2.2.9 Measurement of oxidized LDL concentrations

The concentration of oxidized LDL was measured by the OxiSelect™ human oxidized LDL ELISA commercial Kit (Cell Biolabs, Inc.), using anti-MDA antibody and streptavidin-enzyme conjugate in accordance with manufacturer's instruction (Appendix 9). The detection sensitivity limit of the assay is < 50ng/ml with a standard range of 0 – 1000 ng/ml. Absorbance was measured using an ELx800 Universal plate reader (BioTek Instruments, Inc, VT, USA), and concentrations were calculated using an automated spreadsheet for 4- and 5- parameter logistics curve fitting for bioassay calibrations (©A. Swart 2012-2014). Oxidized LDL/LDL was then derived from laboratory assay results.

2.2.10 Measurement of transforming growth factor β 1, β 2, and β 3 concentrations

Serum TGF β -1, 2 and 3 assays were analyzed using BioPlex Pro(TM) TGF β , Assay kits (Bio-RAD Laboratories, Inc, USA). Assays were carried out in accordance with the manufacturer's instruction (Appendix 11). The sample dilution was 1:16. Absorbance for TGF β -1, TGF β -2, and TGF β -3 were read in the bead region 13, 72, and 66 respectively, on the Bio-Plex™ 200 system (Bio-Rad, Texas, USA) and concentrations were generated automatically with Bio-Plex manager software, version 5.0 (Bio-Rad Laboratories Inc, Hercules, USA).

2.2.11 DNA extraction and cytokine genotyping

All procedures were carried out at room temperature (15-25°C). Genomic DNA was extracted from whole blood using a modified salting out method (315) and the concentrations determined using a NanoDrop™ spectrophotometer (Thermo Scientific, Inqaba Biotech). Cytokine genotyping was performed on the study groups and the controls using a cytokine genotyping tray kit (One Lambda Inc., Los Angeles, USA). Eight single nucleotide polymorphisms (SNPs) of five inflammatory cytokines were typed using the kit. The SNPs that were studied include: Tumour necrosis factor- α (TNF- α) -308 (A/G), transforming

growth factor- β 1 (TGF- β 1) codon 10 (T/C) and 25 (G/C), interleukin-10 (IL-10) -1082 (A/G), -819 (T/C), -592 (A/C), IL-6 -174 (G/C) and interferon- γ (IFN- γ) -874 (T/A). The pre-optimized primers were presented dried in different wells of a 96-well 0.2ml thin-walled tube tray for polymerase chain reaction (PCR), to which DNA samples, recombinant Taq polymerase (Qiagen Taq), and specially formulated dNTP-buffer mix (D-mix) were added. Each genotyping tray included a negative control reaction tube which detects an internal control PCR product. An optimal concentration of 100 ng/ μ l of each DNA sample was used in combination with D-mix and 5U/ μ L of Qiagen Taq polymerase as per the manufacturer's protocol, resulting in a final reaction volume of 10 μ L. The PCR was run on a thermocycler (MJ Mini Thermal cycler, Bio-Rad, USA) using One Lambda PCR program according to manufacturer's instructions (Appendix 12). All the PCR products were visualized on a 2% agarose gel stained with ethidium bromide, with the aid of an image analyser (Gel DocTM EZ Imager, Bio-Rad, USA). All SNPs were assessed based on the sizes of the amplified products for the respective cytokine genes with negative amplifications scored only if the internal control product was present (Figure 2.2).

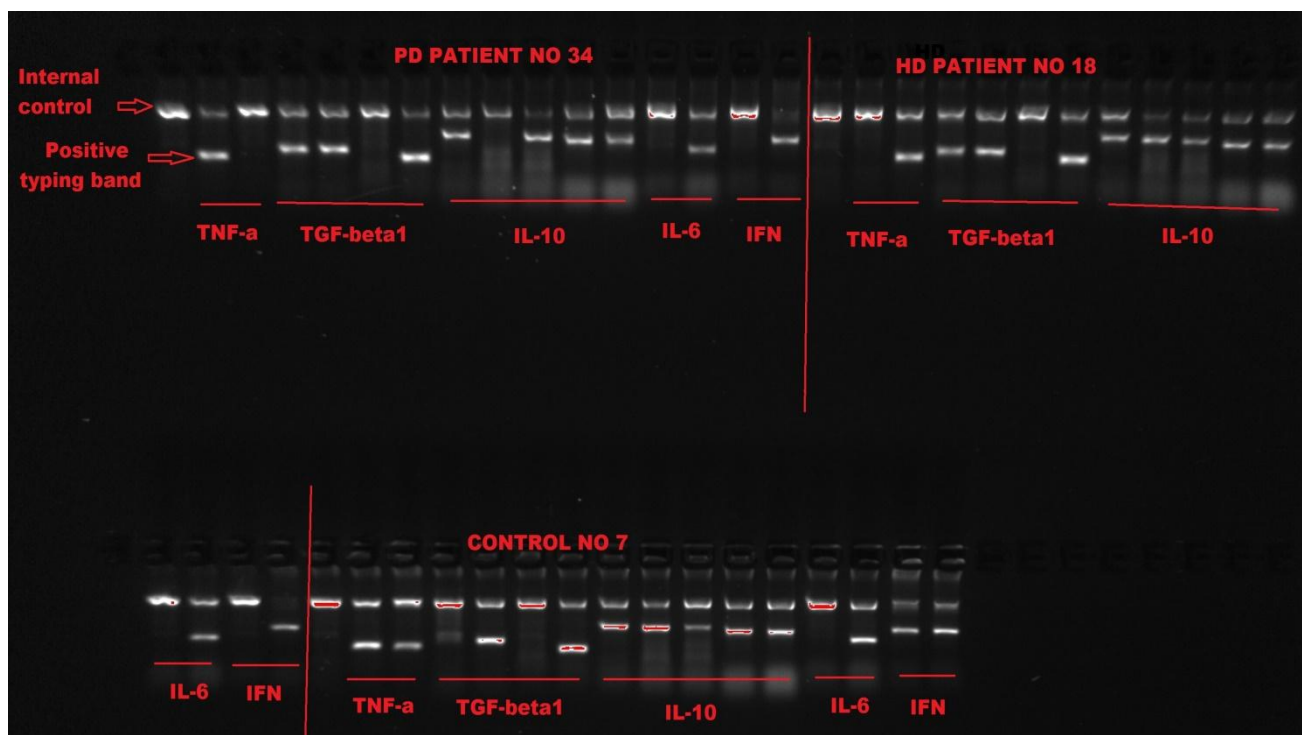


Figure 2.2: Genotype of two patients (PD34 and HD18) and 1control (CR07) according to their TNF- α , TGF- β 1, IL-10, IL-6 and IFN- γ polymorphisms. Arrows show internal control and positive typing bands. The polymerase chain reaction was run on a thermocycler using 1 cycle (96°C for 130 seconds, 63°C for 60 seconds, 9 cycles (96°C for 10 seconds, and 63°C for 60 seconds), followed by 20 cycles (96°C for 10 seconds, 59°C for 50 seconds, and 72°C for 30 seconds). Polymerase chain reaction products were visualised on a 2% agarose gel stained with ethidium bromide.

2.3 Data analysis

All data analyses were performed using the statistical package for social sciences (SPSS) 16 (SPSS, Inc., Chicago IL). Variables were presented as mean $\pm$ SD for continuous data, and percentages and frequencies for categorical data. Results were analysed using a Mann-Whitney test, with the Tukey post-hoc test for normally distributed data and chi-square test and Kruskal Wallis test for nonparametric data. Correlation between variables was assessed by the Pearson correlation coefficients accordingly. The sensitivity and specificity of IVCD (cut-off value: 11.5mm/m²), CI (cut-off value: <40%) and presence of oedema (by physical examination) for fluid overload were determined using receiver operating concentration (ROC) curve analysis. Further analysis was performed after categorising endotoxin levels

into two groups (cut-off value of 50pg/ml; equivalent to 0.5 EU/ml). The categorisation of endotoxin levels (≤ 0.5 EU/ml and > 0.5 EU/ml) was adopted from a previous study(15). Also, CMT measurements as well as serum CD14, IL-8 and MCP-1 concentrations were subdivided into two groups according to the median values. Subclinical atherosclerosis was defined as CMT > 0.55 mm. Strength of associations between endotoxins categories (≤ 0.5 EU/ml, > 0.5 EU/ml) and CMT categories in the presence of variables indicating immune activation (sCD14, IL-8 or MCP-1 categories) were assessed by odds ratios. Factors independently associated with volume overload and atherosclerosis were further explored by a multiple linear regression analysis. Genotype frequencies were determined by gene counting method and expressed as percentages. The frequencies were compared using Fisher's exact test. Further analysis was performed to assess the influence of various genotypes of TGF- β 1 and IL-6 on the serum levels of TGF- β 1 and IL-6 respectively. A P-value <0.05 (two-tailed) was considered significant.

CHAPTER 3

3.0 RESULTS

3.1 Demographic data

This cross-sectional study consisted of 120 patients comprising PD, HD, stage 3 CKD patients, with an average age of 40.6 ± 9.9 , 40.6 ± 10.1 and 42.1 ± 10.6 years respectively. In each group, male patients comprised 55% of the studied population. Of the patients, 106 (88.3%) were black, 8 white (6.7%), 3 (2.5%) Indian while 3 (2.5%) were mixed race. Eighteen patients (15%) were active smokers, 29 (24.2%) reported to have stopped smoking while 73 (60.8%) had never smoked cigarettes. The controls were matched for age and gender. There were 22 (55%) male and 33 (45%) female controls. The mean age for the controls was 42.2 ± 10.1 years. Only 2 (5%) of controls were current smokers. The demographic data are summarised in Table 3.1.

Table 3.1: Demographic data of the study population

	PD	HD	Stage 3 CKD	Control
No of patients	40	40	40	40
Age (years)	40.6 ± 9.9	40.6 ± 10.1	42.1 ± 10.6	42.2 ± 10.1
Sex (M:F)	22:18	22:18	22:18	22:18
Current smokers	9 (22.5%)	5 (12.5%)	4 (4%)	2 (5%)
Race				
Black	35 (87.5%)	37 (92.5%)	34 (85%)	31 (77.5%)
White	4 (10%)	1 (2.5%)	3 (7.5%)	3 (7.5%)
Indian	0 (0%)	0 (%)	3 (7.5%)	1 (2.5%)
Mixed	1 (2.5%)	2 (5%)	0 (0%)	5 (12.5%)

PD, peritoneal dialysis; HD, haemodialysis; CKD, chronic kidney disease. Continuous data were expressed as mean $\pm$ SD and categorical data as frequencies and percentages

3.2 Primary kidney disease

The aetiology of CKD was hypertension-attributed in 59/120 (49.2%), chronic glomerulonephritis in 36/120 (30%), polycystic kidney disease in 8/120 (6.7%), reflux nephropathy in 4/120 (3.3%), congenital abnormalities of the kidneys in 4/120 (3.3%) patients, obstructive uropathy in 3/120 (2.5%), and unknown in 7 (5%) patients (Figure 3.1). Further analysis showed that 52/59 (88.1%) of black patients had hypertension as the primary cause of their kidney disease, while only 7/52 (11.9%) non-blacks had essential hypertension as the aetiology of their CKD.

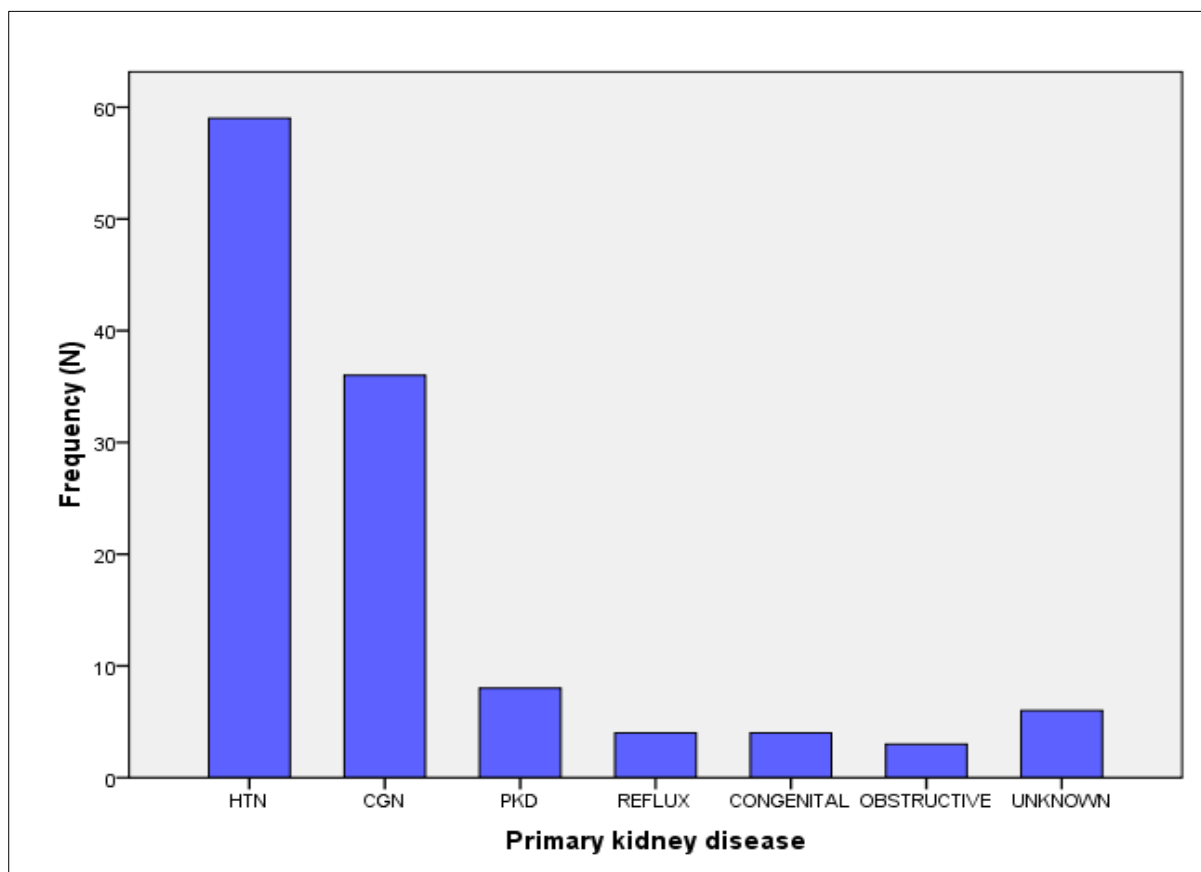


Figure 3.1: The spectrum of primary kidney disease in the chronic kidney disease patients. Bar chart represents number of the CKD patients. HTN, Hypertension; CGN, Chronic glomerulonephritis; PKD, Polycystic kidney disease; Reflux, Reflux nephropathy; Congenital, Congenital anomalies; Obstructive, Obstructive uropathy

3.3 Blood pressure and drug treatment

The mean arterial blood pressure (MABP) was significantly elevated among study patients compared to the controls. As shown in Table 3.2, patients receiving maintenance dialysis had grossly elevated blood pressure (MABP: 147.9 ± 25.3 mmHg in HD and 146.9 ± 17.9 mmHg in PD, $p < 0.001$). One hundred and two patients 102/120 (85%) received various combinations of antihypertensive agents. Calcium channel blockers were taken by in 81/120 (67.5%) of patients, beta blockers by 40/120 (33.3%), angiotensin-II receptor blockers or angiotensin-converting enzyme inhibitors (ACE-I) by 27/120 (22.5%), diuretics by 22/120 (18.3%) and alpha blockers by 16/120 13.3%) of the patients. Regarding patients that were treated with antihypertensive medications; 43/102 (42.2%) were on monotherapy, 39/102 (38.2%) on double, 15/102 (14.7%) on triple, 3/102 (2.9%) on quadruple agents while 2/102 (1.96%) patients received 5 agents in various combination. In addition, patients received other medications for ESRD management including phosphate binders in 104/120 (86.7%), statins in 28/120 (23.3%) and aspirin in 9/120 (7.5%) of cases.

3.4 Clinical and laboratory parameters of the study population

The main clinical and laboratory characteristics of the study population are described in Table 3.2. Chronic kidney disease patients had relatively lower haemoglobin levels compared to the controls (11.8 vs 14.9 g/dL, $p < 0.0001$); with 44/80 (55%) of maintenance dialysis patients at target haemoglobin of 11-12 g/dL (KDIGO) on treatment with erythropoietin stimulating agents. Compared with controls, CKD patients had lower serum albumin (36.5 vs 42.1 g/L, $p < 0.001$) but higher calcium-phosphate product (3.0 vs 2.3 mmol²/L², $p < 0.001$) and severe hyperparathyroidism (intact parathormone level: 69.3 vs 4.3 pmol/L, $p < 0.001$).

Table 3.2: Clinical and Laboratory characteristics of the study population

Parameters	All patients (n=120)	PD (N=40)	HD (N=40)	Stage 3 CKD (N=40)	Control (N=40)	*P value
Clinical						
BMI (kg/m ²)	27.8 ± 6.8	26.3 ± 4.8	25.7 ± 5.1	31.3 ± 7.0	26.6 ± 5.4	NS
Waist Circumference (cm)	84.1 ± 12.8	82.9 ± 9.4	78.4 ± 11.2	90.3 ± 13.9	83.3 ± 12.3	NS
Mean BP (mmHg)	144 ± 22.5	147.9 ± 25.3	146.9 ± 17.9	138.0 ± 22.9	117.3 ± 12.6	<0.001
Renal function						
Serum creatinine (µmol/L)	-	NA	NA	135.0±44.5	73.9±16.8	-
eGFR(ml/min/1.73m ²)	-	NA	NA	50.8 ± 2.2	100.8 ± 23.9	-
Haematological						
Haemoglobin (g/dL)	11.8 ± 2.3	10.9 ± 2.1	11.1 ± 1.7	11.1 ± 1.7	14.9 ± 1.8	<0.001
Biochemical						
Serum albumin (g/L)	38.5 ± 4.9	36.1 ± 4.8	38.1 ± 4.7	41.3 ± 4.1	42.1 ± 3.4	<0.001
Calcium (mmol/L)	2.3 ± 0.2	2.2 ± 0.3	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.1	0.028
Phosphate (mmol/L)	1.3 ± 0.5	1.6 ± 0.6	1.2 ± 0.5	1.1 ± 0.5	1.0 ± 0.2	<0.001
CaPO ₄ (mmol ² /L ²)	3.0 ± 1.3	3.5 ± 1.2	2.8 ± 1.2	2.7 ± 1.2	2.3 ± 0.4	<0.0001
Parathormone (pmol/L)	69.3 ± 69.8	110.8 ± 66.8	84.2 ± 71.8	14.5 ± 19.4	4.3 ± 1.7	<0.001
Lipid profile						
Cholesterol (mmol/L)	4.4 ± 1.3	5.2 ± 1.5	3.4 ± 0.7	4.5 ± 0.9	4.2 ± 1.2	NS
HDL (mmol/L)	1.3 ± 0.9	1.3 ± 0.4	1.3 ± 1.4	1.3 ± 0.5	1.3 ± 0.4	NS
LDL (mmol/L)	2.5 ± 1.0	3.1 ± 1.2	1.9 ± 0.6	2.5 ± 0.8	2.3 ± 1.0	NS
Triglycerides (mmol/L)	1.3 ± 0.8	1.5 ± 0.9	0.9 ± 0.5	1.5 ± 0.7	1.2 ± 0.7	NS

eGFR, estimated glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein; NS, not significant; PD, peritoneal dialysis; HD, haemodialysis; CKD, chronic kidney disease; BMI, body mass index; BP, blood pressure

Continuous data were expressed as mean ± SD

*p-values were calculated by comparing all patients with kidney disease (n=120) to controls (n=40) using Mann Whitney test

3.5 Characteristics of study population

Table 3.3 describes the fluid status, circulating endotoxaemia, severity of inflammatory response, nutritional status, echocardiographic parameters and CIMT measurements in different subgroups of patients.

3.5.1 Fluid status

Chronic kidney disease patients were overhydrated compared to the controls (absolute overhydration: 2.4 vs – 0.6 litres, $p < 0.001$; OH/ECW%: 13.2 vs – 4.5%, $p < 0.001$). Overall, 87.5% (105/120) of the CKD patients were fluid overloaded. There were graded increases in the severity of volume overload across the spectrum of the CKD patients, with dialysis patients characteristically exposed to severe volume overload compared to stage 3 CKD patients who had modest fluid retention (Figure 3.2). Severity of volume overload was not statistically significantly different between PD and HD patients, but was significant between controls and different CKD groups.

3.5.2 Inflammatory markers

Circulating endotoxaemia was also common among CKD stages III-V subjects (HD, PD, and CKD) compared to the controls. Endotoxin concentration was significantly higher among patients on PD (0.59 ± 0.25 EU/ml), HD (0.54 ± 0.34 EU/ml) and CKD stage 3 (0.50 ± 0.22 EU/ml) compared to controls (0.34 ± 0.14 EU/ml) (Figure 3.3). Endotoxin levels were not significantly different between the CKD (non-dialytic) patients and the controls ($p = 0.096$); but they were significantly different between the dialysis group and the controls ($p = 0.013$). There was significant increment in the levels of inflammatory markers in CKD subjects compared to the controls (CRP: 0.87 ± 0.72 versus 0.45 ± 0.48 mg/dL, $p < 0.001$; IL-6: 2.70 ± 3.76 versus 1.03 ± 1.15 pg/ml, $p < 0.001$).

3.5.3 Echocardiographic parameters

Chronic kidney disease patients had severe cardiac chamber enlargement compared to the controls (left ventricular mass index [LVMI]: 118.8 ± 38.8 vs 84.4 ± 15.9 g/m², $p < 0.001$; left atrial dimension: 4.0 ± 0.7 vs 3.1 ± 0.4 cm, $p < 0.001$). Left ventricular ejection fraction was significantly lower in CKD patients compared to the controls (EF: 58.6 ± 8.8 vs 65.0 ± 8.0 %, $p < 0.001$). Patients with kidney failure had impaired left ventricular relaxation, with significantly lower E/A ratio and higher left ventricular filling pressure (E/e'); when compared to the control group; $p < 0.001$. However, there was no significant difference in the deceleration time between CKD patients and those with normal kidney function. There was a weak positive correlation between MABP and LVMI ($r = 0.275$, $p = 0.002$).

3.5.4 Subclinical atherosclerosis

Carotid intima media thickness was significant higher among CKD patients compared to the controls (0.58 ± 0.12 versus 0.45 ± 0.05 mm; $p < 0.001$). Further analysis showed that CIMT was 0.63 ± 0.12 ; 0.56 ± 0.09 and 0.56 ± 0.12 in PD, HD and CKD stage 3 patients respectively (Figure 3.4). Overall, 8/120 (6.7%) CKD patients had carotid plaques. In subgroup analysis, plaques were found in 2/40 (5%), 5/40 (12.5%), and 1/40 (2.5%) of PD, HD and CKD patients respectively. However, carotid plaque was not found among the controls. Chronic kidney disease patients who were smokers had significantly higher CIMT than non-smokers (0.62 ± 0.16 versus 0.53 ± 0.09 mm, $p = 0.03$), however, there was no significant difference in the occurrence of carotid plaque between smokers and non-smokers ($p = 0.507$), as well as CIMT measurements between black and non-black CKD patients (0.58 ± 0.11 versus 0.58 ± 0.13 mm, $p = 0.811$). There was no association between atherosclerosis and SBP ($r = 0.131$, $p = 0.153$), DBP ($r = 0.012$, $p = 0.899$), MABP ($r = 0.029$,

p=0.755), calcium ($r=0.016$, $p=0.920$), phosphate ($r=0.108$, $p=0.507$), calcium-phosphate product ($r=0.117$, $p=0.473$) or parathormone ($r=0.078$, $p=0.631$).

Table 3.3: Fluid status, inflammatory markers, nutrition status and echocardiographic parameters in the study population

Parameter	All patients (N=120)	PD (N=40)	HD (N=40)	Stage 3 CKD (N=40)	Control (N=40)	*P value
Fluid status						
Overhydration (Litres)	2.4 ± 2.3	3.3 ± 3.4	2.9 ± 1.9	1.3 ± 1.2	-0.6 ± 0.94	<0.001
OH/ECW (%)	13.2 ± 10.5	16.1 ± 13.9	15.8 ± 9.5	2.1 ± 9.8	-3.3 ± 6.8	<0.001
Inflammatory markers						
Endotoxin (EU/ml)	0.54 ± 0.28	0.59 ± 0.25	0.54 ± 0.34	0.50 ± 0.22	0.34 ± 0.14	<0.001
C-reactive protein (mg/dL)	0.87 ± 0.72	1.14 ± 0.77	1.05 ± 0.71	0.86 ± 0.71	0.45 ± 0.48	<0.001
Interleukin-6 (pg/ml)	2.7 ± 3.8	3.9 ± 4.9	2.9 ± 3.7	1.3 ± 1.6	1.0 ± 1.2	<0.001
Nutrition status						
Lean Tissue Index(kg/m ²)	14.2 ± 3.2	13.9 ± 2.9	13.8 ± 3.1	14.8 ± 3.6	16.1 ± 3.0	0.002
Fat Tissue Index(kg/m ²)	12.8 ± 7.7	11.2 ± 6.3	10.8 ± 6.1	16.5 ± 9.0	10.9 ± 6.7	0.005
Lean Tissue Mass	37.6 ± 11.1	36.9 ± 10.1	36.3 ± 11.2	39.4 ± 11.9	44.1 ± 11.9	0.029
Fat Tissue Mass	24.4 ± 13.6	21.4 ± 11.3	20.9 ± 11.5	30.2 ± 15.6	21.2 ± 12.8	0.007
Adipose Tissue Mass(kg)	42.0 ± 21.0	29.1 ± 15.4	28.7 ± 14.7	28.9 ± 17.4	33.3 ± 18.2	0.007
Body cell Mass(kg)	21.2 ± 7.2	20.9 ± 6.7	20.5 ± 6.8	22.1 ± 8.0	25.6 ± 7.9	0.027
IVCD measurements						
IVCD (mm/m ²)	7.3 ± 2.7	7.9 ± 2.7	8.2 ± 2.8	5.6 ± 1.5	5.1 ± 1.6	<0.001
Collapsibility index	50.4 ± 23.1	38.0 ± 23.7	50.6 ± 19.5	63.9 ± 18.4	66.6 ± 22.3	<0.001
Echocardiographic parameters						
LVMI (g/m ²)	118.8±38.8	126.9 ±37.6	120.4±36.8	110.4±41.9	84.4±15.9	<0.001
Left atrial dimension (cm)	4.0 ± 0.7	3.9 ± 0.7	4.2 ± 0.7	3.7 ± 0.6	3.1 ± 0.4	<0.001
Ejection fraction (%)	58.6 ± 8.8	58.6±7.8	58.4±12.2	57.9±5.5	65.0±8.0	<0.001
E/A ratio	1.2 ± 0.5	1.0±0.4	1.2±0.5	1.2±0.6	1.36±0.4	<0.001
Deceleration time (msec)	224.9±56.3	226.2±53.6	219.2±64.4	277.0±48.8	209.7±33.9	NS
E/e'	12.5 ± 15.9	11.6±5.5	12.9±5.1	12.9±26.1	6.1±1.6	<0.001
CIMT	0.58 ± 0.11	0.63±0.12	0.56±0.09	0.56±0.12	0.47±0.11	<0.001
Carotid plaque	6.7%	5%	12.5%	2.5%	0%	0.067

NS, not significant; CIMT, carotid intima media thickness; LVMI, left ventricular mass index

Continuous data were expressed as mean ± SD and categorical data as percentages

*p-values were calculated by comparing all patients with chronic kidney disease (n=120) to controls (n=40) using Mann Whitney test

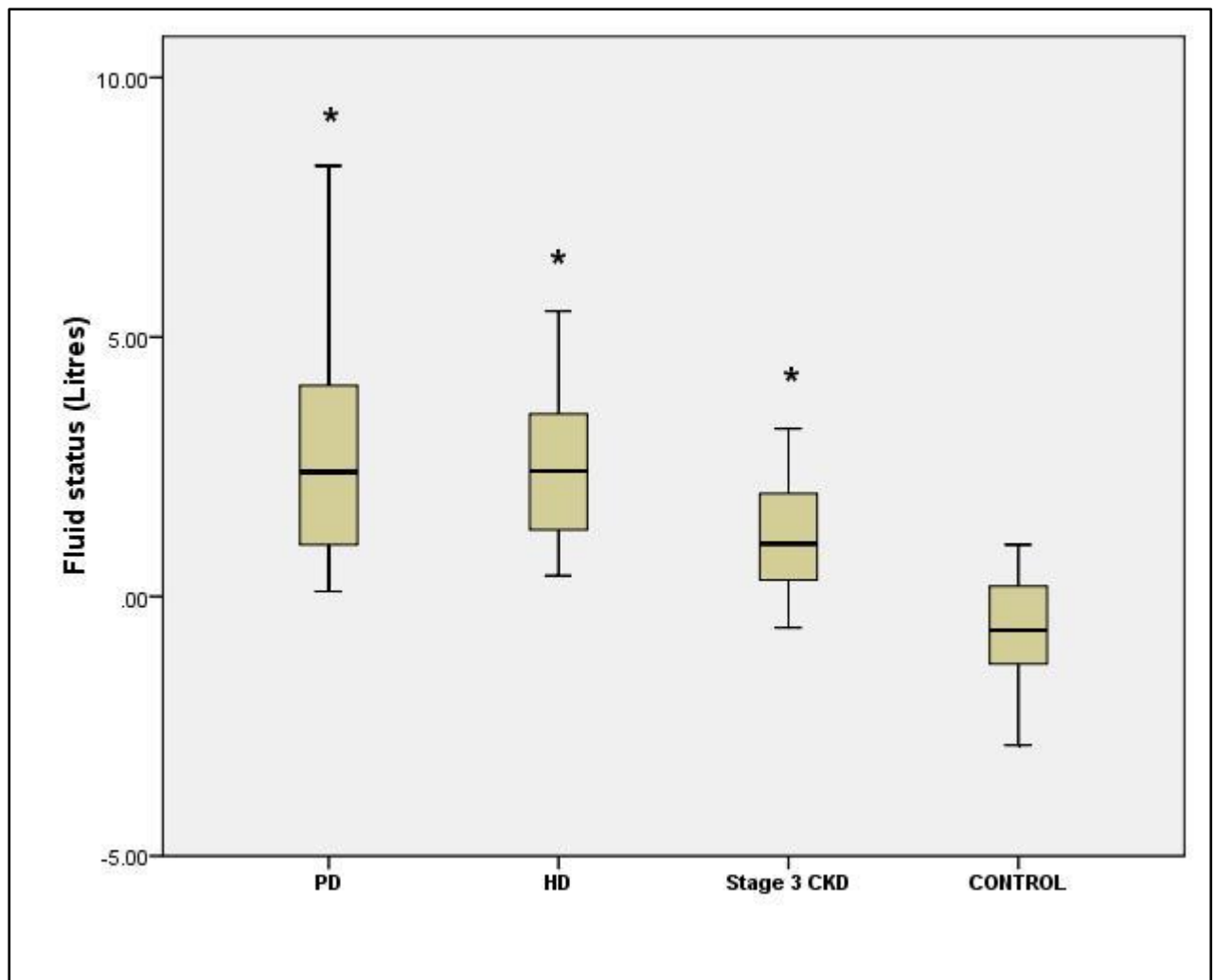


Figure 3.2: Comparison of fluid status between patients on peritoneal dialysis, haemodialysis, chronic kidney disease (stage 3) and the controls. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Fluid status was assessed with body composition monitor, a bio-impedance spectroscopy device (Fresenius Medical Care, Germany). Fluid overload was defined by absolute overhydration (in litres). * $p < 0.001$ compared to control group using Kruskal Wallis test.

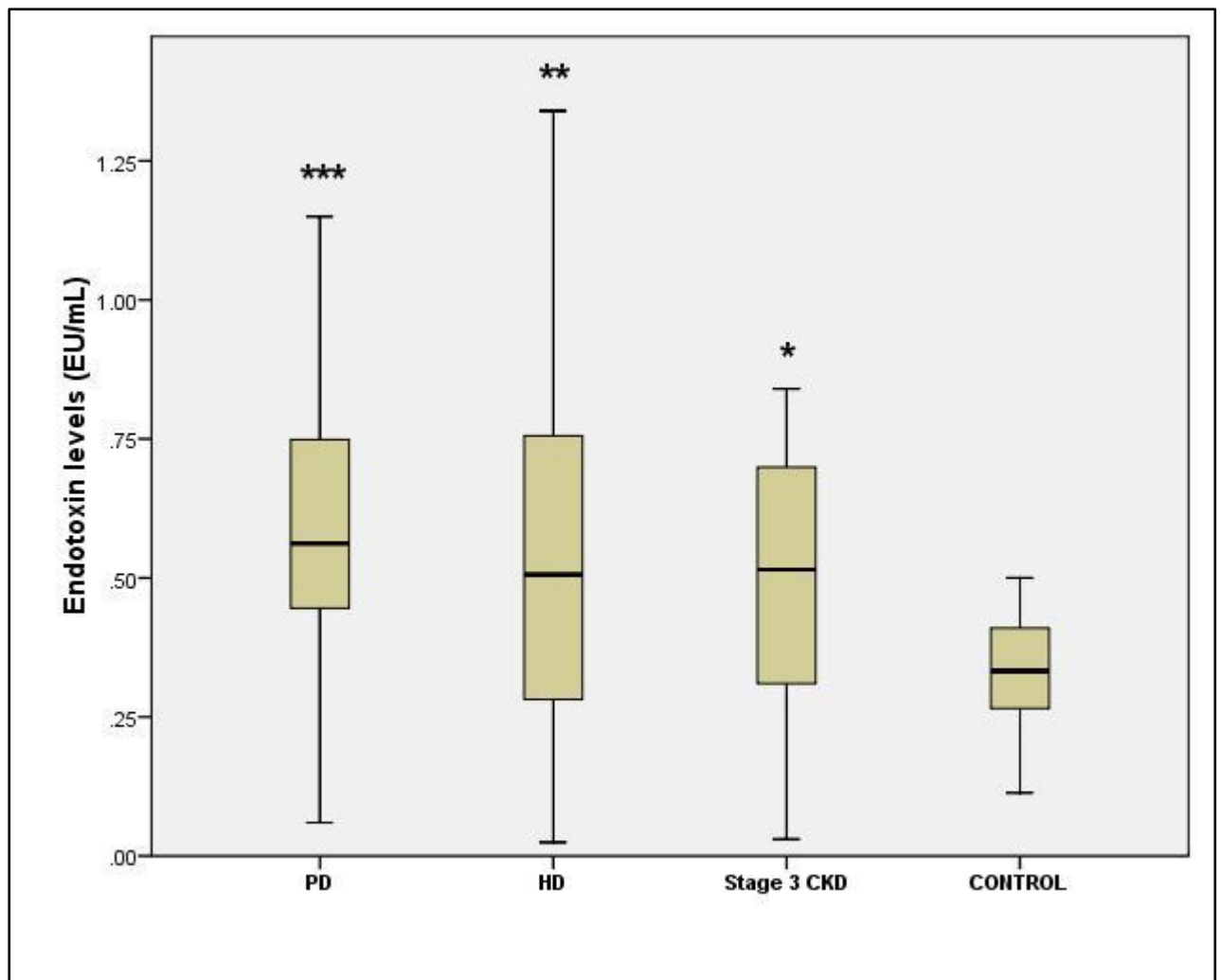


Figure 3.3: Comparison of endotoxin levels between patients on peritoneal dialysis (PD), on haemodialysis (HD) and with chronic kidney disease (CKD) versus controls. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Circulating endotoxin levels were measured using the Limulus amoebocyte lysate kit. *** $p < 0.001$, ** $p = 0.004$, * $p = 0.016$ compared to control group using Kruskal Wallis test.

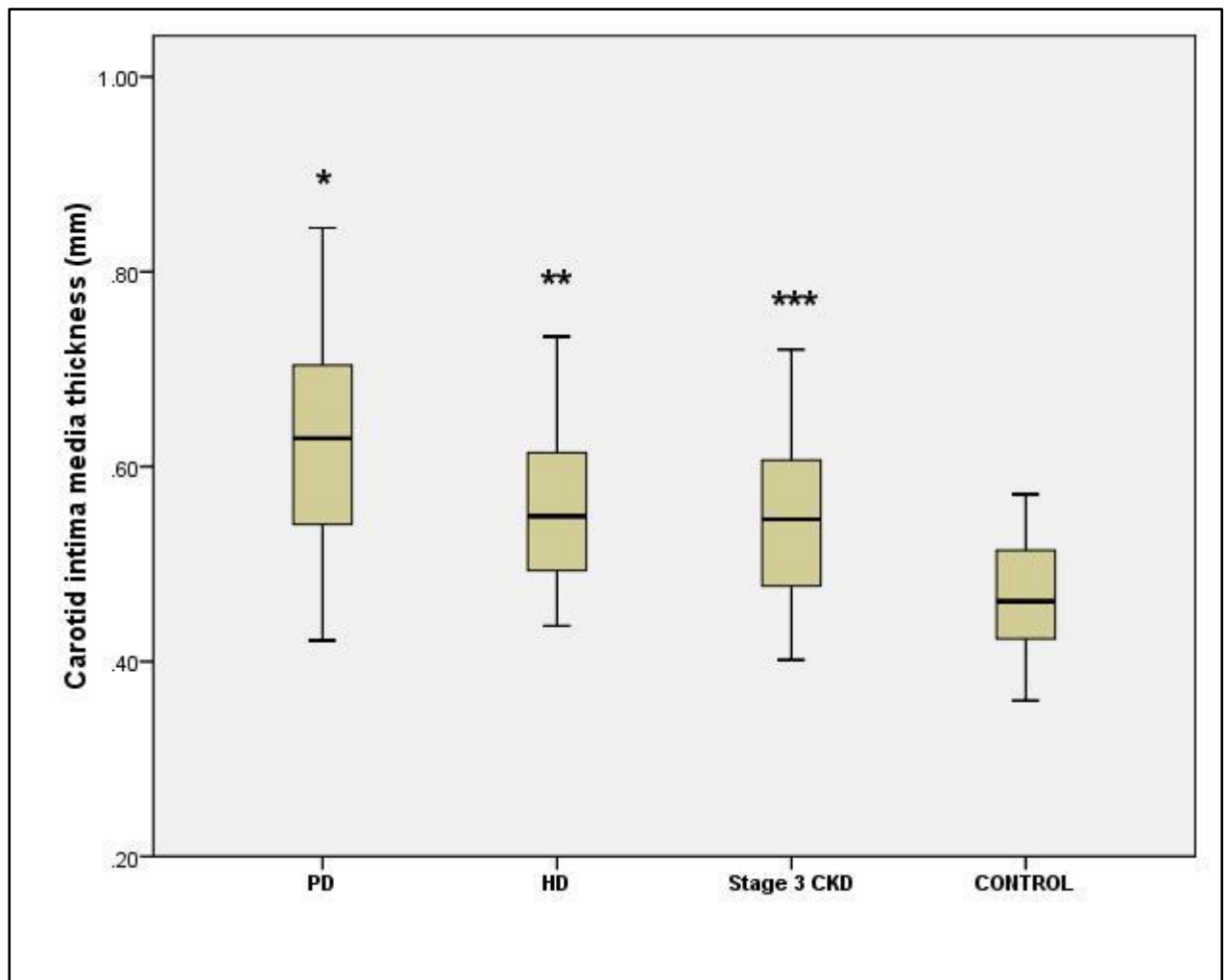


Figure 3.4: Comparison of carotid intima media thickness between patients on peritoneal dialysis, haemodialysis, chronic kidney disease and control. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Carotid intima media thickness was measured using high resolution B-mode ultrasound with aid of L3-11 linear array transducer (Philips corporation USA). Carotid intima media thickness was measured from the far wall of the common carotid artery 1cm proximal to the carotid bulb * $p < 0.001$, ** $p = 0.014$, *** $p = 0.003$ compared to control group using Kruskal Wallis test.

3.6 Prevalence of fluid overload according to various assessment methods

Overhydration (OH) and its percentage in relation to extracellular water (OH/ECW%) and inferior vena cava diameter were higher in CKD patients when compared to the values in the controls, while the collapsibility index was significantly lower in patients compared to the control group (Table 3.3). Overall, fluid overload defined by BIS (absolute overhydration), IVCD, CI and oedema (by physical examination) was present in 96% (115/120), 52% (62/120), 29% (35/120) and 62% (74/120) of all CKD patients respectively. Ninety-eight percent (54/58), 95% (81/85) and 89% (41/46) of the CKD patients initially identified as normovolumic by IVCD, CI and physical examination respectively were diagnosed as volume overloaded by the BIS machine. Specifically, all patients on HD were overloaded while 90% (36/40) and 72.5% (29/40) of PD and stage 3 CKD patients respectively were diagnosed as fluid overloaded by BCM.

3.7 Receiver operating characteristic (ROC) curve analysis of fluid status assessment

As shown in figures 3.5 – 3.7, ROC curve analysis was performed to calculate the sensitivity and specificity of IVCD (cut-off value of 11.5mm/m²), CI (cut-off value of <40%) and oedema (physical examination) as a diagnostic tool to detect volume overload (defined by OH/ECW%≥15%) in the studied patients. The area under the concentration curves (AUC) for IVCD, CI and oedema were 0.663 (sensitivity 0.581, specificity 0.624, p=0.002), 0.662 (sensitivity 0.372, specificity 0.821, p =0.002) and 0.740 (sensitivity 0.814, specificity 0.667, p<0.001) respectively.

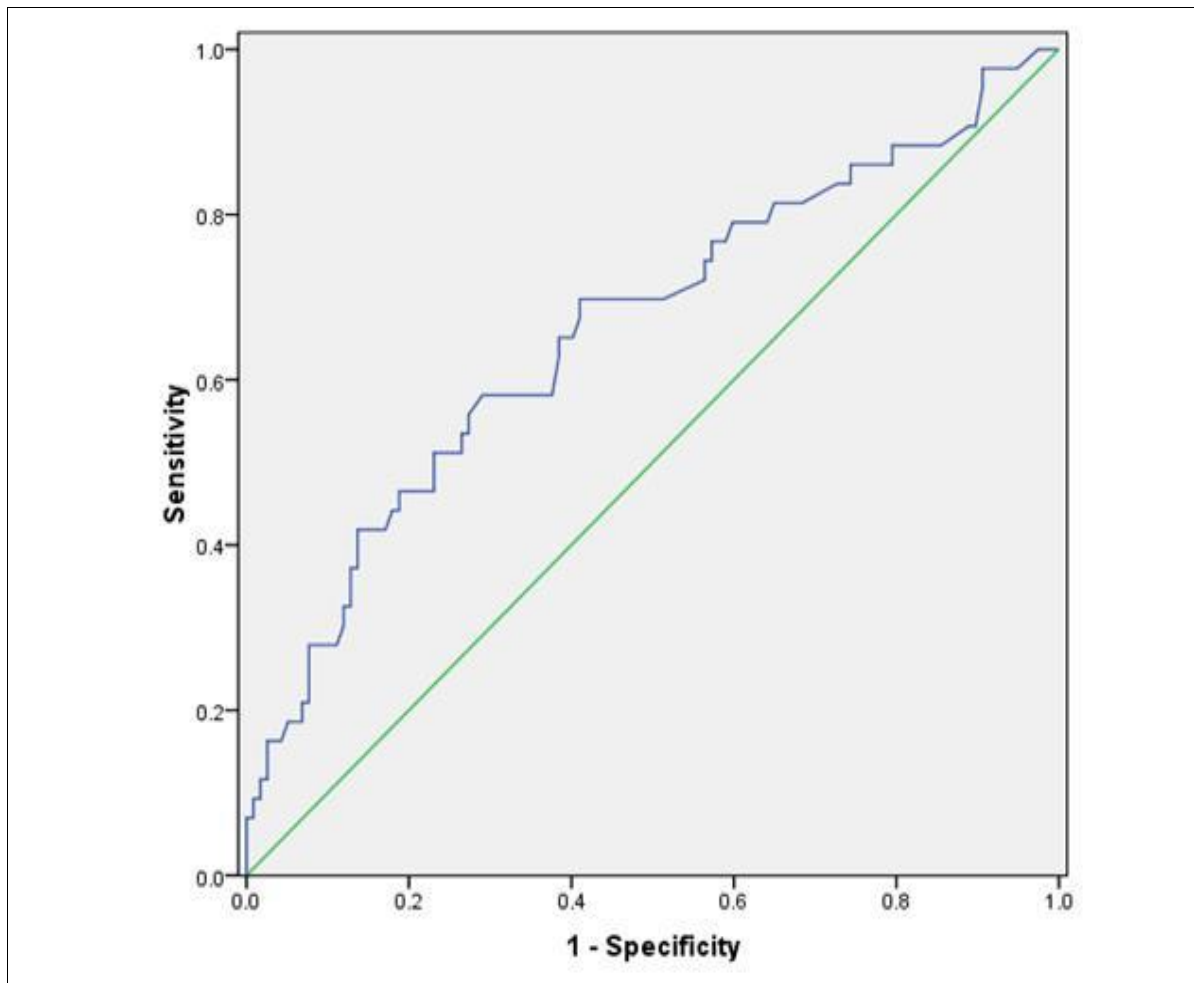


Figure 3.5: Receiver operating characteristics (ROC) for inferior vena cava diameter cut-off value of 11.5mm/m² to predict volume overload using body composition monitor measurements as a positive test (AUC=0.663, confidence interval:0.563–0.763, sensitivity 0.581, specificity 0.624, $p=0.002$). The IVCD measurements is less sensitive when compared to body composition monitor for detection of volume overload in the CKD patients.

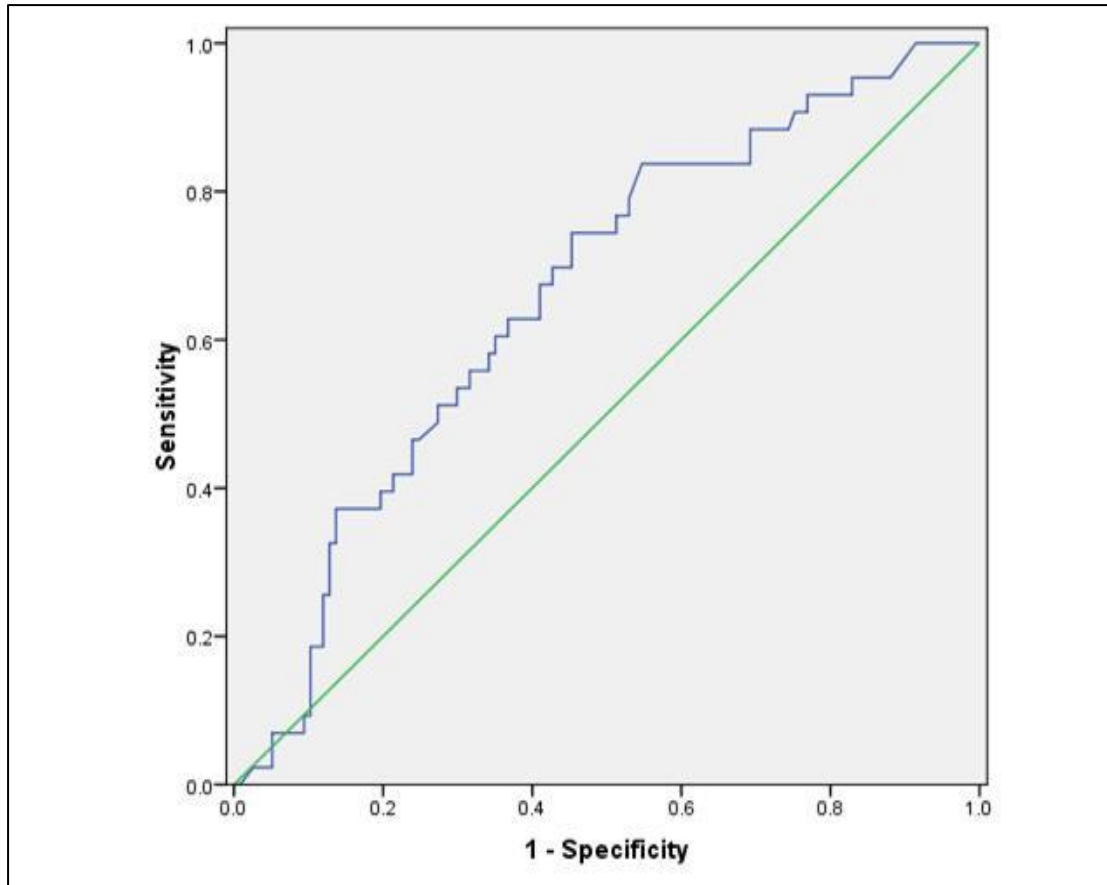


Figure 3.6: Receiver operating characteristics (ROC) for collapsibility index cut-off value of 40% to predict volume overload using Body composition monitor measurements as a positive test (AUC=0.662, confidence interval:0.570–0.754, sensitivity 0.372, specificity 0.821, $p=0.002$). The collapsibility index is less sensitive when compared to body composition monitor for detection of volume overload in the CKD patients.

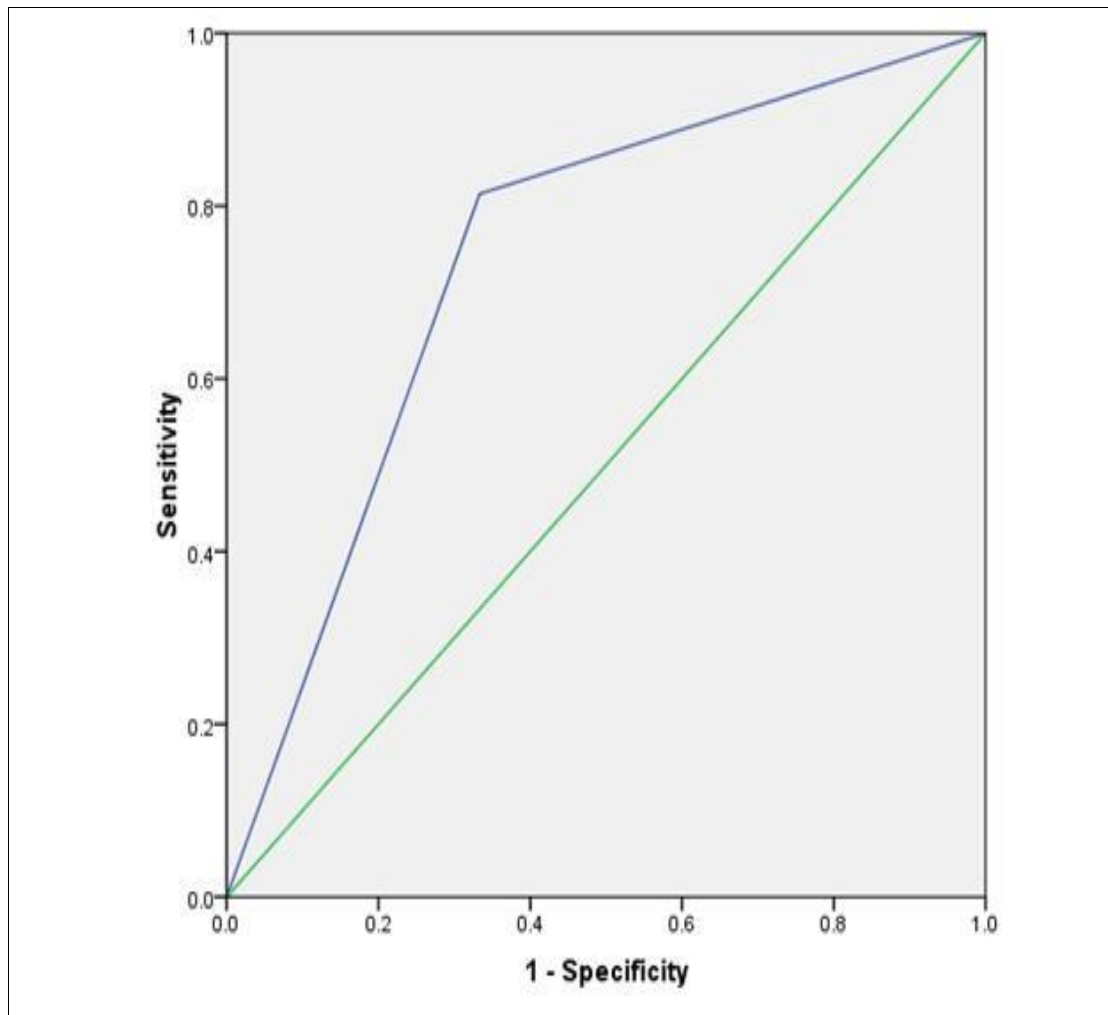


Figure 3.7: Receiver operating characteristic (ROC) analysis of presence of oedema on examination for volume overload using body composition monitor measurements as a positive test (AUC=0.740, sensitivity 0.814, specificity 0.667, $p<0.0001$). Detection of oedema by physical examination is less sensitive when compared to body composition monitor for detection of volume overload in the CKD patients.

3.8 Characteristics of patients with fluid overload (defined by OH/ECW% $\geq 15\%$)

As shown in Table 3.4, measured variables were compared between CKD patients with volume overload (OH/ECW% $\geq 15\%$) and those without volume overload (OH/ECW% $\leq 15\%$). Compared with patients with normal hydration status, patients with volume overload were anaemic (haemoglobin: 10.6 vs 12.5 g/dL, $p < 0.001$), had severe inflammation (CRP: 1.24 ± 0.70 mg/dL vs 0.89 ± 0.72 mg/dL, $p = 0.011$; IL-6: 3.6 vs 2.1 pg/ml, $p = 0.023$), undernourished (LTI: 12.4 vs 15.2 kg/m², $p < 0.001$; FTI: 10.2 vs 14.3 kg/m², $p = 0.002$; BCM: 17.7 vs 23 kg; $p < 0.001$), had lower serum albumin (36.2 vs 39.8 g/L) but had higher cardiovascular morbidity (LVMI: 132 vs 110 g/m², $p = 0.002$; CIMT: 0.61 vs 0.56mm, left atrial enlargement (LAE): 4.2 vs 3.7 cm, $p < 0.001$). Overall, 82/120 (68.3%) of CKD patients had left ventricular hypertrophy (LVH) with concentric LVH being the commonest pattern in 47.5% (Table 3.5).

Table 3.4: Comparison between patients with fluid overload and normal fluid status

Parameter	With Volume Overload (N=43)	Without Volume Overload (N=77)	*P value
Demographic			
Age (Years)	40.4 ± 10.6	41.5 ± 10.2	NS
BMI (kg/m ²)	28.8 ± 6.7	25.8 ± 5.1	0.014
Laboratory			
Serum creatinine (mmol/L)	889.7 ± 460	333.4 ± 396.7	<0.001
Mean BP (mmHg)	146.8 ± 22.0	141.8 ± 22.7	NS
Haemoglobin (g/dL)	10.6 ± 1.86	12.5 ± 2.3	<0.001
Serum albumin (g/L)	36.2 ± 4.2	39.8 ± 4.1	<0.001
CaPO ₄ (mmol ² /L ²)	3.5 ± 1.4	2.7 ± 1.2	0.002
Inflammatory markers			
Endotoxin (EU/m/)	0.59 ± 0.27	0.32 ± 0.15	<0.001
C-reactive protein (mg/dL)	1.24 ± 0.70	0.89 ± 0.72	0.011
IL-6 (pg/ml)	3.6 ± 5.2	2.1 ± 2.5	0.023
Nutrition status			
Lean Tissue Index(kg/m ²)	12.4 ± 2.2	15.2 ± 3.3	<0.001
Fat Tissue Index(kg/m ²)	10.2 ± 6.6	14.3 ± 7.9	<0.002
Lean Tissue Mass (kg)	32.0 ± 8.5	40.7 ± 11.2	<0.001
Fat Tissue Mass (kg)	19.9 ± 11.6	27.0 ± 14.0	0.004
Adipose Tissue Mass(kg)	27.5 ± 15.4	36.5 ± 18.9	0.006
Body cell Mass(kg)	17.7 ± 5.5	23.0 ± 7.3	<0.001
Echocardiographic parameters			
LVMI (g/m ²)	132.8 ± 39.0	110.9 ± 36.7	0.002
Left atrial dimension (cm)	4.2 ± 0.8	3.7 ± 0.6	<0.001
Ejection fraction (%)	56.6 ± 9.9	59.7 ± 7.9	NS
E/A ratio	1.2 ± 0.5	1.1 ± 0.5	NS
Deceleration time (msec)	217.3 ± 63.4	229.3 ± 51.8	NS
E/e'	12.0 ± 5.6	10.5 ± 16.1	0.001
CIMT (mm)	0.61 ± 0.14	0.56 ± 0.09	0.028

* NS, not significant; CIMT, carotid intima media thickness; LVMI, left ventricular mass index. Volume overload defined by OH/ECW% ≥15%; continuous data were expressed as mean ± SD. Markers of nutritional status were calculated and generated automatically by the body composition monitor with the aid of an in-built software. *p-values were calculated by comparing all patients with volume (n=43) to those without volume overload (n=77) using Mann Whitney test

Table 3.5: Pattern of left ventricular hypertrophy in chronic kidney disease patients (n=120)

Pattern	Number of patients	Percentage of patients
Concentric LVH	57	47.5
Eccentric LVH	26	21.7
Concentric remodeling	25	20.8
Normal	12	10

LVH, left ventricular hypertrophy. Four patterns of left ventricular hypertrophy were identified including concentric, eccentric, concentric remodeling and normal pattern. Concentric left ventricular hypertrophy was the commonest pattern among studied CKD patients.

3.9 Nutritional status of the CKD patients

CKD patients were generally malnourished, with lean tissue index (14.2 ± 3.2 vs 16.1 ± 3.0 kg/m², $p=0.002$) and body cell mass (21.2 ± 7.2 vs 25.6 ± 7.9 kg; $p=0.027$) significantly lower than in the controls while fat tissue index (12.8 ± 7.7 vs 10.9 ± 6.7 kg/m², $p=0.005$) and adipose tissue mass (42.0 ± 21.0 vs 33.3 ± 18.2 kg, $p=0.007$) were significantly higher in the controls. As defined by low LTI, 63.3% (73/120) of the CKD patients were malnourished, 65% (78/120) of the patients were undernourished as evidenced by increased FTI, while 50.8% (61/120) of the patients had malnutrition diagnosed by low % BCM. There was a strong correlation between LTI and % BCM ($r=0.785$, $p<0.001$), LTI had a weak correlation with FTI ($r=-0.252$, $p=0.006$) while FTI also weakly correlated with % BCM ($r=-0.311$, $p=0.001$). Malnutrition was diagnosed by low LTI in 55% (22/40), 60% (24/40) and 37.5% (15/40) of the HD, PD and stage 3 CKD patients respectively. Further analysis showed that 81.3% (35/43) of the patients with volume overload had malnutrition, defined by low LTI less than 10th percentile of a reference population. Fat tissue index also showed a strong association with BMI ($r=-0.890$, $p<0.001$).

3.10 Associations between fluid status and measured variables in CKD patients

Table 3.6 shows the correlations between some of the studied parameters in patients with CKD. Markers of inflammation, nutritional status, CIMT, LVMI and LAD were weakly correlated with volume parameters. OH/ECW% showed slight positive correlation with IL-6 ($r=0.313$; $p<0.001$) and LVMI ($r=0.303$; $p<0.001$) (Figures 3.8 & 3.9); and slight inverse correlation with serum albumin ($r=-0.289$, $p<0.001$), lean tissue index ($r=-0.326$, $p<0.001$) and fat tissue index ($r=-0.297$, $p<0.001$). Carotid intima media thickness, a surrogate marker of atherosclerosis correlated with overhydration ($r=0.442$, $p<0.001$) and OH/ECW% ($r=0.421$, $p<0.001$) (Figures 3.10 & 3.11). Carotid intima media thickness was also weakly associated with CRP ($r=0.418$, $p<0.001$) and with serum albumin ($r=-0.206$, $p=0.024$). Left atrial size positively correlated with overhydration ($r=0.454$, $p<0.001$), OH/ECW% ($r=0.489$, $p<0.001$) and IVCD ($r=0.345$, $p<0.001$). Renal dysfunction correlated with all four markers of volume overload (overhydration: $r=0.551$, $p<0.001$; OH/ECW%: $r=0.554$, $p<0.001$; IVCD: $r=0.358$, $p<0.001$; and CI: $r=-0.399$, $p<0.001$). Overhydration was weakly associated with SBP ($r=0.235$, $p=0.010$). However, no correlation was seen between markers of volume overload and age.

Table 3.6: Correlation between fluid parameters, renal function, inflammation and atherosclerosis markers, nutrition status and cardiac size

Parameter	Overhydration (r value)	OH/ECW% (r value)	IVCD (r value)	CI (r value)
Renal function				
Serum creatinine	0.406**	0.396**	0.221*	−0.353**
Markers of inflammation				
C-reactive protein	0.284**	0.277**	0.187*	− 0.181*
Interleukin-6	0.349**	0.313**	0.248**	− 0.284**
Nutrition status				
Albumin	− 0.246**	− 0.289**	− 0.069	0.152
Lean Tissue Index	− 0.254**	− 0.326**	− 0.019	0.024
Fat Tissue Mass	− 0.252**	− 0.297**	− 0.184*	0.140
Adipose Tissue Mass	− 0.219*	− 0.275**	− 0.131	0.139
Body Cell Mass	− 0.230*	− 0.317**	− 0.041	0.027
Cardiac size				
Left Atrial Size	0.454**	0.489**	0.345**	− 0.101
LVMI	0.262**	0.303**	0.190*	− 0.142
Marker of atherosclerosis				
CIMT	0.442**	0.421**	0.112	− 0.138

CI, collapsibility index; BMI, body mass index; LVMI, left ventricular mass index; CIMT, carotid intima media thickness. ** Correlation is significant at the 0.01 (2-tailed), * Correlation is significant at the 0.05 (2-tailed). Correlation between volume parameters and measured variables was assessed by Pearson correlation coefficient

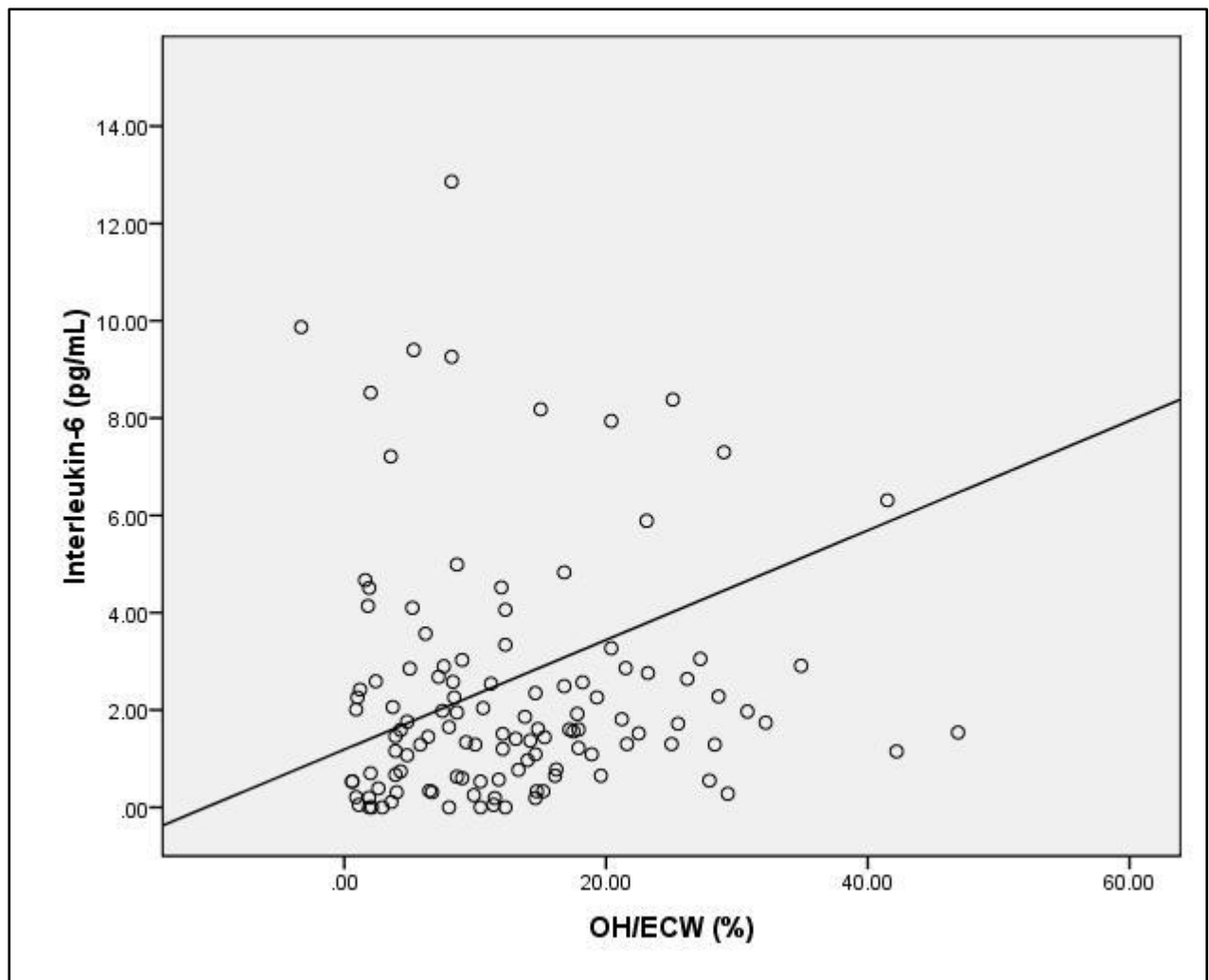


Figure 3.8: Relationship between OH/ECW% (fluid status) and interleukin-6 in CKD patients ($r=0.313$, $p<0.001$). The figure demonstrates a direct relationship between interleukin-6 and OH/ECW%. Association between interleukin-6 concentrations and OH/ECW% was assessed using Pearson correlation coefficient. Interleukin-6 was measured by the Luminex Performance assay kit while OH/ECW% was measured with body composition monitor.

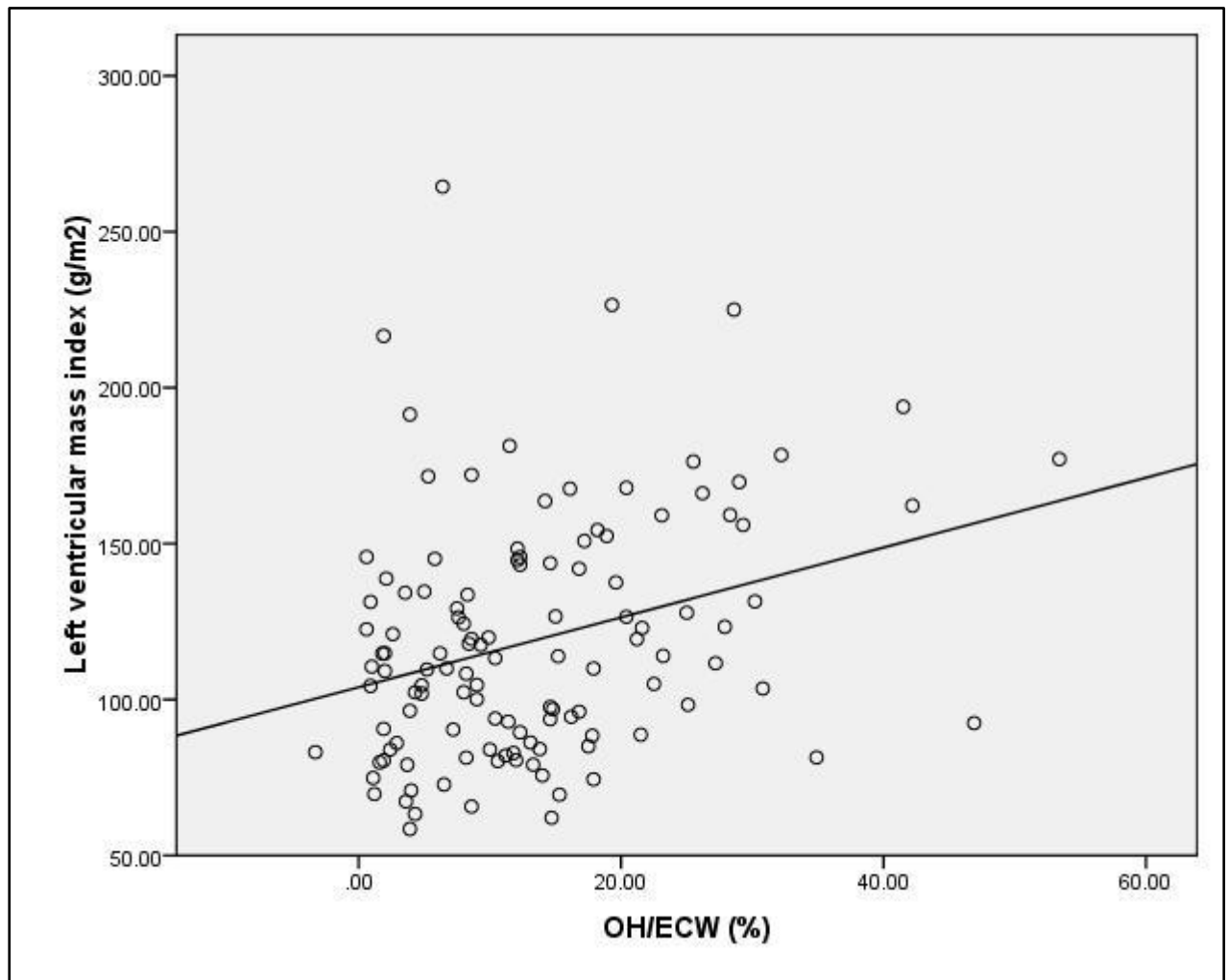


Figure 3.9: Relationship between OH/ECW% (fluid status) and left ventricular mass index in CKD patients ($r=0.303$, $p<0.001$). The figure demonstrates a direct relationship between OH/ECW% and left ventricular mass index. Association between OH/ECW% and left ventricular mass index was assessed using Pearson correlation coefficient. OH/ECW% was measured by body composition monitor while left ventricular mass index was computed by dividing left ventricular mass (generated automatically from echocardiography) by body surface area.

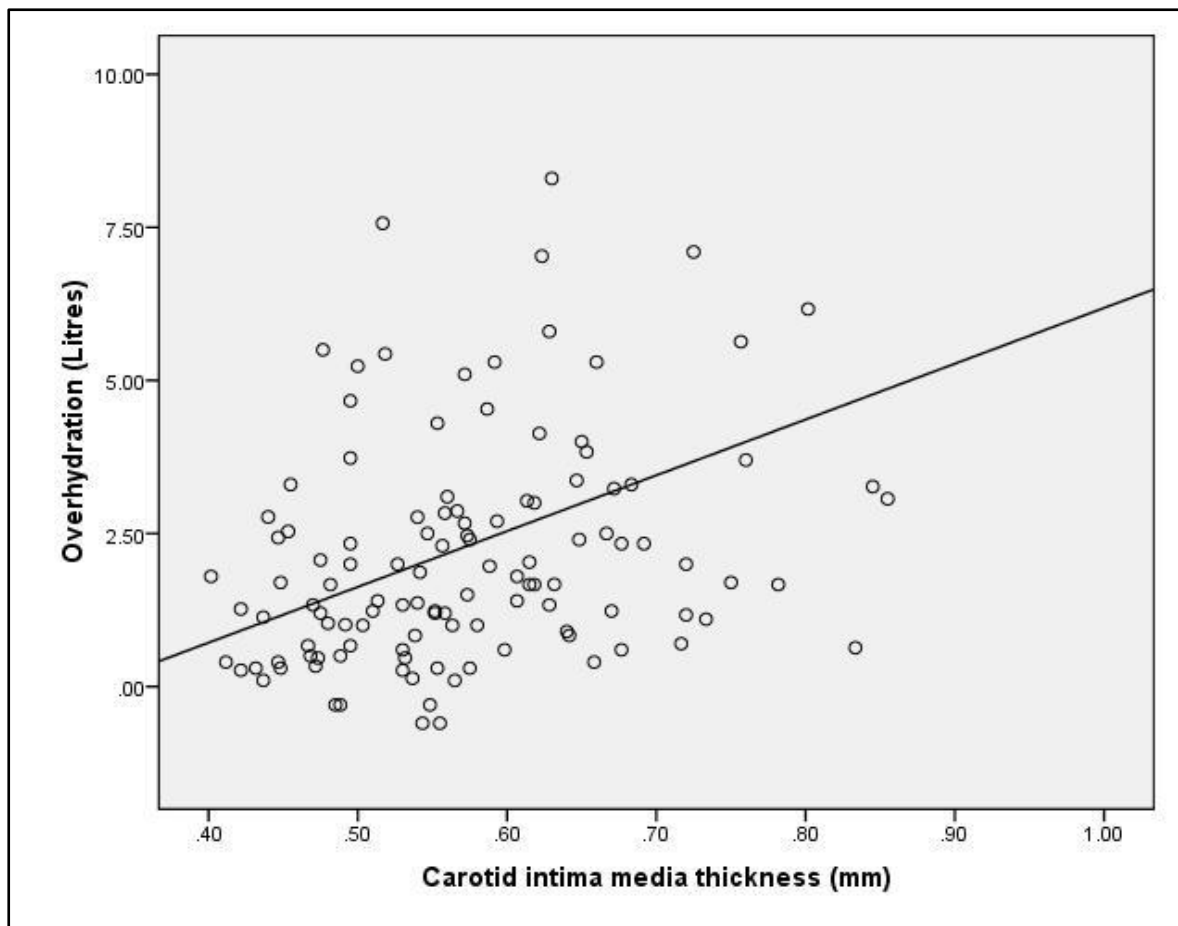


Figure 3.10: Relationship between overhydration and carotid intima media thickness in CKD patients ($r=0.442$, $p<0.001$). The figure demonstrates a direct relationship between overhydration and carotid intima media thickness. Association between overhydration and carotid intima media thickness was assessed using Pearson correlation coefficient. Overhydration was measured by body composition monitor while carotid intima media thickness was assessed with B-mode ultrasound.

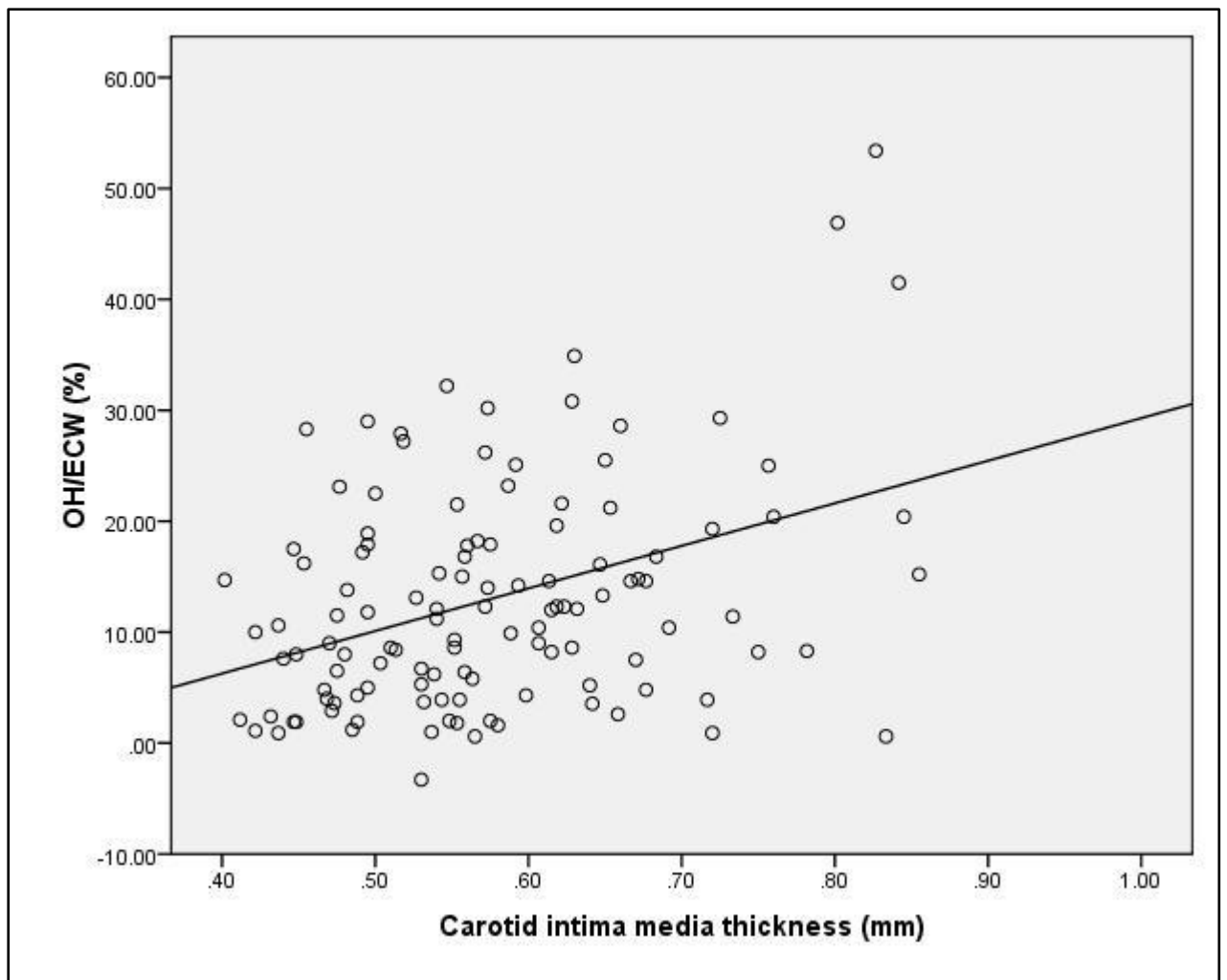


Figure 3.11: Relationship between OH/ECW% (fluid status) and carotid intima media thickness in CKD patients ($r=0.442$, $p<0.001$). The figure demonstrates a direct relationship between OH/ECW% and carotid intima media thickness. Association between OH/ECW% and carotid intima media thickness was assessed using Pearson correlation coefficient. OH/ECW% was measured by body composition monitor while carotid intima media thickness was assessed with B-mode ultrasound.

Multiple linear regression analysis was performed to further determine which of the variables independently predicted volume overload in CKD patients. Only serum creatinine ($\beta=0.152$, $p<0.001$), LTI ($\beta=-0.328$, $p<0.001$), FTI ($\beta=-0.261$, $p<0.001$), LAD ($\beta=0.336$, $p<0.001$) and CIMT ($\beta=0.238$, $p=0.002$) were identified as independent determinants of volume overload (Table 3.7), with overall model $r^2 = 0.55$. When looking at the effect of volume overload on the markers of inflammation, OH/ECW% significantly affect CRP and IL-6 levels ($p<0.002$ and $p<0.001$ respectively) in CKD patients.

Table 3.7: Multiple linear regression analysis of determinant of volume overload

Variables	Unstandardized Coefficients (B)	Standardized Coefficient Beta	P value	Confidence Interval
Serum creatinine	0.003	0.152	0.044*	0.000-0.007
Albumin	- 0.117	- 0.055	0.449	- 0.421-0.188
IL-6	0.312	0.113	0.150	- 0.114-0.739
CRP	0.205	0.014	0.861	- 2.114-2.524
LTI	- 1.072	- 0.328	< 0.001**	- 1.524- - 0.619
FTI	- 0.357	- 0.261	< 0.001**	- 0.552- - 0.163
LVMI	- 0.021	- 0.077	0.336	- 0.063-0.022
LAD	5.153	0.336	< 0.001**	2.830-7.476
CIMT	21.720	0.238	0.002**	8.248-35.192

Notes: * Significant at P value <0.05, ** Significant at P value <0.01. IL-6, interleukin-6; CRP, C-reactive protein; LTI=lean tissue index; LTI=lean tissue index; FTI, fat tissue index; LVMI, left ventricular mass index; LAD, left atrial dimension; CIMT, carotid intima media thickness. Serum creatinine, lean tissue index, fat tissue index, left atrial enlargement and presence of subclinical atherosclerosis were identified as independent predictors of volume overload in CKD patients.

3.11 Relationship between renal function, volume overload and endotoxaemia

Chronic kidney disease patients were significantly overloaded and exposed to elevated levels of endotoxins compared to the controls (Figures 3.12 and 3.13). Volume overloaded CKD patients were exposed to circulating endotoxaemia compared to those without fluid overload (Figure 3.14). Endotoxin levels correlated with absolute overhydration ($r=0.513$; $p<0.001$) (Figure 3.15) and weakly with OH/WCW% ($r=0.490$; $p<0.001$). In HD cohorts, endotoxin levels weakly correlated with OH/ECW% ($r=0.351$, $p=0.026$) and absolute overhydration ($r=0.396$, $p=0.011$). Nine (22.5%) out of 40 HD patients suffered dialysis-induced hypotension, with significantly increased levels of post-dialysis endotoxin in hypotensive patients compared to normotensive patients (0.83 ± 0.25 vs 0.52 ± 0.29 EU/ml, $p=0.006$) (Figure 3.16). Post-dialysis endotoxin levels were also significantly elevated in patients that had high volume ultrafiltration compared to those with low ultrafiltration volume (Figure 3.17). However, there was no significant difference between pre-dialysis and post-dialysis endotoxin levels (0.53 ± 0.37 EU/ml versus 0.57 ± 0.31 EU/ml; $p=0.933$), and neither of the endotoxin levels correlated with intra-dialysis SBP (pre-dialysis: $r=0.162$, $p=0.311$; post-dialysis: $r=0.086$, $p=0.590$) or DBP (pre-dialysis: $r=0.027$, $p=0.870$; post dialysis: $r=0.238$, $p=139$). Among PD patients, endotoxaemia strongly correlated with OH/ECW% ($r=0.715$, $p<0.001$) as well as absolute overhydration ($r=0.674$, $p<0.001$) and weakly correlated with serum creatinine ($r=0.331$, $p=0.037$); while in stage 3 CKD cohorts, there was also an association between circulating endotoxaemia and OH/ECW% ($r=0.535$, $p<0.001$) and absolute overhydration ($r=0.680$, $p<0.001$), but no correlation between endotoxaemia and serum creatinine. Overall, circulating endotoxaemia did not correlate with serum creatinine ($r=0.158$, $p=0.085$) and was not different between PD, HD and stage 3 CKD patients ($p>0.05$). In PD cohorts, endotoxaemia modestly correlated with haemoglobin levels ($r= - 0.340$, $p=0.032$); however in the sub analysis of HD and non-dialytic CKD patients, no association was found between circulating endotoxaemia and haemoglobin levels. In addition, haemoglobin had a weak but significant

association with IL-6 ($r = -0.301$, $p = 0.001$) and CRP ($r = -0.274$, $p = 0.003$). Multiple regression analysis showed hydration status to be significantly associated with endotoxin levels (β coefficient = 0.058 , $r^2 = 0.28$, $p < 0.001$) while in a sub analysis of HD patients, ultrafiltration volume was the only identified predictor of circulating endotoxaemia (β coefficient = 0.4 , $r^2 = 0.47$, $p < 0.001$).

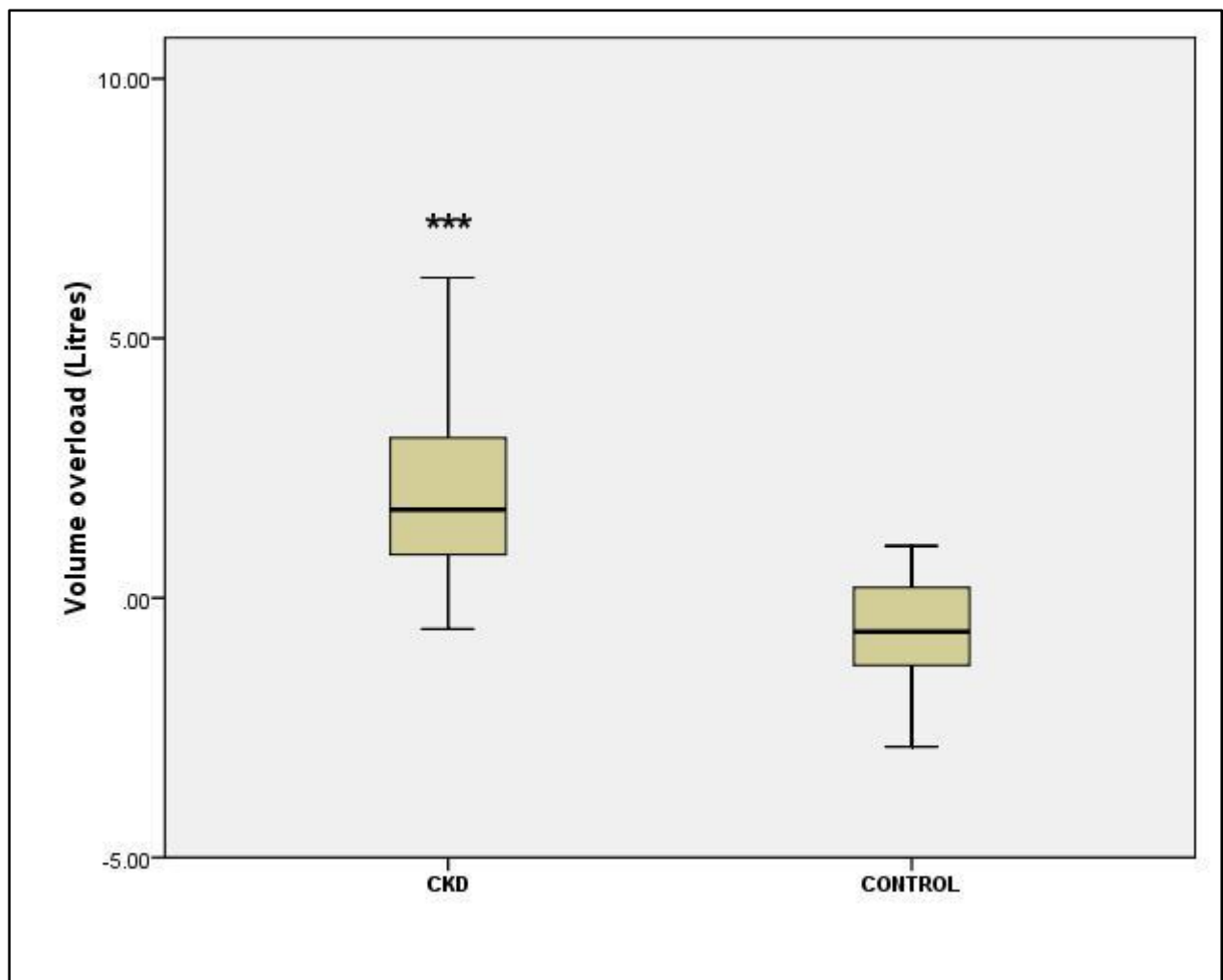


Figure 3.12: Comparison of hydration status between CKD and controls. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Fluid status was assessed with body composition monitor. Fluid status was compared between patients with CKD and those without CKD. Volume overload was severe among CKD patients compared to the controls (2.4 ± 2.3 versus -0.6 ± 0.94 EU/ml).

*** $P < 0.001$ compared to group without CKD.

Notes: CKD = chronic kidney disease

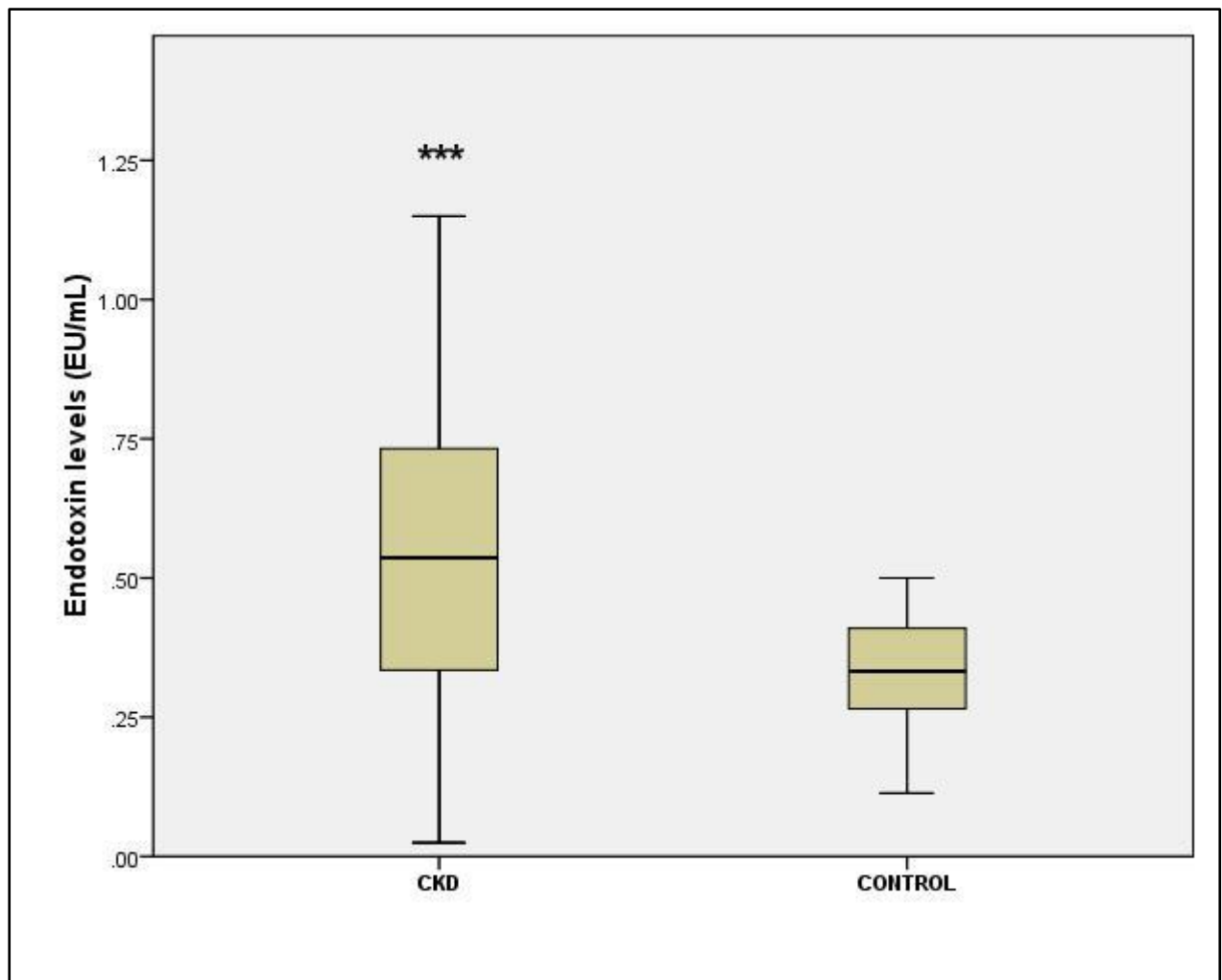


Figure 3.13: Comparison of endotoxin levels between CKD and controls. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Fluid status was assessed with body composition monitor. Fluid status was compared between patients with CKD and those without CKD. Endotoxin levels were significantly different between patients with CKD and the controls (0.54 ± 0.28 versus 0.34 ± 0.14 EU/ml). *** $P < 0.001$ compared to group without CKD. Notes: CKD = chronic kidney disease

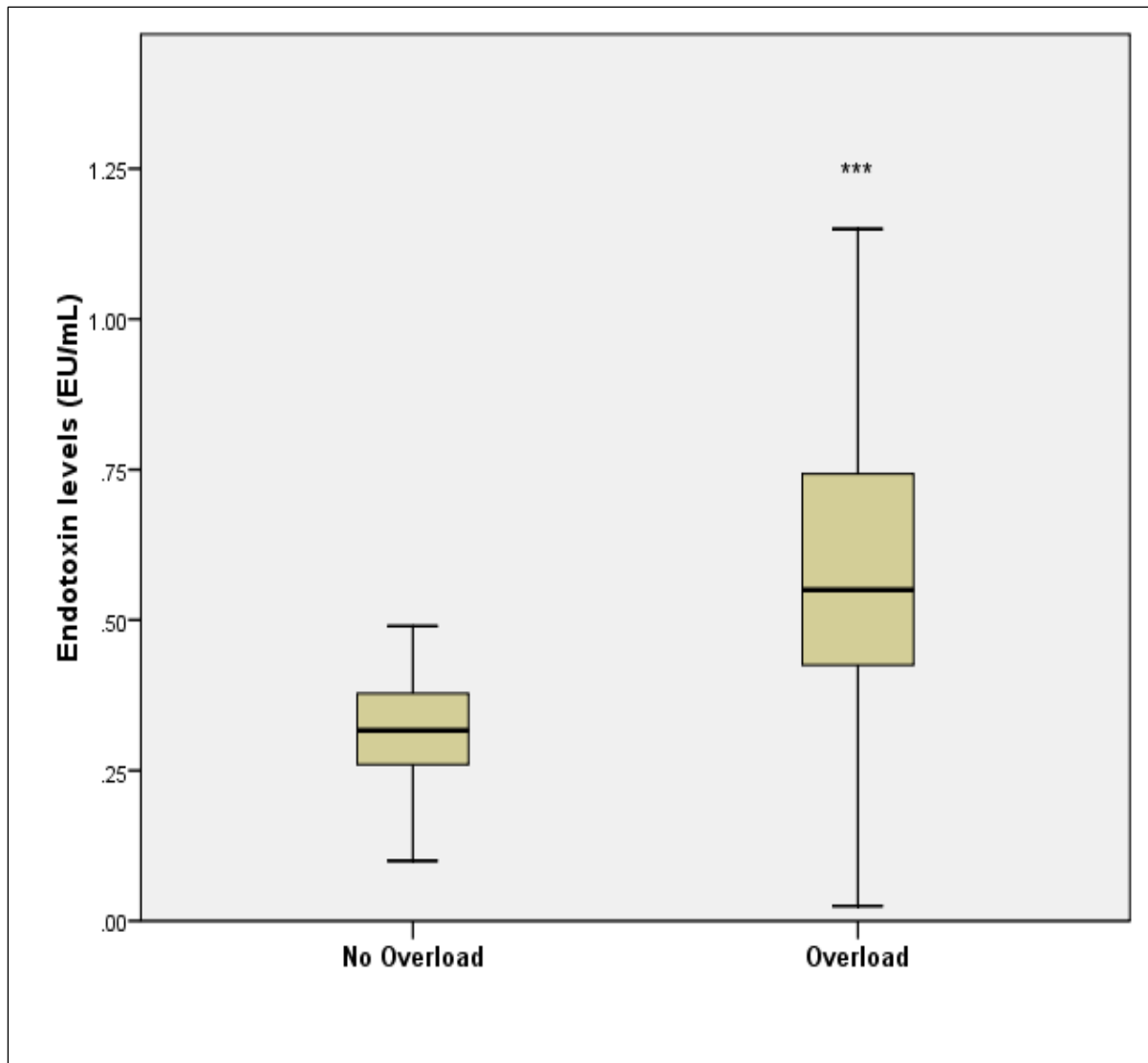


Figure 3.14: Comparison of endotoxin levels between patient without fluid overload and those with fluid overload. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Endotoxin was measured using Limulus amebocyte lysate kit. Volume status was assessed using body composition monitor. Fluid overload was defined as OH/ECW% > 15%. Patients with fluid overload and those without fluid overload were compared. *** P < 0.001 compared to group without volume overload.

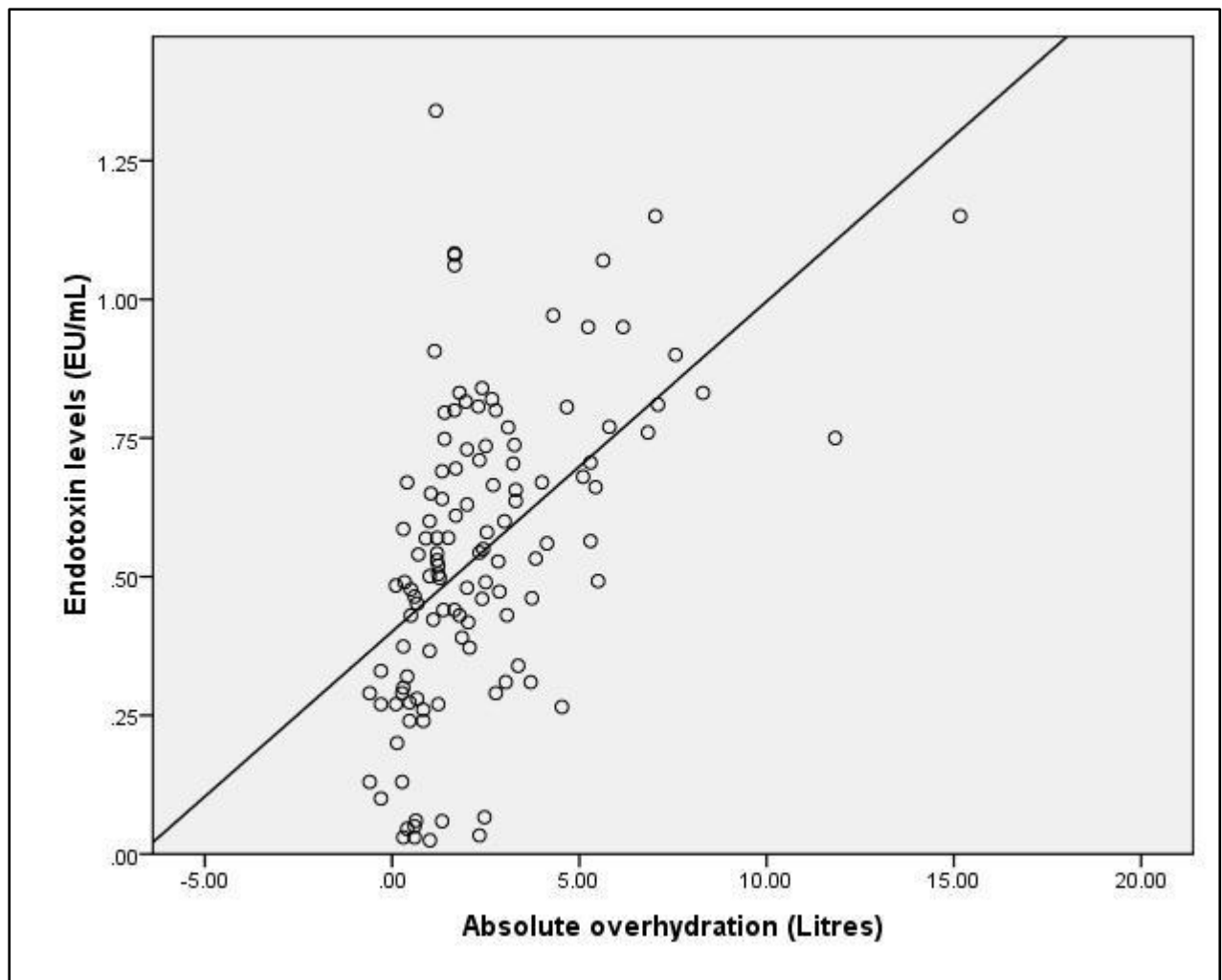


Figure 3.15: Relationship between endotoxin levels and absolute overhydration ($r=0.513$, $p<0.001$). The figure demonstrates a direct relationship between circulating endotoxaemia and hydration status. Association between endotoxin levels and overhydration was assessed using Pearson correlation coefficient. Fluid status was measured with body composition monitor while endotoxin levels were assessed with Limulus Amoebocyte Lysate test kit.

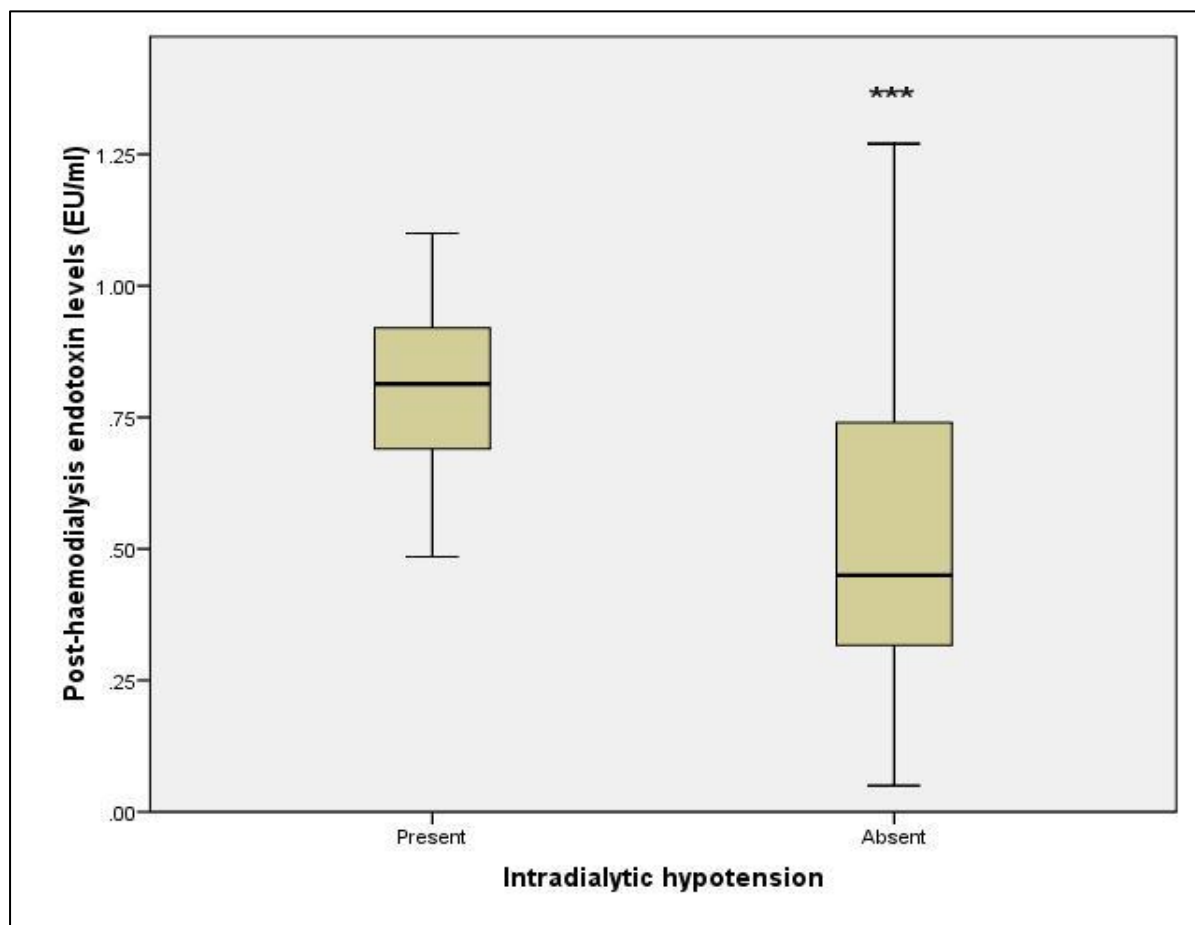


Figure 3.16: Comparison of post-haemodialysis endotoxin levels between patients that had intradialytic hypotension and those without hypotension. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Endotoxin was measured using Limulus amebocyte lysate kit. Patients were diagnosed to have hypotension when systolic blood pressure was lower than 100 mmHg or a fall in systolic blood pressure more than 40 mmHg. Post-dialysis endotoxin levels were compared between patients with intradialytic hypotension and non-hypotensive patients. *** P =0.006 compared to group without intradialytic hypotension.

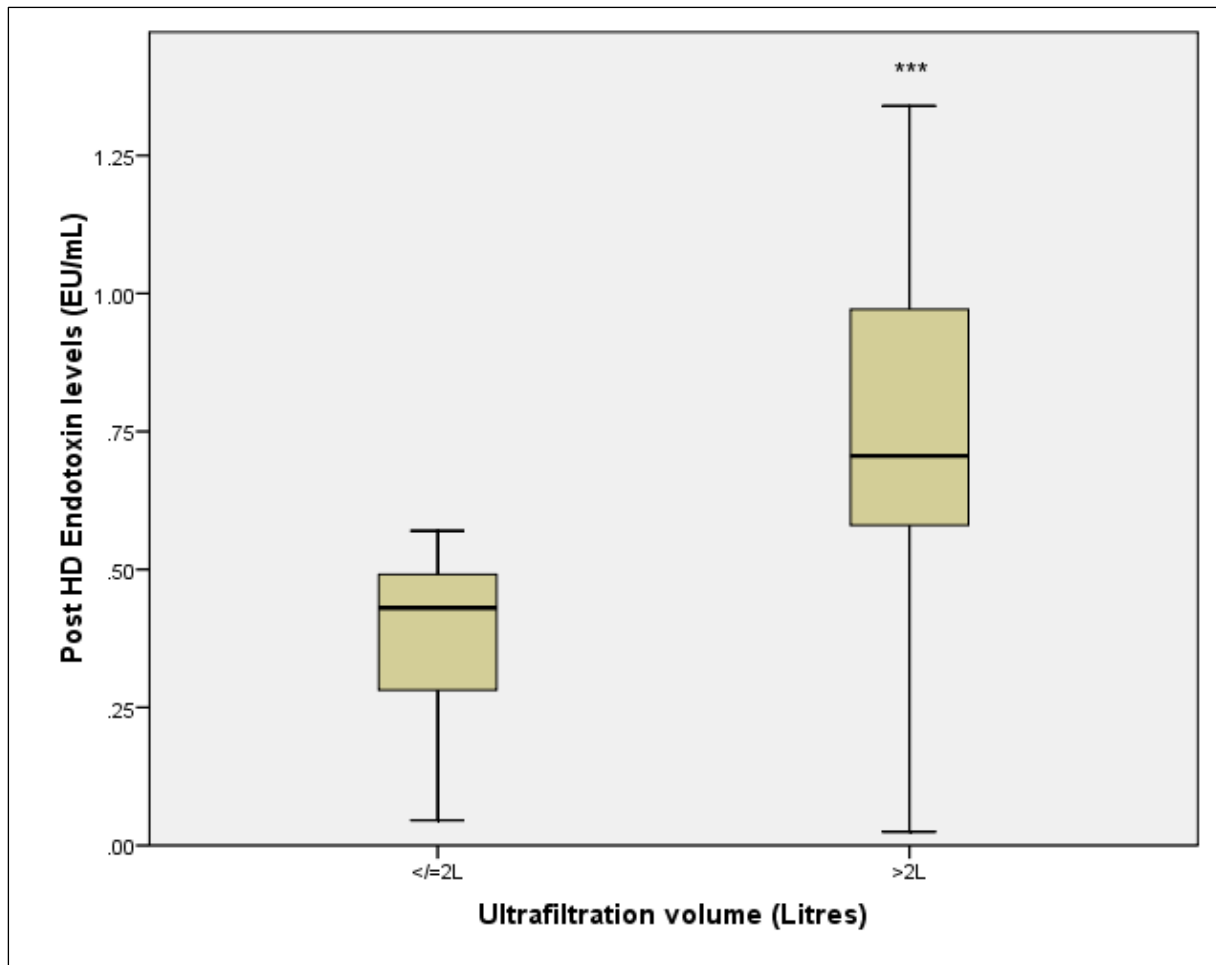


Figure 3.17: Comparison of post-haemodialysis endotoxin level between patients with low ultrafiltration volume and those with high ultrafiltration volume. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Endotoxin was measured using Limulus amoebocyte lysate kit. Patients were categorized based on cut-off ultrafiltration value of 2 litres and were compared. *** $p = 0.003$ compared to group with ultrafiltration volume ≤ 2 litres

HD- Haemodialysis

3.12 Subclinical atherosclerosis, endotoxemia, and inflammatory markers

Chronic kidney disease patients with elevated circulating endotoxaemia (> 0.5 EU/ml) had significantly higher CIMT compared to patients with lower endotoxin levels (≤ 0.5 EU/ml) (Figure 3.18). When divided into two groups, CKD patients with high CIMT values (>0.55 mm) had significantly elevated levels of serum IL-6 and CRP compared to those with normal CIMT (≤ 0.55 mm) (Figure 3.19 and 3.20). However, endotoxin levels did not correlate with IL-6 and CRP levels. Serum IL-6 was associated with serum CRP ($r=0.329$, $p<0.001$). There was a very weak but significant correlation between endotoxin levels and some nutritional indices [FTI ($r=-0.229$, $p=0.012$), FTM ($r=-0.209$, $p=0.022$)]. Eight (6.7%) of the 120 CKD patients had carotid plaques, with endotoxin levels significantly higher among these patients compared to those without carotid plaques (0.98 ± 0.24 versus 0.52 ± 0.26 EU/ml, $p=0.002$) (Figure 3.21). Carotid plaques were present in 5% (2/40) of PD, 12.5% (5/40) of HD and 2.5% (1/40) of non-dialytic CKD patients (Table 3.3). No carotid plaque was identified in any of the controls. Endotoxin levels were weakly associated with CIMT, a surrogate marker of atherosclerosis ($r=0.313$, $p=0.001$). Carotid intima media thickness had a weak but significant correlation with serum creatinine ($r=0.240$, $p=0.008$) and serum albumin ($r=-0.206$, $p<0.0001$). Multiple linear regression analysis showed that endotoxin levels ($\beta=0.178$, $p=0.045$) and age of the patients ($\beta=0.251$, $p=0.005$) were the independent predictors of increased CIMT (Table 3.8).

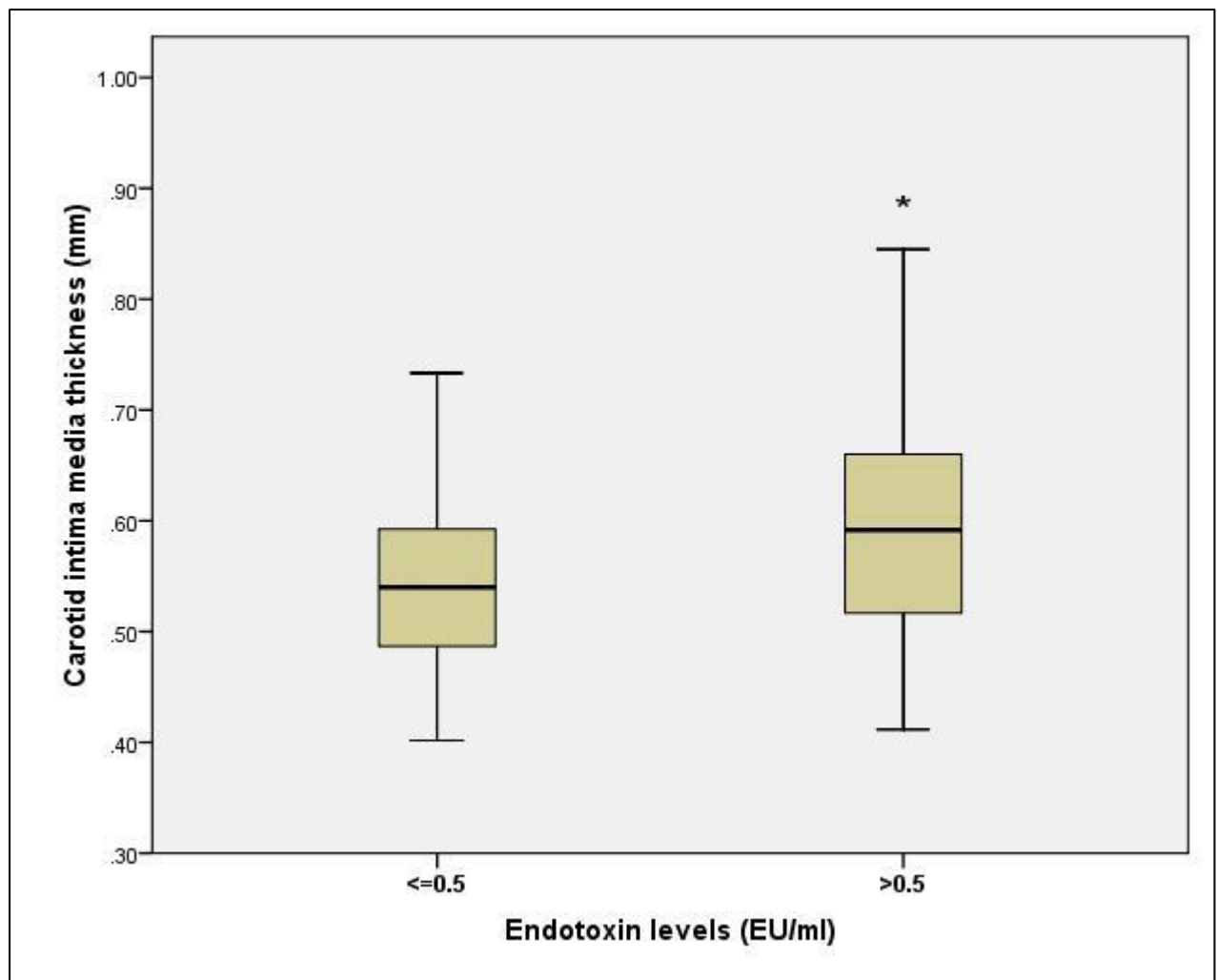


Figure 3.18: Comparison of carotid intima media thickness between CKD patients with elevated endotoxin level (>0.5 EU/ml) and those with low endotoxin levels (≤ 0.5 EU/ml). The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Endotoxin levels were measured using Limulus amoebocyte lysate kit while carotid intima media thickness was measured using B-mode ultrasound. Carotid intima media thickness was compared between patients with high endotoxin levels and those with low endotoxin levels, * $P < 0.001$ compared to group without CKD.

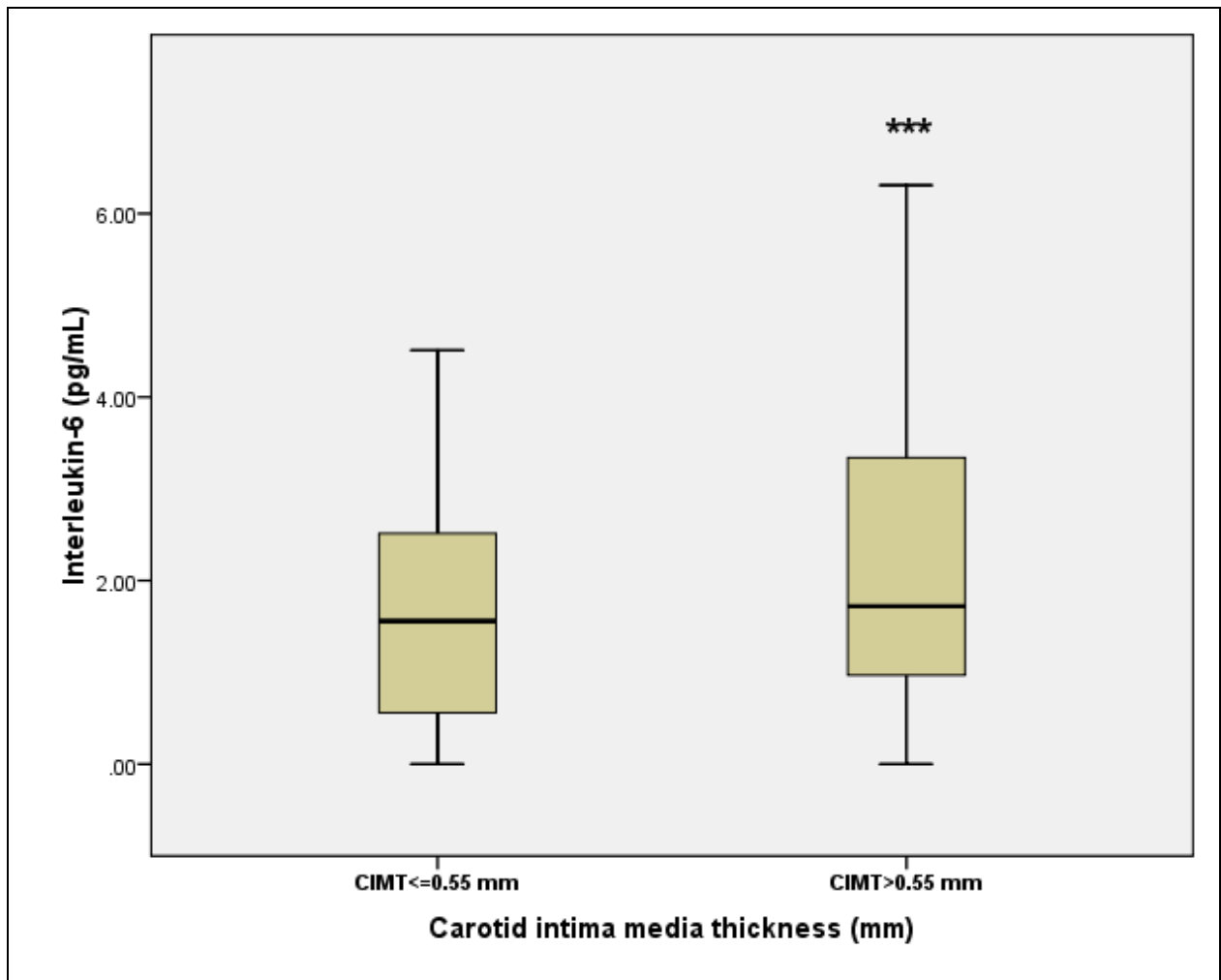


Figure 3.19: Serum interleukin-6 levels in CKD patients with high carotid intima media thickness (> 0.55 mm) compared to those with normal carotid intima media thickness (≤ 0.55 mm). The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Serum interleukin-6 levels were analyzed with R&D Luminex assay kit while carotid intima media thickness was measured using B-mode ultrasound. Interleukin-6 levels were compared between patients with $\text{CIMT} \leq 0.55$ mm and those with $\text{CIMT} > 0.55$ mm, *** $P < 0.001$ compared to the group with $\text{CIMT} \leq 0.55$ mm.

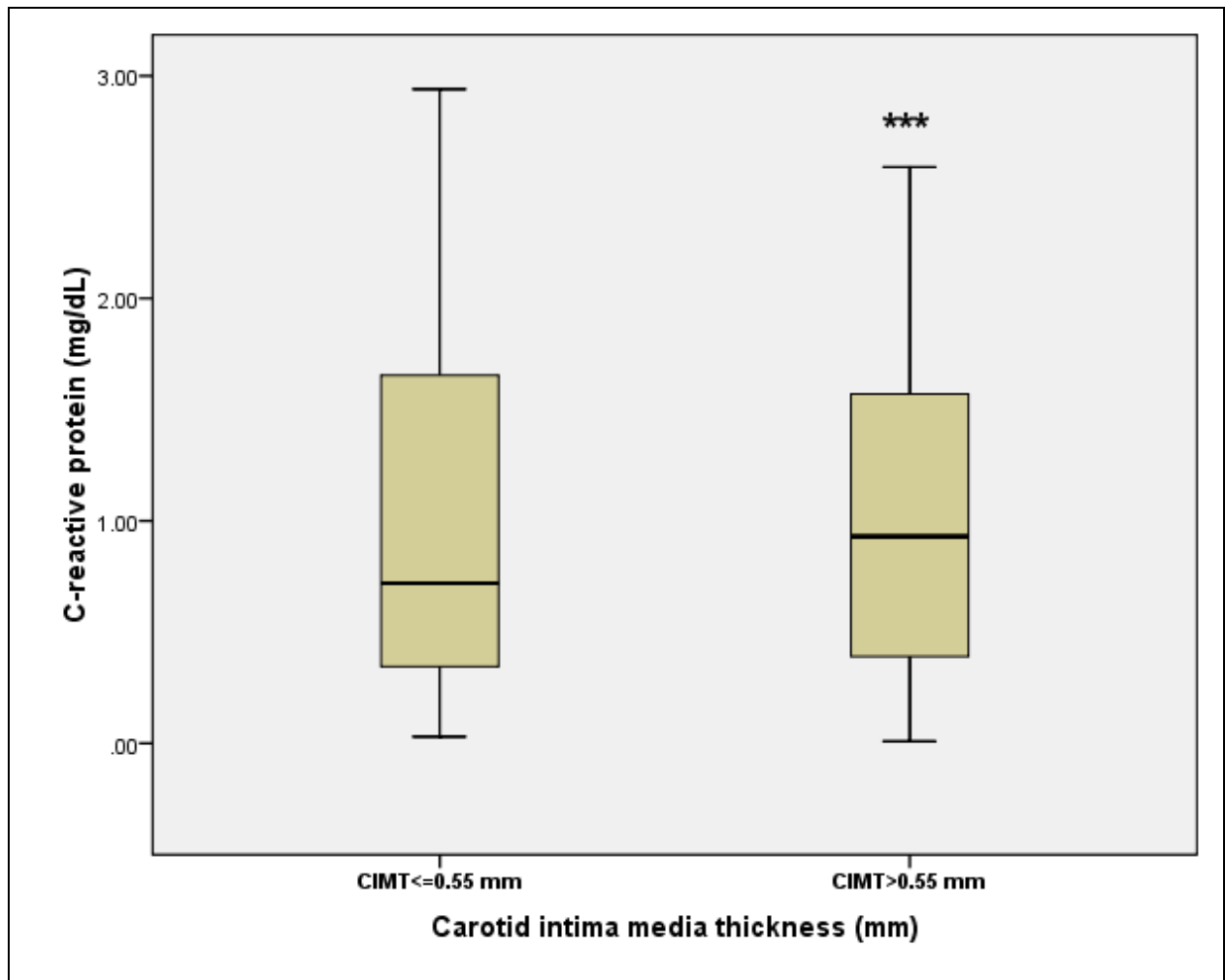


Figure 3.20: Serum C-reactive protein levels in CKD patients with high carotid intima media thickness (> 0.55 mm) compared to those with normal carotid intima media thickness (≤ 0.55 mm). The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Serum C-reactive protein levels were analyzed with R&D Luminex assay kit while carotid intima media thickness was measured using B-mode ultrasound. C-reactive protein levels were compared between patients with CIMT ≤ 0.55 mm and those with CIMT > 0.55 mm, *** P = 0.001 compared to the group with CIMT ≤ 0.55 mm.

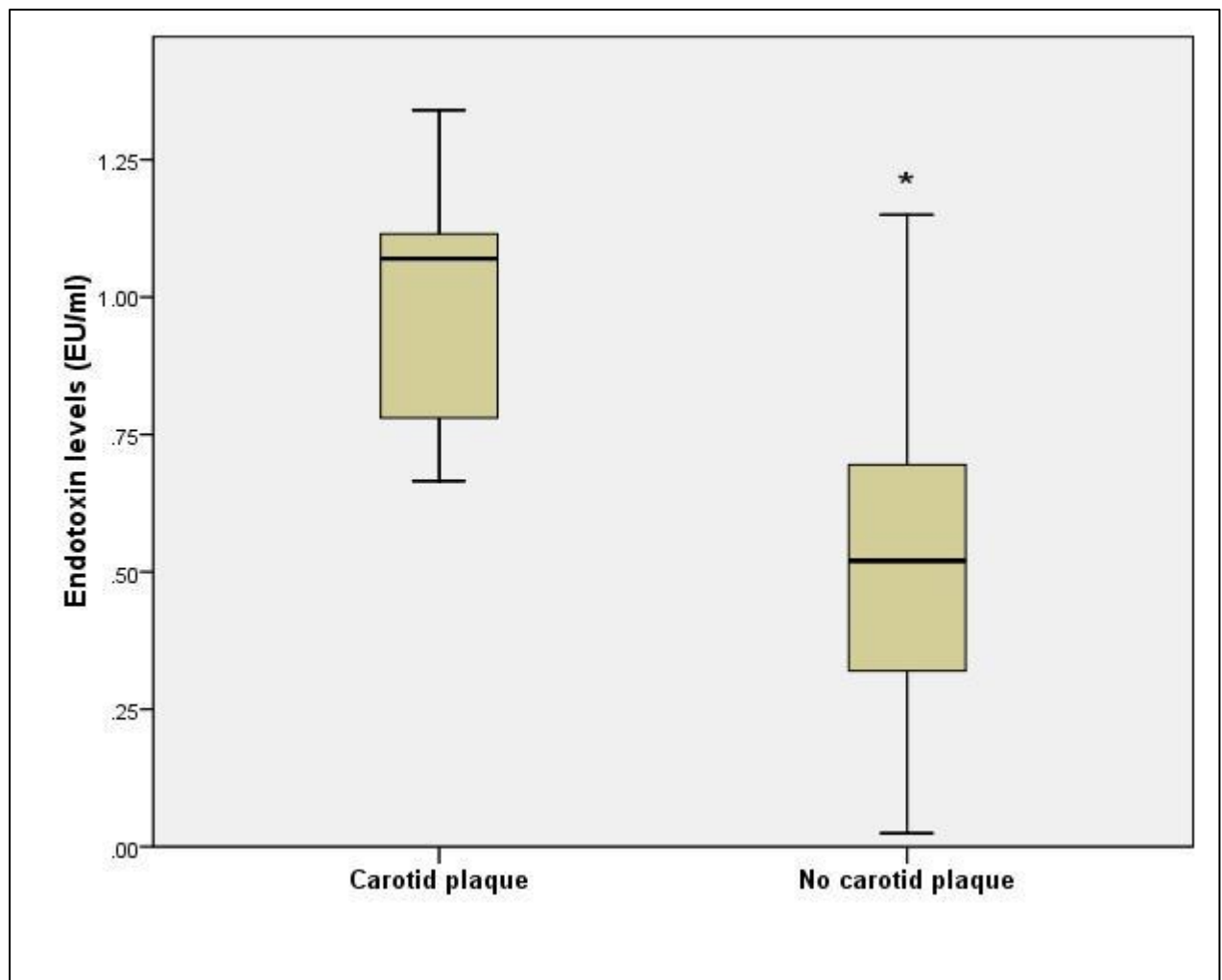


Figure 3.21: Comparison of endotoxin levels between patients with carotid plaque and those without carotid plaque. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Endotoxin was measured using Limulus amoebocyte lysate kit. Carotid plaque was defined as the echogenic structure protruding into the lumen with the distance between the media adventitia interface and the internal side of the lesion ≥ 1.2 mm. Endotoxin levels were compared between patients with carotid plaques and those without carotid plaques. * $P < 0.001$ compared to group without CKD

Table 3.8: Multiple linear regression analysis of determinants of carotid intima media thickness

	Unstandardized Coefficients (B)	Standardized Coefficient Beta	P value	Confidence Interval
Endotoxin levels	0.074	0.178	0.045	0.002-0.147
Age	0.003	0.251	0.005	0.001-0.005
Interleukin-6	0.004	0.129	0.145	– 0.001-0.009
Smoking	0.022	0.144	0.107	0.005-0.050
Cholesterol	0.002	0.018	0.840	– 0.014-0.017
CaPO ₄	0.006	0.071	0.428	– 0.010-0.023

Endotoxin levels and age were the independent risk factors for subclinical atherosclerosis

3.13 Markers of immune activation and oxidative stress

Table 3.9 showed markers of immune activation and oxidative stress in the CKD patients and controls. Serological markers were compared between the CKD patients and controls and some statistically significant differences were observed; CKD patients had more LBP ($1.39 \times 10^5 \pm 0.59 \times 10^5$ vs $0.96 \times 10^5 \pm 0.30 \times 10^5$ ng/ml, $p < 0.001$), sCD14 ($1.86 \times 10^6 \pm 0.8 \times 10^6$ vs $1.2 \times 10^6 \pm 0.3 \times 10^6$ pg/ml, $p < 0.001$), IL-8 (36.7 ± 78.5 vs 8.4 ± 12.4 pg/ml, $p < 0.001$) and MCP-1 (11.8 ± 11.1 vs 4.3 ± 2.9 pg/ml, $p < 0.001$) in their sera than controls. While the LBP/sCD14 ratio was lower in CKD patients when compared to the controls (0.086 ± 0.08 vs 0.11 ± 0.16 , $p = 0.522$), the difference was not statistically significant. On univariate analysis, serum LBP showed a positive correlation with sCD14 ($r = 0.674$, $p < 0.001$) and MCP-1 ($r = 0.359$, $p < 0.001$). Oxidized LDL concentrations were significantly higher in dialysis and CKD groups than in the control group ($p < 0.001$). Oxidized LDL concentrations were 360.8 ± 132.7 , 369.5 ± 81.3 , 340.3 ± 123.2 and 266.5 ± 100.2 ng/ml in the PD, HD, CKD stage 3 patients and the controls respectively. However, no significant difference was observed between peritoneal dialysis, haemodialysis and CKD groups on post-hoc analysis. Similarly, the ratio of oxidized LDL to LDL was significantly different between kidney disease patients and the controls ($p < 0.05$).

Table 3.9: Comparison of endotoxin, markers of immune activation and oxidized LDL between CKD patients and controls

Parameters	All patients (n=120)	PD (n=40)	HD (n=40)	Stage 3 CKD (n=40)	Control (n=40)	*P-value
Markers of immune activation						
LBP (ng/ml)	$1.39 \times 10^5 \pm 0.59 \times 10^5$	$1.5 \times 10^5 \pm 0.70 \times 10^5$	$1.4 \times 10^5 \pm 0.61 \times 10^5$	$1.2 \times 10^5 \pm 0.36 \times 10^5$	$0.96 \times 10^5 \pm 0.30 \times 10^5$	<0.001
sCD14 (pg/ml)	$1.86 \times 10^6 \pm 0.8 \times 10^6$	$2.1 \times 10^6 \pm 0.8 \times 10^6$	$1.9 \times 10^6 \pm 0.7 \times 10^6$	$1.6 \times 10^6 \pm 0.9 \times 10^6$	$1.2 \times 10^6 \pm 0.3 \times 10^6$	<0.001
LBP/sCD14 ratio	0.086±0.08	0.076±0.02	0.079±0.03	0.082±0.02	0.11±0.16	0.522
IL-8 (pg/ml)	36.7±78.5	39.9±72.3	49.3±107.4	20.9±40.1	8.4±12.4	<0.001
MCP-1 (pg/ml)	11.8±11.1	15.4±9.9	9.9±10.6	10.0±12.1	4.3±2.9	<0.001
Markers of oxidative stress						
Oxidized LDL (ng/ml)	362.6±119.5	360.8±132.7	369.5±81.3	340.3±123.2	266.5±100.2	<0.001
Oxidized LDL/LDL ratio	175.1±106.6	147.9±124.3	220.6±84.7	156.9±93.9	137.9±80.9	0.029

Notes: LBP=lipopolysaccharide binding protein, sCD14=serum CD14, IL-8=interleukin-8, MCP-1=monocyte chemoattractant protein-1, Oxidized LDL=Oxidized low density lipoprotein. Continuous data were expressed as mean ± SD. *p-values were calculated by comparing all patients with kidney disease (n=120) to controls (n=40) using Mann Whitney test

3.14 Associations between endotoxin levels, markers of immune activation and carotid intima media thickness

On univariate analysis, endotoxin levels showed a weak correlation with sCD14 ($r=0.381$, $p<0.001$) and MCP-1 ($r=0.340$, $p<0.001$). Carotid intima media thickness was also associated with MCP-1 ($r=0.557$, $p<0.001$) (Figure 3.22), sCD14 levels ($r=0.556$, $p<0.001$) (Figure 3.23), LBP ($r=0.311$, $p=0.001$), IL-8 ($r=0.256$, $p<0.001$) and LBP/sCD14 ratio ($r=-0.251$, $p=0.006$).

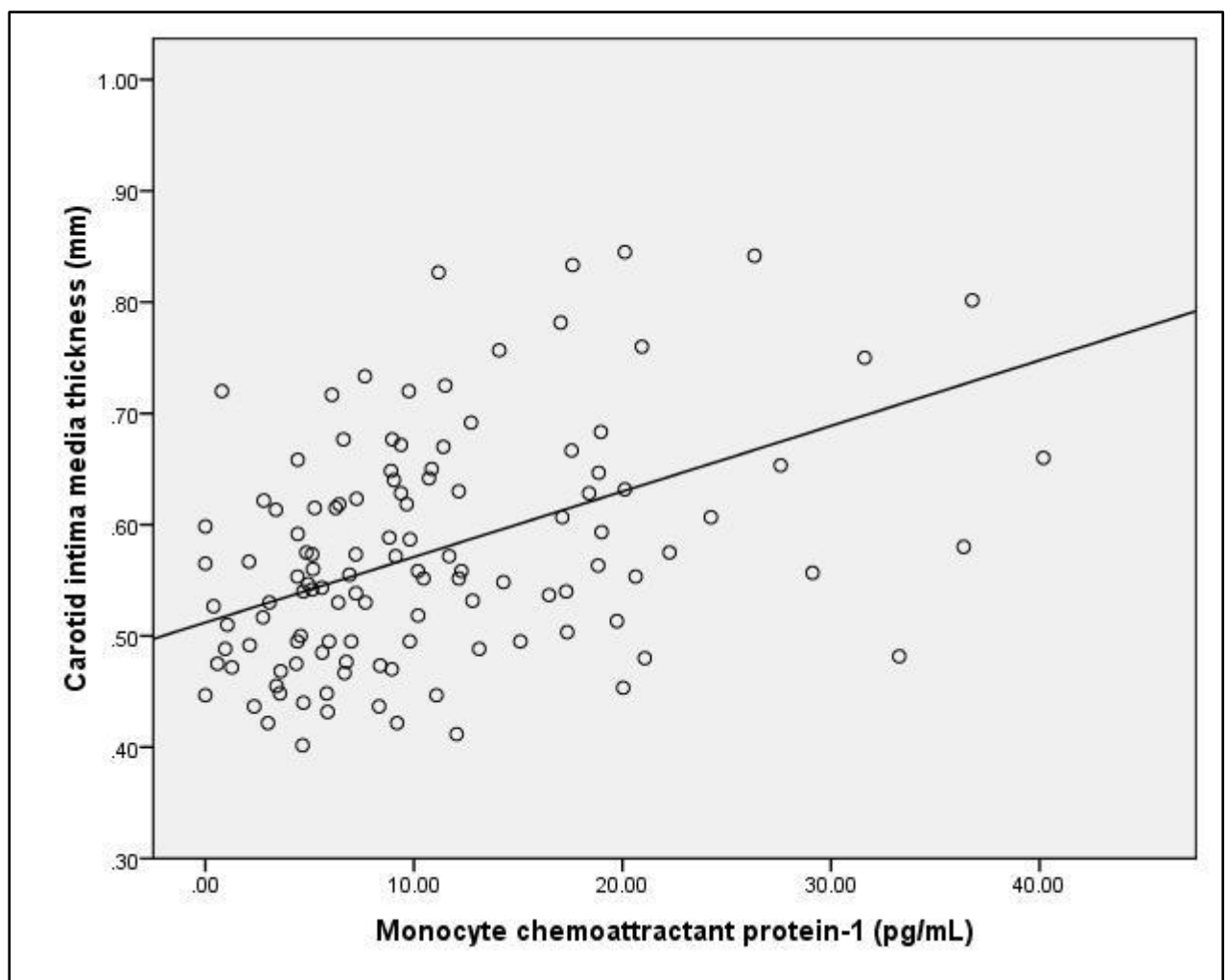


Figure 3.22: Relationship between carotid intima media thickness and monocyte chemoattractant protein-1 ($r=0.556$, $p<0.001$). The figure demonstrates a direct relationship between carotid intima media thickness and monocyte chemoattractant protein-1. Association between carotid intima media thickness and monocyte chemoattractant protein-1 was assessed using Pearson correlation coefficient. Carotid intima media thickness was measured by B-mode ultrasound while monocyte chemoattractant protein-1 levels were assessed with R&D Luminex performance assay kit.

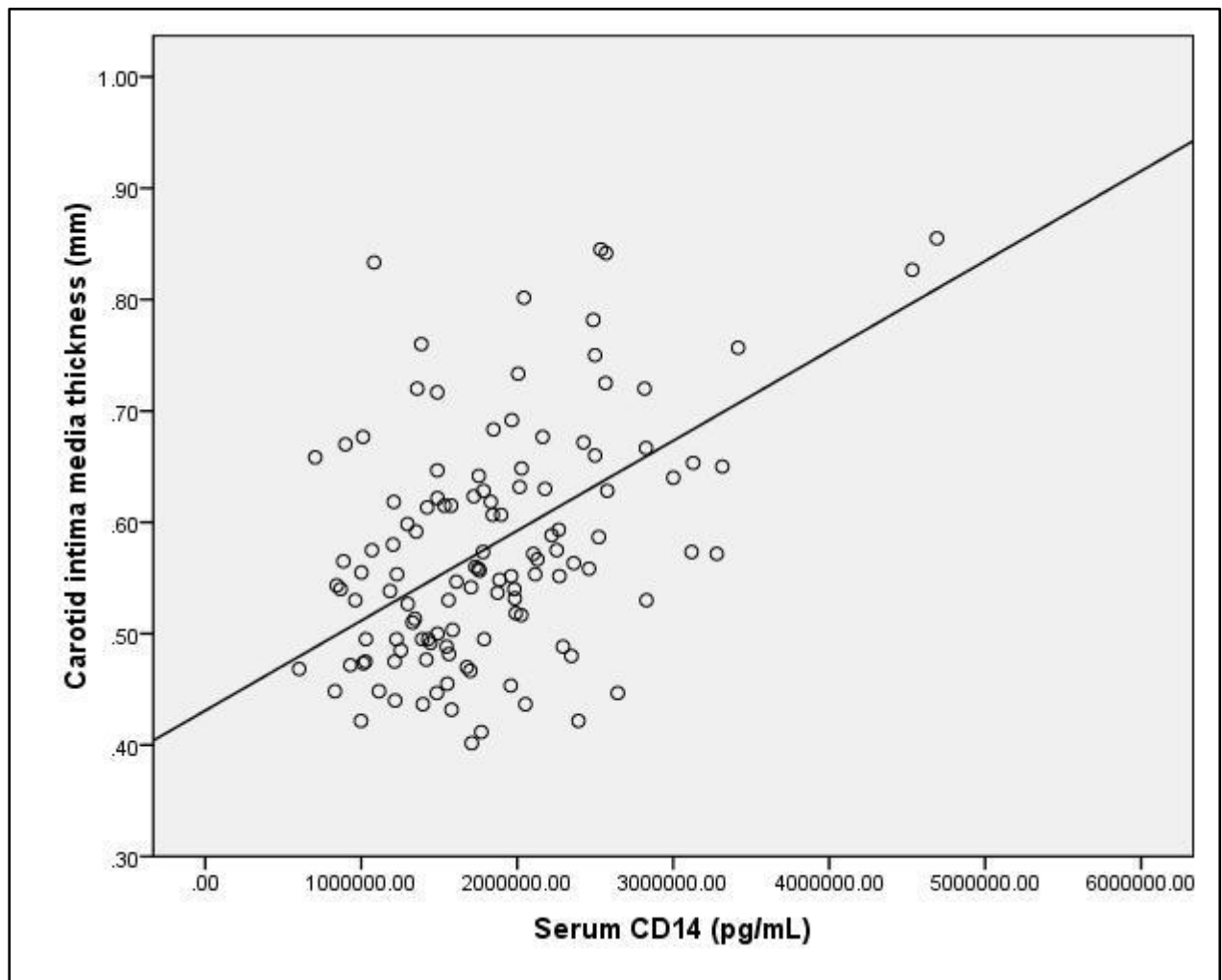


Figure 3.23: Relationship between carotid intima media thickness and serum CD14 ($r=0.556$, $p<0.001$). The figure demonstrates a direct relationship between carotid intima media thickness and serum CD14. Association between carotid intima media thickness and serum CD14 was assessed using Pearson correlation coefficient. Carotid intima media thickness was measured by B-mode ultrasound while serum CD14 levels were assessed with R&D Luminex performance assay kit.

3.15 Effect of immune activation on risk of endotoxin-induced atherosclerosis

Overall, the risk of atherosclerosis was associated with elevated levels of endotoxins (odd ratio: 2.34; confidence interval: 1.26-4.35; $p=0.007$), with a more than two-fold rise in the risk of subclinical atherosclerosis among patients with high circulating endotoxaemia (> 0.5 EU/ml) compared with the reference group, patients with low endotoxaemia (≤ 0.5 EU/ml). In order to determine whether the presence of markers of immune activation in the CKD patients enhanced the predictive power of circulating endotoxaemia, concentrations of IL-8, MCP-1 and sCD14 as well as CIMT measurements were subdivided into two equal groups. The median concentrations of IL-8, MCP-1 and sCD14 were 7.99pg/ml, 8.46pg/ml and 1.7×10^6 respectively, while the median CIMT measurement was 0.55 mm. As shown in Table 3.10, the prevalence of atherosclerosis was influenced in patients with low endotoxaemia (≤ 0.5 EU/ml) by the presence of elevated concentrations of IL-8 or MCP-1 or sCD14. Similarly, in the group with high endotoxin levels, patients with high endotoxin levels (> 0.5 EU/ml) in combination with elevated levels of IL-8 or MCP-1 or sCD14 had a markedly increase risk of subclinical atherosclerosis when compared to the group with low endotoxin levels that was associated with low levels of IL-8 or MCP-1 or sCD14.

Table 3.10: Risk of early atherogenesis by interleukin-8, monocyte chemoattractant protein-1 and serum CD14 levels

Categories	Serum concentration of immune mediators (Mean ± SD)	Number of patients at risk of subclinical atherosclerosis (CIMT > 0.5 mm)	Odds ratio (CI)	P-value
Interleukin-8				
Endotoxin ≤ 0.5 EU/ml				
Interleukin-8 ≤ 7.99 pg/ml	4.9 ± 1.9	14/36	1.31 (0.87 – 1.97)	0.144
Interleukin-8 > 7.99 pg/ml	60.0 ± 122.2	11/19	2.16 (0.69 – 6.69)	
Endotoxin > 0.5 EU/ml				
Interleukin-8 ≤ 7.99 pg/ml	5.2 ± 2.6	9/20	2.75 (1.36 – 5.57)	0.006
Interleukin-8 > 7.99 pg/ml	66.4 ± 90.3	36/45	4.89 (1.56 – 15.35)	
Monocyte chemoattractant protein-1				
Endotoxin ≤ 0.5 EU/ml				
MCP-1 ≤ 8.46 pg/ml	4.0 ± 2.4	13/36	1.47 (0.96 – 2.26)	0.051
MCP-1 > 8.46 pg/ml	17.9 ± 12.1	12/19	3.03 (0.95 – 9.62)	
Endotoxin > 0.5 EU/ml				
MCP-1 ≤ 8.46 pg/ml	5.5 ± 1.9	9/21	3.0 (1.51 – 5.95)	0.002
MCP-1 > 8.46 pg/ml	18.4 ± 11.8	36/44	6.0 (1.89 – 19.04)	
Serum CD14				
Endotoxin ≤ 0.5 EU/ml				
Serum CD14 ≤ 1.7 x 10 ⁶ pg/ml	1.2 × 10 ⁶ ± 0.3 × 10 ⁶	16/39	1.19 (0.84 – 1.71)	0.232
Serum CD14 > 1.7 x 10 ⁶ pg/ml	2.3 × 10 ⁶ ± 0.7 × 10 ⁶	9/16	1.85 (0.57 – 5.99)	
Endotoxin > 0.5 EU/ml				
Serum CD14 ≤ 1.7 x 10 ⁶ pg/ml	1.4 × 10 ⁶ ± 0.3 × 10 ⁶	7/18	3.54 (1.61 – 7.77)	0.002
Serum CD14 > 1.7 x 10 ⁶ pg/ml	2.4 × 10 ⁶ ± 0.8 × 10 ⁶	38/47	6.64 (2.01 – 21.90)	

Risk of subclinical atherosclerosis associated with levels of endotoxin according to serum concentrations of interleukin-8, monocyte chemoattractant protein-1 and sCD14. The P value, Odds ratios and 95% confidence interval were derived from analyses of the strength of association between subclinical atherosclerosis and endotoxin as well as elevated levels interleukin-8, monocyte chemoattractant protein-1 and sCD14. CIMT, carotid intima media thickness; CI, confidence interval.

3.16 Associations between oxidized LDL, lipid profile and inflammatory markers

Oxidized LDL was closely associated with oxidized LDL/LDL ratio ($r=0.641$, $p<0.001$) (Figure 3.24). However, there was no significant correlation between oxidized LDL and total cholesterol ($r=-0.134$, $p=0.091$), LDL ($r=-0.110$, $p=0.166$), HDL ($r=-0.100$, $p=0.209$) as well as triglycerides ($r=0.056$, $p=0.478$). There was a weak but significant association between oxidized LDL and inflammatory markers (IL-6: $r=0.350$, $p<0.001$; CRP: $r=0.226$, $p=0.013$). However, circulating endotoxaemia did not correlate with oxidized LDL ($r=0.080$, $p=0.387$), IL-6 ($r=0.127$, $p=0.167$) or CRP ($r=0.010$, $p=0.914$). Similarly, no association was observed between endotoxin levels and total cholesterol ($r=0.085$, $p=0.283$), LDL ($r=0.113$, $p=0.155$), HDL ($r=-0.019$, $p=0.816$) as well as triglycerides ($r=0.100$, $p=0.209$). When the lipid profile was compared between the CKD groups, there was no statistically significant difference between the CKD patients and the controls. However, when comparing the PD to the HD group, total cholesterol, LDL and triglycerides were significantly higher ($p<0.001$, $p<0.001$ and $p=0.001$ respectively) in PD than in HD patients.

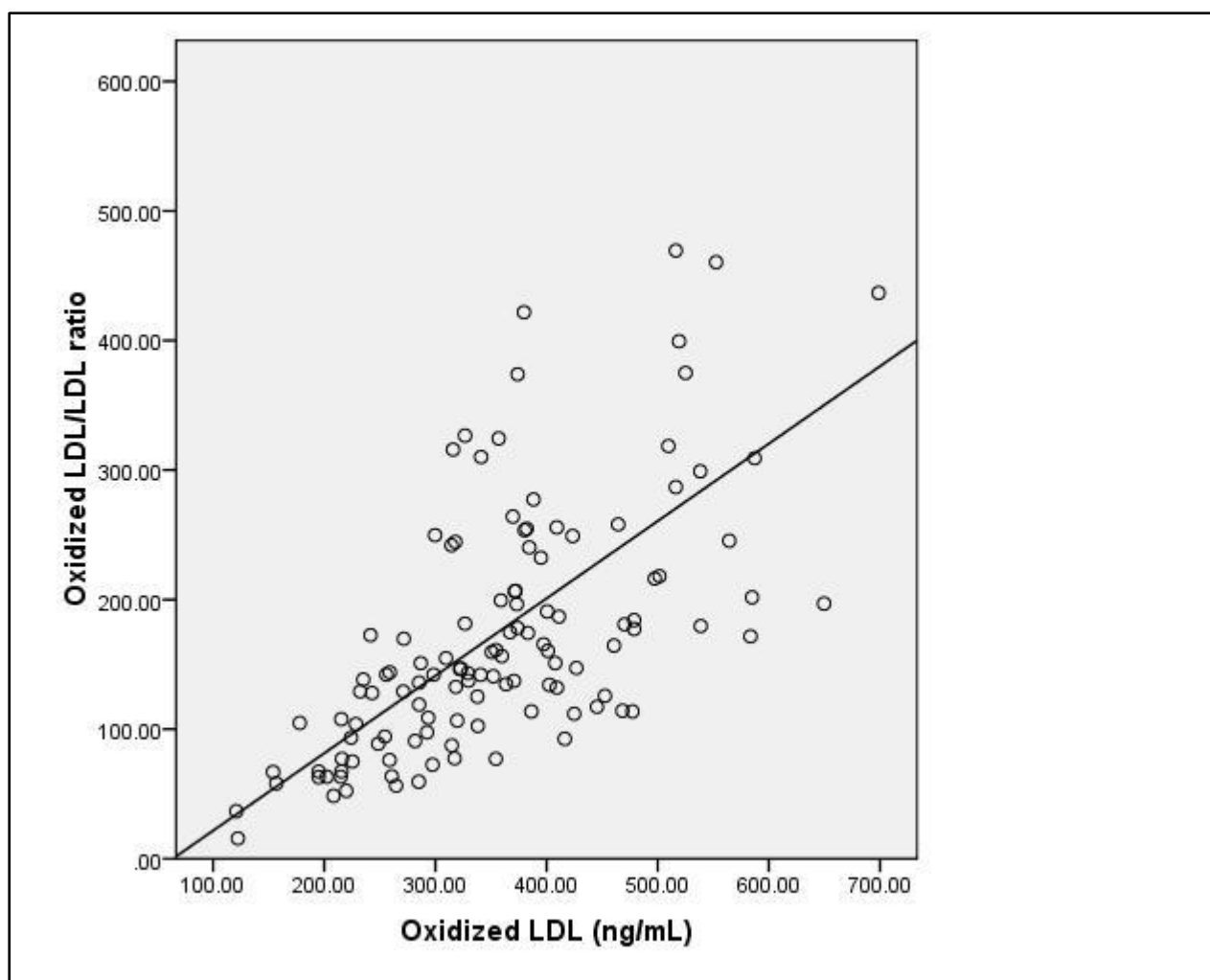


Figure 3.24: Relationship between oxidized LDL/LDL ratio and oxidized LDL ($r=0.641$, $p<0.001$). The figure demonstrates a direct relationship between oxidized LDL/LDL ratio and oxidized LDL concentrations. Association between oxidized LDL/LDL ratio oxidized LDL concentrations was assessed using Pearson correlation coefficient. Oxidized LDL was measured by the OxiSelect™ human oxidized LDL ELISA kit.

3.17 Associations between oxidized LDL and cardiovascular disease

Patients with subclinical atherosclerosis (CIMT >0.55 mm) had significantly elevated levels of oxidized LDL, compared to patients with normal CIMT (CIMT $\leq$ 0.55 mm); 388.2 ± 118.8 versus 313.0 ± 92.1 ng/ml, $p < 0.001$ (Figure 3.25). Oxidized LDL levels were significantly higher in patients with carotid plaques compared to patients without carotid plaques (472.6 ± 104.6 versus 348.6 ± 110.9 ng/ml, $p = 0.012$) (Figure 3.26). On bivariate analysis, oxidized LDL showed a weak but significant correlation with CIMT ($r = 0.377$; $p < 0.001$) and carotid plaque ($r = 0.317$, $p < 0.001$), and was inversely related to E/A ratio ($r = -0.319$; $p = 0.001$). In the sub analysis of non-dialytic CKD cohorts (CKD stage 3), oxidized LDL showed a stronger association with CIMT ($r = 0.664$, $p < 0.001$) (Figure 3.27) and E/A ratio ($r = -0.468$, $p = 0.002$); whereas oxidized LDL concentration was not correlated to CIMT and E/A ratio in dialysis patients. There was no correlation between total cholesterol ($r = -0.103$; $p = 0.187$), triglycerides ($r = 0.057$; $p = 0.471$), LDL ($r = -0.083$; $p = 0.291$), HDL ($r = -0.088$; $p = 0.264$) and CIMT.

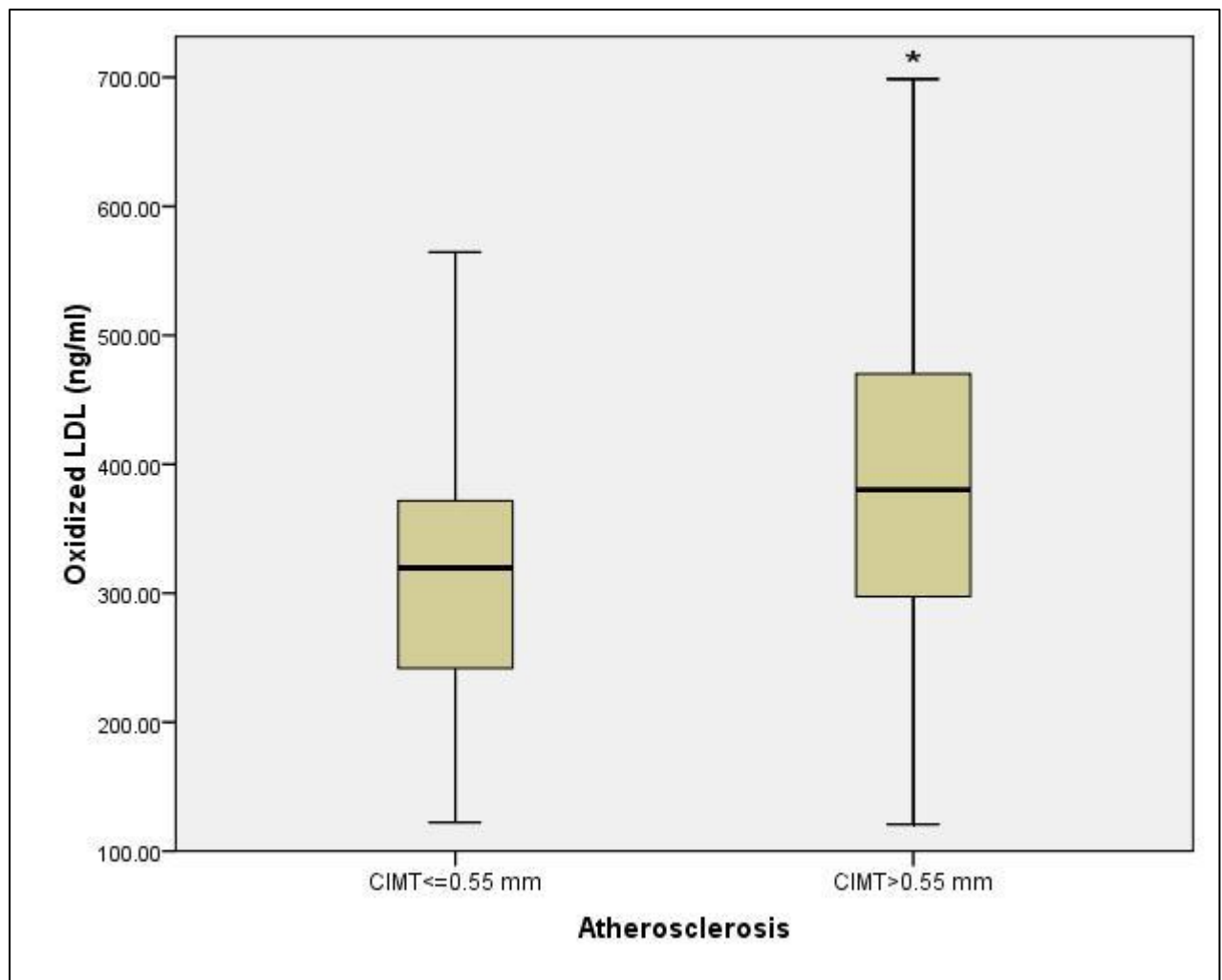


Figure 3.25: Comparison of oxidized LDL levels between CKD patients with early atherosclerosis and those without atherosclerosis. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Oxidized LDL was measured by the OxiSelect™ human oxidized LDL ELISA kit. Carotid intima media thickness was measured using B-mode ultrasound. Oxidized LDL levels were compared between patients with subclinical atherosclerosis and those without atherosclerosis. *P < 0.001 compared to group without subclinical atherosclerosis.

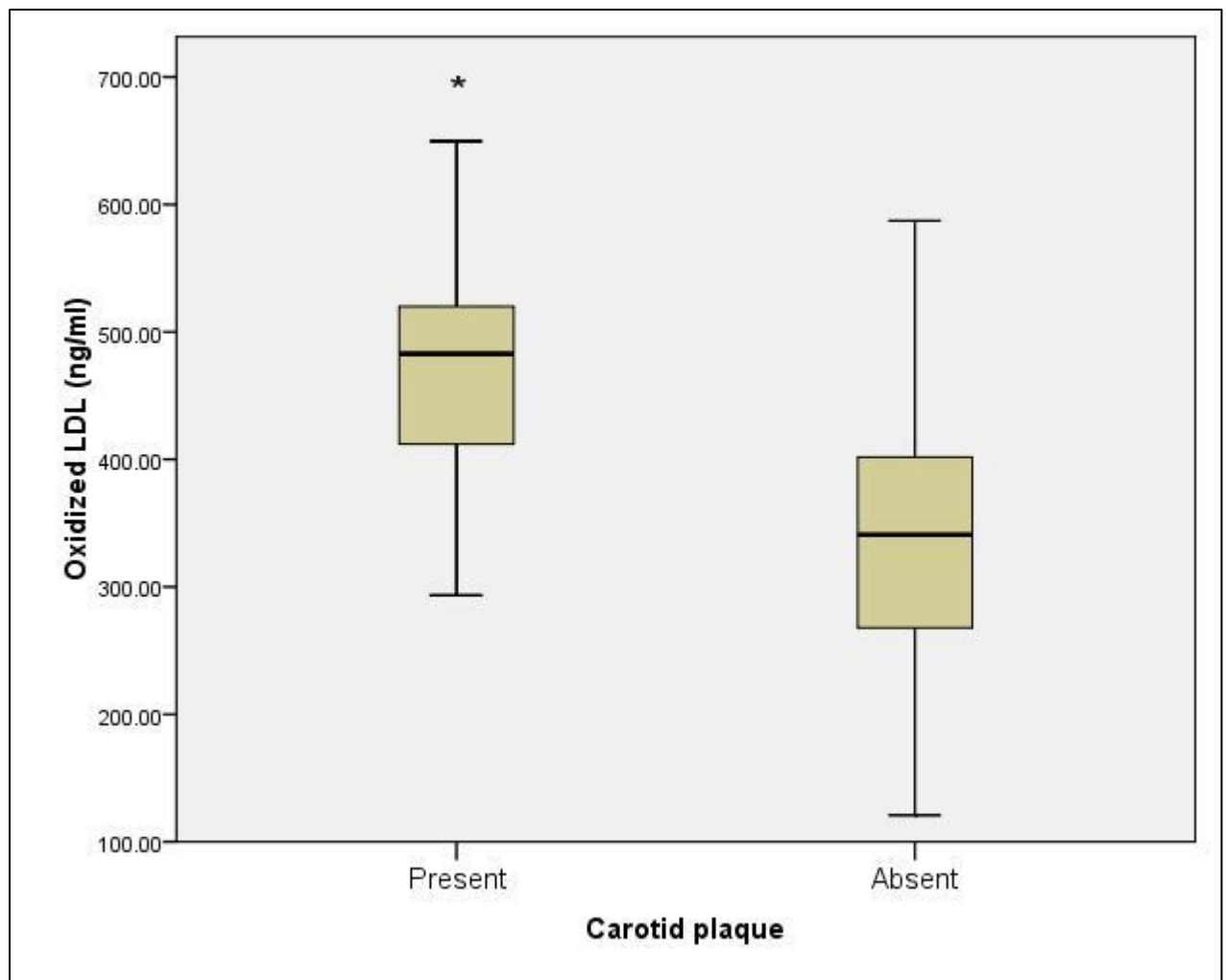


Figure 3.26: Comparison of oxidized levels between patients with carotid plaque and those without carotid plaque. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Oxidized LDL was measured by the OxiSelect™ human oxidized LDL ELISA kit. Carotid plaque was defined as the echogenic structure protruding into the lumen with the distance between the media adventitia interface and the internal side of the lesion ≥ 1.2 mm. Oxidized LDL levels were compared between patients with carotid plaques and those without carotid plaques. *P =0.012 compared to group without carotid plaque.

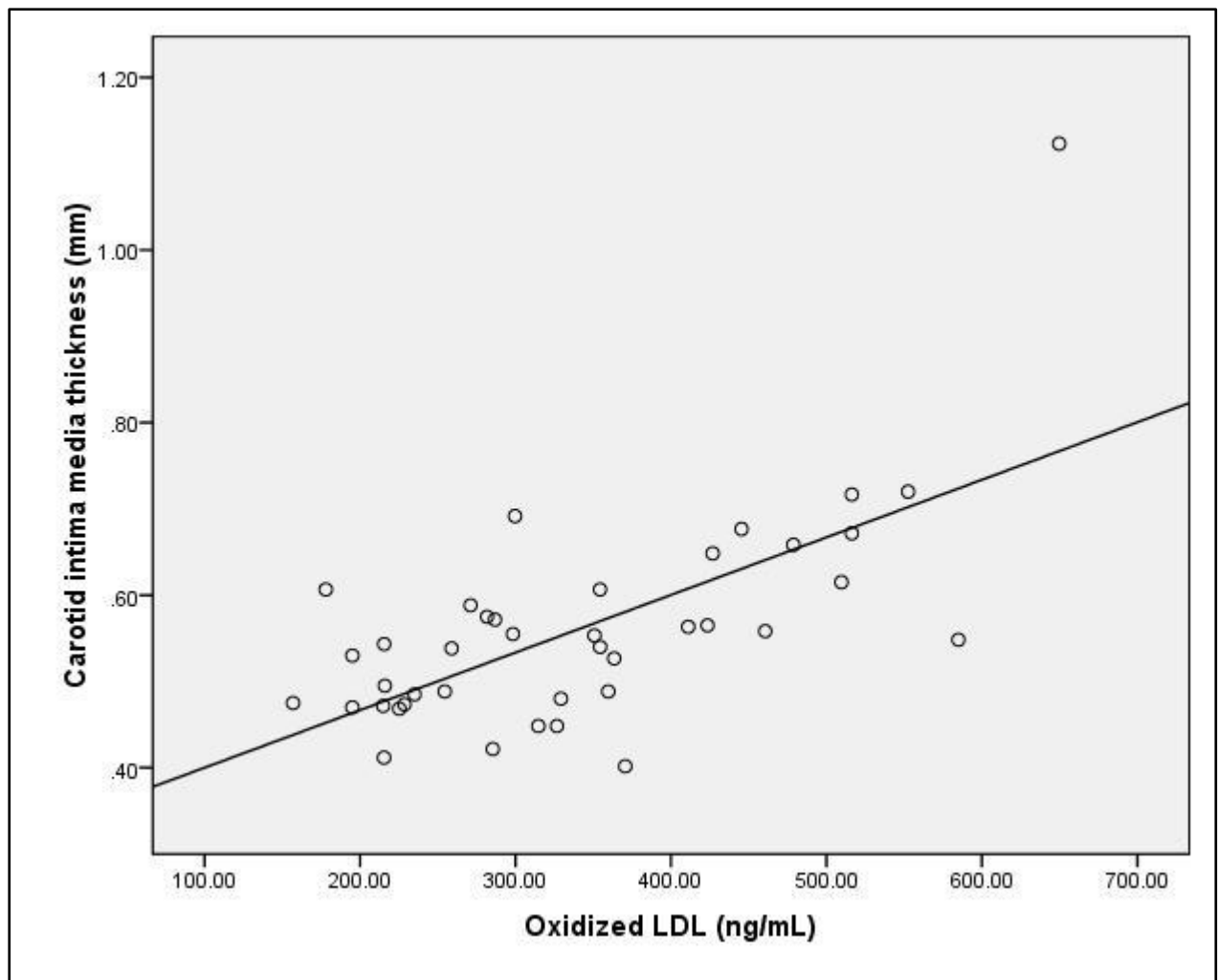


Figure 3.27: Relationship between oxidized LDL and carotid intima media thickness in non-dialytic CKD (stage 3) patients ($r=0.664$, $p<0.001$). The figure demonstrates a direct relationship between oxidized LDL concentrations and carotid intima media thickness measurements. Association between oxidized LDL concentrations and carotid intima media thickness was assessed using Pearson correlation coefficient. Oxidized LDL was measured by the OxiSelect™ human oxidized LDL ELISA kit while carotid intima media thickness was measured using B-mode ultrasound.

3.18 Transforming growth factor-beta (TGF- β) isoforms levels in CKD patients

Of the three TGF- β isoforms, TGF- β 1 has the highest concentrations. The lowest TGF- β isoform concentrations were present in haemodialysis patients compared to the PD, CKD and controls (Figure 3.28a-c). The mean concentrations of the three TGF- β isoforms are presented in Table 3.11. There was a statistically significant difference in the levels of TGF- β 1 between male and female CKD patients, with higher TGF- β 1 levels measured in female patients compared to the male patients (TGF- β 1: $4.9 \times 10^4 \pm 1.4 \times 10^4$ versus $4.2 \times 10^4 \pm 1.7 \times 10^4$ pg/ml, $p=0.009$). No relationship was found between any of the TGF- β isoforms and age. Regarding TGF- β 1 and TGF- β 3 isoforms concentrations, there was statistically significant difference between HD patients and other study groups including the controls ($p<0.05$), while in the sub analysis of TGF- β 2 concentrations, there was no difference between HD and PD patients ($p>0.05$). In CKD patients (HD, PD and stage 3 CKD), no significant difference was found between TGF- β isoforms and the aetiology of the CKD. When comparing patients with CKD and the controls, CKD patients had significantly reduced concentrations of TGF- β 1 ($4.6 \times 10^4 \pm 1.5 \times 10^4$ versus $6.6 \times 10^4 \pm 1.5 \times 10^4$ pg/ml) and TGF- β 3 ($4.5 \times 10^4 \pm 1.0 \times 10^2$ versus $5.6 \times 10^2 \pm 1.0 \times 10^2$ pg/ml) compared to the controls, while there was no difference in the concentration of TGF- β 2 isoforms between CKD patients and controls ($p>0.05$) (Figure 3.29a-c).

Table 3.11: Concentrations of TGF- β isoforms in the sub-groups of chronic kidney patients and the controls

TGF-β isoforms	All Patients (n=120) Mean $\pm$ SD	PD (n=40) Mean $\pm$ SD	HD (n=40) Mean $\pm$ SD	Stage 3 CKD (n=40) Mean $\pm$ SD	Controls (n=40) Mean $\pm$ SD	*P-value
TGF- β 1 (pg/ml)	$4.6 \pm 1.5 \times 10^4$	$4.5 \pm 1.3 \times 10^4$	$3.6 \pm 1.3 \times 10^4$	$5.7 \pm 1.4 \times 10^4$	$6.6 \pm 1.5 \times 10^4$	<0.001
TGF- β 2 (pg/ml)	$1.7 \pm 0.3 \times 10^3$	$1.7 \pm 0.3 \times 10^3$	$1.6 \pm 0.2 \times 10^3$	$1.8 \pm 0.3 \times 10^3$	$1.8 \pm 1.5 \times 10^3$	0.057
TGF- β 3 (pg/ml)	$4.6 \pm 1.0 \times 10^2$	$4.5 \pm 0.9 \times 10^2$	$3.9 \pm 0.9 \times 10^2$	$5.2 \pm 0.9 \times 10^2$	$5.6 \pm 1.0 \times 10^2$	<0.001

Transforming growth factor- β isoforms levels between chronic kidney disease patients and the controls.*p-values were calculated by comparing all patients with kidney disease (n=120) to controls (n=40). Transforming growth factor-beta1, 2, and 3 levels were analysed with Bio-Plex Pro™ TGF- β Assays kit. PD, peritoneal dialysis; HD, haemodialysis; CKD, chronic kidney disease; TGF- β , transforming growth factor- β . Continuous data were expressed as mean $\pm$ SD.

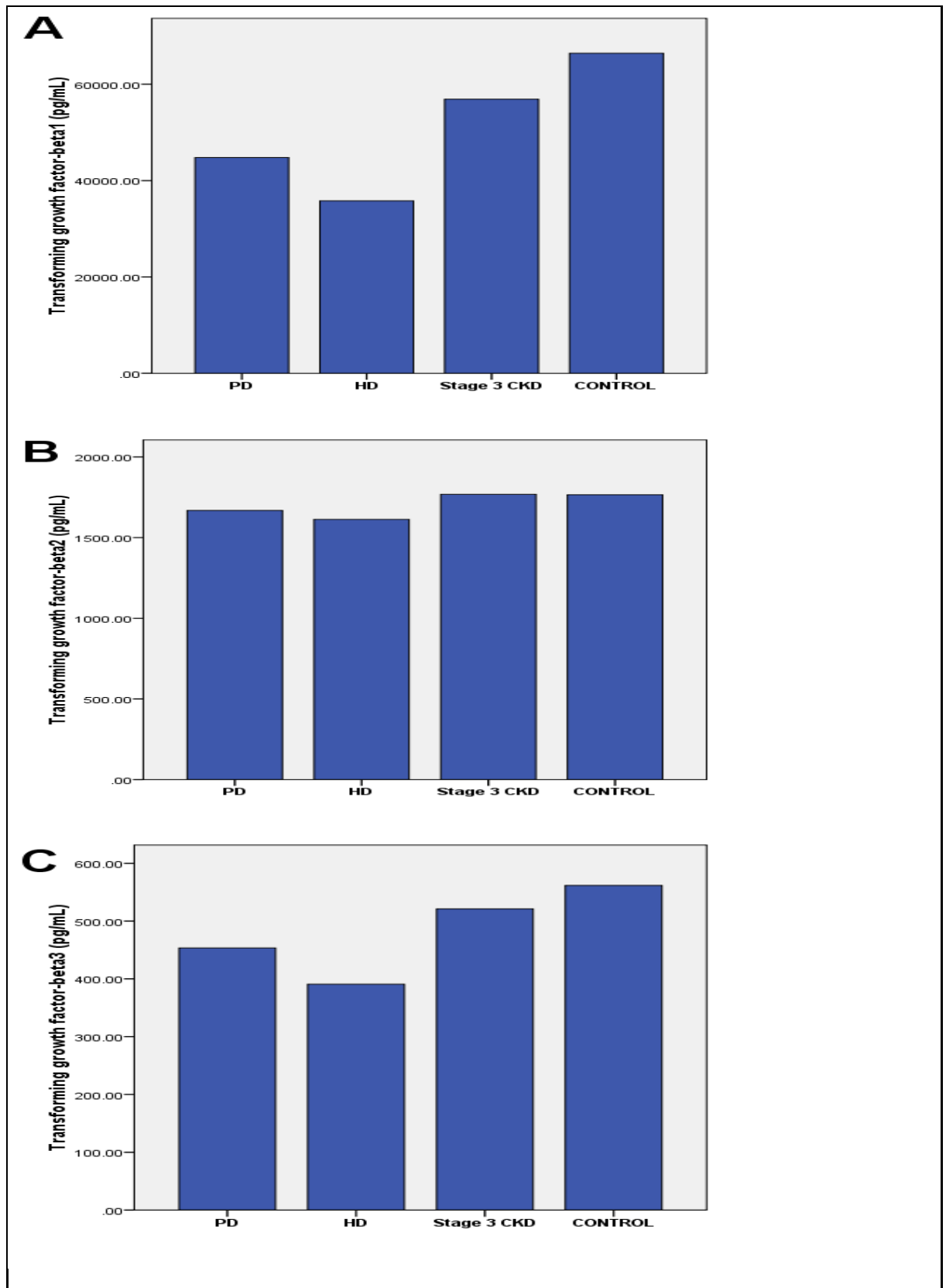


Figure 3.28: Serum transforming growth factor- β 1 (A), β 2 (B) & β 3 (C) in the CKD patients groups and the controls. Bar chart represents mean values for transforming growth factor beta-1, 2 & 3 levels. Transforming growth factor- β 1, 2 & 3 levels were analyzed with Bio-Plex ProTM TGF- β Assays kit.

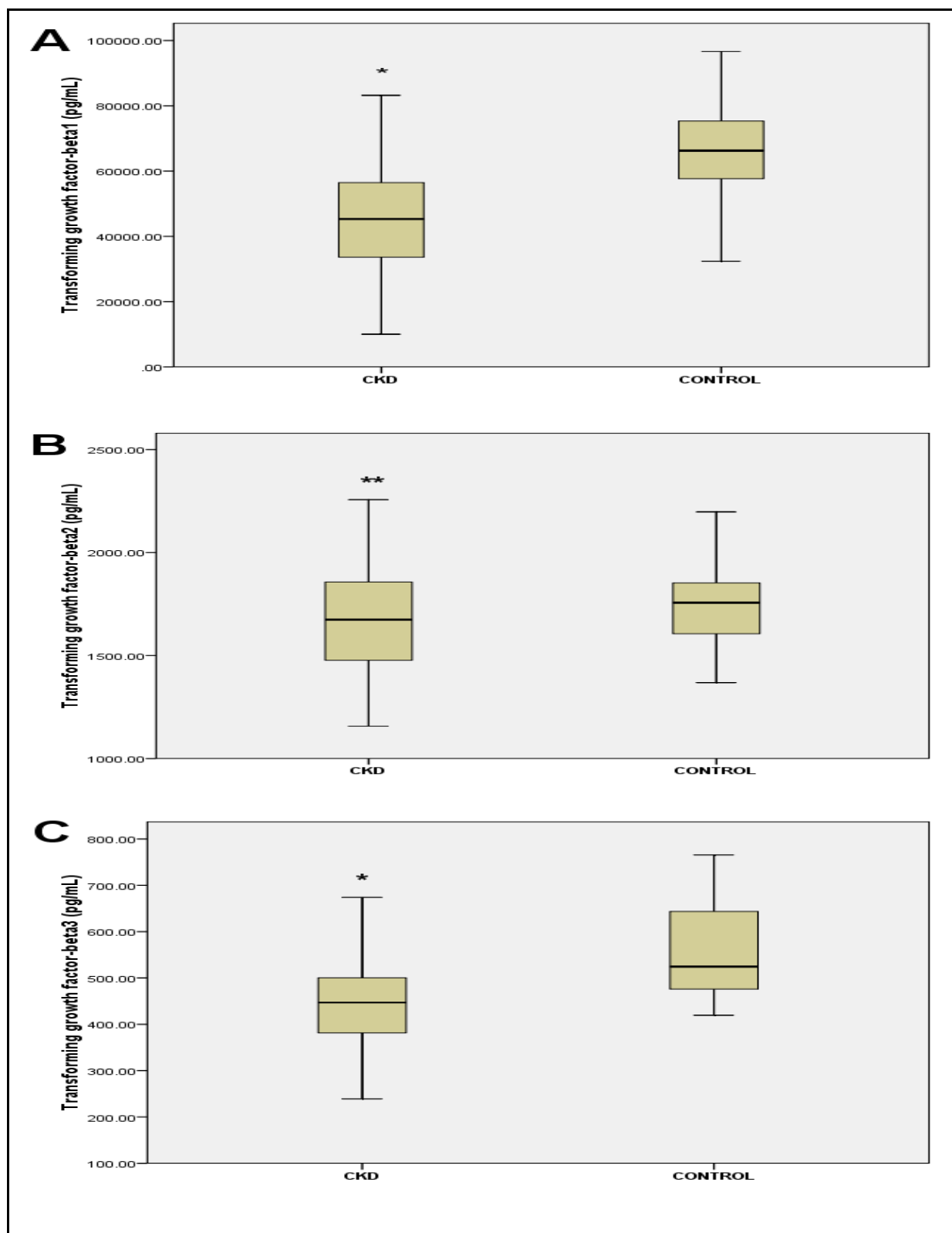


Figure 3.29: Comparison of serum transforming growth factor- β 1 (A), β 2 (B) & β 3 (C) between CKD patients and controls. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Transforming growth factor- β 1, 2 & 3 levels were analyzed with Bio-Plex ProTM TGF- β Assays kit. Serum transforming growth factor- β 1, 2 & 3 levels were compared between CKD patients and controls, * $P < 0.001$, ** $P > 0.05$ compared to controls.

3.19 Transforming growth factor- β 1 isoform and Atherosclerosis

Transforming growth factor- β isoform concentrations were compared between patients with subclinical atherosclerosis and those without atherosclerosis; TGF- β isoform concentrations were significantly lower in the patients with subclinical atherosclerosis compared to patients without atherosclerosis (Figure 3.30a-c). Furthermore, TGF- β 1 and TGF- β 2 levels were significantly lower in patients with carotid plaques compared to those without carotid plaque (TGF- β 1: $3.1 \times 10^4 \pm 1.7 \times 10^4$ versus $4.7 \times 10^4 \pm 1.7 \times 10^4$ pg/ml, $p=0.035$; and TGF- β 2: $1.5 \times 10^3 \pm 0.2 \times 10^3$ versus $1.7 \times 10^3 \pm 0.2 \times 10^3$ pg/ml, $p<0.001$), while there was no difference in the concentration of TGF- β 3 between CKD patients with carotid plaque and those without plaque ($p>0.05$) (Figure 3.31a-c). Overall, TGF- β isoforms had an inverse relationship with CIMT (TGF- β 1: $r=-0.562$, $p<0.001$, (Figure 3.32); TGF- β 2: $r=-0.477$, $p<0.001$; TGF- β 3: $r=-0.442$, $p<0.001$).

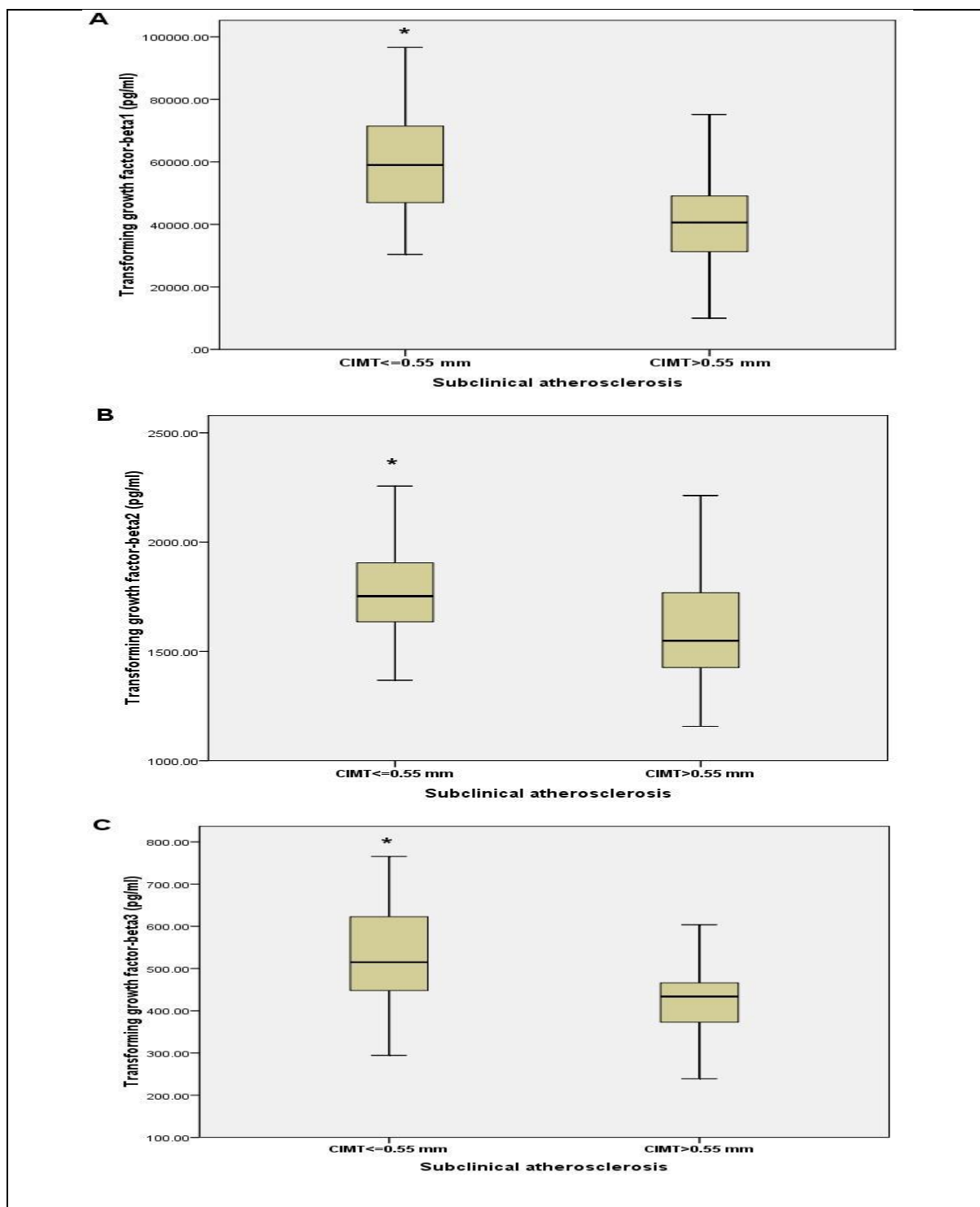


Figure 3.30: Comparison of serum transforming growth factor- β 1 (A), β 2 (B) & β 3 (C) between patients with subclinical atherosclerosis and those without atherosclerosis. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Transforming growth factor- β 1, 2 & 3 levels were analyzed with Bio-Plex Pro™ TGF- β Assays kit. Carotid intima media thickness was measured using B-mode ultrasound. Serum transforming growth factor- β 1, 2 & 3 levels were compared between CKD patients and controls, * $P < 0.001$ compared to controls.

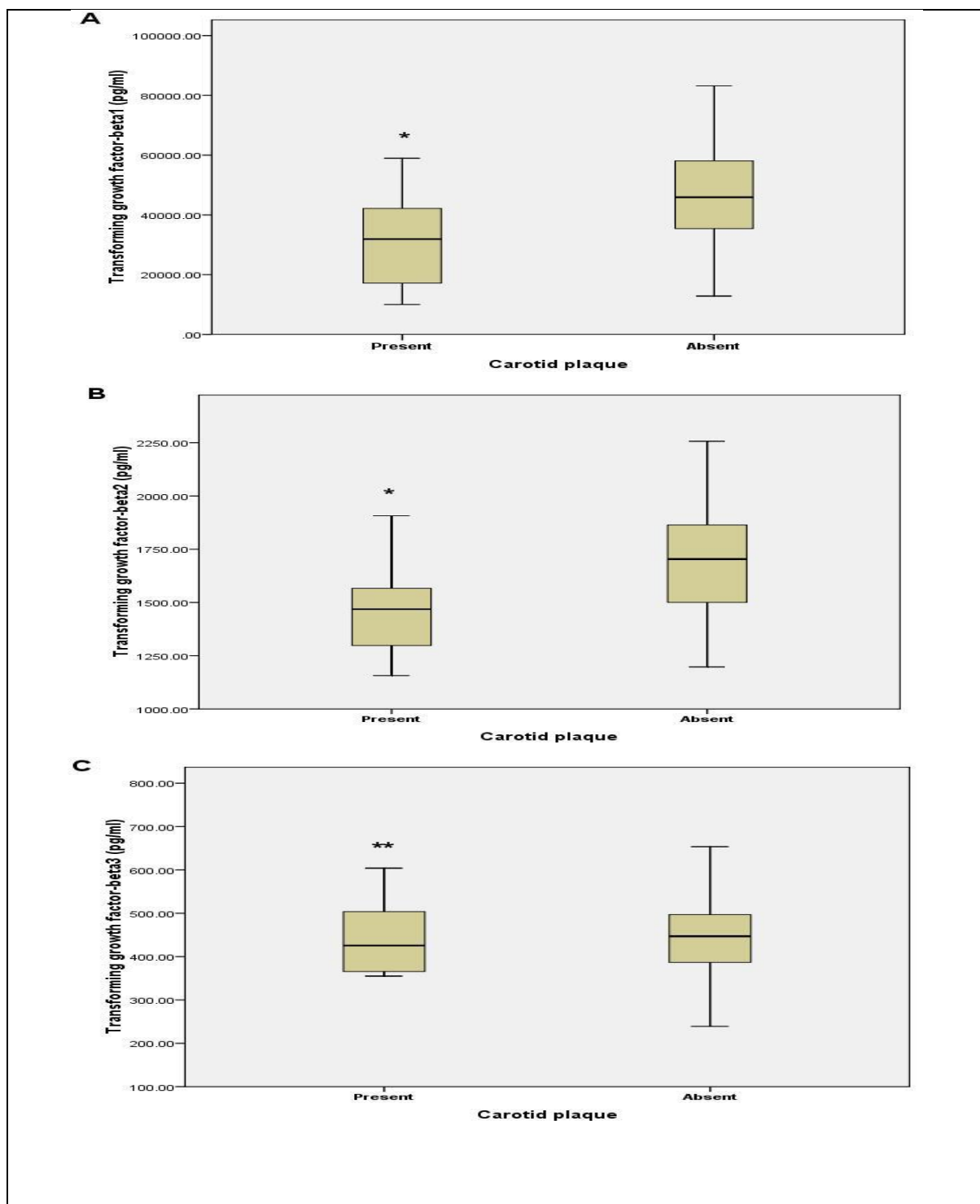


Figure 3.31: Comparison of serum transforming growth factor- β 1 (A), β 2 (B) & β 3 (C) between patients with carotid plaque and those without plaques. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile. Transforming growth factor- β 1, 2 & 3 levels were analyzed with Bio-Plex Pro™ TGF- β Assays kit. Carotid plaque was defined as the echogenic structure protruding into the lumen with the distance between the media adventitia interface and the internal side of the lesion ≥ 1.2 mm. Serum transforming growth factor- β 1, 2 & 3 levels were compared between patients with carotid plaques and those without plaque, * $P < 0.05$, ** $P > 0.05$ compared to those patients without plaque.

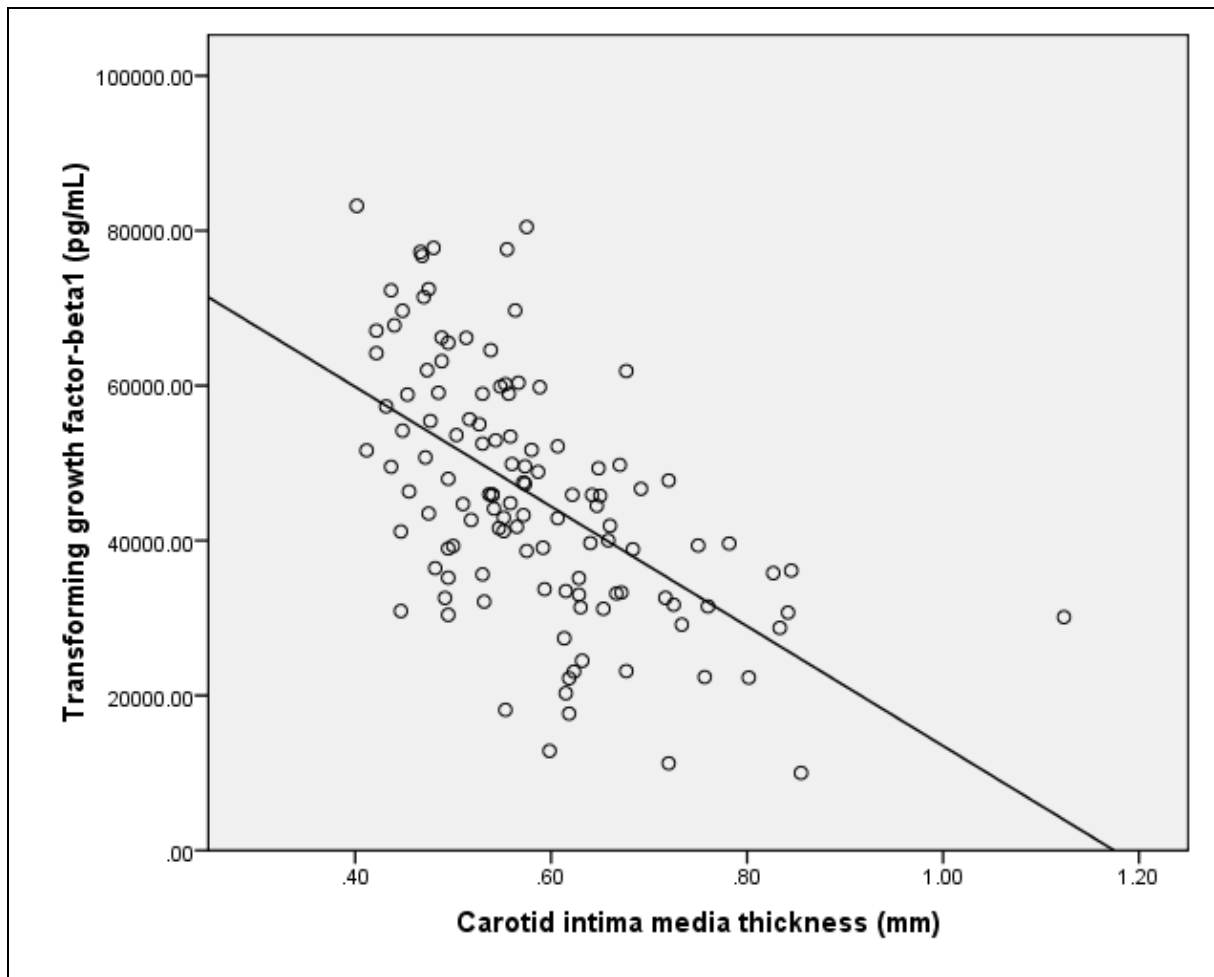


Figure 3.32: Relationship between Transforming growth factor- β 1 and carotid intima media thickness in CKD patients ($r=-0.562$, $p<0.001$). The figure demonstrates an inverse relationship between transforming growth factor- β 1 concentrations and carotid intima media thickness measurements. Association between transforming growth factor- β 1 concentrations and carotid intima media thickness was assessed using Pearson correlation coefficient. Transforming growth factor- β 1 levels were analyzed with Bio-Plex Pro™ Assays kit while carotid intima media thickness was measured using B-mode ultrasound.

3.20 Relationship between TGF- β , pro-inflammatory cytokines and cardiovascular risk factors

In the study population, TGF- β 1 correlated with TGF- β 2 ($r=0.561$, $p<0.001$) and TGF- β 3 ($r=0.645$, $p<0.001$). When TGF- β 1 was correlated with mediators of the endotoxin signaling pathway, a modest relationship was demonstrated between TGF- β 3 and LBP ($r=-0.414$, $p<0.001$), and serum CD14 ($r=-0.350$, $p<0.001$); (Figure 3.33 and 3.34). Transforming growth factor- β showed a weak relationship with oxidized LDL ($r=-0.308$, $p=0.001$) and MCP-1 ($r=-0.246$, $p=0.007$). It also demonstrated a weak negative correlation with CRP ($r=-0.243$, $p=0.007$) and systolic blood pressure ($r=-0.256$, $p=0.005$). TGF- β 3 showed a weak positive correlation with albumin ($r=0.236$, $p=0.010$), while a negative correlation was demonstrated between albumin and CRP ($r=-0.243$, $p=0.007$). Multiple linear regression analysis showed that subclinical atherosclerosis and blood pressure are the independent predictors of TGF- β 1 concentrations ($r^2=0.37$, $p<0.05$) while atherosclerosis and LBP, a marker of circulating endotoxaemia, are the only independent determinants of serum TGF- β 2 and TGF- β 3 levels ($p<0.05$), with $r^2=0.34$ and $r^2=0.32$ respectively.

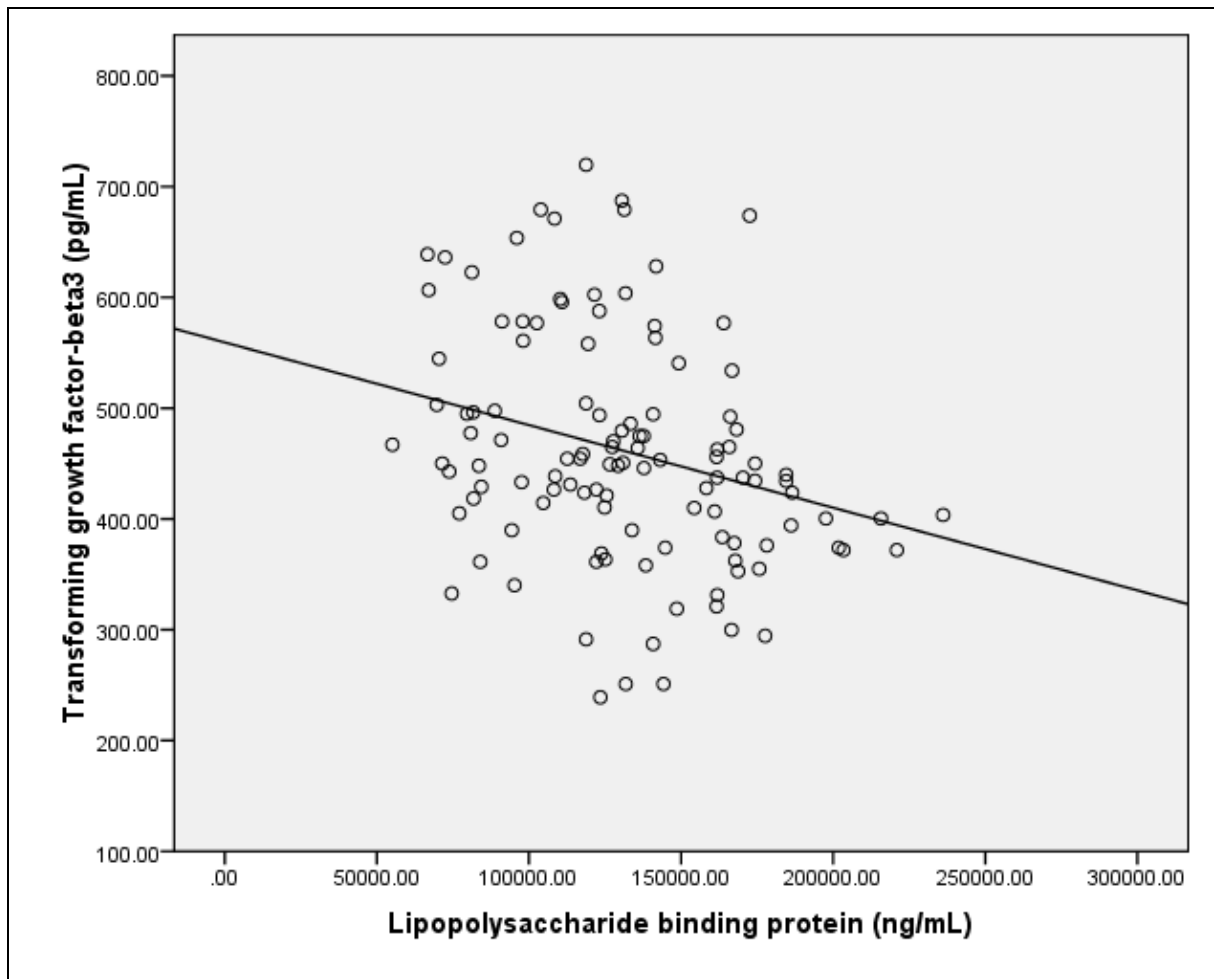


Figure 3.33: Relationship between Transforming growth factor- β 3 and lipopolysaccharide binding protein in CKD patients ($r=-0.414$, $p<0.001$). The figure demonstrates an inverse relationship between transforming growth factor-beta3 concentration and lipopolysaccharide binding protein levels. Association between transforming growth factor- β 3 concentrations and lipopolysaccharide binding protein was assessed using Pearson correlation coefficient. Transforming growth factor- β 3 levels were analyzed with Bio-Plex ProTM Assays kit while lipopolysaccharide binding protein was measured with a human LBP ELISA kit.

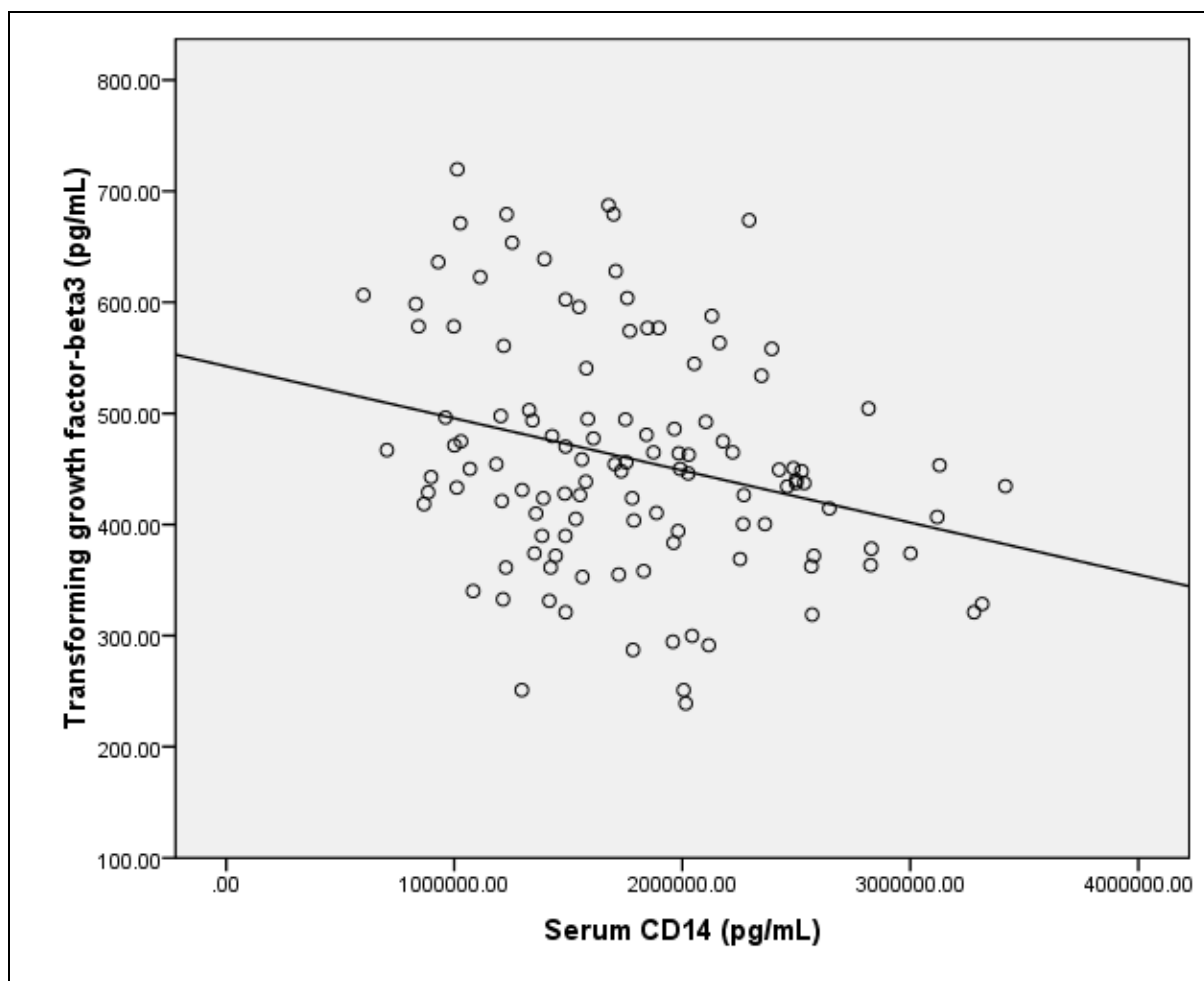


Figure 3.34: Relationship between Transforming growth factor- β 3 and serum sCD14 in CKD patients ($r=-0.350$, $p<0.001$). The figure demonstrates an inverse relationship between transforming growth factor- β 1 concentrations and serum CD14 levels. Association between transforming growth factor- β 1 concentrations and serum CD14 was assessed using Pearson correlation coefficient. Transforming growth factor-beta1 and serum CD14 levels were analyzed with Bio-Plex Pro™ Assays kit.

3.21 Inflammatory cytokine polymorphisms in chronic kidney disease patients

A total of 95, 106, 85, 93, and 101 single nucleotide polymorphisms were successfully genotyped for TNF- α , TGF- β 1, IL-10, IL-6 and IFN- γ respectively. One SNP was genotyped for TNF- α , 2 SNPs for TGF- β 1, 3 SNPs for IL-10, 1 SNP for IL-6 and 1 SNP for IFN- γ . The distribution of the single nucleotide polymorphisms and their genotyping frequencies in the chronic kidney disease patients and the controls are shown in Table 3.12. The TNF- α , TGF- β 1, IL-10, IL-6 and IFN- γ genotypes did not differ between the controls and the CKD patients ($p>0.05$). Similarly, no significant difference was observed in the occurrence of any SNPs between individual CKD subgroups and the controls ($p>0.05$). Further analysis was done to determine whether carriage of genotypes of TGF- β 1 and IL-10 influence the levels of these inflammatory cytokines in the serum of the study participants. In the CKD patients, there was no significant difference in the levels of TGF- β 1 between 58 (71%) of high producers, $4.8 \pm 1.5 \times 10^4$ pg/ml, the 17 (21%) intermediate producers, $4.3 \pm 1.3 \times 10^4$ pg/ml, and the 7 (9%) low producers, $4.9 \pm 1.6 \times 10^4$ pg/ml, Table 3.13. In the control group, similar percentage distribution was found: 15 (63%) of high producers, $6.9 \pm 1.6 \times 10^4$ pg/ml, the 18 (33%) intermediate producers, $6.7 \pm 1.3 \times 10^4$ pg/ml, and the 1 (4%) low producers, 8.9×10^4 pg/ml. In contrast, the IL-6 (high producers) genotype was significantly associated with elevated serum levels of IL-6 in CKD patients when compared with low producer CKD patients, 69 (95%) of high producers, 3.9 ± 4.2 pg/ml and the 4 (5%) low producers, 2.3 ± 1.6 pg/ml, $p=0.048$ (Table 3.14). In the control group, there was no difference in the serum IL-6 between high producer and low producer, 19 (95%) of high producers, 1.6 ± 1.4 pg/ml and the 1 (5%) low producer, 1.4 pg/ml, $p=0.892$. The IL-6 and TGF- β 1 genotypes were not associated with the presence of subclinical atherosclerosis ($p>0.05$).

Table 3.12: Results of TNF α , TGF- β 1, IL-10, IL-6 and IFN- γ genotyping showing genotype distribution and frequency in CKD patients and controls.

Cytokine	Genotype /Producer	All patients	HD	PD	Stage 3 CKD	Control	*P-value
TNF- α		n=75	n=17	n=30	n=28	n=20	
	G/G (low)	46 (61.3%)	11 (64.7%)	15 (50%)	20 (71.4%)	16 (80%)	0.186
	G/A (high)	24 (32%)	5 (29.4%)	12 (40%)	7 (25%)	4 (20%)	0.410
	A/A (high)	5 (6.7%)	1 (5.9%)	3 (10%)	1 (3.6%)	0 (0%)	0.580
TGF- β 1		n=82	n=20	n=32	n=30	n=24	
	T/T G/G (high)	17 (20.7%)	2 (10%)	6 (18.7%)	9 (30%)	2 (8.3%)	0.231
	T/C G/G (high)	41 (50%)	12 (60%)	15 (46.9%)	14 (46.7%)	13 (54.2%)	0.818
	T/C G/C (intermediate)	6 (7.3%)	1 (5%)	2 (6.3%)	3 (10%)	4 (16.7%)	0.229
	C/C G/G (intermediate)	11 (13.4%)	3 (15%)	6 (18.7%)	2 (6.7%)	4 (16.7%)	0.352
	T/T G/C (intermediate)	1 (1.2%)	0 (0%)	1 (3.1%)	0 (0%)	0 (0%)	1.000
	C/C G/C (low)	5 (6.1%)	2 (10%)	1 (3.1%)	2 (6.7%)	1 (4.2%)	0.586
	T/C C/C (low)	1 (1.2%)	0 (0%)	1 (3.1%)	0 (0%)	0 (0%)	1.000
IL-10		n=65	n=13	n=27	n=25	n=20	
	GCC/GCC (high)	10 (15.4%)	2 (15.4%)	4 (14.8%)	4 (16%)	2 (10%)	0.723
	GCC/ACC (intermediate)	12 (18.5%)	2 (15.4%)	5 (18.5%)	5 (20%)	5 (25%)	0.533
	GCC/ATA (intermediate)	9 (13.8%)	1 (7.7)	4 (14.8%)	4 (16%)	2 (10%)	1.000
	ACC/ACC (low)	4 (6.2%)	2 (15.4%)	1 (3.7%)	1 (4%)	2 (10%)	0.622
	ACC/ATA (low)	20 (30.8%)	4 (0330.8)	6 (22.2%)	6 (24%)	7 (35%)	0.786
	ATA/ATA (low)	10 (15.4%)	2 (15.4%)	5 (18.5%)	5 (20%)	2 (10%)	0.723
1L-6		n=73	n=19	n=29	n=25	n=20	
	G/G (high)	65 (89%)	18 (94.7%)	24 (82.8%)	23 (92%)	17 (85%)	0.697
	G/C (high)	4 (5.5%)	1 (5.3%)	1 (3.4%)	2 (8%)	2 (10%)	0.606
	C/C (low)	4 (5.5%)	0 (0%)	4 (13.8%)	0 (0%)	1 (5%)	1.000
IFN- γ		n=78	n=18	n=31	n=29	n=23	
	T/T (high)	3 (3.8%)	0 (0%)	3 (9.7%)	0 (0%)	0 (0%)	1.000
	T/A (intermediate)	24 (30.8%)	5 (27.8%)	9 (29%)	10 (34.5%)	11 (47.8%)	0.143
	A/A (low)	51 (65.4)	13 (72.2%)	19 (61.3%)	19 (65.5%)	12 (52.2%)	0.328

TNF- α , Tumour necrosis factor- α , TGF- β 1, Transforming growth factor- β 1, IL-10, Interleukin-10, IL-6, interleukin-6, IFN- γ , Interferon- γ . A total of 95, 106, 85, 93, and 101 single nucleotide polymorphisms were successfully genotyped for TNF- α , TGF- β 1, IL-10, 1L-6 and IFN respectively. *P-values were calculated by comparing all patients with kidney disease to the controls for each single nucleotide polymorphism (SNP) using Fisher's exact test. From the analysis of the SNPs, there was no difference between patients with chronic kidney disease and the controls ($p>0.05$).

Table 3.13: Producer status and transforming growth factors-β1 in genotyped chronic kidney disease patients (n=82)

Producer status	High	Intermediate	Low	*P-value
Frequency	58/82 (70.7%)	17/82 (20.7%)	7/82 (8.5%)	
TGF-β1 levels (pg/ml)	$4.8 \pm 1.5 \times 10^4$	$4.3 \pm 1.3 \times 10^4$	$4.9 \pm 1.6 \times 10^4$	0.420

DNA samples were genotyped for single nucleotide polymorphism in the transforming growth factor-β1 gene by polymerase chain reaction (PCR) single strand polymorphism (SSP) methodology with the Cytokine Genotyping Tray Kit (One Lambda Inc., Los Angeles, USA). The majority (70.7%) of the CKD patients were high producer of transforming growth factor-β1 while only few were low producer (8.5%). There was no statistically significant difference between high producer and low producer CKD patients. *P-value compared low producer to high producer using Mann-Whitney test.

Table 3.14: Producer status and serum interleukin-6 levels in genotyped chronic kidney disease patients (n=73)

Producer status	High	Low	*P-value
Frequency	69/73 (95%)	5% (4/73)	
IL-6 levels (pg/ml)	3.9 ± 4.2	2.3 ± 1.6	0.048

DNA samples were genotyped for single nucleotide polymorphism in the transforming growth factor-β1 gene by polymerase chain reaction (PCR) single strand polymorphism (SSP) methodology with the Cytokine Genotyping Tray Kit (One Lambda Inc., Los Angeles, USA). The majority (95%) of the CKD patients were high producer of IL-6 while few were low producers (5%). There was statistically significant difference between high IL-6 producers and low IL-6 producers CKD patients. *P-value compared low producer to high producer using Mann-Whitney test.

CHAPTER 4

4.0 DISCUSSION

Inflammation is increasingly recognised as a novel risk factor for CVD in CKD patients (16, 18, 307). Inflammatory markers are directly related to a range of complications in CKD patients including insulin resistance, vascular calcification, accelerated atherosclerosis and increased peritoneal permeability (316). Although data suggests that chronic low grade inflammation occurs frequently in the setting of CKD and CVD (131), possible triggers and mechanisms of immune activation in CKD patients are yet to be fully established. Available evidence suggests a role for circulating endotoxaemia as an inflammatory mediator of CVD in patients with CKD (317-320). However, this hypothesis has never been tested in an indigenous African CKD population. This study therefore investigated endotoxaemia among African CKD patients and also objectively assessed volume overload as a possible trigger for endotoxaemia-related inflammation using a body composition monitor and IVCD measurements. In addition, this study also explored the potential role of the endotoxin signaling pathway to explain the association between circulating endotoxaemia and subclinical atherosclerosis in CKD patients.

The majority of the CKD patients that participated in this study were blacks, constituting 88% of the total study population. This observation is not unexpected because the South African population is predominantly black. There is a slight male preponderance in this study, which is similar to that reported by previous studies (16, 18); however this study differs from the study by Goncalves *et al* (307) which reported a slight female preponderance. Moreover, the study population is relatively younger compared to previously studied CKD population (16, 18, 307). This difference in age could be explained by the fact that, due to the limited resources in South Africa, patients who are older than 60 years, patients with advanced CVD, and diabetic patients with CVD are not accepted onto chronic

dialysis program in public sector institutions. Hypertension is the commonest aetiology of CKD in this study, and was present in about 50% of the patients. This may be due to the predominance of black patients in this study, as well as exclusion of diabetic patients from the study.

This study demonstrated that volume overload was very common across the spectrum of CKD patients. Fluid overload by bio-impedance spectroscopy method occurred in all HD patients (100%), 90% of PD patients and 73% of stage 3 CKD patients in this study and was exceptionally high when compared to previously reported prevalence (using BCM) of 39%, 66.8% and 50% among HD (321), PD (284), and non-dialytic CKD patients (322) respectively, possibly because this study involved predominantly black CKD cohorts, with hypertension (49.2%) and presence of LVH (68%) in a large proportion of the study population.

BCM measurements significantly improved the sensitivity in diagnosis of volume overload when compared with echocardiographic measurements and physical examination. The prevalence of fluid overload using BCM in this study was substantially higher than the diagnostic rates of 52%, 29% and 62% by IVCD, CI and physical examination respectively. This further supports the use of the BIS method in the earlier detection of occult volume overload in 'clinically normovolemic' non-dialytic CKD patients, since strict volume control has been documented to play a crucial role in the amelioration of cardiovascular complications in CKD patients (102, 284) as well as improve survival of dialysis patients (323).

Although a previous report had suggested that IVCD measurements are useful markers for volume overload in CKD patients (307), this study demonstrated that the sensitivity and specificity of IVCD measurements as a diagnostic tool for fluid overload was

relatively low compared to BIS measurement, which was used as the confirmatory test. Perhaps, IVCD measurements could have overestimated the underhydrated state as a result of delay of refilling from the interstitial fluid compartment into the intravascular space (324). In addition, bio-impedance spectroscopy and inferior vena cava diameters measure fluid in different compartments (294, 295).

Volume overload appears to be exacerbated by worsening renal function and was severe in patients on dialysis with significantly reduced residual renal function. Available evidence suggests that the degree of renal impairment was associated with fluid overload (307). This observation was confirmed by this present study; a close relationship was observed between serum creatinine and all markers of volume status, suggesting that impaired excretion of salt and water by the kidneys may contribute to retention of fluid in CKD patients. This had been previously reported in non-dialytic CKD and continuous ambulatory peritoneal dialysis patients (284, 307).

This study established a relationship between volume overload and nutritional status (low serum albumin and nutritional parameters measured by BCM). Overhydrated patients were undernourished, and had lower serum albumin, lean tissue index and fat tissue index. There was an association between markers of volume and malnutrition. There was also a negative correlation between markers of fluid overload and serum albumin. Theoretically, hypoalbuminaemia in an undernourished patient not only brings about reduced plasma oncotic pressure, leading to fluid retention in interstitial space; it may also be a consequence of dilution resulting from volume overload as well as systemic inflammation. Even though this study suggests that hypoalbuminaemia may contribute to fluid retention in CKD patients, it remains to be seen whether nutritional rehabilitation will improve cardiovascular outcomes in CKD patients.

There is increasing evidence that fluid overload is an important and independent predictor of inflammatory activation in CKD. This present study established a close relationship between volume overload and inflammation. This is in keeping with a previous study in PD cohorts that showed an association between hypervolaemia and inflammation (325). This study showed that relative overhydration was common in PD patients with inflammation compared with those without inflammation (325). Similarly, in this present study, it was observed that fluid overloaded patients had elevated mean CRP and IL-6 values, and low serum albumin when matched with those without fluid overload.

On multiple linear regression analysis, serum creatinine, lean tissue index, fat tissue index, CIMA and LAD were identified as independent determinants of fluid overload in CKD patients. In addition, volume overload significantly predicted inflammation in the CKD patients. These findings corroborated earlier studies that showed a link between volume overload and the “malnutrition-inflammation-atherosclerosis” syndrome (326-330). Stenvinkel *et al*, found a strong association between malnutrition, inflammation and occurrence of CVD in patients starting dialysis (330). Likewise, Dermirci *et al* reported a close association between inflammation, atherosclerosis and volume parameters in peritoneal dialysis patients (296). The same group identified LAD as a surrogate marker of fluid overload, confirming the findings of this present study.

This study demonstrated that endotoxaemia is common among CKD patients, and the degree of endotoxaemia is related to the severity of kidney failure; CKD patients had grossly elevated endotoxin levels compared to the controls. This observation is in support of previous studies by Szeto *et al* (16) in peritoneal dialysis patients and McIntyre *et al* (18) involving a spectrum of CKD patients including haemodialysis patients (18). The two studies showed that endotoxin levels were significantly higher among maintenance dialysis patients compared to controls. On the other hand, our study showed that there was no significant

difference in endotoxin levels between the CKD stage 3 cohort and controls. This observation was supported by a previous report that showed no correlation between endotoxin levels and GFR (307). Endotoxin, a macromolecule with a molecular weight of about 1000kD, is normally removed from the circulation by binding to the lipopolysaccharide binding protein, resulting in eventual clearance in the liver and ultimately excreted in bile (159, 331, 332). Correspondingly, grossly elevated levels of endotoxins have been reported in patients with severe liver disease (332).

This study suggests that the haemodialysis treatment modality seems to be responsible for increased exposure to translocated intestinal endotoxin. Post-dialysis endotoxin levels correlated with ultrafiltration volume during haemodialysis therapy. This finding further support the hypothesis that, as a result of increasing shear stress with ultrafiltration, the enteric circulation is exposed to ischaemia, resulting in increased gut permeability, bacteria translocation and circulating endotoxaemia (18, 333). However, this study did not find a significant increment in circulating endotoxin levels following haemodialysis treatment. Clearly, several factors could have accounted for this in the haemodialysis cohort. Notably, the type of dialyzer membrane could have played a role, with the possibility of endotoxin directly binding to the dialyzer membrane during dialysis treatment. Polysulfone-based dialysis membranes, the only membrane type in use at the dialysis unit of CMJAH, have the ability to adsorb endotoxin and other products of complement activation, thereby preventing endotoxaemia during haemodialysis therapy (334). The cellulose-acetate dialyser on the other hand is less biocompatible and was associated with increased circulating endotoxin during dialysis (335). Moreover, heparin (used in prevention of blood clotting during haemodialysis) as well as ethylenediamine tetra-acetic acid (EDTA) have been linked with dose-dependent inhibition of the endotoxin assay using Limulus Amebocyte Lysate (LAL) reagent (18, 336). Furthermore, dilution is the most widely used technique employed to overcome interference

in the samples during endotoxin assay. It is possible to dilute the samples to a point when the interfering factor could no longer affect the test, but at which the endotoxin concentration threshold is still detectable, and this has been helpful in most situations (336, 337). Alternatively, interfering protein in the samples can be denatured through heat treatment, thereby allowing heat-resistant endotoxin to be detected (336, 337). In this work, the samples were diluted until the inhibition was overcome (338). Nevertheless, the aforementioned factors may have accounted for relatively lower endotoxin levels detected in haemodialysis compared to peritoneal dialysis patients.

This study demonstrated that the majority of patients with kidney failure presented with endotoxaemia that was associated with fluid overload. Furthermore, this study also established a close association between volume overload and endotoxaemia, thus supporting the hypothesis that volume overload is associated with generation of the chronic inflammatory response, through endotoxin translocation in patients with gut oedema as a result of volume overload (133, 307). Although previous evidence showed that endotoxaemia was associated with fluid overload, the investigators used inferior vena cava diameter measurement and collapsibility index as a marker of fluid overload (307), both of which are limited by intra- and inter-observer variability (283). The current study, on the other hand, used a body composition monitor – an accurate, safe and inexpensive tool for body fluid measurement in ESRD patients (339). In addition, bio-impedance spectroscopy measures total body water and extracellular water, while inferior vena cava diameter assesses plasma volume and right atrial pressure (294, 295), hence the choice of bio-impedance spectroscopy to assess hydration status in this study.

Although there was a negative correlation between circulating endotoxaemia and haemoglobin levels in PD cohorts, however this study did not demonstrate the same relationship between endotoxin levels and haemoglobin in HD and non-dialytic CKD

patients. Furthermore, there was also a weak association between markers of inflammation and haemoglobin levels in the CKD groups, thus suggesting that the chronic inflammatory state may contribute to severity of anaemia in the studied CKD patients. Available evidence suggests that inflammation is an important predictor of baseline haemoglobin and response to erythropoietin stimulating agents [ESAs] therapy (340). However, this study was not designed to examine the influence of circulating endotoxaemia and inflammatory markers on the doses of ESAs that were administered in the dialysis cohorts.

This study also showed that circulating endotoxins were associated with subclinical atherosclerosis as measured by CIMT, thus corroborating previous reports that suggested that endotoxaemia may be pathogenically linked to accelerated atherosclerosis in CKD patients (16, 341). Furthermore, Stenvinkel *et al.* reported a strong association between malnutrition, inflammation and occurrence of CVD among patients initiating peritoneal dialysis (330). Similarly, our study also demonstrated an association between markers of inflammation (defined by elevated serum interleukin-6 and CRP) and CIMT, a surrogate marker of atherosclerosis. In addition, our study showed an inverse correlation between circulating endotoxaemia and some of the nutritional indices – fat tissue index and fat tissue mass. This data further supports the theory that circulating endotoxaemia may be a component of the so-called “malnutrition-inflammation-atherosclerosis syndrome”.

The findings of this study support the hypothesis that endotoxaemia is related to atherosclerosis in the CKD patients. Multiple linear regression analysis showed that circulating endotoxaemia is an independent predictor of CIMT in the CKD patients. Even though endotoxaemia independently predicts atherosclerosis and was also related to systemic inflammation, this study was not able to establish an association between endotoxaemia and markers of inflammation as well as low serum albumin, an established marker of inflammation, thus suggesting possible multifaceted mechanisms leading to chronic low

grade inflammation in CKD patients. Nevertheless, current evidence suggests that the mechanism by which endotoxaemia causes atherosclerosis is a complex process, initiated and perpetuated by interaction of immune cells with the vascular endothelium, leading to vascular inflammation mediated by cytokines and adhesion molecules, ultimately resulting in plaque formation and CVD (132, 159, 342). Therefore, further prospective study is needed to elucidate the preferential relationship between systemic inflammation and accelerated atherosclerosis in CKD patients.

This study also demonstrated an association between inflammation and LVH, as well as between CIMT and volume overload which is in keeping with the report of Wu *et al* that found a strong association between inflammation and CVD in PD patients (343). The presence of LVH in CKD patients is variable. It has been documented in 50%-97% of ESRD patients (102, 344-347). In this study, LVH was present in 68% of the CKD patients. Foley *et al* described concentric hypertrophy and eccentric hypertrophy in 44% and 30% of their studied population respectively (34). Concentric LVH was the commonest LVH pattern, present in 48% of the CKD population in our study. Concentric LVH is associated with pressure overload (typically accompanying hypertension, arteriosclerosis or occasionally aortic stenosis), and is characterized by increased wall thickness and normal or decreased left ventricular chamber (34). Eccentric LVH is characterized by an increase in myocyte length that is proportional to the increase in left ventricular diameter (34). Risk factors for eccentric LVH include volume overload secondary to salt and water retention, anaemia and arteriovenous fistula (6, 34).

The prevalence of carotid plaques was approximately 7%, which was lower than 14% previously reported by another study that investigated CIMT and CVD risk factors among kidney transplant recipients (KTRs) cohorts receiving treatment at the same health facility (348). This difference may be related to the existence of other CVD risk factors such as

diabetes mellitus, previous history of cardiovascular events as well as long-term exposure to steroid-based immunosuppressive medications in the KTRs cohorts. Thus, these other risk factors might have played a role in KTRs with increased prevalence of carotid plaques. However, it remains to be seen whether oxidative stress and the chronic inflammatory state contribute to the burden and the severity of CVD in renal transplant recipients.

Signs of immune dysfunction and elevated levels of inflammatory mediators were observed in our patients, and increased with severity of CKD. This study demonstrated that serum concentrations of LBP, sCD14, IL-8 and MCP-1 were higher in CKD patients compared with the controls, increased with the degree of renal failure; and correlated with CIMT. Thus, a possible link between cellular immune activation (as measured by serum LBP, sCD14, MCP-1 and IL-8) and atherosclerosis in CKD patients is proposed.

Serum LBP was associated with elevated endotoxin levels. This finding is in support of previous studies that suggested that LBP is a clinical marker of circulating endotoxaemia (230, 231). Also, serum LBP was associated with serum levels of monocyte chemoattractant protein-1, a chemotactic factor, thus providing evidence that is consistent with the role of LBP as an acute phase reactant protein (232, 233). In addition, serum LBP levels had a strong association with sCD14 levels, a pattern recognition receptor, which plays a central immunomodulatory role in response to signaling by the LPS-LBP complex (133, 159), thus supporting a previous study that demonstrated that increased endotoxaemia was associated with raised sCD14 concentrations in healthy humans (237). Furthermore, in support of a previous study that showed that elevated LBP levels are an independent predictor of CAD, our study also established an association between elevated LBP levels and CIMT (349). Similarly, this study showed a positive correlation between CIMT measurements and sCD14, MCP-1 and LBP levels. Taken together, these findings could indicate that innate

inflammatory responses are related to atherogenesis, and may thus be a triggering cascade for accelerated atherosclerosis in CKD patients.

The association of CIMT measurements with lower LBP/sCD14 ratios in combination with relatively lower LBP/sCD14 ratio observed in CKD patients is in support of the hypothesis that chronic low grade inflammatory-response related atherosclerosis is associated with a lower LBP/sCD14 ratio, thus providing the evidence that the capacity of endotoxin to activate vascular cells might be relatively greater in these group of patients. This suggests that lower concentrations of LBP play an enhancing role in the endotoxin-induced activation of peripheral monocytes, tissue macrophages and neutrophils (350). The findings of statistically insignificant difference in LBP/sCD14 ratio between CKD patients and the controls suggests a possible role for a CD14-independent signalling pathways, particularly at endotoxin concentrations that are higher than physiological levels (159).

Furthermore, this study demonstrated that the risk of atherogenesis was associated with higher circulating endotoxaemia. Further analysis also revealed that the risk was significantly predicted by the serum levels of sCD14, IL-8 as well as MCP-1. The data suggested that elevated levels of immune mediators, as measured by sCD14, IL-8 and MCP-1 play a critical role in predicting atherogenic potential of circulating endotoxaemia in CKD patients. This observation corroborated previous reports that identified endotoxaemia associated with high levels of neopterin or soluble interleukin-2 receptors, which are markers of immune activation, as the only independent predictor of vascular risk in a prospective population based study (341).

Oxidized LDL was closely related to CIMT and the occurrence of carotid plaques in the CKD patients. To further assess the association between oxidized LDL and development of early atherosclerosis, patients were subdivided into two groups according to CIMT measurements. Patients with high CIMT measurements had significantly higher oxidized

LDL levels when compared with patients with normal CIMT. These findings taken together are in support of previous studies that demonstrated a significant association between circulating oxidized LDL and the development of atherosclerosis (351, 352). Oxidized LDL may play a significant role in the pathogenesis of acute coronary syndrome by upregulating release of inflammatory cytokines and increasing CRP levels, ultimately leading to plaque rupture and cardiovascular events (133, 159, 246).

This study also clearly demonstrated that peritoneal dialysis patients had markedly elevated cholesterol and triglycerides levels when compared to haemodialysis and stage 3 CKD patients. This pattern of dyslipidaemia observed in peritoneal dialysis patients may be due to chronic exposure to glucose in the PD fluids and concurrent insulin resistance that facilitates transportation of free fatty acids (FFAs) to the liver and subsequent conversion of FFAs to triglycerides or very low density lipoproteins (169). However, this study did not find significant associations between total cholesterol, LDL, HDL and triglycerides and oxidized LDL levels. It is therefore plausible that the serum oxidized LDL and LDL-cholesterol may be under different metabolic control in CKD patients.

The data generated from this study showed that uraemia *per se* may induce an oxidative state in CKD patients. Circulating oxidized LDL was more markedly elevated in CKD patients than the age- and sex-matched controls, with the haemodialysis group exhibiting the highest oxidative burst, demonstrated by significantly elevated levels of circulating oxidized LDL. This seems to support a previous report that stated that dialysis therapy may also contribute to the imbalance between oxidative stress and activity of the anti-oxidative enzymes as well as the anti-oxidant defence mechanisms in patients on chronic dialysis (169). Haemodialysis may further contribute to the severity of oxidative stress through dialyzer incompatibility, leading to activation of macrophages and eventually resulting in oxidative burst. Although peritoneal dialysis is regarded to be more

biocompatible than haemodialysis, elevated levels of markers of oxidative stress have been reported in peritoneal dialysis patients (169). Nevertheless, the contribution of dialysis therapy to the oxidative milieu in ESRD patients is multifaceted; and therefore further studies are needed to delineate this complex and not yet fully understood component of the innate immune response.

This study demonstrated that serum levels of TGF β -1, 2, and 3 are significantly reduced in CKD patients compared to the control group, especially in patients with subclinical atherosclerosis and carotid plaques. This is in agreement with previous studies in non-CKD patients (266) and haemodialysis patients (267). Kempf *et al* (353) also demonstrated reduced expression of TGF β -1 by peripheral leucocytes in patients who had acute myocardial infarction. Similarly, Erren *et al* showed that TGF β -1 concentrations are low in patients with coronary artery disease and peripheral vascular disease (354). Theoretically, the inverse relation TGF β isoforms and accelerated atherosclerosis in the CKD patients may be related to the anti-proliferative and cardio-protective properties of these immuno-modulatory cytokines. Transforming growth factor- β 1, the most extensively studied of the three closely related isoforms of TGF β , counteracts vascular inflammation by inhibiting the synthesis of tumour necrosis factor- α or by down-regulating the pro-inflammatory effects of IL-1 β and interferon- γ (262), ultimately leading to reduction of inflammatory cytokine-induced VCAM-1, chemotaxis and leucocyte adhesion to vascular endothelial lining, and decreased macrophage activity (263, 264). These are consistent with the hypothesis that TGF β -1 plays a role in the maintenance of normal vascular integrity.

There is no consensus about the role of TGF β -1 in the process of atherosclerosis and restenosis. Some studies have reported an association between elevated TGF β -1 levels and vascular restenosis lesions (355, 356). In contrast, other authors have showed that decreased activation of TGF β -1 contributes to progression of atherosclerosis (265, 266). Nevertheless,

the absence of the anti-proliferative effects of TGF- β 1 in the blood vessels leads to increased chemotaxis, deposition of extracellular matrix, proliferation of vascular smooth muscle cells and decreased apoptosis, thereby facilitating progression of atherosclerosis or restenotic vascular lesions (357, 358).

The negative associations between TGF- β 1 and some of the mediators of the endotoxin-signaling pathway (LBP, sCD14, MCP-1, oxidized LDL) which have been shown to contribute to immune activation and subclinical atherosclerosis in this study, further highlight the cardiovascular protective effect of TGF- β 1. This suggests that reduction of protective signaling of TGF- β 1 results in increased susceptibility to atherosclerosis in response to pro-inflammatory stimuli. Clearly, more studies are needed to determine the role of the endotoxin-signaling pathway on TGF- β isoforms levels.

Hypertension which is an established risk factor for myocardial infarction seems to influence TGF- β 1 levels partially. This observation was supported by the report of an inverse relationship between TGF- β 1 polymorphisms and hypertension by Cambien *et al* (359). Gender, also to some extent, affected TGF- β 1 concentrations in the study population. There was a weak association between TGF- β 1 and gender. This finding is in support of a previous study in non-CKD patients that reported an association between TGF- β 1 levels and gender (266). The author postulated that serum TGF- β 1 levels in women may be under the control of antioestrogen hormones, ultimately resulting in the secretion of TGF- β 1 by foetal human fibroblasts.

The finding that TGF- β 1 levels showed a modest significant correlation with CRP and albumin (a marker of malnutrition) was consistent with the report of Stefoni *et al* (267). Stenvinkel *et al* (328) reported a link between malnutrition, inflammation and cardiovascular disease. Therefore, the association between serum TGF- β 1 levels and CRP may suggest the

degree of vascular inflammation, as with low serum albumin, TGF- β 1 levels may suggest a state of malnutrition in CKD patients.

In agreement with a previous report (328), this study also demonstrated lower levels of TGF- β levels in haemodialysis patients compared to the controls. According to the report, the low levels of TGF- β in haemodialysis patients may be due to subclinical endothelial damage or a result of heparin-mediated activation of TGF- β signaling pathways which may lead to reduced serum levels of TGF- β isoforms, as a consequence of exhaustion resulting from the repeated binding of TGF- β to various TGF- β receptors. However, heparin-mediated activation of TGF- β pathway might not explain the low levels of TGF- β in peritoneal dialysis patients, since this group of patients were not exposed to heparin. Multiple linear regression analysis showed that subclinical atherosclerosis is an independent determinant of TGF- β levels in all CKD patients. It is therefore plausible that subclinical endothelial damage leading to progression of atherosclerosis may hold the key to interpreting the lower levels of TGF- β isoforms in the CKD patients compared to the controls. Nonetheless, additional studies are needed to explore the complex biology of TGF- β signaling pathways in CKD patients. The contributory role of TGF- β to severity of atherosclerosis as well as correlations between TGF- β levels and other CVD risk factors in CKD patients should be subjected to further evaluation by larger longitudinal controlled studies.

A major finding of this study is the significant association between the IL-6 polymorphisms and elevated serum levels of IL-6 in the CKD patients. The majority of the study population had G/G or G/C genotypes (otherwise referred to as high producers) and had significantly elevated IL-6 levels compared to CC genotype subjects with lower IL-6 levels. These findings are supported by a previous study that showed that IL-6 levels are lower in individuals with CC genotype, compared with GG or GC subjects (360). In addition, this study did not find any association between IL-6 polymorphism and the presence of

subclinical atherosclerosis in the CKD patients, and this may be explained by the fact that the majority of the study population had G/G or GC genotype which seems to protect against the development of atherosclerosis; more so, no correlation was found between IL-6 levels and subclinical atherosclerosis in the CKD patients. There are no conclusive data in the literature on the correlation of IL-6 polymorphism to atherosclerosis. Some studies have shown that individuals who are homozygous for the C alleles had a higher risk for significant coronary artery stenosis (361, 362). On the contrary, another study has reported a close association between the G-allele and carotid intima media thickness, another surrogate marker of atherosclerosis(363). These contradictory findings may be due to the different methods of assessing carotid atherosclerosis, the severity of the disease as well as the racial difference among the study population.

Available evidence from this work rule out the possibility that genetic patterns of production ultimately affect serum levels of TGF- β 1 in the CKD patients. The prevalence of low producers among the CKD patients was only 8%, and there was no difference in the levels of TGF- β 1 between low producers and high producers. This is in keeping with previous findings (267). Although there is no explanation for this result however, given the complexity and the variety of the TGF- β family responses, future studies targeted at exploring potential defects in the activation and signalling pathway of TGF- β 1 might well hold the key to understanding the mechanisms leading to low serum TGF- β 1 levels in CKD patients.

A major limitation of this study is the relatively small number of successfully genotyped SNPs in the patients and the controls as well as the predominance of black patients in the study. This might have limited the statistical power of the study to detect any difference in the genetic associations between the CKD patients and the controls, thus

potentially biasing the outcome of the study. These results should be replicated in a larger and more diverse CKD population to determine if these findings are generalizable.

This is a single-center descriptive study where volume overload and nutritional status were defined by a body composition monitor device as designed by the manufacturer. There is lack of local data on validation of BIS in healthy populations. Nevertheless, all output parameters of the body composition monitor have thus far been validated against the gold standard reference method (by isotope dilution) in both patients and healthy individuals (299, 300, 364, 365). Most importantly, the BIS measurement technique is highly reproducible and very specific in the detection of both nutritional and fluid status (301-303). Future longitudinal studies are therefore needed to determine the impact of guided optimization of fluid status on malnutrition, inflammation and CVD in CKD patients.

Even though this study provided strong evidence linking endotoxaemia to CIMT, an important risk factor for cardiovascular morbidity and mortality in CKD patients on maintenance dialysis (366, 367) as well as pre-dialytic CKD patients (368), the findings of this study can only be regarded as preliminary considering the study design, which was cross sectional. This study did not allow for evaluation of cause and effect relationship between endotoxaemia-related immune activation and atherosclerosis. A prospective epidemiological study is needed to determine the potential contributory role of endotoxaemia-induced immune activation to the risk of incident atherosclerosis in the African CKD populations. Also, additional studies are needed to determine whether elevated circulating endotoxaemia could predict cardiovascular outcomes in CKD patients with an attendant high burden of CVD.

Measurement of TLR-4 levels or genetic analysis for TLR-4 polymorphism was not done in this study. Recently, TLR-4 polymorphism was shown to be associated with reduced incidence of CVD in a Caucasian population (369). It remains to be seen whether expression

of TLR-4 or mutations in TLR-4 gene will predict susceptibility to atherosclerosis and CVD in African CKD patients.

Another aspect that requires further study is the investigation of therapeutic strategies that could potentially lead to the reduction of systemic inflammation in CKD patients through anti-inflammatory properties. Available evidence suggested that sevelamer and statins may have additional protective roles in blocking some of the endotoxin signaling pathways, leading to a decrease in inflammatory response (248, 256). Therefore, a larger and sufficiently powered randomized controlled trial may possibly give an insight into whether controlling inflammation may improve cardiovascular outcomes or not.

Another possible limitation is the use of malondialdehyde (MDA)-LDL as the only marker of oxidized LDL in this study. The study was not designed to evaluate the presence and significance of other markers of oxidized LDL such as 4-hydroxynonenal (HNE)-LDL and oxidized phospholipids in patients with CKD. Moreover, oxidized LDL is heterogeneous in nature. Possibly, measurement of a single marker of oxidized LDL could have resulted in underestimation of oxidative stress in the CKD population. Perhaps, other markers of oxidized LDL may be more associated with mechanisms of endotoxaemia-induced atherosclerosis than MDA-LDL; thus, providing a plausible explanation for the inability of this study to establish a direct link between circulating endotoxin levels and oxidized LDL (MDA-LDL). Therefore additional studies are needed to evaluate the occurrence and to determine the importance of other epitopes of oxidized LDL in the pathogenesis of subclinical atherosclerosis in African CKD patients. In addition, because this was a cross-sectional study, the causal relationship between oxidized LDL and early atherosclerosis remains to be proven. Larger longitudinal studies are therefore also needed to elucidate the relationship between circulating oxidized LDL levels and subclinical atherosclerosis.

In summary, volume overload was common across the spectrum of the South African CKD patients. Bio-impedance spectroscopy device and IVCD measurements are useful, inexpensive and simple tools for evaluation of hydration status in CKD patients. Bio-impedance spectroscopy measurement is a more sensitive method of detecting volume overload in ‘clinically euvolaemic’ non-dialytic CKD patients. Moreover, technical and time-based limitations should be considered during IVCD measurements. Fluid overload was aggravated by deteriorating renal function. Volume overload was independently predicted by uraemia, inflammation, malnutrition, atherosclerosis and left atrial dimension. The correlation between inflammation, malnutrition, atherosclerosis and markers of volume overload suggests that volume overload may play a role in the pathogenesis of the “malnutrition-inflammation-atherosclerosis” syndrome in CKD patients

Chronic kidney disease patients presented with overt fluid overload and significant endotoxaemia, which increases in advanced stages of CKD. Specifically, haemodialysis may play a role in the generation of endotoxaemia, through the induction of circulatory haemodynamic stress, leading to intestinal ischaemia and bacteria translocation from the gut. This study explored the association between endotoxaemia and accelerated atherosclerosis among an indigenous black CKD population. Circulating endotoxaemia was related to systemic inflammation and subclinical atherosclerosis as measured by CIMT. This study also provided additional evidence that circulating endotoxaemia is possibly a component of the “malnutrition-inflammation-atherosclerosis syndrome”.

The study also showed that endotoxaemia was related to the degree of severity of immune activation and atherosclerosis. Additional findings from this study suggested that the risk of subclinical atherosclerosis was determined by elevated serum levels of sCD14, IL-8 as well as MCP-1, thereby providing evidence that severe immune activation may amplify the role of endotoxin as a pro-inflammatory mediator of atherosclerosis in CKD patients. There is

an association between oxidized LDL, CIMT and occurrence of carotid plaques in the CKD patients. Oxidized LDL may play an important role in the pathogenesis of accelerated atherosclerosis in CKD patients. It is plausible that the concentrations of circulating oxidized LDL in the CKD patients are independent of serum lipids levels, indicating that the serum oxidized LDL and LDL-cholesterol may be under different metabolic control.

This study also demonstrated a significantly lower serum levels of TGF β -1, 2, and 3 in the CKD patients compared to the control group, especially in patients with subclinical atherosclerosis and carotid plaques, thus suggesting that reduced levels of TGF β isoform may contribute to the susceptibility and severity of atherosclerosis in CKD patients. Our study also confirmed a close association between IL6 polymorphisms (-174G/G and -174G/C) and elevated IL-6 levels in CKD patients.

CHAPTER 5

5.0 CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated that volume overload was common across the spectrum of CKD patients and was associated with the severity of kidney failure, inflammation, malnutrition, atherosclerosis and cardiac enlargement. Bio-impedance spectroscopy device is a more sensitive tool than IVCD measurement for the assessment of fluid status in clinically euvolaemic non-dialytic CKD patients. Circulating endotoxaemia is also common among the CKD patients and is related to volume overload and aggravated by initiation of dialysis.

This study is the first to explore the potential role of some elements of the endotoxin signaling receptor complex – to explain the association between circulating endotoxin and atherosclerosis in African CKD patients. Clearly, circulating endotoxaemia was related to accelerated atherosclerosis as measured by CIMT, a surrogate marker of atherosclerosis. The risk of atherosclerosis was associated with serum levels of endotoxins, with excess risk confined to the group with high endotoxin levels. This study also provided evidence to suggest that circulating endotoxaemia may contribute to atherosclerosis by maintaining a heightened state of inflammatory response in CKD patients with volume overload. CKD patients with high circulating endotoxaemia in combination with high levels of sCD14, IL-8 or MCP-1 showed markedly elevated risk of early atherogenesis. The findings therefore suggest that the atherogenic predictive power of endotoxin is significantly increased by the presence of high concentrations of immune mediators in CKD patients. Even though endotoxaemia independently predicted atherosclerosis, this study however, was not able to establish an association between endotoxaemia and inflammatory markers, thus suggesting possible multifaceted mechanisms leading to chronic low grade inflammation in CKD

patients. Furthermore, the additive contribution from the low serum TGF- β levels may increase the severity of atherosclerosis in CKD patients.

Oxidized LDL concentrations were associated with CIMT, carotid plaques and left ventricular dysfunction. Elevated levels of oxidized LDL – indicative of oxidative stress – may play an important role in the pathogenesis of CVD in CKD patients. Notably, the data showed that oxidized LDL levels in CKD patients are independent of serum lipids levels, indicating that the serum oxidized LDL and LDL-cholesterol may be under a different metabolic control. Finally, there is a close association between IL-6 polymorphisms (G/G and G/C) and elevated serum IL-6 levels in CKD patients.

Limitations of this study

The major limitation of this study was the cross sectional design. The findings can be regarded as a preliminary report. The cause and effect relationship between endotoxin levels and accelerated atherosclerosis remain to be established. Moreover, measurement of TLR-4 levels or genetic analysis for TLR-4 polymorphism was not done in this study. This could have helped to ascertain whether the presence of mutation in TLR-4, an integral part of endotoxin signalling, will influence occurrence of atherosclerosis among indigenous African CKD patients. Although this study provided evidence that suggests that chronic inflammatory state may contribute to severity of anaemia in the studied CKD patients, however it did not evaluate the influence of circulating endotoxaemia and inflammatory markers on the doses of ESAs received by the dialysis patients. Additional future studies will be needed to evaluate the influence of circulating endotoxaemia on the response to ESAs therapy in end stage renal disease patients. Moreover, the use of malondialdehyde (MDA)-LDL as the sole marker of oxidized LDL in this study could have resulted in underestimation of oxidized LDL in the studied CKD population since oxidized LDL is heterogeneous in nature, comprising other epitopes such as 4-hydroxynonenal (HNE)-LDL and oxidized phospholipids.

The predominance of black patients in the study might have limited the statistical power of the study to detect any difference in the genetic associations between the CKD patients and the controls, thus potentially biasing the outcome of the study.

Recommendations

1. As endotoxaemia is common in CKD patients, and related to accelerated atherosclerosis, it is recommended that, prevention of endotoxaemia, through treating foci of endotoxin (periodontal disease, catheters, vascular access) or reducing bacteria translocation from the gut and/or endotoxin-induced vascular inflammation (by cholestyramine, sevelamer carbonate and statins) may potentially reduce the chronic inflammatory state in CKD patients.
2. The data provided evidence that haemodialysis may play a role in inducing haemodynamic stress during haemodialysis therapy, thereby predisposing to reduction of intestinal perfusion and exposure to circulating endotoxaemia. It is recommended that focus should be on reduction of circulatory stress during haemodialysis therapy as a potential therapeutic strategy to reduce cardiovascular morbidity and mortality.
3. Volume overload was common across the spectrum of studied CKD patients, and is related to subclinical atherosclerosis and cardiac enlargement. It is therefore recommended that guided optimization of control of fluid status and ideal dry weight be adhered to, especially in patients on dialysis, using a body composition monitor, as this study clearly demonstrated that body composition monitor measurements are useful in the early detection of volume overload in ‘clinically euvolemic’ non-dialytic stage 3 CKD patients.
4. A prospective epidemiological study is needed to determine the potential contributory role of immune activation and oxidative burst on the risk of incident atherosclerosis and CVD among indigenous African CKD populations.

5. A larger and sufficiently powered randomized controlled trial may possibly give insight into whether controlling endotoxin-related inflammation may improve cardiovascular outcomes or not.
6. A study is needed to determine the importance of other epitopes of oxidized LDL in the pathogenesis of subclinical atherosclerosis in CKD patients.
7. The results of the cytokine genotyping should be reproduced in a larger CKD population from different ethnic groups to determine if the findings of this study are generalizable.

REFERENCES

1. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis.* 2002;39(2 Suppl. 1):S1-266.
2. Hajhosseiny R, Khavandi K, Goldsmith DJ. Cardiovascular disease in chronic kidney disease: untying the Gordian knot. *Int J Clin Pract.* 2013;67(1):14-31.
3. Naicker S. End-stage renal disease in sub-Saharan Africa. *Ethn Dis* 2009;19:S1-S15.
4. Matsha T, Yako Y, Rensburg M, Hassan M, Kengne A, Erasmus R. Chronic kidney diseases in mixed ancestry South African populations: prevalence, determinants and concordance between kidney function estimators. *BMC Nephrology* 2013;14:75.doi:10.1186/471-2369-14-75.
5. Daly C. Is early chronic kidney disease an important risk factor for cardiovascular disease? A background paper prepared for the UK Consensus Conference on early chronic kidney disease. *Neph Dial Transplant.* 2007;22(Suppl.9):19-25.
6. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, et al. Kidney disease as a risk factor for development of cardiovascular disease: A statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation.* 2003;108:2154–69.
7. Yeun JY, Levine RA, Mantadilok V, Kaysen GA. C-reactive protein predicts all-cause and cardiovascular mortality in hemodialysis patients. *Am J Kidney Dis.* 2000;35:469-76.
8. Stehouwer C, Smulders Y. Microalbuminuria and risk for cardiovascular disease: analysis of potential mechanisms. *J Am Soc Nephrol.* 2006; 17:2106-11.
9. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem.* 2002;71:635–700.

10. Andreassen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Møller K. Human Endotoxemia as a Model of Systemic Inflammation. *Current Medicinal Chemistry*. 2008;15:1697-705.
11. Li L, Messas E, Batista EL, Jr., Levine RA, Amar S. Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E deficient murine model *Circulation* 2002;105:861-7.
12. Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E, Oberhollenzer F, et al. Chronic infections and the risk of carotid atherosclerosis: Prospective results from a large population study. *Circulation*. 2001;103:1064-70.
13. Wiedermann CJ, Kiechl S, Dunzendorfer S, Schratzberger P, Egger G, Oberhollenzer F, et al. Association of endotoxemia with carotid atherosclerosis and cardiovascular disease. Prospective results from the Bruneck study. *J Am Coll Cardiol*. 1999;34: 1975-81.
14. Lehr HA, Sagban TA, Ihling C, Zahringer U, Hungerer KD, Blumrich M, et al. Immunopathogenesis of atherosclerosis: endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet. *Circulation*. 2001;104:914-20.
15. Wiedermann CJ, Kiechl S, Schratzberger P, Dunzendorfer S, et al. The role of immune activation in endotoxin-induced atherogenesis. *Journal of Endotoxin Research*. 2001;7:322-6.
16. Szeto C, Kwan BC, Chow K, Lai K, Chung K, et al. Endotoxaemia is related to systemic inflammation and atherosclerosis in peritoneal dialysis patients. *Clin J Am Soc Nephrol*. 2008; 3: 431-6.
17. Terawaki H, Yokoyama K, Yamada Y, Maruyama Y, Lida R, et al. Low-grade endotoxaemia contributes to chronic inflammation in haemodialysis patients: Examination with a novel lipopolysaccharide detection method. *Therapeutic Apheresis and Dialysis*. 2010;14(5):477-82.

18. McIntyre CW, Harrison LEA, Eldehni MT, Jefferies HJ, Szeto C, et al. Circulating Endotoxaemia: A Novel Factor in Systemic inflammation and Cardiovascular Disease in Chronic Kidney Disease. *Clin J Am Soc Nephrol*. 2011;6:133-41.
19. Di Angelantonio E, Chowdhury R, Sarwar N, Aspelund T, Danesh J, Gudnason V. Chronic kidney disease and risk of major cardiovascular disease and non-vascular mortality: prospective population based cohort study. *BMJ*. 2010;341:c4986. doi:10.1371/journal.pmed.0050207.
20. Matsushita K, van der Velde M, Astor BC, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet*. 2010;375:2073-81.
21. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and risks of death, cardiovascular events, and hospitalisation. *N Engl J Med*. 2004;351:1296-305.
22. Perkovic V, Verdon C, Ninomiya T, et al. The relationship between proteinuria and coronary risk: a systematic review and meta-analysis. *PLoS Med*. 2008; 5: e207. doi: 10.1371/journal.pmed.0050207.
23. Ljungman S, Wikstrand J, Hartford M, Berglund G. Urinary albumin excretion- a predictor of risk of cardiovascular disease. A prospective 10-year follow-up of middle-aged nondiabetic normal and hypertensive men. *Am J Hypertens*. 1996;9:770-8.
24. Miettinen H, Haffner SM, Lehto S, Ronnema T, Pyorala K, Laakso M. Proteinuria predicts stroke and other atherosclerotic vascular disease events in non-insulin-dependent diabetic subjects. *Stroke*. 1996;27:2033-9.
25. Schmieder RE, Mann JF, Schumacher H, et al. Changes in albuminuria predict mortality and morbidity in patients with vascular disease. *J Am Soc Nephrol*. 2011;22:1353-64.
26. National Institute of Diabetes and Digestive and Kidney Diseases USRDS. USRDS 2009 annual data report: atlas of chronic kidney disease and end-stage renal disease in the

- United States. Bethesda, Md: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. 2009.
27. Goldsmith DJ, Covic A. Coronary artery disease in uremia: etiology, diagnosis, and therapy. *Kid Int.* 2001;60:2059-78.
28. Manske CL, Thomas W, Wang Y, Wilson RF. Screening diabetic transplant candidates for coronary artery disease - identification of a low-risk subgroup. *Kid Int.* 1993;44: 617-21.
29. Liu H, Yan L, Ma GS, Zhang LP, Gao M, Wang YL, et al. Association of chronic kidney disease and coronary artery disease in 1,010 consecutive patients undergoing coronary angiography. *J Nephrol.* 2011;25(2):219-24.
30. Fishbein GA, Fishbein MC. Arteriosclerosis: rethinking the current classification. *Arch Pathol Lab Med.* 2009 133:1309-16.
31. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation.* 1995 92:1355-74.
32. Micheletti RG, Fishbein GA, Currier JS, et al. Mönckeberg sclerosis revisited: a clarification of the histologic definition of Mönckeberg sclerosis. *Arch Pathol Lab Med.* 2008;132: 43-7.
33. Essig M, Escoubet B, de Zuttere D, et al. Craefeiesockd. Cardiovascular remodelling and extracellular fluid excess in early stages of chronic kidney disease. *Nephrol Dial Transplant.* 2008;23:239-48.
34. Foley RN, Parfrey PS, Harnett JD, et al. Clinical and echocardiographic disease in patients starting end-stage renal disease therapy. *Kidney Int.* 1995;47:186-92.

35. Kannel WB, Dawber TR, Kagan A, Revotskie N, Stokes J 3rd. Factors of risk in the development of coronary heart disease—six year follow-up experience: the Framingham Study *Ann Intern Med.* 2009;55:33–50.
36. McGill HC Jr., McMahan CA, Gidding SS. Preventing heart disease in the 21st century: implications of the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study *Circulation.* 2008;117:1216–27.
37. Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, et al. American Heart Association Expert Panel on Subclinical Atherosclerotic Diseases and Emerging Risk Factors and the Stroke Council. Criteria for evaluation of novel markers of cardiovascular risk: a scientific statement from the American Heart Association. *Circulation.* 2009;119:2408-16.
38. Edmunds ME, Russell GI. Hypertension in renal failure. In: Swales JD, editor. *Textbook of hypertension.* Oxford, UK: Blackwell Scientific Publications; 1994. p. 831-57.
39. Boudville N, Prasad GV, Knoll G, Muirhead N, Thiessen-Philbrook H, Yang RC, et al. Meta-analysis: risk for hypertension in living kidney donors. *Ann Intern Med.* 2006;145:185-96.
40. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R. Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* 2002;360:1903–13.
41. Cushman WC, Evans GW, Byington RP, Goff DC Jr, Grimm RH Jr, Cutler JA, et al. Effects of intensive blood-pressure control in type 2 diabetes mellitus. *N Engl J Med* 2010;362(17):1575-85.

42. Myers OB, Adams C, Rohrscheib MR, Servilla KS, Miskulin D, Bedrick EJ, et al. Age, race, diabetes, blood pressure, and mortality among hemodialysis patients. *J Am Soc Nephrol.* 2010; 21(11):1970-8.
43. KDIGO Clinical Practice Guideline for the Management of Blood Pressure in Chronic Kidney Disease. *Kidney International Supplements.* 2012;2(5):337-414.
44. Wright JT Jr, Bakris G, Greene T, Agodoa LY, Appel LJ, Charleston J, et al. Effect of blood pressure lowering and antihypertensive drug class on progression of hypertensive kidney disease: results from the AASK trial. *JAMA.* 2002;288(19):2421-31.
45. <http://www.renal.org/guidelines/modules/cardiovascular-disease-in-ckd>. [accessed 1 February 2014].
46. Shoji T, Tsubakihara Y, Fujii M, Imai E. Hemodialysis-associated hypotension as an independent risk factor for two-year mortality in hemodialysis patients. *Kid Int.* 2004;66:1212-20.
47. Burton JO, Jefferies HJ, Selby NM, McIntyre CW. Hemodialysis-induced repetitive myocardial injury results in global and segmental reduction in systolic cardiac function. *Clin J Am Soc Nephrol.* 2009;4:1925-31.
48. McIntyre CW, Burton JO, Selby NM, et al. Hemodialysis-induced cardiac dysfunction is associated with an acute reduction in global and segmental myocardial blood flow. *Clin J Am Soc Nephrol.* 2008;3:19-26.
49. Goldsmith DJ, Covic A. Blood pressure control in CKD stage 5D patients - are we more or less certain what to do in 2009? *Nephrol Dial Transplant.* 2009;24:3597-601.
50. Majumdar A, Wheeler DC. Lipid abnormalities in renal disease. *J R Soc Med.* 2000; 93:178-82.

51. Lamarche B, St-Pierre AC, Ruel IL, Cantin B, Dagenais GR, Després JP. A prospective, population-based study of low density lipoprotein particle size as a risk factor for ischemic heart disease in men. *Can J Cardiol*. 2001;17(8):859-65.
52. Chaturvedi N, Fuller JH, Taskinen MR. Differing associations of lipid and lipoprotein disturbances with macrovascular and microvascular complications of type 1 diabetes. *Diabetes Care*. 2001;24:2071-7.
53. Kahri J, Groop PH, Elliott T, Viberti G, Taskinen MR. Plasma cholesteryl ester transfer protein and its relationship to plasma lipoproteins and apolipoproteins A-1-containing lipoproteins in IDDM patients with microalbuminuria and clinical nephropathy. *Diabetes Care*. 1994;17:412-9.
54. Castellani LW, Lusis AJ. ApoA-II versus ApoA-I: two for one is not always a good deal. *Arterioscler Thromb Vasc Biol*. 2001;21:1870-2.
55. Tsutsumi K. Lipoprotein lipase and atherosclerosis. *Curr Vasc Pharmacol*. 2003;1:11-7.
56. Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. *Atherosclerosis*. 2003;170:1-9.
57. Barter P. CETP and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2000;20:2029-31.
58. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, et al. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA*. 2009;302:1993-2000.
59. Cholesterol Treatment Trialists' (CTT) Collaboration, Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, et al. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet*. 2010;376:1670-81.
60. Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) Collaborative Group, Armitage J, Bowman L, Wallendszus K, Bulbulia R, Rahimi K, et al. Intensive lowering of LDL cholesterol with 80 mg versus 20 mg

- simvastatin daily in 12,064 survivors of myocardial infarction: a double-blind randomised trial *Lancet*. 2010;376:1658-69.
61. Sechi LA, Zingaro L, De Carli S, Sechi G, Catena C, Falletti E, et al. Increased serum lipoprotein(a) levels in patients with early renal failure. *Ann Intern Med*. 1998;129(6):457-61.
 62. Kronenberg F, König P, Lhotta K, Ofner D, Sandholzer C, Margreiter R, et al. Apolipoprotein(a) phenotype-associated decrease in lipoprotein(a) plasma concentrations after renal transplantation. *Arterioscler Thromb Vasc Biol*. 1994;14(9):1399-404.
 63. Clarke R, Peden JF, Hopewell JC, Kyriakou T, Goel A, Heath SC, et al. Genetic variants associated with Lp(a) lipoprotein level and coronary disease. *N Engl J Med*. 2009 361(26):2518-28.
 64. Wahn F, Daniel V, Kronenberg F, Opelz G, Michalk DV, Querfeld U. Impact of apolipoprotein(a) phenotypes on long-term renal transplant survival. *J Am Soc Nephrol* 2001 12(5):1052-8.
 65. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, et al. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet* 2011;378(9785):31-40.
 66. Kalk WJ, Joannou J, Ntsepo S, Mahomed I, Mahanlal P, Becker PJ. Ethnic differences in the clinical and laboratory associations with retinopathy in adult onset diabetes: studies in patients of African, European and Indian origins. *J Intern Med* 1997 241(1):31-7.
 67. Panzram G. Mortality and survival in type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*. 1987;30:123-31.
 68. Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, Di Angelantonio E, et al. The Emerging Risk Factors Collaboration. Diabetes mellitus, fasting blood glucose

- concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet* 2010;375(9733):2215-22.
69. Naicker S. End-Stage Renal Disease in Sub-Saharan Africa. *Ethnicity and Disease*. 2009;19(Suppl 1):S1-13-S1-5.
 70. Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. *BMJ* 2004 328(7455):1519.
 71. O'Seaghdha CM, Hwang SJ, Upadhyay A, Meigs JB, Fox CS. Predictors of incident albuminuria in the Framingham Offspring cohort. *Am J Kidney Dis* 2010;56(5):852-60.
 72. Yacoub R, Habib H, Lahdo A, Al Ali R, Varjabedian L, Atalla G, et al. Association between smoking and chronic kidney disease: a case control study. *BMC Public Health* 2010;10:731.
 73. Landray MJ, Emberson JR, Blackwell L, Dasgupta T, Zakeri R, Morgan MD, et al. Prediction of ESRD and death among people with CKD: the Chronic Renal Impairment in Birmingham (CRIB) prospective cohort study. *Am J Kidney Dis*. 2010;56(6):1082-94.
 74. Franklin SS, Larson MG, Khan SA, Wong ND, Leip EP, Kannel WB, et al. Does the relation of blood pressure to coronary heart disease risk change with aging? The Framingham Heart Study. *Circulation* 2001;103(9):1245-9.
 75. Harris T, Cook EF, Kannel WB, Goldman L. Proportional hazards analysis of risk factors for coronary heart disease in individuals aged 65 or older. The Framingham Heart Study. *J Am Geriatr Soc* 1988; 36(11):1023-8.
 76. Wilson PW, Kannel WB. Hypercholesterolemia and Coronary Risk in the Elderly: The Framingham Study. *Am J Geriatr Cardiol* 1993;2(2):56.
 77. Maas AHEM, Appelman YEA. Gender differences in coronary heart disease. *Netherlands Heart Journal*. 2010;18(12):598-602.

78. Towfighi A, Zheng L, Ovbiagele B. Sex-specific trends in midlife coronary heart disease risk and prevalence. *Arch Intern Med.* 2009;169:1762-6.
79. Ossewaarde ME, Bots ML, Verbeek AL, Peeters PH, van der Graaf Y, Grobbee DE, et al. Age at menopause, cause-specific mortality and total life expectancy. *Epidemiology* 2005 16(4):556-62.
80. Matthews KA, Meilahn EN, Kuller LH, Kelsey SF, Caggiula AW, RR W. Menopause and risk factors for coronary heart disease. *N Engl J Med.* 1989;321:641-6.
81. Grundtvig M, Hagen TP, German M, Reikvam A. Sex-based differences in premature first myocardial infarction caused by smoking: twice as many years lost by women as by men. *Eur J Cardiovasc Prev Rehab.* 2009;16:174-9.
82. Huxley R, Barzi F, Woodward M. Excess risk of fatal coronary heart disease associated with diabetes in men and women: metaanalysis of 37 prospective cohort studies. *BMJ.* 2006;332:73-8.
83. Dubey RK, Oparil S, Imthurn B, Jackson EK. Sex hormones and hypertension. *Cardiovasc Res.* 2002;53:688-708.
84. Barton M, Meyer MR. Postmenopausal hypertension. Mechanisms and therapy. *Hypertension.* 2009; 54(1):11-8.
85. Vasan RS, Larson MG, Leip EP, Evans JC, O'Donnell CJ, Kannel WB, et al. Impact of high-normal blood pressure on the risk of cardiovascular disease. *N Engl J Med.* 2001;345:1291-7.
86. Kannel WB, Castelli WP, Gordon T, McNamar PM. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham Study. *Ann Intern Med.* 1971;74: 1-12.

87. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med*. 2008;359:2195-207.
88. Powell KE, Thompson PD, Caspersen CJ, Kendrick JS. Physical activity and the incidence of coronary heart disease. *Annu Rev Public Health* 1987; 8:253-87.
89. Berlin JA, Colditz GA. Meta-analysis of physical activity in the prevention of coronary heart disease. *Am J Epidemiol*. 1990;132:612-28.
90. Wannamethee SG, Shaper AG, Walker M. Changes in physical activity, mortality, and incidence of coronary heart disease in older men. *Lancet* 1998; 351:1603-8.
91. Fletcher GF, Blair SN, Blumenthal J, Caspersen C, Chaitman B, Epstein S, et al. Statement on exercise. Benefits and recommendations for physical activity programs for all Americans. A statement for health professionals by the Committee on Exercise and Cardiac Rehabilitation of the Council on Clinical Cardiology, American Heart association. *Circulation*. 1992; 86:340-4.
92. Paffenbarger RS Jr, Hyde RT, Wing AL, Hsieh CC. Physical activity, all cause mortality and longevity of college alumni. *N Engl J Med*. 1986;314:605-13.
93. Manson JE, Rimm EB, Stampfer MJ, Colditz GA, Willett WC, Krolewski AS, et al. Physical activity and incidence of non-insulin-dependent diabetes mellitus in women. *Lancet* 1991; 338:774-8.
94. Oldridge N, Gottlieb M, Guyatt G, Jones N, Streiner D, Feeny D. Predictors of health-related quality of life with cardiac rehabilitation after acute myocardial infarction. *J Cardiopulm Rehabil* 1998;18: 95-103.
95. Wannamethee SG, Shaper AG, Walker M. Physical activity and mortality in older men with diagnosed coronary heart disease. *Circulation* 2000;102:1358-63.

96. Al-Mamari A. Atherosclerosis and Physical Activity. *Oman Medical Journal*. 2009.;24:173-8.
97. Durstine JL, Haskell WL. Effects of exercise training on plasma lipids and lipoproteins. *Exerc Sport Sci Rev* 1994;22:477-521.
98. Kokkinos PF, Holland JC, Narayan P, Collieran JA, Dotson CO, Papademetriou V. Miles run per week and high-density lipoprotein cholesterol levels in healthy, middle-aged men. A dose-response relationship. *Arch Intern Med* 1995;155:415-20.
99. Hambrecht R, Fiehn E, Weigl C, Gielen S, Hamann C, Kaiser R, et al. Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. *Circulation*. 1998;98:2709-15.
100. Hornig B, Maier V, Drexler H. Physical training improves endothelial function in patients with chronic heart failure. *Circulation*. 1996;93: 210-4.
101. Stewart GA, Gansevoort RT, Mark PB, Rooney E, McDonagh TA, Dargie HJ, et al. Electrocardiographic abnormalities and uremic cardiomyopathy. *Kidney Int* 2005;67:217-26.
102. Levin A, Singer J, Thompson C, Ross H, Lewis M. Prevalent left ventricular hypertrophy in the predialysis population: identifying opportunities for intervention. *Am J Kidney Dis*. 1996 27:347-54.
103. Deo R, Lin F, Vittinghoff E, Tseng ZH, Hulley SB, Shlipak MG. Kidney dysfunction and sudden cardiac death among women with coronary heart disease. *Hypertension*. 2008;51:1578-82.
104. Pun PH, Smarz TR, Honeycutt EF, Shaw LK, Al-Khatib SM, Middleton JP. Chronic kidney disease is associated with increased risk of sudden cardiac death among patients with coronary artery disease. *Kidney Int*. 2009;76:652-8.

105. Soman SS, Sandberg KR, Borzak S, Hudson MP, Yee J, McCullough PA. Chest The independent association of renal dysfunction and arrhythmias in critically ill patients. 2002;122: 669-77.
106. McPherson R, Pertsemlidis A, Kavaslar N, Stewart A, Roberts R CD, Hinds DA, et al. A common allele on chromosome 9 associated with coronary heart disease. Science. 2007;316:1488-91.
107. Helgadóttir A, Thorleifsson G, Manolescu A, Gretarsdóttir S, Blondal T, Jonasdóttir A, et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. Science. 2007;316:1491-3.
108. Schunkert H, Gotz A, Braund P, McGinnis R, Tregouet D-A, Mangino M, et al. Repeated Replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease. Circulation. 2008;117: 1675-84.
109. Thanassoulis G, Vasan RS. Genetic Cardiovascular Risk Prediction: Will We Get There? Circulation 2010;122(22):2323-34. doi: 10.1161/CIRCULATIONAHA.109.909309.
110. Adeyemo A, Gerry N, Chen G, Herbert A, Doumatey A, Huang H, et al. A genome-wide association study of hypertension and blood pressure in African Americans. PLoS Genet. 2009;5:e1000564.
111. Levy D, Ehret GB, Rice K, Verwoert GC, Launer LJ, Dehghan A, et al. Genome-wide association study of blood pressure and hypertension. Nat Genet. 2009; 41: 677- 87.
112. Kathiresan S, Willer CJ, Peloso GM, Demissie S, Musunuru K, Schadt EE, et al. Common variants at 30 loci contribute to polygenic dyslipidemia. Nat Genet. 2009; 41(1):56-65.
113. Aulchenko YS, Ripatti S, Lindqvist I, Boomsma D, Heid IM, Pramstaller PP, et al. Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. Nat Genet. 2009;41:47-55.

114. Ito K, Bick AG, Flannick J, Friedman DJ, Genovese G, Parfenov MG, et al. Increased Burden of Cardiovascular Disease in Carriers of APOL1 Genetic Variants *Circ Res*. 2014;114(5):845-50.
115. Genovese G, Friedman DJ, Ross MD, Lecordier L, Uzureau P, Freedman BI, et al. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. *Science*. 2010;329(5993): 841-5.
116. Friedman DJ, Pollak MR. Genetics of kidney failure and the evolving story of APOL1. *J Clin Invest*. 2011; 121:3367-74.
117. Tzur S, Rosset S, Shemer R, Yudkovsky G, Selig S, Tarekegn A, et al. Missense mutations in the APOL1 gene are highly associated with end stage kidney disease risk previously attributed to the MYH9 gene. *Hum Genet*. 2010;128: 345-50.
118. Davidson WS, Silva RA, Chantepie S, Lagor WR, Chapman MJ, Kontush A. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler Thromb Vasc Biol* 2009;29(870-876).
119. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317-25.
120. Hoy A, Leininger-Muller B, Poirier O, Siest G, Gautier M, Elbaz A, et al. Myeloperoxidase polymorphisms in brain infarction. Association with infarct size and functional outcome. *Atherosclerosis*. 2003;167: 223-30.
121. Makela R, Laaksonen R, Janatuinen T, Vesalainen R, Nuutila P, Jaakkola O. Myeloperoxidase gene variation and coronary flow reserve in young healthy men. *J Biomed Sci* 2004;11:59-64.
122. Humphries SE, Luong LA, Ogg MS, Hawe E, Miller GJ. The interleukin-6 -174 G/C promoter polymorphism is associated with risk of coronary heart disease and systolic blood pressure in healthy men. *Eur Heart J* 2001;22:2243-52.

123. Liu Y, Berthier-Schaad Y, Fallin MD, Fink NE, Tracy RP, Klag MJ, et al. IL-6 haplotypes, inflammation, and risk for cardiovascular disease in a multiethnic dialysis cohort. *J Am Soc Nephrol* 2006;17:863-70.
124. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, et al. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med*. 2002; 347:185-92.
125. Sorensen GL, Madsen J, Kejlting K, Tornoe I, Nielsen O, Townsend P, et al. Surfactant protein D is proatherogenic in mice. *Am J Physiol Heart Circ Physiol* 2006;290:2286-94.
126. Simeoni E, Winkelmann BR, Hoffmann MM, Fleury S, Ruiz J, Kappenberger L, et al. Association of RANTES G-403A gene polymorphism with increased risk of coronary arteriosclerosis. *Eur Heart J*. 2004 25:1438-46.
127. Ioannidis JP. Prediction of cardiovascular disease outcomes and established cardiovascular risk factors by genome-wide association markers. *Circ Cardiovasc Genet*. 2009; 2:7-15.
128. Lloyd-Jones DM, Nam BH, D'Agostino RB Sr, Levy D, Murabito JM, Wang TJ, et al. Parental cardiovascular disease as a risk factor for cardiovascular disease in middle-aged adults: a prospective study of parents and offspring. *JAMA*. 2004;291:2204 -11.
129. Murabito JM, Pencina MJ, Nam BH, D'Agostino RB Sr, Wang TJ, Lloyd-Jones D, et al. Sibling cardiovascular disease as a risk factor for cardiovascular disease in middle-aged adults. *JAMA*. 2005;294:3117-23.
130. Ridker PM, Paynter NP, Rifai N, Gaziano JM, Cook NR. C-reactive protein and parental history improve global cardiovascular risk prediction: the Reynolds Risk Score for men. *Circulation*. 2008; 118:2243-51.
131. Stehouwer CD, Smulders YM. Microalbuminuria and risk for cardiovascular disease: analysis of potential mechanisms. *J Am Soc Nephrol*. 2006;17:2106-11.

132. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105:1135-43.
133. Hauser AB, Stinghen AEM, Goncalves SM, Bucharles S, Pecoits-Filho R. A gut feeling on endotoxaemia: causes and consequences in chronic kidney disease. *Nephron Clin Pract*. 2011;118:c165-c72.
134. Hauser AB, Stinghen AE, Kato S, Bucharles S, Aita C, Yuzawa Y, et al. Characteristics and causes of immune dysfunction related to uremia and dialysis. *Perit Dial Int*. 2008;28(suppl 3):S183-S7.
135. Hansson G. Immune mechanisms in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2001; 21:1876–90.
136. Schonbeck U, Libby P. CD40 signaling and plaque instability. *Circ Res*. 2001;89: 1092-103.
137. Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. *JAMA*. 2002; 288:2724–31.
138. Espinola-Klein C, Rupprecht H, Blankenberg S, Bickel C, Kopp H, Rippin G, et al. Are Morphological or Functional Changes in the Carotid Artery Wall Associated With Chlamydia pneumoniae, Helicobacter pylori, Cytomegalovirus, or Herpes Simplex Virus Infection? *Stroke*. 2000;31:2127-33.
139. Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Rippin G, et al. Impact of infectious burden on extent and long-term prognosis of atherosclerosis. *Circulation*. 2002;105:15-21.
140. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med*. 2002;347(20):1557-65.

141. Stehouwer CD, Gall MA, Twisk JW, Knusden E, Emeis JJ, Parving HH. Increased urinary albumin excretion, endothelial dysfunction, and chronic low-grade inflammation in type 2 diabetes: progressive, interrelated, and independently associated with risk of death. *Diabetes Care*. 2002;51:1157-65.
142. Schram MT, Chaturvedi N, Schalkwijk CG, Fuller JH, Stehouwer CD. Markers of inflammation are cross-sectionally associated with microvascular complication and cardiovascular disease in type 1 diabetes - the EURODIAB Prospective Complications Study. *Diabetologia*. 2005;48:370-8.
143. Vaccaro F, Mulè G, Cottone S, Soresi M, Giannitrapani L, Vadalà A, et al. Circulating levels of adhesion molecules in chronic kidney disease correlate with the stage of renal disease and with C-reactive protein. *Arch Med Res*. 2007;38(5):534-8.
144. Costa E, Lima M, Alves JM, Rocha S, Rocha-Pereira P, Castro E, et al. Inflammation, T-cell phenotype, and inflammatory cytokines in chronic kidney disease patients under hemodialysis and its relationship to resistance to recombinant human erythropoietin therapy. *J Clin Immunol*. 2008;28(3): 268-75.
145. Libby P, Ridker PM, Hansson GK. Inflammation in atherosclerosis: From pathophysiology to practice. *J Am Coll Cardiol*. 2009;54(23):2129-38.
146. Ridker PM, Libby P. Risk markers for atherothrombotic disease. In: Bonow RO, Mann DL, Zipes DP, Libby P, Braunwald E, editors. *Braunwald's heart disease: a textbook of cardiovascular medicine*. Philadelphia: Elsevier Saunders; 2012. p. 914-34.
147. Ridker PM. C-reactive protein: eighty years from discovery to emergence as a major risk marker for cardiovascular disease. *Clin Chem*. 2009;55(2):209-15.
148. Ridker PM. C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: moving an inflammatory hypothesis toward consensus. *J Am Coll Cardiol* 2007;49(21):2129-38.

149. Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG, et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet* 2010;375(9709):132-40.
150. Boekholdt SM, Hack CE, Sandhu MS, Luben R, Bingham SA, Wareham NJ, et al. C-reactive protein levels and coronary artery disease incidence and mortality in apparently healthy men and women: the EPIC-Norfolk prospective population study 1993-2003. *Atherosclerosis*. 2006;187(2):415-22.
151. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med* 2004;350(14):1387-97.
152. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO 3rd, Criqui M, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation* 2003;107(3):499-511.
153. Wilson PW, Pencina M, Jacques P, Selhub J, D'Agostino R Sr, O'Donnell CJ. C-reactive protein and reclassification of cardiovascular risk in the Framingham Heart Study. *Circ Cardiovasc Qual Outcomes* 2008;1(2):92-7.
154. Ridker PM, Cook N. Clinical usefulness of very high and very low levels of C-reactive protein across the full range of Framingham Risk Scores. *Circulation*. 2004;109(16):1955-9.
155. Evans J, Goldfine I, Maddux B, Grodsky G. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes*. 2003; 52:1-8.

156. Coombes J, Fassett R. Antioxidant therapy in hemodialysis patients: a systematic review. *Kidney Int* 2011; 81:233-46.
157. Stocker R, Keaney JJ. Role of oxidative modifications in atherosclerosis *Physiol Rev.* 2004; 84:1381-478.
158. Himmelfarb J, Stenvinkel P, Ikizler T, Hakim R. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int.* 2002;62:1524-38.
159. Stoll LL, Denning GM, Weintraub NL. Potential Role of Endotoxin as a Proinflammatory Mediator of Atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004 24(12):2227-36.
160. Tousoulis D, Psaltopoulou T, Androulakis E, Papageorgiou N, Papaioannou S, Oikonomou E, et al. Oxidative Stress and Early Atherosclerosis: Novel Antioxidant Treatment. *Cardiovasc Drugs Ther* 2014;DOI 10.1007/s10557-014-6562-5. Epub ahead of print.
161. Sorescu D, Weiss D, Lassegue B, Clempus R, Szocs K, et al. Superoxide production and expression of Nox family proteins in human atherosclerosis. *Circulation.* 2002;105: 1429-35.
162. Barry-Lane P, Patterson C, van der Merwe M, Hu Z, Holland S, et al. P47phox is required for atherosclerotic lesion progression in ApoE^{-/-} mice. *J Clin Investig.*108:1513-22.
163. Vendrov A, Hakim Z, Madamanchi N, Rojas M, Madamanchi C, Runge M. Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells. *Arterioscler Thromb Vasc Biol.* 2007;27: 2714-21.
164. Freedman J. Oxidative stress and platelets. *Arterioscler Thromb Vasc Biol.* 2008 28: s11-6.
165. Schulz E, Jansen T, Wenzel P, Daiber A, Münzel T. Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension *Antioxid Redox Signal.* 2008;10:1115-26.

166. Kao M, Ang D, Pall A, Struthers A. Oxidative stress in renal dysfunction: mechanisms, clinical sequelae and therapeutic options. *J Hum Hypertens*. 2010;24: 1-8.
167. Vaziri N, Oveisi F, Ding Y. Role of increased oxygen free radical activity in the pathogenesis of uremic hypertension. *Kidney Int*. 1998;53:1748-54.
168. Cachofeiro V, Goicochea M, de Vinuesa S, Oubiña P, Lahera V, Luño J. Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. *Kidney Int Suppl* 2008 111:S4-9. doi: 10.1038/ki.2008.516.
169. Samouilidou E, Karpouza A, Kostopoulos V, Bakirtzi T, Pantelias K, Petras D, et al. Lipid abnormalities and oxidized LDL in chronic kidney disease patients on hemodialysis and peritoneal dialysis. *Renal Failure* 2012;34(2):160-4.DOI: 10.3109/0886022X.2011.641515.
170. Vaziri N. Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. *Curr Opin Nephrol Hypertens*. 2004;13:93-9.
171. Coombes J, Fassett R. Antioxidant therapy in hemodialysis patients: a systematic review. *Kidney Int*. 2011;81:233-46.
172. Galle J. Oxidative stress in chronic renal failure. *Nephrol Dial Transplant*. 2001;16: 2135-7.
173. Ha T, Sattar N, Talwar D, Cooney J, Simpson K, O'Reilly D, et al. Abnormal anti-oxidant vitamin and carotenoid status in chronic renal failure. *QJM*. 1996;89:765-9.
174. Cheung A. Biocompatibility of hemodialysis membranes. *J Am Soc Nephrol* 1990;1: 150-61.
175. Jha P, Flather M, Lonn E, Farkouh M, Yusuf S. The antioxidant vitamins and cardiovascular disease. A critical review of epidemiologic and clinical trial data. *Ann Intern Med*. 1995;123(11):860-72.

176. Fan L, Li J. Evaluation of methods of detecting cell reactive oxygen species production for drug screening and cell cycle studies. *J Pharmacol Toxicol Method* 2014 70(1):40-7. doi: 10.1016/j.vascn.2014.03.173.
177. Ho E, Karimi Galougahi K, Liu C, Bhindi R, Figtree G. Biological markers of oxidative stress: Applications to cardiovascular research and practice. *Redox Biol* 2013;1(1):483-91. doi: 10.1016/j.redox.2013.07.006.
178. Annuk M, Zilmer M, Fellstrom B. Endothelium-dependent vasodilation and oxidative stress in chronic renal failure: Impact on cardiovascular disease. *Kidney Int.* 2003; 63 Suppl 84:S50-S3.
179. Mune M, Yukawa S, Kishino M, Otani H, Kimura K, Nishikawa O, et al. Effect of vitamin E on lipid metabolism and atherosclerosis in ESRD patients. *Kidney int.* 1999;56 (Suppl 71): S126-S9.
180. Lee K. Oxidative stress markers in Korean subjects with insulin resistance syndrome. *Diabetes Res Clin Pract.* 2001;54(suppl 2):S29-S33.
181. Hansel B, Giral P, Nobecourt E, Bruckert E, Chapman M, Kontush A. Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. *J Clin Endocrinol Metab.* 2004;89: 4963-71.
182. Faxon D, Fuster V, Libby P, Beckman J, Hiatt W, Robert W, et al. Atherosclerotic Vascular Disease Conference Writing Group III: Pathophysiology. *Circulation.* 2004;109:2617-25.
183. Luft F. Proinflammatory effects of angiotensin II and endothelin: targets for progression of cardiovascular and renal diseases. *Curr Opin Nephrol Hypertens.* 2002;11:59-6.
184. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med.* 1993;329: 2002-12.

185. Stehouwer CD. Endothelial dysfunction in diabetic nephropathy: state of the art and potential significance for non-diabetic renal disease. *Nephrol Dial Transplant*. 2004 19(4): 778-81.
186. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*. 1992;339:572-5.
187. Schwedhelm E, Boger R. The role of asymmetric and symmetric dimethylarginines in renal disease. *Nat Rev Nephrol* 2011;7:275-85.
188. Schepers E, Glorieux G, Dhondt A, Leybaert L, Vanholder R. Role of symmetric dimethylarginine in vascular damage by increasing ROS via storeoperated calcium influx in monocytes. *Nephrol Dial Transplant*. 2009;24: 1429-35.
189. Stühlinger M, Conci E, Haubner B, Stocker E, Schwaighofer J, Cooke J, et al. Asymmetric Dimethyl L-Arginine (ADMA) is a critical regulator of myocardial reperfusion injury. 2007. *Cardiovasc Res*;75:417-25.
190. Ganji V, Kafai MR. Demographic, health, lifestyle, and blood vitamin determinants of serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey, 1988-1994. *Am J Clin Nutr*. 2003;77:826-33.
191. Heinz J, Domröse U, Luley C, Westphal S, Kropf S, Neumann K, et al. Influence of a supplementation with vitamins on cardiovascular morbidity and mortality in patients with endstage renal disease: design and baseline data of a randomized clinical trial. *Clin Nephrol* 2009;71:363-5.
192. Ducloux D, Motte G, Challier B, Gibey R, Chalopin J. Serum total homocysteine and cardiovascular disease occurrence in chronic, stable renal transplant recipients: a prospective study. *J Am Soc Nephrol*. 2000;11:134-7.
193. <http://www.renal.org/guidelines/modules/cardiovascular-disease-in-ckd> [updated 6 August 2010, accessed 30 November 2014].

194. van Guldener C, Stehouwer C. Homocysteine and methionine metabolism in renal failure. *Semin Vasc Med.* 2005; 5: 201-8.
195. Arnadottir M, Hultberg B, Nilsson-Ehle P, Thysell H. The effect of reduced glomerular filtration rate on plasma total homocysteine concentration. *Scand J Clin Lab Invest.* 1996;56:41-6.
196. Danesh J, Lewington S. Plasma homocysteine and coronary heart disease: systematic review of published epidemiological studies. *J Cardiovasc Risk.* 1998; 5:229-32.
197. Klerk M, Verhoef P, Clarke R, Blom H, Kok F, Schouten E, et al. MTHFR 677C→T polymorphism and risk of coronary heart disease: a meta-analysis. *JAMA.* 2002;288: 2023-31.
198. Marcus J, Sarnak M, Menon V. Homocysteine lowering and cardiovascular disease risk: lost in translation. *Can J Cardiol.* 2007;23:707-10.
199. Wrone E, Hornberger J, Zehnder J, McCann L, Coplon N, Fortmann S. Randomized trial of folic acid for prevention of cardiovascular events in end-stage renal disease (vol 15, pg 420, 2004). *J Am Soc Nephrol.* 2004;15:420-6.
200. Abbasi F, Chu J, Lamendola C, McLaughlin T, Hayden J, Reaven G, et al. Discrimination between obesity and insulin resistance in the relationship with adiponectin. *Diabetes.* 2004;53:585-90.
201. Jeffery AN, Murphy MJ, Metcalf BS, Hosking J, Voss LD, English P, et al. Adiponectin in childhood. *Int J Pediatr Obes.* 2008;3:130-40.
202. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* 2006;116:1784-92.

203. Amira O, Naicker S, Manga P, Sliwa K, Mia A, Raal F, et al. Adiponectin and atherosclerosis risk factors in African hemodialysis patients: A population at low risk for atherosclerotic cardiovascular disease. *Hemodialysis International*. 2012;16:59-68.
204. Beltowski J. Adiponectin and resistin-new hormones of white adipose tissue. *Med Sci Monit*. 2003;9:55-61.
205. Zoccali C, Mallamaci F, Tripepi G. Adipose tissue as a source of inflammatory cytokines in health and in disease: Focus on end-stage renal disease. *Kidney Int*. 2003;63:S65-S8.
206. Tentolouris N, Doulgerakis D, Moyssakis I, Kyriaki D, Makrilakis K, Kosmadakis G, et al. Plasma adiponectin concentrations in patients with chronic renal failure: Relationship with metabolic risk factors and ischemic heart disease. *Horm Metab Res*. 2004;36:721-7.
207. Guebre-Egziabher F, Bernhard J, Funahashi T, Hadj-Aissa A, Fouque D. Adiponectin in chronic kidney disease is related more to metabolic disturbances than to decline in renal function. *Nephrol Dial Transplant*. 2005;20:129-34.
208. Díez J, Iglesias P, Fernández-Reyes M, Aguilera A, Bajo M, Alvarez-Fidalgo P, et al. Serum concentrations of leptin, adiponectin and resistin, and their relationship with cardiovascular disease in patients with end-stage renal disease. *Clin Endocrinol (Oxf)*. 2005;62:242-9.
209. Seiler S, Heine G, Fliser D. Clinical relevance of FGF-23 in chronic kidney disease. *Kidney Int Suppl* 2009;76:S34-42.
210. Baum M, Schiavi S, Dwarakanath V, Quigley R. Effect of fibroblast growth factor-23 on phosphate transport in proximal tubules. *Kidney Int* 2005;68:1148-53.
211. Marsell R, Grundberg E, Krajsnik T, Mallmin H, Karlsson M, Mellström D, et al. Fibroblast growth factor-23 is associated with parathyroid hormone and renal function in a population-based cohort of elderly men. *Eur J Endocrinol*. 2008;158:125-9.

212. Fliser D, Kollerits B, Neyer U, Ankerst D, Lhotta K, Lingenhel A, et al. Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the mild to moderate kidney disease (MMKD) study. *J Am Soc Nephrol.* 2007;18:2600-8.
213. Razzaque M. Does FGF23 toxicity influence the outcome of chronic kidney disease? *Nephrol Dial Transplant* 2009;24:4-7.
214. Faul C, Amaral A, Oskoue B, Hu M, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. *J Clin Invest* 2011;121:4393-408.
215. Libby P. Inflammation and cardiovascular disease mechanisms. *Am J Clin Nutr.* 2006;83(2):456S-60S.
216. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem.* 1997;272(34):20963-6.
217. Mallika V, Goswami B, Rajappa M. Atherosclerosis Pathophysiology and the Role of Novel Risk Factors: A Clinicobiochemical Perspective. *Angiology* 2007; 58:513-22.
218. Weber C, Noel H. Atherosclerosis: current pathogenesis and therapeutic options. *Nature Medicine.* 2011;17(11):1410-22.
219. Andreasen A, Krabbe K, Krogh-Madsen R, Taudorf S, Pedersen B, Møller K. Human Endotoxemia as a Model of Systemic Inflammation. *Current Medicinal Chemistry* 2008;15:1697-705.
220. Kabanov D, Prokhorenko I. Structural Analysis of Lipopolysaccharides from Gram Negative Bacteria. *Biochemistry (Moscow).* 2010;75 (4):383-404.
221. Raetz C, Reynolds C, Trent M, Bishop R. Lipid A Modification Systems in Gram-Negative Bacteria. *Annu Rev Biochem.* 2007;76:295-329.
222. Bang F. The toxic effect of a marine bacterium on *Limulus* and the formation of blood clots. *Biol Bull (Woods Hole, MA).* 1953;105:361-2.

223. Levin J, Bang F. Clottable protein in *Limulus*: its localization and kinetics of its coagulation by endotoxin. *Thromb Diath Haemorrh*. 1968;19:186-97.
224. Hochstei H. The LAL test versus the rabbit pyrogen test for endotoxin detection: Update 87. *Pharm Technol*. 1987;11(6):124-9.
225. Zhang G, Ghosh S. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. *J Endotoxin Res*. 2000;6(6):453-7.
226. Darveau R, Pham T, Lemley K, Reife R, Bainbridge B, Coats S, et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun*. 2004;72(9):5041-51.
227. Levi M, ten Cate H, van der Poll T, van Deventer SJ. Pathogenesis of disseminated intravascular coagulation in sepsis. *JAMA*. 1993;25:975-9.
228. Bannerman D, Goldblum S. Direct effects of endotoxin on the endothelium: barrier function and injury. *Lab Invest*. 1999;79:1181-99.
229. Fenton M, Golenbock D. LPS-binding proteins and receptors. *J Leukocyte Biol*. 1998;64:25-32.
230. Lequier L, Nikaidoh H, Leonard S, Bokovoy J, White M, Scannon P, et al. Preoperative and postoperative endotoxemia in children with congenital heart disease. *Chest*. 2000 117(6):1706-12.
231. Sun L, Yu Z, Ye X, Zou S, Li H, Yu D, et al. A marker of endotoxemia is associated with obesity and related metabolic disorders in apparently healthy Chinese. *Diabetes Care*. 2010;33 (9):1925-32.10. doi: 10.2337/dc10-0340.
232. Schumann R. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. *Biochem Soc Trans* 2011;39:989-93. doi: 10.1042/bst0390989.

233. Tobias P, Ulevitch R. Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation. *Immunobiology*. 1993;187:227-32. doi: 10.1016/s0171-2985(11)80341-4.
234. Read T, Harris H, Grunfeld C, Feingold K, Kane J, Rapp J. The protective effect of serum lipoproteins against bacterial lipopolysaccharide. *Eur Heart J*. 1993;14(Suppl. K):125-9.
235. Parker T, Levine D, Chang J, Laxer J, Coffin C, Rubin A. Reconstituted high-density lipoprotein neutralizes Gram-negative bacterial lipopolysaccharides in human whole blood. *Infect Immun*. 1995;63:253-8.
236. Antal-Szalmas P. Evaluation of CD14 in host defense. *Eur J Clin Invest*. 2000;30:167-79.
237. Laugerette F, Vors C, G  lo  n A, Chauvin M, Soulage C, Lambert-Porcheron S, et al. Emulsified lipids increase endotoxemia: possible role in early postprandial low-grade inflammation. *J Nutr Biochem* 2011;22:53-9. doi: 10.1016/j.jnutbio.2009.11.011.
238. Unkelbach K, Gardemann A, Kostrzewa M, Philipp M, Tillmanns H, Haberbosch W. A new promoter polymorphism in the gene of lipopolysaccharide receptor CD14 is associated with expired myocardial infarction in patients with low atherosclerotic risk profile. *Arterioscler Thromb Vasc Biol*. 1999;19:932-8.
239. Amar J, Ruidavets J, Bal Dit Sollier C, Bongard V, Boccalon H, Chamontin B, et al. Soluble CD14 and aortic stiffness in a population-based study. *J Hypertens*. 2003; 21:1869 -77.
240. Raj DS, Shah VO, Rambod M, Kovesdy CP, Kalantar-Zadeh K. Association of soluble endotoxin receptor CD14 and mortality among patients undergoing hemodialysis. *Am J Kidney Dis*. 2009;54:1062-71.
241. Means T, Golenbock D, Fenton M. Structure and function of Toll-like receptor proteins. *Life Sci*. 2000;68:241-58.

242. Jin M, Lee J. Structures of the toll-like receptor family and its ligand complexes. *Immunity*. 2008;29:182-91.
243. Vandewalle A. Toll-like receptors and renal bacterial infections. *Chang Gung Med J*. 2008;31:525-37.
244. Gibbs L, Del Vecchio P, Shaffer J. Mn and Cu/Zn SOD expression in cells from LPS-sensitive and LPS-resistant mice. *Free Radical Biol & Med*. 1992;12:107-11.
245. Suzuki K, Tatsumi H, Satoh S, Senda T, Nakata T, Jujii J, et al. Manganese-superoxide dismutase in endothelial cells: localization and mechanism of induction. *Am J Physiol*. 1993;265:H1173-H8.
246. Griendling K, Sorescu D, Ushio-Fukai M. NAD(P)H Oxidase. Role in cardiovascular biology and disease. *Circ Res*. 2000;86:494 -501.
247. Wiesner P, Choi S, Almazan F, Benner C, Huang W, Diehl C, et al. Low Doses of Lipopolysaccharide and Minimally Oxidized Low-Density Lipoprotein Cooperatively Activate Macrophages via Nuclear Factor B and Activator Protein-1. Possible Mechanism for Acceleration of Atherosclerosis by Subclinical Endotoxemia. *Circ Res* 2010;107:56-65. doi: 10.1161/CIRCRESAHA.110.218420.
248. Rice JB, Stoll LL, Li W-G, Denning GM, Weydert J, Charipar E, et al. Low level endotoxin induces potent inflammatory activation of human blood vessels: inhibition by statins. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2003;23:1576 -82.
249. Reape T, Groot P. Chemokines and atherosclerosis *Atherosclerosis*. 1999;147:213-25.
250. Diks S, van Deventer S, Peppelenbosch M. Lipopolysaccharide recognition, internalisation, signalling and other cellular effects. *J Endotoxin Res*. 2001;7:335-48.
251. Dickensheets H, Freeman S, Smith M, Donnelly R. Interleukin-10 up regulates tumor necrosis factor receptor type-II (p75) gene expression in endotoxin-stimulated human monocytes. *Blood*. 1997;90:4162- 71.

252. Suliman M, Qureshi A, Heimbürger O, Lindholm B, Stenvinkel P. Soluble adhesion molecules in end-stage renal disease: a predictor of outcome. *Nephrol Dial Transplant.* 2006;21:1603-10.
253. Papayianni A, Alexopoulos E, Giamalis P, Gionanlis L, Belechri A, Koukoudis P, et al. Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidaemia, and vascular events. *Nephrol Dial Transplant.* 2002;17:435-41.
254. Ferramosca E, Burke S, Chasan-Taber S, Ratti C, Chertow G, Raggi P. Potential antiatherogenic and anti-inflammatory properties of sevelamer in maintenance hemodialysis patients. *Am Heart J* 2005;149:820-5.
255. Phan O, Ivanovski O, Nguyen-Khoa T, Mothu N, Angulo J, Westenfeld R, et al. Sevelamer prevents uremia-enhanced atherosclerosis progression in apolipoprotein E-deficient mice. *Circulation* 2005;112: 2875-82.
256. Stinghen A, Gonçalves S, Bucharles S, Branco F, Gruber B, Hauser A, et al. Sevelamer decreases systemic inflammation in parallel to a reduction in endotoxemia. *Blood Purif.* 2010;29: 352-6.
257. Sparrow C, Burton C, M H, Mundt S, Hassing H, Patel S, et al. Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol.* 2001;21:115-21.
258. Diomedede L, Albani D, Sottocorno M, Donati M, Bianchi M, Fruscella P, et al. In vivo anti-inflammatory effect of statins is mediated by nonsterol mevalonate products. *Arterioscler Thromb Vasc Biol.* 2001;21:1327-32.
259. Casey P. Protein lipidation in cell signaling. *Science.* 1995;268:221-5.

260. Gordon L, Kleinman M, Miller D, Neuberg D, Giobbie-Hurder A, Gerhard-Herman M, et al. Clinical trial of a farnesyltransferase inhibitor in children with Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci USA*. 2012;109(41):16666-71.
261. Blobel GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. *N Engl J Med*. 2000;342:1350-8.
262. Park SK, Yang WS, Lee SK, Ahn H, Park JS, Hwang O, et al. TGF- β 1 down-regulates inflammatory cytokine-induced VCAM-1 expression in cultured human glomerular endothelial cells. *Nephrol Dial Transplant* 2000;15(5):596-604.
263. Gamble JR, Knew-Goodall Y, Vadas MA. Transforming growth factor β 1 inhibits E-selectin expression on human endothelial cells. *J Immunol*. 1993;150: 4494-503.
264. Wahl SM, McCartney-Francis N, Mergenhagen SE. Inflammatory and immunomodulatory roles of TGF β 1. *Immunol Today*. 1989;10:258-61.
265. Tashiro H, H S, Yamamoto K, Momohara M, Tada H, Takeshita A. Altered plasma levels of cytokines in patients with ischemic heart disease. *Coron Artery Dis*. 1997; 8(3-4): 143-7.
266. Grainger DJ, Kemp PR, Metcalfe JC, Liu AC, Lawn RM, Williams NR, et al. The serum concentration of active transforming growth factor- β is severely depressed in advanced atherosclerosis. *Nat Med*. 1995;1(1):74-9.
267. Stefoni S, Cianciolo G, Donati G, Dormi A, Silvestri MG, Coli L, et al. Low TGF- β 1 serum levels are a risk factor for atherosclerosis disease in ESRD patients. *Kidney Int* 2002;61(1):324-35.
268. Yeun J, Levine R, Mantadilok V, Kaysen G. C-reactive protein predicts all-cause and cardiovascular mortality in hemodialysis patients. *Am J Kidney Dis*. 2000;35:469-76.

269. Foley RN, Murray AM, Li S, et al. Chronic kidney disease and the risk for cardiovascular disease, renal replacement, and death in the United States Medicare population, 1998 to 1999. *J Am Soc Nephrol*. 2005;16:489-95.
270. Pecoits-Filho R, Gonçalves S, Barberato S, et al. Impact of residual renal function on volume status in chronic renal failure. *Blood Purif* 2004;22:285-92.
271. Sato Y, Takatsu Y, Kataoka K, T Y, Taniguchi R, Sasayama S, et al. Serial circulating concentrations of C-reactive protein, interleukin (IL)-4, and IL-6 in patients with acute left heart decompensation *Clin Cardiol* 1999;22:811-3.
272. Mookadam F, Moustafa S, Lester S, Warsame T. Subclinical Atherosclerosis: Evolving Role of Carotid Intima-Media Thickness. *Prev Cardiol*. 2010;13:186-97.
273. Rosvall M, Janzon L, Berglund G, Engström G, Hedblad B. Incident coronary events and case fatality in relation to common carotid intima-media thickness. *J Intern Med*. 2005;257(5):430-7.
274. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation*. 1997;96:1432-7.
275. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, et al. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987- 1993. *Am J Epidemiol* 1997;146:483-94.
276. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr, et al. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. *N Engl J Med*. 1999;340:14-22.

277. Oren A, Vos LE, Uiterwaal CS, Grobbee DE, Bots ML. Cardiovascular risk factors and increased carotid intima-media thickness in healthy young adults: the Atherosclerosis Risk in Young Adults (ARYA) Study. *Arch Intern Med.* 2003;163:1787-92.
278. Krishnan P, Balamurugan A, Urbina E, Srinivasan SR, Bond G, Tang R, et al. Cardiovascular risk profile of asymptomatic healthy young adults with increased carotid artery intima-media thickness: the Bogalusa Heart Study. *J La State Med Soc.* 2003;155:165-9.
279. Blankenhorn DH, Selzer RH, Crawford DW, Barth JD, Liu CR, Liu CH, et al. Beneficial effects of colestipolnicin therapy on the common carotid artery. Two- and four-year reduction of intima-media thickness measured by ultrasound. *Circulation.* 1993; 88:20-8.
280. Espeland MA, O'Leary DH, Terry JG, Morgan T, Evans G, Mudra H. Carotid intimal-media thickness as a surrogate for cardiovascular disease events in trials of HMG-CoA reductase inhibitors. *Curr Control Trials Cardiovasc Med.* 2005;6(1):3. doi: 10.1186/468-6708-6-3.
281. Stein J, Korcarz C, Hurst R, Lonn E, Kendall C, Mohler E, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr* 2008;21(2): 93-111.
282. Bots ML, Baldassarre D, Simon A, de Groot E, O'Leary DH, Riley W, et al. Carotid intima-media thickness and coronary atherosclerosis: weak or strong relations? *Eur Heart J* 2007 28(4):398-406.
283. Jaeger JQ, Mehta RL. Assessment of dry weight in haemodialysis: an overview. *J AM Soc Nephrol.* 1999;10:392-403.

284. Guo Q, Yi C, Li J, Wu X, Yang X, Yu X. Prevalence and risk factors of fluid overload in Southern Chinese continuous ambulatory peritoneal dialysis patients. *PLoS One*. 2013;8(1):e53294. doi: 10.1371/journal.pone.0053294. Epub 2013 Jan 14.
285. De Vecchis R, Ciccarelli A, Ariano C. Inferior Vena Cava collapsibility and heart failure signs and symptoms: new insights about possible links *Arq Bras Cardiol* [online]. 2012;98(6):544-52.
286. Cheriex E, Leunissen K, Janssen J, Mooy JM, van Hooff J. Echography of the inferior vena cava is a simple and reliable tool for estimation of 'dry weight' in haemodialysis patients. *Nephrol Dial Transplant*. 1989;4:563-8.
287. Katzarski K, Charra B, Luik A, Nisell J, Divino Filho J, Leypoldt J, et al. Fluid state and blood pressure control in patients treated with long and short haemodialysis. *Nephrol Dial Transplant* 1999 14(2):369-75.
288. Brennan J, Ronan A, Goonewardena S, Blair J, Hammes M, Shah D, et al. Hand carried Ultrasound Measurement of the Inferior Vena Cava for Assessment of Intravascular Volume Status in the Outpatient Hemodialysis Clinic. *Clin J Am Soc Nephrol*. 2006;1:749-53.
289. Chanchairujira T, Mehta R. Bioimpedance and Its Application. *Saudi J Kidney Dis Transplant* 2005;16(1):6-16.
290. Segal K, Burastero S, Chun A, Coronel P, Pierson RJ, Wang J. Estimation of extracellular and total body water by multiple frequency bioelectrical-impedance measurement. *Am J Clin Nutr*. 1991;54:26-9.
291. Kurtin P, Shapiro A, Tomita H, Raizman D. Volume status and body composition of chronic dialysis patients: utility of bioelectrical impedance plethysmography. *Am J Nephrol* 1990;10:363-7.

292. Machek P, Jirka T, Moissl U, Chamney P, Wabel P. Guided optimization of fluid status in haemodialysis patients. *Nephrol Dial Transplant* 2010;25:538-44.
293. Kushner R, Schoeller D. Estimation of total body water by bioelectrical impedance analysis. *Am J Clin Nutr* 1988;42:101-6.
294. Segal KR, Gutin B, Presta E, Wang J, Van Itallie TB. Estimation of human body composition by electrical impedance method: A comparative study. *J Appl Physiol*. 1985;58:1565-71.
295. Cox-Reijven PL, Kooman JP, Soeters PB, van der Sande F. Role of bioimpedance spectroscopy in assessment of body water compartments in haemodialysis patients. *Am J Kidney Dis*. 2001;38:832-8.
296. Demirci M, Demirci C, Ozdogan O, Kircelli F, Akcicek F, Basci A, et al. Relations between malnutrition–inflammation–atherosclerosis and volume status. The usefulness of bioimpedance analysis in peritoneal dialysis patients. *Nephrol Dial Transplant* 2011; 26:1708-16.
297. Cridlig J, Nadi M, Kessler M. Bioimpedance Measurement in the Kidney Disease Patient. In: Penido MG, editor. *Technical Problems in Patients on Hemodialysis*. Rijeka, Croatia: InTech; 2011. p. 165-84.
298. Dilorio BR, Terracciano V, Bellizzi V. Bioelectrical Impedance measurement: errors and artifacts. *J Renal Nutr* 1999; 9:192-7.
299. Moissl U, Wabel P, Chamney P, et al. Body fluid volume determination via body composition spectroscopy in health and disease. *Physiol Meas* 2006;27:921-33.
300. Cooper BA, Aslani A, Ryan M, Zhu FY, Ibels LS, et al. Comparing different methods of assessing body composition in end-stage renal failure. *Kidney Int*. 2000;58: 408-16.

301. Wabel P, Chamney P, Moissl U, et al. Reproducibility of bioimpedance spectroscopy (BIS) for the assessment of body composition and dry weight. *J Am Soc Nephrol*. 2007;18: 255A.
302. Kraemer M, Rode C, Wizemann V. Detection limit of methods to assess fluid status changes in dialysis patients. *Kidney Int* 2006;69:1609-20.
303. Wabel P, Rode C, Moissl U, Chamney P, Wizemann V. Accuracy of Bioimpedance Spectroscopy (BIS) to detect fluid status changes in hemodialysis patients *Nephrol Dial Transplant* 2007;VI(22 Supplement 6):129.
304. Barbosa-Silva MCG, Barros AJD, Post CLA, Waitzberg DL, Heymsfield SB. Can bioelectrical impedance analysis identify malnutrition in preoperative nutrition assessment? *Nutrition*. 2003 19:422-6.
305. Wizemann V, Wabel P, Chamney P, Zaluska W, Moissl U, Rode C, et al. The mortality risk of overhydration in haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(5):1574-9.
306. <http://www.bcm-fresenius.com/23.htm>. [10 March 2015].
307. Gonçalves S, Pecoits-Filho R, Perreto S, Barberato SH, Stinghen AEM, Lima EGA, et al. Associations between renal function, volume status and endotoxaemia in chronic kidney disease patients. *Nephrol Dial Transplant* 2006;21:2788-94.
308. Lang R, Bierig M, Devereux R, Flachskampf F, Foster E, Pellikka P, et al. Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J Am Soc Echocardiogr* 2005;18(12):1440-63.

- 309.Lang R, Bierig M, Devereux R, Flachskampf F, Foster E, Pellikka P, et al. Recommendations for chamber quantification. *Eur J Echocardiogr.* 2006; 7(2):79-108.
- 310.Devereux R, Reichek N. Echocardiographic determination of left ventricular mass in man. Anatomic validation of the method. *Circulation.* 1977;55:613-8.
- 311.Foppa M, Duncan B, Rohde L. Echocardiography-based left ventricular mass estimation. How should we define hypertrophy? *Cardiovasc Ultrasound* 2005;3:17. doi: 0.1186/476-7120-3-17.
- 312.Ganau A, Devereux R, Roman M, de Simone G, Pickering T, Saba P, et al. Patterns of left ventricular hypertrophy and geometric remodeling in essential hypertension. *J Am Coll Cardiol.* 1992 19(7):1550-8.
- 313.Wikstrand J. Methodological considerations of ultrasound measurement of carotid artery intima-media thickness and lumen diameter. *Clin Physiol Funct Imaging.* 2007;27:341-5.
- 314.Brenchley J, Price D, Schacker T, Asher T, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med.* 2006;12: 1365-71.
- 315.Nasiri H, Forouzandeh M, Rasaee MJ, Rahbarizadeh F. Modified salting-out method: high-yield, high-quality genomic DNA extraction from whole blood using laundry detergent. *J Clin Lab Anal.* 2005;19(6):229-32.
- 316.Pecoits-Filho R, Stenvinkel P, Wang A, Heimbürger O, Lindholm B. Chronic inflammation in peritoneal dialysis: the search for the holy grail? *Perit Dial Int.* 2004;24:327-39.
- 317.Hauser A, Azevedo I, Gonçalves S, Stinghen A, Aita C, Pecoits-Filho R. Sevelamer carbonate reduces inflammation and endotoxemia in an animal model of uremia. *Blood Purif.* 2010;30:153-8.

318. Sandek A, Bauditz J, Swidsinski A, Buhner S, Weber-Eibel J, von Haehling S, et al. Altered intestinal function in patients with chronic heart failure. *J Am Coll Cardiol.* 2007;50:1561-9.
319. Sharma R, Bolger A, Li W, Davlourous P, Volk H, Poole-Wilson P, et al. Elevated circulating levels of inflammatory cytokines and bacterial endotoxin in adults with congenital heart disease. *Am J Cardiol.* 2003:188-93.
320. Wang A, Wang M, Woo J, Law M, Chow K, Li P, et al. A novel association between residual renal function and left ventricular hypertrophy in peritoneal dialysis patients. *Kidney Int* 2002:639-47.
321. Antlanger M, Hecking M, Haidinger M, Werzowa J, Kovarik J, Paul G, et al. Fluid overload in hemodialysis patients: a cross-sectional study to determine its association with cardiac biomarkers and nutritional status. *BMC Nephrology* 2013; 14:266.doi:10.1186/471-2369-14-266.
322. Tsai Y-C, Chiu Y-W, Kuo H-T, Chen S-C, Hwang S-J, Chen T-H, et al. Fluid Overload, Pulse Wave Velocity, and Ratio of Brachial Pre-Ejection Period to Ejection Time in Diabetic and Non-Diabetic Chronic Kidney Disease. *PLoS ONE.* 2014;9(11):1-8.
323. Ozkahya M, Ok E, Toz H, Asci G, Duman S, Basci A, et al. Long-term survival rates in haemodialysis patients treated with strict volume control. *Nephrol Dial Transplant.* 2006; 21 (12): 3506-13. doi: 10.1093/ndt/gfl487.
324. Ando Y, Tabei K, Shiina A, Asano Y, Hosoda S. Ultrasonographic evaluation of changes in the inferior vena cava configuration during hemodialysis: relationship between the amount of water removed and the diameter of the inferior vena cava. *Journal of Japanese Society for Dialysis Therapy.* 1985;18:173-9.

325. Vicenté-Martínez M, Martínez-Ramírez L, Muñoz R, Avila M, Ventura M, Rodríguez E, et al. Inflammation in patients on peritoneal dialysis is associated with increased extracellular fluid volume. *Arch Med Res.* 2004;35:220-4.
326. Stenvinkel P, Heimbürger O, Lindholm B, Kaysen G, Bergström J. Are there two types of malnutrition in chronic renal failure? Evidence for relationships between malnutrition, inflammation and atherosclerosis (MIA syndrome). *Nephrol Dial Transplant.* 2000 15(7):953-60.
327. Aguilera A, Sánchez-Tomero J, Bajo M, Ruiz-Caravaca M, Alvarez V, del Peso G, et al. Malnutrition-inflammation syndrome is associated with endothelial dysfunction in peritoneal dialysis patients. *Adv Perit Dial.* 2003;19:240-5.
328. Stenvinkel P. Malnutrition and chronic inflammation as risk factors for cardiovascular disease in chronic renal failure. *Blood Purif.* 2001;19(2):143-51.
329. Tonbul H, Demir M, Altintepe L, Güney I, Yeter E, Türk S, et al. Malnutrition-inflammation-atherosclerosis (MIA) syndrome components in hemodialysis and peritoneal dialysis patients. *Ren Fail.* 2006;28(4):287-94.
330. Stenvinkel P, Chung S, Heimbürger O, Lindholm B. Malnutrition, inflammation, and atherosclerosis in peritoneal dialysis patients. *Perit Dial Int.* 2001;21 (Suppl 3):S157-62.
331. Caridis D, Reinhold R, Woodruff P, Fine J. Endotoxaemia in man. *Lancet* 1972;299:1381-6.
332. Lumsden A, Henderson J, Kutner M. Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood in patients with cirrhosis. *Hepatology.* 1988;8: 232-6.
333. Grum C. Tissue oxygenation in low flow states and during hypoxaemia. *Crit Care Med.* 1993;21:S44-S9.

334. Takemoto Y, Nakatani T, Sugimura K, Yoshimura R, Tsuchida K. Endotoxin adsorption of various dialysis membranes : In vitro study. *Artif Organs*. 2003;27:1134-7.
335. Urena P, Herbelin A, Basile C, Zingraff J, Man N, Drueke T. In vitro studies of endotoxin transfer across cellulosic and noncellulosic dialysis membranes. I. Radiolabeled endotoxin. *Contrib Nephrol*. 1989;74:71-8.
336. Dawson ME. Interference with the LAL test and how to address it. *LAL Update*. 2005;22(3):1-5.
337. Roth RI, Levin FC, Levin J. Optimization of detection of bacterial endotoxin in plasma with the Limulus test. *J Lab Clin Med* 1990 116(2): 153-61.
338. Dawson ME. Maximum valid dilution and minimum valid concentration. *LAL Update*. 1995;13 (4):1-3.
339. Kushner RF, Schoeller DA. Estimation of total body water by bioelectrical impedance analysis. *Am J Clin Nutr*. 1988;42:101-6.
340. de Francisco AL, Stenvinkel P, Vaulont S. Inflammation and its impact on anaemia in chronic kidney disease: from haemoglobin variability to hyporesponsiveness. *NDT Plus* 2009; 2(Suppl 1):i18-i26.
341. Wiedermann CJ, Kiechl S, Schratzberger P, Dunzendorfer S, Weiss G, Willeit J. The role of immune activation in endotoxin-induced atherogenesis. *Journal of Endotoxin Research* 2001;7: 322-6.
342. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res*. 2002; 91:281-91.
343. Wu A, Wu H, Cheng M, Tseng C, Huang Y, Huang J. Relation of malnutrition, inflammation and cardiovascular disease in chronic peritoneal dialysis patients. *Acta Nephrologica*. 2007;21:40-7.

344. Middleton R, Parfrey P, Foley R. Left ventricular hypertrophy in the renal patient. *J Am Soc Nephrol*. 2001;12(5):1079-84.
345. Ha S, Park H, Kim S, Park C, Kim D, Kim H. Prevalence and patterns of left ventricular hypertrophy in patients with predialysis chronic renal failure. *J Korean Med Sci*. 1998;13(5):488-94.
346. Tucker B, Fabbian F, Giles M, Thuraisingham R, Raine A, Baker L. Left ventricular hypertrophy and ambulatory blood pressure monitoring in chronic renal failure. *Nephrol Dial Transplant* 1997;12(4):724-8.
347. Foley R. Clinical epidemiology of cardiac disease in dialysis patients: left ventricular hypertrophy, ischemic heart disease, and cardiac failure. *Semin Dial* 2003;16(2):111-7.
348. Muhammad AS, Naidoo S, Wambugu MB, Britz R, Manga P, Shehu NM, et al. Carotid intima-media thickness and cardiovascular risk factors in kidney transplant recipients at a South African transplant center. *Int J Biol Med Res* 2014;5(3):4340-5.
349. Lepper P, Schumann C, Triantafilou K, Rasche F, Schuster T, Frank H, et al. Association of lipopolysaccharide-binding protein and coronary artery disease in men. *J Am Coll Cardiol* 2007;50(1):25-31.
350. Gutschmann T, Müller M, Carroll S, MacKenzie R, Wiese A, Seydel U. Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. *Infect Immun* 2001;69(11):6942-50.
351. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, et al. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2001;21:844-8.
352. Toshima S, Hasegawa A, Kurabayashi M, Itabe H, Takano T, Sugano J, et al. Circulating oxidized low density lipoprotein levels: a biochemical risk marker for coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2000;20:2243-7.

353. Kempf K, Haltern G, Futh R, Herder C, Muller-Scholze S, Gulker H, et al. Increased TNF-alpha and decreased TGF-beta expression in peripheral blood leukocytes after acute myocardial infarction. *Horm Metab Res.* 2006;38:346-51.
354. Erren M, Reinecke H, Junker R, Fobker M, Schulte H, Schurek JO, et al. Systemic inflammatory parameters in patients with atherosclerosis of the coronary and peripheral arteries. *Arterioscler Thromb Vasc Biol* 1999;19(10):2355-63.
355. Nikol S, Isner JM, Pickering JG, Kearney M, Leclerc G, Weir L. Expression of transforming growth factor-beta 1 is increased in human vascular restenosis lesions. *J Clin Invest* 1992; 90(4):1582-92.
356. Wolf YG, Rasmussen LM, Ruoslahti E. Antibodies against transforming growth factor-beta 1 suppress intimal hyperplasia in a rat model. *J Clin Invest* 1994 93(3):1172-8.
357. Gamble JR, Vadas MA. Endothelial adhesiveness for blood neutrophils is inhibited by transforming growth-factor beta 1. *Science.* 1998;242:97-9.
358. Bauriedel G, Schluckebier S, Hutter R, Welsch U, Kandolf R, Lüderitz B, et al. Apoptosis in restenosis versus stable-angina atherosclerosis: implications for the pathogenesis of restenosis. *Arterioscler Thromb Vasc Biol* 1998 18(7):1132-9.
359. Cambien F, Ricard S, Troesch A, Mallet C, Generenaz L, Evans A, et al. Polymorphisms of the transforming growth factor-beta 1 gene in relation to myocardial infarction and blood pressure. The Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) Study. *Hypertension* 1996;28:881-7.
360. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, et al. The Effect of Novel Polymorphisms in the Interleukin-6 (IL-6) Gene on IL-6 Transcription and Plasma IL-6 Levels, and an Association with Systemic-Onset Juvenile Chronic Arthritis. *J Clin Invest.* 1998;102 (7):1369-76.

361. Berg KK, Madsen HO, Garred P, Wiseth R, Gunnes S, Videm V. The Additive Contribution from Inflammatory Genetic Markers on the Severity of Cardiovascular Disease. *Scandinavian Journal of Immunology* 2009;69:36-42.
362. Humphries SE, Luong LA, Ogg MS, Hawe E, Miller GJ. The interleukin-6 - 174 G/C promoter polymorphism is associated with risk of coronary heart disease and systolic blood pressure in healthy men. *Eur Heart J*. 2001;22:2243-52.
363. Rundek T, Elkind MS, Pittman J, Boden-Albala B, Martin S, Humphries SE. Carotid intima-media thickness is associated with allelic variants of stromelysin-1, interleukin-6, and hepatic lipase genes: the Northern Manhattan Prospective Cohort Study. *Stroke*. 2002; 33:1420-3.
364. Moissl U, Bosaeus I, Lemmey A, et al. Validation of a 3C model for determination of body fat mass. *J Am Soc Nephrol*. 2007;18:257A.
365. Moissl U, Wabel P, Chamney P, et al. Validation of a bioimpedance spectroscopy method for the assessment of fat free mass. *NDT Plus*. 2008;1(Suppl 2): ii215.
366. Nishizawa Y, Shoji T, Maekawa K, Nagasue K, Okuno S, Kim M, et al. Intima-media thickness of carotid artery predicts cardiovascular mortality in hemodialysis patients. *Am J Kidney Dis*. 2003;41(Suppl 1):S76-S9.
367. Ekart R, Hojs R, Hojs-Fabjan T, Balon B. Predictive value of carotid intima media thickness in hemodialysis patients. *Artif Organs*. 2005;29: 615-9.
368. Szeto C, Chow K, Woo K, Chook P, Kwan B, Leung C, et al. Carotid intima media thickness predicts cardiovascular diseases in Chinese pre-dialysis chronic kidney diseases patients. *J Am Soc Nephrol* 2007;18:1966-72.
369. Kiechl S, Lorenz E, Reindl M, Wiedermann C, Oberhollenzer F, Bonora E, et al. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med*. 2002;347:185-92.

APPENDIX 1

Submitted Manuscript

ASSESSMENT OF VOLUME OVERLOAD AND ITS RISK FACTORS IN SOUTH AFRICAN CHRONIC KIDNEY DISEASE PATIENTS: AN APPRAISAL OF BIO-IMPEDANCE SPECTROSCOPY AND INFERIOR VENA CAVA DIAMETER MEASUREMENTS

Muzamil Olamide Hassan¹, Raquel Duarte², Therese Dix-Peek², Ahmed Vachiat³, Caroline Dickens², Sacha Grinter³, Sagren Naidoo¹, Pravin Manga³ and Saraladevi Naicker⁴

¹Division of Nephrology, Department of Internal Medicine, University of the Witwatersrand, South Africa; ²Department of Internal Medicine Research Laboratories, Department of Internal Medicine, University of the Witwatersrand, South Africa; ³Division of Cardiology, Department of Internal Medicine, University of the Witwatersrand, South Africa; ⁴School of Clinical Medicine, University of the Witwatersrand, South Africa

Correspondence: Muzamil Olamide Hassan, Renal Unit, Department of Medicine,

Obafemi Awolowo University/Teaching Hospitals Complex, PMB 5538 Ile-Ife, Osun State Nigeria.

Telephone number: +2348034399839

E-mail: muzlamide@yahoo.com

Abstract

Background: Fluid overload occurs early in chronic kidney disease (CKD); driving inflammation, left ventricular hypertrophy (LVH) and atherosclerosis. This study is aimed to assess volume overload in South African CKD patients and determine the relationship between malnutrition, inflammation, cardiovascular disease (CVD) and volume overload using bio-impedance spectroscopy (BIS) analysis. We also evaluated the usefulness of bio-impedance spectroscopy (BIS) and inferior vena cava diameter (IVCD) measurements in assessing volume overload.

Methods: One hundred and sixty patients comprising haemodialysis (HD), peritoneal dialysis (PD), stage 3 CKD patients and controls (40 in each group) were studied. Bio-impedance spectroscopy method, Body Composition Monitor (BCM) was used to assess fluid and nutritional status. Echocardiographic studies were done for the presence of LVH, IVCD and carotid intima media thickness (CIMT). Serum interleukin-6 (IL-6) levels and C-reactive protein (CRP) were measured as markers of inflammation.

Results: Volume overload and malnutrition were present in 88% and 63% of the CKD patients. Nutritional markers, inflammation, atherosclerosis and left atrial dimension were associated with volume overload. Receiver operating concentration (ROC) curves for IVCD (11.5mm/m^2) in predicting fluid overload using BIS as confirmatory tool showed AUC of 0.727 (sensitivity 0.504, specificity 0.818, $p < 0.0001$).

Conclusions: Volume overload was common across the spectrum of South African CKD cohorts and was associated with malnutrition, inflammation. BIS is a more sensitive tool than IVCD measurements for the assessment of fluid status in clinically euvolumic non-dialytic CKD patients.

Keywords: Atherosclerosis, chronic kidney disease, inflammation, malnutrition, volume overload

Summary

Volume overload and malnutrition are not uncommon among predominantly black stage 3 CKD cohorts. Volume overload was independently predicted by uraemia, inflammation, malnutrition, atherosclerosis and left atrial dimension in South African CKD patients. Bio-impedance spectroscopy measurement is more sensitive than IVCD measurements in detecting volume overload in ‘clinically euvolaemic’ non-dialytic CKD patients.

Introduction

Fluid retention occurs early in CKD, resulting in chronic fluid overload which is closely associated with LVH (1), hypertension (2), cardiac dysfunction (2), inflammation (3) and mortality in dialysis patients (4). Malnutrition adversely affect prognosis in CKD patients, aggravating existing inflammation and accelerating atherosclerosis (5), and is closely associated with an increase morbidity and mortality in these patients (6, 7).

Carotid intima media thickness is an independent risk factor for CVD events and mortality (8, 9). Wealth of evidence from observational and interventional studies has validated CIMT as a good surrogate marker for CVD (10-14). The predictive association between baseline CIMT and increased CVD risk has been demonstrated in healthy young adult populations (15, 16).

Body composition monitor, is fast, non-invasive and cheap method for detecting malnutrition and volume overload (17). It is based on the principle of BIS, a method that measures opposition of alternating electric current by body cells and tissues over a wide range of frequencies, and also incorporates three-compartments (3C) model of body composition (17). The BCM has been validated against various gold standard methods to detect total body water, extracellular water (ECW) and intracellular water (ICW) based on the assumption that in healthy individual, regardless of the body composition, a predetermined mass of tissue represent fixed amount of ICW and ECW, thus implying that the ratio of ECW to ICW in a fixed mass of tissue is constant (18). This 3C compartments model detects excess fluid, differentiates normal hydrated lean tissue mass from normal hydrated fat tissue mass and also provides a specific method for assessing nutritional and fluid status in dialysis patients (19). In addition, body cell mass, an indicator of both visceral and somatic protein in the body, have also been validated in HD patients (20). Even though the use of BIS for the assessment of nutritional status has been described previously in HD (21, 22) and PD (3, 23) patients, its

usefulness in the assessment of malnutrition in patients with early CKD (stage 3) is yet to be reported in the literature.

Furthermore, BIS, provides a precise and specific method for obtaining information about fluid status in healthy population, HD, PD and renal transplant patients (17, 24-26). However, there is paucity of local data on the usefulness of BIS analysis for detection of volume overload in patients with CKD but not yet requiring dialysis. Although there is a wealth of data suggesting that malnutrition, inflammation and atherosclerosis are major predictors of mortality in CKD patients (3, 27-29), it remains to be established whether volume overload contributes to malnutrition, inflammation and atherosclerosis in a predominantly black African CKD population; with traditionally lower risk of atherosclerotic CVD. We aimed to assess volume overload and malnutrition using a BCM among the spectrum of black South African CKD patients and also investigate relationship between malnutrition, inflammation, subclinical atherosclerosis and volume overload in CKD patients. We also compared the utility of BIS and echocardiographic measurements in assessing volume overload.

Subjects and Methods

A cross-sectional study of 120 CKD patients treated at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), Johannesburg; comprising 40 each of PD, HD and stage 3 CKD patients as well as 40 age and sex-matched controls. The study was approved by the University of the Witwatersrand, human research ethics committee (Protocol M130127) and was in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Information regarding demographic data was documented. Height was measured to the nearest 0.5 cm and weight to the nearest 0.1 kg using the Seca 220 telescopic measuring rod (Seca gmbh GmbH & Co, Germany) and body mass index (BMI) was calculated as the ratio

of weight to height squared. The dry weight was used to calculate BMI in haemodialysis patients. Estimated glomerular filtration rate (eGFR) was calculated using CKD-EPI formula.

Haemoglobin concentration, creatinine, albumin, calcium, phosphate and intact parathormone were analysed in the National Health Laboratory as part of the routine standard of care using ADVIA^R auto-analysers (Siemens Healthcare Diagnostics Inc, USA).

C-reactive protein and Interleukin-6 assay

Serum CRP and IL-6 assays were analyzed using Luminex® Performance Assay multiplex kits (R&D Systems, Inc. Minneapolis, USA). Assays were in accordance with the manufacturer's instructions. Absorbance for CRP and IL-6 were read in the bead region 8 and 19 respectively, on the Bio-PlexTM 200 system (Bio-Rad, Texas, USA) and concentrations were generated automatically with Bio-Plex manager software, version 5.0 (Bio-Rad Laboratories Inc, Hercules, USA).

Bio-impedance spectroscopy analysis

Fluid and nutritional status were evaluated with BCM (Fresenius Medical Care, Germany). Malnutrition was defined as lean tissue index (LTI) less than the 10th percentile of a reference population (30). Percentage body cell mass (%BCM) was computed using the formula: $100 \times \text{BCM} / \text{current weight}$. Male and female patients with %BCM less than 35% and 30% respectively were considered malnourished (31). Fluid overload was defined by absolute overhydration (in litres) or as $\text{OH/ECW\%} \geq 15\%$ (32). Exclusion criteria included patients with amputated limbs, patients with pacemakers and/or implants; and patients with incomplete measurements. In addition to the fluid overload measurement by the BCM, patient's fluid status was also assessed by evaluating for the presence of oedema.

Echocardiography

Echocardiography was performed according to the American Society of Echocardiography recommendations (33, 34) using the Philips iE33 machine equipped with a S5-1 1-5 MHz transducer (Philips Corporation USA).

Carotid intima media thickness

As previously described (35), CIMT was assessed using high resolution B-mode ultrasonography with the aid of L3-11 MHz linear array transducer (Philips Corporation USA).

Inferior vena cava diameter measurement

The anteroposterior dimension of IVCD was measured using two-dimensional and Doppler recordings 1.5 cm below the diaphragm in the hepatic segment. The IVCD was expressed as IVCD in expiration, adjusted for body surface area (BSA). Fluid overload was defined as IVCD $>11.5\text{mm/m}^2$ (36). The collapsibility index (CI) was calculated as the percentage of decrease in IVCD in inspiration versus expiration. Patients with a CI lower than 40% were considered to be fluid overloaded (37).

Data analysis

Data analyses were performed using the statistical package for social sciences (SPSS) version 16 (SPSS, Inc., Chicago IL). Patients' parameters were presented as mean $\pm$ SD for continuous data, and percentages and frequencies for categorical data. Categorical data were compared using the chi-square test and continuous data using Mann Whitney and Kruskal Wallis test. We ascertained sensitivity and specificity of IVCD (cut-off value: 11.5mm/m^2), CI (cut-off value: $<40\%$) and the presence of oedema (by physical examination) for fluid overload using receiver operating concentration (ROC) curve analysis. Correlation between

variables was assessed by the Spearman or Pearson correlation coefficients accordingly. Factors independently associated with fluid overload were further explored by a multiple linear regression analysis. A $P < 0.05$ (two-tailed) was considered significant.

Results

Demographic, clinical and laboratory data

A total of 160 participants, include HD, PD, stage 3 CKD patients and controls (40 in each group) were enrolled for this study. Demographic, clinical and laboratory parameters of participants were shown in Table 1.

Characteristics of the study population

Fluid status

Fluid status, inflammatory response, nutritional status, echocardiographic parameters and CIMT measurements in different subgroups of patients were described in Table 2. Overhydration and its percentage in relation to extracellular water (OH/ECW %) and IVCD were higher in CKD patients when compared to the values in the controls, while the collapsibility index was significantly lower in patients compared to the control group. Overall, fluid overload defined by BIS, IVCD, CI and oedema (by physical examination) was present in 87.5% (105/120), 52% (62/120), 29% (35/120) and 62% (74/120) of all CKD patients respectively. Ninety-eight percent (54/58), 95% (81/85) and 89% (41/46) of the CKD patients initially identified as normovolumic by IVCD, CI and physical examination respectively were diagnosed as volume overloaded by BIS machine. Specifically, all patients on HD were overloaded while 90% (36/40) and 72.5% (29/40) of PD and stage 3 CKD patients respectively were diagnosed as fluid overloaded by BCM.

Nutritional status

CKD patients were generally malnourished, with LTI (14.2 ± 3.2 vs 16.1 ± 3.0 kg/m², $p=0.002$) and BCM (21.2 ± 7.2 vs 25.6 ± 7.9 kg; $p=0.027$) significantly lower than in the controls while FTI (12.8 ± 7.7 vs 10.9 ± 6.7 kg/m², $p=0.005$) and ATM (42.0 ± 21.0 vs 33.3 ± 18.2 kg, $p=0.007$) were significantly higher in the controls. As defined by low LTI, 63.3% (73/120) of the CKD patients were malnourished, 65% (78/120) of the patients were undernourished as evidenced by increased FTI, while 50.8% (61/120) of the patients had malnutrition diagnosed by low %BCM. There was a strong correlation between LTI and %BCM ($r=0.785$, $p<0.001$), LTI had a weak correlation with FTI ($r=-0.252$, $p=0.006$) while FTI also weakly correlated with %BCM ($r=-0.311$, $p=0.001$). Malnutrition was diagnosed by low LTI in 55% (22/40), 60% (24/40) and 37.5% (15/40) of the HD, PD and stage 3 CKD patients respectively. Further analysis showed that 81.3% (35/43) of the patients with volume overload had malnutrition. Fat tissue index also showed a strong association with BMI ($r=-0.890$, $p<0.001$).

Inflammatory markers

There was significant increment in the levels of inflammatory markers in the CKD subjects compared to the controls (CRP: 0.87 ± 0.72 versus 0.45 ± 0.48 mg/dL, $p<0.001$; IL-6: 2.70 ± 3.76 versus 1.03 ± 1.15 pg/ml, $p<0.001$).

Echocardiographic and carotid ultrasound

Chronic kidney disease patients had severe cardiac chamber enlargement compared to the controls (left ventricular mass index: 118.8 ± 38.8 vs 84.4 ± 15.9 g/m², $p<0.001$; left atrial dimension: 4.0 ± 0.7 vs 3.1 ± 0.4 cm, $p<0.001$). Left ventricular ejection fraction was significantly lower in CKD patients compared to the controls (EF: 58.6 ± 8.8 vs 65.0 ± 8.0 %,

p<0.001). Patients with kidney failure had significantly lower E/A ratio and higher left ventricular filling pressure (E/e'); when compared to the control group; p<0.001. However, there was no significant difference in the deceleration time between CKD patients and those with normal kidney function. Carotid intima media thickness was significant higher among CKD patients compared to the controls (0.58 ± 0.12 versus 0.45 ± 0.05 mm; p<0.001). Further analysis showed that CIMT was 0.63 ± 0.12 ; 0.56 ± 0.09 and 0.56 ± 0.12 in PD, HD and CKD stage 3 patients respectively (Figure 1).

Receiver operating characteristic curve analysis of fluid status assessment

Receiver operating characteristic curve analysis was performed to calculate the sensitivity and specificity of IVCD (cut-off value of 11.5mm/m^2), CI (cut-off value of <40%) and oedema (physical examination) as a diagnostic tool to detect volume overload (defined by $\text{OH/ECW}\% \geq 15\%$) in the studied patients. The area under concentration curve (AUC) for IVCD, CI and edema were 0.663 (sensitivity 0.581, specificity 0.624, p=0.002), 0.662 (sensitivity 0.372, specificity 0.821, p =0.002) and 0.740 (sensitivity 0.814, specificity 0.667, p<0.0001) respectively.

Characteristics of patients with fluid overload (defined by $\text{OH/ECW}\% \geq 15\%$)

As shown in Table 3, compared with patients with normal hydration status, patients with volume overload were anaemic (haemoglobin: 10.6 vs 12.5 g/dL, p<0.0001), had severe renal failure (eGFR: 7.6 vs 30.4 ml/min/ 1.73m^2 , p<0.001), had severe inflammation (CRP: 1.24 ± 0.70 mg/dL vs 0.89 ± 0.72 mg/dL, p=0.011) (Figure 2); IL-6: 3.6 vs 2.1 pg/ml, p=0.023), undernourished (LTI: 12.4 vs 15.2 kg/ m^2 , p<0.0001; FTI: 10.2 vs 14.3 kg/ m^2 , p=0.002; BCM: 17.7 vs 23 kg; p<0.0001), had lower serum albumin (36.2 vs 39.8 g/L) but had higher cardiovascular morbidity (LVMI: 132 vs 110 g/ m^2 , p=0.002; CIMT: 0.61 vs 0.56mm, left

atrial enlargement (LAE): 4.2 vs 3.7 cm, $p<0.0001$). Overall, 68% of CKD patients had left ventricular hypertrophy (LVH) with concentric LVH being the commonest pattern (Table 4).

Association between fluid status and some measured variables in CKD patients

Table 5 shows the correlations between some of the studied parameters in patients with CKD. Markers of inflammation, nutritional status, CIMT, LVMI and LAD were weakly correlated with volume parameters. OH/ECW% showed slight positive correlation with IL-6 ($r=0.313$; $p<0.001$) and LVMI ($r=0.303$; $p<0.001$); and slight inverse correlation with serum albumin ($r=-0.289$, $p<0.001$), lean tissue index ($r=-0.326$, $p<0.001$) and fat tissue index ($r=-0.297$, $p<0.001$). Carotid intima media thickness correlated with overhydration ($r=0.442$, $p<0.001$) and OH/ECW% ($r=0.421$, $p<0.001$). Carotid intima media thickness was also weakly associated with CRP ($r=0.418$, $p<0.001$). Left atrial size positively correlated with overhydration ($r=0.454$, $p<0.001$), OH/ECW% ($r=0.489$, $p<0.001$) and IVCD ($r=0.345$, $p<0.001$). Serum creatinine correlated with all four markers of volume overload (overhydration: $r=0.551$, $p<0.001$; OH/ECW%: $r=0.554$, $p<0.001$; IVCD: $r=0.358$, $p<0.001$; and CI: $r=-0.399$, $p<0.001$). No correlation was seen between markers of volume overload and age. Multiple linear regression analysis was performed to further determine which of the variables independently predicted volume overload in CKD patients. Only serum creatinine ($\beta=0.152$, $p<0.001$), LTI ($\beta=-0.328$, $p<0.001$), FTI ($\beta=-0.261$, $p<0.001$), LAD ($\beta=0.336$, $p<0.001$) and CIMT ($\beta=0.238$, $p=0.002$) were identified as independent determinants of volume overload (Table 6), with overall model $r^2 = 0.55$. When looking at the effect of volume overload on the markers of inflammation, OH/ECW% significantly affect CRP and IL-6 levels ($p<0.002$ and $p<0.001$ respectively) in CKD patients.

Discussion

This study demonstrated that volume overload was very common across the spectrum of our CKD patients. Fluid overload by BCM occurred in all HD patients (100%), 90% of PD patients and 73% of stage 3 CKD patients in this study and was exceptionally high when compared to previously reported prevalence of 39%, 66.8% and 50% among HD (38), PD (39), and non-dialytic CKD patients (40) respectively, possibly because this study involved predominantly black CKD cohorts, with hypertension (49.2%) and presence of LVH (68%) in a large proportion of the study population.

Body composition monitor significantly improved the sensitivity in diagnosis of volume overload when compared with echocardiographic measurements and physical examination. The prevalence of fluid overload using BCM in this study was substantially higher than the diagnostic rates of 52%, 29% and 62% by IVCD, CI and physical examination respectively. This supports the use of BCM in the earlier detection of occult volume overload in 'clinically normovolemic' non-dialytic CKD patients, since strict volume control has been documented to play a crucial role in the amelioration of cardiovascular complications in CKD patients (39, 41) as well as improve the survival of dialysis patients (42).

Although a previous report had suggested that IVCD measurements are useful markers for volume overload in CKD patients (37), this study demonstrated that the sensitivity and specificity of IVCD measurements as a diagnostic tool for fluid overload was relatively low compared to BCM which was used as the confirmatory test. Perhaps, IVCD measurements could have overestimated the underhydrated state as a result of delay of refilling from the interstitial fluid compartment into the intravascular space (43). In addition, bio-impedance spectroscopy and inferior vena cava diameters measure fluid in different compartments (44, 45).

Available evidence suggests that the degree of renal impairment was associated with overhydration (37). We observed a close relationship between serum creatinine and all markers of volume status, suggesting that impaired excretion of salt and water by the kidneys may contribute to retention of fluid in CKD patients. This had been previously reported in non-dialytic CKD and continuous ambulatory peritoneal dialysis patients (CAPD) (37, 39).

The prevalence of malnutrition as expressed by low LTI was present in 63% of our CKD patients, and this is consistent with the report of a previous study which established undernutrition in about 60% of the study population (22). However, when %BCM was used, 50.8% of our CKD patients were classified as malnourished and this was slightly higher than the previously reported prevalence of 43.9% by Oliveira *et al* (21). Assessment of nutritional status by %BCM is more specific than LTI because the lean tissue mass contains extracellular water which is typically increased in CKD, thereby resulting in overestimation of nutritional status in CKD patients (21). Thus, explaining the variation between the prevalence of malnutrition measured by low LTI and %BCM.

Remarkably, our study demonstrated a high prevalence of malnutrition of about 40% among stage 3 CKD patients. This highlights the significance of protein-energy malnutrition in early CKD as well as the usefulness of whole body BIS device (BCM) for early detection of malnutrition in non-dialytic CKD patients. Bio-impedance spectroscopy may be useful for regular monitoring of hydration and nutritional status in non-dialytic CKD patients; thus allowing early recognition of a high-risk patient that may benefit from aggressive treatment of malnutrition and overhydration.

This study further demonstrated high prevalence of volume overload (81%) among CKD patients with low LTI. This study also showed that overhydrated patients had lower serum albumin and serum albumin showed a modest but significant association with markers of

fluid overload. Theoretically, hypoalbuminaemia in undernourished patients not only brings about reduced plasma oncotic pressure, leading to fluid retention in interstitial space; it may also be a consequence of dilution from resulting volume overload. Even though our study suggests that hypoalbuminaemia may contribute to fluid retention in our CKD cohorts, it remains to be seen whether nutritional rehabilitation will improve cardiovascular outcomes in CKD patients.

The presence of LVH in CKD patients is variable. It was documented in 50%-97% of end stage renal disease patients (41, 46-49). In this study, we found LVH in 68% of the CKD patients, with concentric LVH being the commonest LVH pattern present in 48% of our CKD population. Foley *et al* had previously described concentric hypertrophy and eccentric hypertrophy in 44% and 30% of their studied population respectively. We also demonstrated an association between inflammation and LVH as well as between CIMT and volume overload which is in keeping with the study of Wu *et al* that found a strong association between inflammation and cardiovascular disease in PD patients (29).

There is increasing evidence that fluid overload is an important and independent predictor of inflammatory activation in CKD. We established a relationship between volume overload and markers of inflammation. This is in keeping with a previous study in PD cohorts that showed an association between hypervolaemia and inflammation (50); this study showed that relative overhydration was common in PD patients with inflammation compared with those without inflammation (50). Similarly, in our study, we demonstrated that fluid overloaded patients had elevated mean CRP and IL-6 values when matched with those without fluid overload.

On multiple linear regression analysis, serum creatinine, LTI, FTI, CIMT and LAD were identified as independent determinants of fluid overload in CKD patients. In addition, volume overload significantly predicted inflammation in the CKD patients. These findings

corroborated report of earlier studies that showed a link between volume overload and the “malnutrition-inflammation-atherosclerosis” syndrome (27, 28, 51-53). Stenvinkel *et al*, found a strong association between malnutrition, inflammation and occurrence of cardiovascular disease in patients starting dialysis (28). Likewise, Dermirci *et al* reported a close association between inflammation, atherosclerosis and volume parameters in peritoneal dialysis patients (3). The same group identified LAD as a surrogate marker of fluid overload, confirming the findings of our study.

This study is a single-center descriptive cross-sectional study. Volume overload and nutritional status were defined by a body composition monitor device as designed by the manufacturer. There is lack of local data on validation of BIS in healthy populations. Nevertheless, all output parameters of the body composition monitor have thus far been validated against the gold standard reference method by isotope dilution in both patients and healthy individuals (54-57). Most importantly, the BIS technique is highly reproducible and very specific in the detection of both nutritional and fluid status (58-60). Future longitudinal studies are needed to determine the impact of guided optimization of fluid status on inflammation and cardiovascular disease in CKD patients.

In conclusion, volume overload and malnutrition was highly prevalent across the spectrum of the South African CKD patients. Bio-impedance spectroscopy measurement is more sensitive than IVCD measurements in detecting volume overload in ‘clinically euvolaemic’ non-dialytic CKD patients. Volume overload was independently predicted by uraemia, inflammation, malnutrition, atherosclerosis and left atrial dimension.

Acknowledgements

The authors would like to thank the National Research Fund, Division of Nephrology and the Faculty Research Committee, Faculty of Health Sciences, University of the Witwatersrand Johannesburg for providing funds for CRP and IL-6 assay, and Fresenius Medical Care, South Africa for donating body composition monitor used for assessment of nutritional and fluid parameters.

Dr Hassan was an ISN Fellow at the University of the Witwatersrand and this study contributed to research towards his PhD degree program in the University of the Witwatersrand.

References

1. Fagugli R, Pasini P, Quintaliani G, Pasticci F, Ciao G, Cicconi B, et al. Association between extracellular water, left ventricular mass and hypertension in haemodialysis patients. *Nephrol Dial Transplant*. 2003;18(11):2332-8.
2. Konings C, Kooman J, Schonck M, Dammers R, Cheriex E, Palmans Meulemans A, et al. Fluid status, blood pressure, and cardiovascular abnormalities in patients on peritoneal dialysis. *Perit Dial Int*. 2002;22(4):477-87.
3. Demirci M, Demirci C, Ozdogan O, Kircelli F, Akcicek F, Basci A, et al. Relations between malnutrition–inflammation–atherosclerosis and volume status. The usefulness of bioimpedance analysis in peritoneal dialysis patients. *Nephrol Dial Transplant* 2011; 26:1708-16.
4. Paniagua R, Ventura M, Avila-Díaz M, Hinojosa-Heredia H, Méndez-Durán A, Cueto-Manzano A, et al. NT-proBNP, fluid volume overload and dialysis modality are independent predictors of mortality in ESRD patients. *Nephrol Dial Transplant*. 2010;25(2):551-7.
5. Pecoits-Filho R, Lindholm B, Stenvinkel P. The malnutrition, inflammation, and atherosclerosis (MIA) syndrome - the heart of the matter. *Nephrol Dial Transplant* 2002;17(Suppl 11): 28-31.
6. Lowrie EG, Lew NL, . Death risk in hemodialysis patients: the predictive value of commonly measured variables and an evaluation of death rate differences between facilities. *Am J Kidney Dis*. 1990;15:458-82.
7. Owen WF Jr, Lew NL, Liu Y, Lowrie EG, Lazarus JM. The urea reduction ratio and serum albumin concentration as predictors of mortality in patients undergoing hemodialysis. *N Eng J Med*. 1993;329:1001-6.

8. Mookadam F, Moustafa S, Lester S, Warsame T. Subclinical Atherosclerosis: Evolving Role of Carotid Intima-Media Thickness. *Prev Cardiol.* 2010;13:186-97.
9. Rosvall M, Janzon L, Berglund G, Engström G, Hedblad B. Incident coronary events and case fatality in relation to common carotid intima-media thickness. *J Intern Med.* 2005;257(5):430- 7.
10. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation.* 1997;96:1432-7.
11. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, et al. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987-1993. . *Am J Epidemiol* 1997;146:483-94.
12. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr, et al. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. *N Engl J Med.* 1999;340:14-22.
13. Blankenhorn DH, Selzer RH, Crawford DW, Barth JD, Liu CR, Liu CH, et al. Beneficial effects of colestipolnicin therapy on the common carotid artery. Two- and four-year reduction of intima-media thickness measured by ultrasound. *Circulation.* 1993; 88:20-8.
14. Espeland MA, O'Leary DH, Terry JG, Morgan T, Evans G, Mudra H. Carotid intimal-media thickness as a surrogate for cardiovascular disease events in trials of HMG-CoA reductase inhibitors. *Curr Control Trials Cardiovasc Med.* 2005;6(1):3. doi: 10.1186/468-6708-6-3.
15. Oren A, Vos LE, Uiterwaal CS, Grobbee DE, Bots ML. Cardiovascular risk factors and increased carotid intima-media thickness in healthy young adults: the

- Atherosclerosis Risk in Young Adults (ARYA) Study. Arch Intern Med. 2003;163:1787-92.
16. Krishnan P, Balamurugan A, Urbina E, Srinivasan SR, Bond G, Tang R, et al. Cardiovascular risk profile of asymptomatic healthy young adults with increased carotid artery intima-media thickness: the Bogalusa Heart Study. J La State Med Soc. 2003;155:165-9.
 17. Chanchairujira T, Mehta R. Bioimpedance and Its Application. Saudi J Kidney Dis Transplant 2005;16(1):6-16.
 18. Moissl UM, Wabel P, Chamney PW, Bosaeus I, Levin NW, Bosy-Westphal A. Body fluid volume determination via body composition spectroscopy in health and disease. Physiol Meas 2006;27(9):921-33.
 19. Chamney P, Wabel P, Moissl U, et al. A whole-body model to distinguish excess fluid from the hydration of major body tissues Am J Clin Nutr. 2007;85:80-9.
 20. Chertow GM, Lowrie EG, Wilmore DW, Gonzalez J, Lew NL, et al., et al. Nutritional assessment with bioelectrical impedance analysis in maintenance hemodialysis patients. J Am Soc Nephrol 1995; 6:75-81.
 21. Oliveira CMC, Kubrusly M, Mota RS, Silva CAB, Oliveira VN. Malnutrition in chronic kidney failure: what is the best diagnostic method to assess. J Bras Nefrol. 2010;32(1):55-68.
 22. Valtuille R, Casos ME, Fernandez EA, Guinsburg A, Marelli C. Nutritional Markers and Body Composition in Hemodialysis Patients. International Scholarly Research Notices. 2015;2015:7.
 23. Fein PA, Gundumalla G, Jorden A, Matza B, Chattopadhyay J, Avram MM. Usefulness of bioelectrical impedance analysis in monitoring nutrition status and survival of peritoneal dialysis patients. Adv Perit Dial 2002;18:195-9.

24. van den Ham EC, Kooman JP, Christiaans MH, Nieman FH, Van Kreel BK, et al. Body composition in renal transplant patients: bioimpedance analysis compared to isotope dilution, dual energy X-ray absorptiometry, and anthropometry. *J Am Soc Nephrol*. 1999;10:1067-79.
25. Jones CH, Smye SW, Newstead CG, Will EJ, Davison AM. Extracellular fluid volume determined by bioelectric impedance and serum albumin in CAPD patients. *Nephrol Dial Transplant* 1998;13:393-7.
26. Cooper BA, Aslani A, Ryan M. Comparing different methods of assessing body composition in end-stage renal failure. *Kidney Int* 2000;58:408-16.
27. Stenvinkel P. Malnutrition and chronic inflammation as risk factors for cardiovascular disease in chronic renal failure. *Blood Purif*. 2001;19(2):143-51.
28. Stenvinkel P, Chung S, Heimbürger O, Lindholm B. Malnutrition, inflammation, and atherosclerosis in peritoneal dialysis patients. *Perit Dial Int*. 2001;21 (Suppl 3):S157-62.
29. Wu A, Wu H, Cheng M, Tseng C, Huang Y, Huang J. Relation of malnutrition, inflammation and cardiovascular disease in chronic peritoneal dialysis patients. *Acta Nephrologica*. 2007;21:40-7.
30. <http://www.bcm-fresenius.com/23.htm>.
31. Barbosa-Silva MCG, Barros AJD, Post CLA, Waitzberg DL, Heymsfield SB. Can bioelectrical impedance analysis identify malnutrition in preoperative nutrition assessment? *Nutrition*. 2003 19:422-6.
32. Wizemann V, Wabel P, Chamney P, Zaluska W, Moissl U, Rode C, et al. The mortality risk of overhydration in haemodialysis patients. *Nephrol Dial Transplant* 2009; 24(5):1574-9.
33. Lang R, Bierig M, Devereux R, Flachskampf F, Foster E, Pellikka P, et al. Recommendations for chamber quantification: a report from the American Society of

Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J Am Soc Echocardiogr* 2005;18(12):1440-63.

34. Lang R, Bierig M, Devereux R, Flachskampf F, Foster E, Pellikka P, et al. Recommendations for chamber quantification. *Eur J Echocardiogr*. 2006; 7(2):79-108.
35. Stein J, Korcarz C, Hurst R, Lonn E, Kendall C, Mohler E, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr* 2008;21(2): 93-111.
36. Cheriex E, Leunissen K, Janssen J, Mooy JM, van Hooff J. Echography of the inferior vena cava is a simple and reliable tool for estimation of 'dry weight' in haemodialysis patients. *Nephrol Dial Transplant*. 1989;4:563-8.
37. Gonçalves S, Pecoits-Filho R, Perreto S, Barberato SH, Stinghen AEM, Lima EGA, et al. Associations between renal function, volume status and endotoxaemia in chronic kidney disease patients. *Nephrol Dial Transplant* 2006;21:2788-94.
38. Antlanger M, Hecking M, Haidinger M, Werzowa J, Kovarik J, Paul G, et al. Fluid overload in hemodialysis patients: a cross-sectional study to determine its association with cardiac biomarkers and nutritional status. *BMC Nephrology* 2013; 14:266.
39. Guo Q, Yi C, Li J, Wu X, Yang X, Yu X. Prevalence and risk factors of fluid overload in Southern Chinese continuous ambulatory peritoneal dialysis patients. *PLoS One*. 2013;8(1):e53294. doi: 10.1371/journal.pone.0053294. Epub 2013 Jan 14.
40. Tsai Y-C, Chiu Y-W, Kuo H-T, Chen S-C, Hwang S-J, Chen T-H, et al. Fluid Overload, Pulse Wave Velocity, and Ratio of Brachial Pre-Ejection Period to Ejection

Time in Diabetic and Non-Diabetic Chronic Kidney Disease. PLoS ONE. 2014;9(11):e111000. doi:10.1371/journal.pone.0111000.

41. Levin A, Singer J, Thompson C, Ross H, Lewis M. Prevalent left ventricular hypertrophy in the predialysis population: identifying opportunities for intervention. *Am J Kidney Dis.* 1996 27(3).
42. Ozkahya M, Ok E, Toz H, Asci G, Duman S, Basci A, et al. Long-term survival rates in haemodialysis patients treated with strict volume control. *Nephrol Dial Transplant.* 2006; 21 (12): 3506-13. doi: 10.1093/ndt/gfl487.
43. Ando Y, Tabei K, Shiina A, Asano Y, Hosoda S. Ultrasonographic evaluation of changes in the inferior vena cava configuration during hemodialysis: relationship between the amount of water removed and the diameter of the inferior vena cava. *Journal of Japanese Society for Dialysis Therapy.* 1985;18:173-9.
44. Segal KR, Gutin B, Presta E, Wang J, Van Itallie TB. Estimation of human body composition by electrical impedance method: A comparative study. *J Appl Physiol.* 1985;58:1565-71.
45. Cox-Reijven PL, Kooman JP, Soeters PB, van der Sande F. Role of bioimpedance spectroscopy in assessment of body water compartments in haemodialysis patients. *Am J Kidney Dis.* 2001;38:832-8.
46. Middleton R, Parfrey P, Foley R. Left ventricular hypertrophy in the renal patient. *J Am Soc Nephrol.* 2001;12(5):1079-84.
47. Ha S, Park H, Kim S, Park C, Kim D, Kim H. Prevalence and patterns of left ventricular hypertrophy in patients with predialysis chronic renal failure. *J Korean Med Sci.* 1998;13(5):488-94.
48. Tucker B, Fabbian F, Giles M, Thuraisingham R, Raine A, Baker L. Left ventricular hypertrophy and ambulatory blood pressure monitoring in chronic renal failure. *Nephrol Dial Transplant* 1997;12(4):724-8.

49. Foley R. Clinical epidemiology of cardiac disease in dialysis patients: left ventricular hypertrophy, ischemic heart disease, and cardiac failure. *Semin Dial* 2003;16(2):111-7.
50. Vicenté-Martínez M, Martínez-Ramírez L, Muñoz R, Avila M, Ventura M, Rodríguez E, et al. Inflammation in patients on peritoneal dialysis is associated with increased extracellular fluid volume. *Arch Med Res*. 2004;35:220-4.
51. Stenvinkel P, Heimbürger O, Lindholm B, Kaysen G, Bergström J. Are there two types of malnutrition in chronic renal failure? Evidence for relationships between malnutrition, inflammation and atherosclerosis (MIA syndrome). *Nephrol Dial Transplant*. 2000 15(7):953-60.
52. Aguilera A, Sánchez-Tomero J, Bajo M, Ruiz-Caravaca M, Alvarez V, del Peso G, et al. Malnutrition-inflammation syndrome is associated with endothelial dysfunction in peritoneal dialysis patients. *Adv Perit Dial*. 2003;19:240-5.
53. Tonbul H, Demir M, Altintepe L, Güney I, Yeter E, Türk S, et al. Malnutrition-inflammation-atherosclerosis (MIA) syndrome components in hemodialysis and peritoneal dialysis patients. *Ren Fail*. 2006;28(4):287-94.
54. Moissl U, Bosaeus I, Lemmey A, et al. Validation of a 3C model for determination of body fat mass. *J Am Soc Nephrol*. 2007;18:257A.
55. Moissl U, Wabel P, Chamney P, et al. Validation of a bioimpedance spectroscopy method for the assessment of fat free mass. *NDT Plus*. 2008;1(Suppl 2): ii215.
56. Moissl U, Wabel P, Chamney P, et al. Body fluid volume determination via body composition spectroscopy in health and disease. *Physiol Meas* 2006;27:921-33.
57. Cooper BA, Aslani A, Ryan M, Zhu FY, Ibels LS, et al. Comparing different methods of assessing body composition in end-stage renal failure. *Kidney Int*. 2000;58: 408-16.

58. Wabel P, Chamney P, Moissl U, et al. Reproducibility of bioimpedance spectroscopy (BIS) for the assessment of body composition and dry weight. *J Am Soc Nephrol.* 2007;18: 255A.
59. Kraemer M, Rode C, Wizemann V. Detection limit of methods to assess fluid status changes in dialysis patients. *Kidney Int* 2006;69:1609-20.
60. Wabel P, Rode C, Moissl U, Chamney P, Wizemann V. Accuracy of Bioimpedance Spectroscopy (BIS) to detect fluid status changes in hemodialysis patients *Nephrol Dial Transplant* 2007;VI(22 Supplement 6):129.

Table 1: Demographic, clinical and laboratory characteristics of the study population

Parameters	All patients (n=120)	PD (N=40)	HD (N=40)	CKD (N=40)	Control (N=40)	*P value
Demographic data						
Age (years)	41.4 ± 10.2	40.6±9.9	40.6±10.1	42.1±10.6	42.2±10.1	0.761
Sex (male/female)	88:72	22:18	22:18	22:18	22:18	0.985
Clinical						
BMI (kg/m ²)	27.8 ± 6.8	26.3 ± 4.8	25.7 ± 5.1	31.3 ± 7.0	26.6 ± 5.4	NS
Mean BP (mmHg)	144 ± 22.5	147.9 ± 25.3	146.9 ± 17.9	138.0 ± 22.9	117.3 ± 12.6	<0.001
Renal function						
Serum creatinine (mmol/L)	619.2±484.6	1149.8±371.4	572.9±212.6	135.0±44.5	73.9±16.8	<0.001
eGFR(ml/min/1.73m ²)	-	NA	NA	50.8 ± 22.4	100.8 ± 23.9	-
Haematological						
Haemoglobin (g/dL)	11.8 ± 2.3	10.9 ± 2.1	11.1 ± 1.7	11.1 ± 1.7	14.9 ± 1.8	<0.001
Biochemical						
Serum albumin (g/L)	38.5 ± 4.9	36.1 ± 4.8	38.1 ± 4.7	41.3 ± 4.1	42.1 ± 3.4	<0.001
Calcium (mmol/L)	2.3 ± 0.2	2.2 ± 0.3	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.1	0.028
Phosphate (mmol/L)	1.3 ± 0.5	1.6 ± 0.6	1.2 ± 0.5	1.1 ± 0.5	1.0 ± 0.2	<0.001
CAPO4(mmol ² /L ²)	3.0 ± 1.3	3.5 ± 1.2	2.8 ± 1.2	2.7 ± 1.2	2.3 ± 0.4	<0.0001
Parathormone (pmol/L)	69.3 ± 69.8	110.8 ± 66.8	84.2 ± 71.8	14.5 ± 19.4	4.3 ± 1.7	<0.001

eGFR, estimated glomerular filtration rate; NS, not significant; PD, peritoneal dialysis; HD, haemodialysis; CKD, chronic kidney disease, BMI, body mass index; BP, blood pressure

Continuous data were expressed as mean ± SD and categorical data as number

*p-values were calculated by comparing all patients with kidney disease (n=120) to controls (n=40) using Mann-Whitney test

Table 2: Fluid status, inflammatory markers, nutrition status and echocardiographic parameters in the study population

Parameter	All patients (N=120)	PD (N=40)	HD (N=40)	CKD (N=40)	Control (N=40)	*P value
Fluid status						
Overhydration (Litres)	2.4 ± 2.3	3.3 ± 3.4	2.9 ± 1.9	1.3 ± 1.2	-0.6 ± 0.94	<0.001
OH/ECW (%)	13.2 ± 10.5	16.1 ± 13.9	15.8 ± 9.5	2.1 ± 9.8	-3.3 ± 6.8	<0.001
Fluid overloaded	105/120 (87.5%)	36/40 (90%)	40/40 (100%)	29/40 (72.5.5%)	0%	<0.001
IVCD measurements						
IVCD (mm/m ²)	7.3 ± 2.7	7.9 ± 2.7	8.2 ± 2.8	5.6 ± 1.5	5.1 ± 1.6	<0.001
Collapsibility index	50.4 ± 23.1	38.0 ± 23.7	50.6 ± 19.5	63.9 ± 18.4	66.6 ± 22.3	<0.001
Inflammatory markers						
C-reactive protein (mg/dL)	0.87 ± 0.72	1.14 ± 0.77	1.05 ± 0.71	0.86 ± 0.71	0.45 ± 0.48	<0.001
Interleukin-6 (pg/ml)	2.7 ± 3.8	3.9 ± 4.9	2.9 ± 3.7	1.3 ± 1.6	1.0 ± 1.2	<0.001
Nutrition status						
Lean Tissue Index(kg/m ²)	14.2 ± 3.2	13.9 ± 2.9	13.8 ± 3.1	14.8 ± 3.6	16.1 ± 3.0	0.002
Fat Tissue Index(kg/m ²)	12.8 ± 7.7	11.2 ± 6.3	10.8 ± 6.1	10.9 ± 6.7	16.5 ± 9.0	0.005
Lean Tissue Mass	37.6 ± 11.1	36.9 ± 10.1	36.3 ± 11.2	39.4 ± 11.9	44.1 ± 11.9	0.029
Fat Tissue Mass	24.4 ± 13.6	21.4 ± 11.3	20.9 ± 11.5	21.2 ± 12.8	30.2 ± 15.6	0.007
Adipose Tissue Mass(kg)	33.3 ± 18.2	29.1 ± 15.4	28.7 ± 14.7	28.9 ± 17.4	42.0 ± 21.0	0.007
Body cell Mass(kg)	21.2 ± 7.2	20.9 ± 6.7	20.5 ± 6.8	22.1 ± 8.0	25.6 ± 7.9	0.027
Echocardiographic parameters						
LVMI (g/m ²)	118.8±38.8	126.9 ±37.6	120.4±36.8	110.4±41.9	84.4±15.9	<0.001
Left atrial dimension (cm)	4.0 ± 0.7	3.9 ± 0.7	4.2 ± 0.7	3.7 ± 0.6	3.1 ± 0.4	<0.001
Ejection fraction (%)	58.6 ± 8.8	58.6±7.8	58.4±12.2	57.9±5.5	65.0±8.0	<0.001
E/A ratio	1.2 ± 0.5	1.0±0.4	1.2±0.5	1.2±0.6	1.36±0.4	<0.001
Deceleration time (msec)	224.9±56.3	226.2±53.6	219.2±64.4	277.0±48.8	209.7±33.9	NS
E/e'	12.5 ± 15.9	11.6±5.5	12.9±5.1	12.9±26.1	6.1±1.6	<0.001
CIMT	0.58 ± 0.11	0.63±0.12	0.56±0.09	0.56±0.12	0.47±0.11	<0.001

NS, not significant; CIMT, carotid intima media thickness; LVMI, left ventricular mass index

Continuous data were expressed as mean ± SD and categorical data as percentages

*p-values were calculated by comparing all patients with chronic kidney disease (n=120) to controls (n=40) using Mann-Whitney test

Table 3: Comparison between patients with fluid overload and normal fluid status

Parameter	With Volume Overload (N=43)	Without Volume Overload (N=77)	P value
Demographic			
Age (Years)	40.4 ± 10.6	41.5 ± 10.2	NS
BMI (kg/m ²)	28.8 ± 6.7	25.8 ± 5.1	0.014
Laboratory			
Serum creatinine (mmol/L)	889.7 ± 460	333.4 ± 396.7	<0.001
Mean BP (mmHg)	146.8 ± 22.0	141.8 ± 22.7	NS
Haemoglobin (g/dL)	10.6 ± 1.86	12.5 ± 2.3	<0.001
Serum albumin (g/L)	36.2 ± 4.2	39.8 ± 4.1	<0.001
CAPO ₄ (mmol ² /L ²)	3.5 ± 1.4	2.7 ± 1.2	0.002
Inflammatory markers			
C-reactive protein (mg/dL)	1.24 ± 0.70	0.89 ± 0.72	0.011
IL-6 (pg/ml)	3.6 ± 5.2	2.1 ± 2.5	0.023
Nutrition status			
Lean Tissue Index(kg/m ²)	12.4 ± 2.2	15.2 ± 3.3	<0.001
Fat Tissue Index(kg/m ²)	10.2 ± 6.6	14.3 ± 7.9	<0.002
Lean Tissue Mass (kg)	32.0 ± 8.5	40.7 ± 11.2	<0.001
Fat Tissue Mass (kg)	19.9 ± 11.6	27.0 ± 14.0	0.004
Adipose Tissue Mass(kg)	27.5 ± 15.4	36.5 ± 18.9	0.006
Body cell Mass(kg)	17.7 ± 5.5	23.0 ± 7.3	<0.001
Echocardiographic parameters			
LVMI (g/m ²)	132.8 ± 39.0	110.9 ± 36.7	0.002
Left atrial dimension (cm)	4.2 ± 0.8	3.7 ± 0.6	<0.001
Ejection fraction (%)	56.6 ± 9.9	59.7 ± 7.9	NS
E/A ratio	1.2 ± 0.5	1.1 ± 0.5	NS
Deceleration time (msec)	217.3 ± 63.4	229.3 ± 51.8	NS
E/e'	12.0 ± 5.6	10.5 ± 16.1	0.001
CIMT (mm)	0.61 ± 0.14	0.56 ± 0.09	0.028

* NS, not significant; CIMT, carotid intima media thickness; LVMI, left ventricular mass index

Volume overload defined by OH/ECW% ≥15%; continuous data were expressed as mean ± SD

*p-values were calculated by comparing all patients with volume (n=43) to those without volume overload (n=77) using Mann-Whitney test

Table 4: Pattern of left ventricular hypertrophy in chronic kidney disease patients (n=120)

Pattern	Number of patients	Percentage of patients
Concentric LVH	57	47.5
Eccentric LVH	26	21.7
Concentric remodeling	25	20.8
Normal	12	10

Table 5: Correlation between fluid parameters, renal function, inflammation and atherosclerosis markers, nutrition status and cardiac size

Parameter	Overhydration (r value)	OH/ECW% (r value)	IVCD (r value)	CI (r value)
Renal function				
Serum creatinine	0.406**	0.396**	0.221*	-0.353**
Markers of inflammation				
C-reactive protein	0.284**	0.277**	0.187*	-0.181*
Interleukin-6	0.349**	0.313**	0.248**	-0.284**
Nutrition status				
Albumin	-0.246**	-0.289**	-0.069	0.152
Lean Tissue Index	-0.254**	-0.326**	-0.019	0.024
Fat Tissue Mass	-0.252**	-0.297**	-0.184*	0.140
Adipose Tissue Mass	-0.219*	-0.275**	-0.131	0.139
Body Cell Mass	-0.230*	-0.317**	-0.041	0.027
Cardiac size				
Left Atrial Size	0.454**	0.489**	0.345**	-0.101
LVMI	0.262**	0.303**	0.190*	-0.142
Marker of atherosclerosis				
CIMT	0.442**	0.421**	0.112	-0.138

CI, collapsibility index; BMI, body mass index; LVMI, left ventricular mass index; CIMT, carotid intima media thickness. ** Correlation is significant at the 0.01 (2-tailed), * Correlation is significant at the 0.05 (2-tailed). Correlation between volume parameters and measured variables was assessed by Pearson correlation coefficient

Table 6: Multiple linear regression analysis of determinant of volume overload

Variables	Unstandardized Coefficients (B)	Standardized Coefficient Beta	P value	Confidence Interval
Serum creatinine	0.003	0.152	0.044*	0.000-0.007
Albumin	− 0.117	− 0.055	0.449	− 0.421-0.188
IL-6	0.312	0.113	0.150	− 0.114-0.739
CRP	0.205	0.014	0.861	− 2.114-2.524
LTI	− 1.072	− 0.328	< 0.001**	− 1.524- − 0.619
FTI	− 0.357	− 0.261	< 0.001**	− 0.552- − 0.163
LVMI	− 0.021	− 0.077	0.336	− 0.063-0.022
LAD	5.153	0.336	< 0.001**	2.830-7.476
CIMT	21.720	0.238	0.002**	8.248-35.192

Notes: * Significant at P value <0.05, ** Significant at P value <0.01. IL-6, interleukin-6; CRP, C-reactive protein; LTI=lean tissue index; LTI=lean tissue index; FTI, fat tissue index; LVMI, left ventricular mass index; LAD, left atrial dimension; CIMT, carotid intima media thickness.

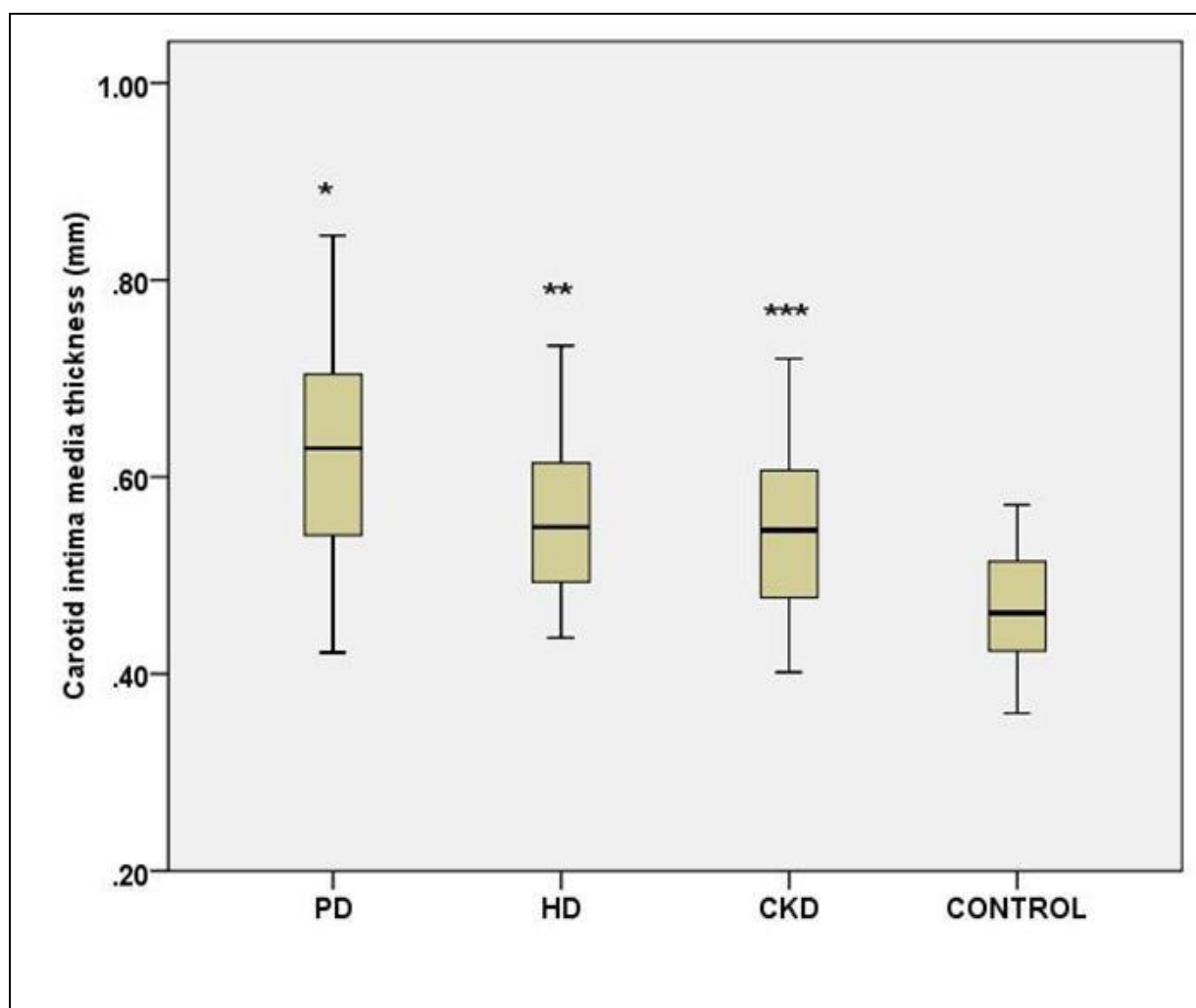


Figure 1: Comparison of carotid intima media thickness between patients on peritoneal dialysis, haemodialysis, chronic kidney disease and control. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile.

* $p < 0.001$, ** $p = 0.014$, *** $p = 0.003$ compared to control group using Kruskal Wallis test

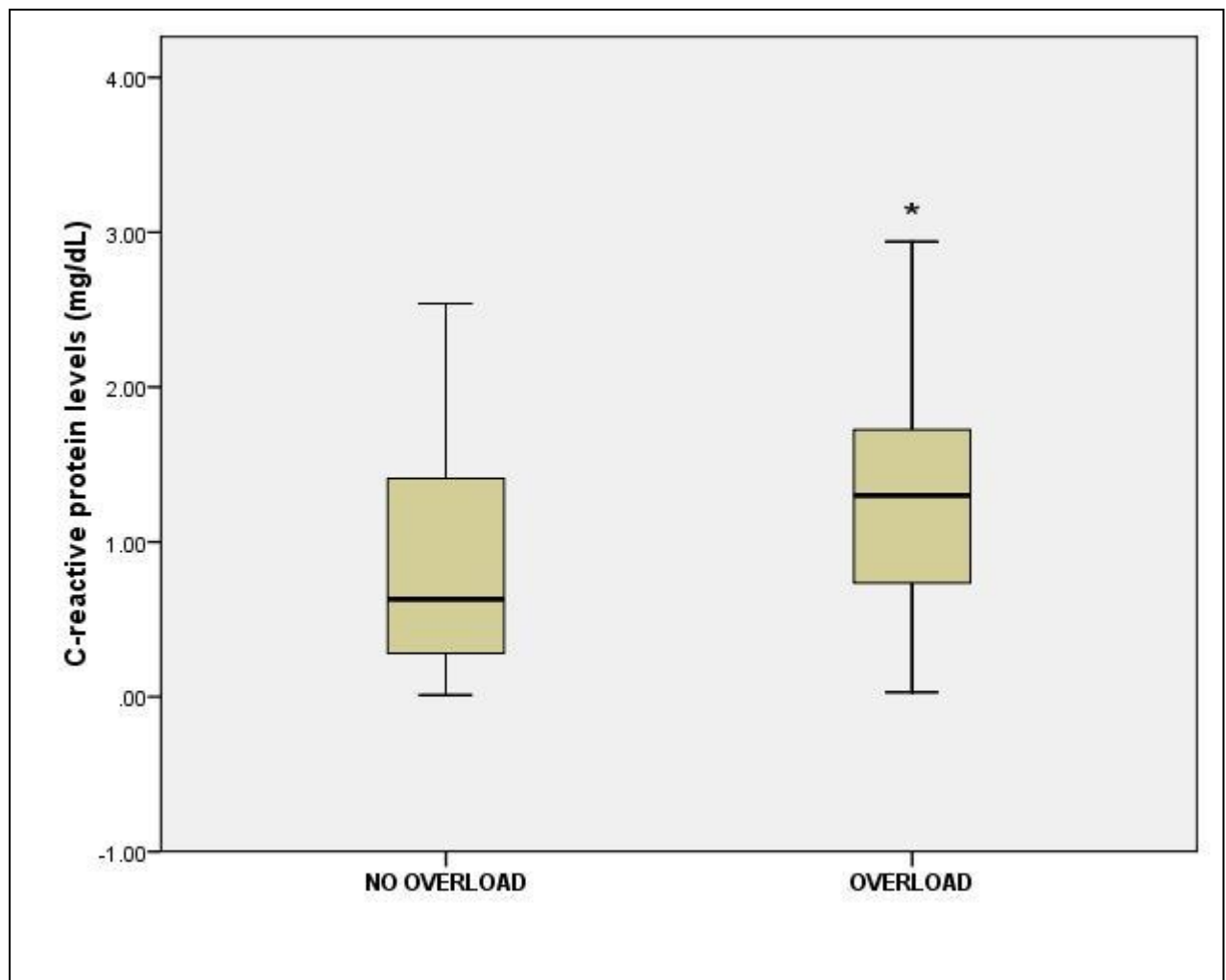


Figure 2: Comparison of C-reactive protein between patients on with volume overload and those without volume overload. The boxes indicate median, 25th, and 75th percentile; whiskers represent data range; whisker caps indicate 5th and 95th percentile.

*p = 0.011 compared to control group using Kruskal Wallis test

APPENDIX 2

ETHIC CLEARANCE CERTIFICATE



R14/49 Dr Muzamil Hassan

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130127

NAME: Dr Muzamil Hassan
(Principal Investigator)

DEPARTMENT: Internal Medicine
Charlotte Maxeke Johannesburg Academic Hospital

PROJECT TITLE: The Role of Circulating Endotoxaemia as a
Pro-Inflammatory Mediator of Atherosclerosis in
Chronic Kidney Disease Patients

DATE CONSIDERED: 25/01/2013

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Prof S Naicker

APPROVED BY: 
Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 30/04/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature _____

Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 3

PARTICIPANT INFORMATION SHEET AND CONSENT FORM

Part A

STUDY NUMBER: M130127

TITLE: The role of circulating endotoxaemia as a pro-inflammatory mediator of atherosclerosis in chronic kidney disease patients.

INVESTIGATOR: Dr. Muzamil Hassan

INSTITUTION: University of Witwatersrand, Internal medicine

TELEPHONE NUMBER: Mobile – 083 547 4789

Landline (Departmental) – 011-488-3672

Part B

Introduction:

Good day. My name is Muzamil Hassan. I am a medical doctor, training as a kidney specialist at the Charlotte Maxeke Johannesburg Hospital in the Division of Nephrology. I am also doing my PhD with the University of Witwatersrand. I am performing a research study. Before you participate in this study, I would like to make sure you read all the information about the study and fully understand what the study is all about. If you do not read or understand English, an interpreter will be provided for you.

Purpose of the study:

Patients with chronic kidney disease (CKD) are at high risk of developing heart disease. Heart diseases also cause significant health problems in kidney disease patients. It follows that kidney disease patients are more likely to die from cardiovascular disease than from kidney disease. Inflammation plays a major role in the development of vascular diseases (atherosclerosis) among advanced CKD patients. However, the causes and mechanism of inflammation and how it leads to heart disease are not known. This study is designed to investigate the source of inflammation and the role of inflammation and genetic variant carrier status in the development of heart disease in CKD patients.

To find out why both vascular and heart disease are very common among kidney disease patients, I will look at some proteins as well as genes that are responsible for production of proteins that help the body to fight and protect against infections. Genes are a set of instructions that decide what a human is like, how he/she survives and behaves. I will be looking at changes in five specific genes called TNF- α , TGF- β 1, IL-10, IL-6, and IFN- γ genes that have been shown to be associated with development of both vascular and heart disease. These changes are called mutations (a mutation is a permanent change that may affect the way the instructions are interpreted by the body). From this, I will be able to find out whether there are differences in these proteins and genes in people who get cardiovascular disease and in those that do not.

Participation:

Your participation in this study is entirely voluntary. Before you agree to take part, it is important that you read and understand the explanation of the purpose of this study and the procedures that will be performed. This information sheet will help you decide if you would like to take part or not. If you have any questions, do not hesitate to ask me. You are free to take part or not. If you do agree to take part, you are still free to withdraw from the study at any time and this will not be held against you. If you decide to take part, you will be asked to sign this document to confirm that you understand the nature of the study and agree to take part and you will be given a copy to keep. Should you decide to take part in this study; each study visit will not take more than 3 hours of your time.

Procedures:

If you agree to take part in the study; you will be asked to sign the informed consent form, you will be examined, and your weight, height, waist circumference and blood pressure will be taken. You will also have some blood drawn (about 2 teaspoons). The blood will be used to tests for markers of infection and some will be stored until genetic testing is carried out.

You will require ultrasound imaging of your heart and blood vessels. Ultrasound imaging involves the use of a small transducer (a device that resembles a computer mouse) and ultrasound gel to expose the body to high-frequency sound waves. Ultrasonography examination of the heart is called echocardiography. You will lie on an examination table, and a cardiologist will hold a transducer against your chest, slowly sliding it back and forth.

You may be asked to roll on your side during the test, or hold your breath for a few seconds. The test takes 30 to 60 minutes to complete.

Also, doppler ultrasound which is a special ultrasound technique that evaluates blood flow through a blood vessel will be performed. You will be positioned lying face-up on an examination table that can be tilted or moved. After you are positioned on the examination table, sonographer will apply some warm Vaseline-like gel on your skin and then place the transducer against your neck, moving it back and forth over the area of interest until the desired images are captured. This ultrasound examination is usually completed within 30 to 45 minutes.

Your hydration status will be determined with the aid of Bioimpedance analysis (BIA) which is a valuable tool for measuring your body composition. Measurements are taken with the bioimpedance analyzer, which uses electrodes similar to electrocardiography (ECG) electrodes. The analyzer calculates your tissue and fluid compartments using an imperceptible electrical current passed through pads placed on one hand and foot as you lie comfortably on a treatment couch.

Benefits:

The benefits of this study are that we will be able to find out early if you are at high risk of developing cardiovascular disease. We will also be able to determine if you are fluid overloaded. You may also receive additional treatment based on the findings or special counselling on lifestyle changes.

Possible risks:

Drawing blood is normally done as a part of routine medical care. It will cause some pain at the site and some physical discomfort. However, this is usually mild and transient; lasting a few second to minutes.

Confidentiality:

All the information we will get during the course of this study, including your clinic records, will be kept strictly confidential. The information may be reported in the research report and scientific journals but will not have any information that identifies you or your names. There

are no names on the information sheets, they will contain codes that only I, the researcher will have access to.

Ethical consideration:

This study has been approved by the Human Research Ethics Committee (HREC). It is supported by the Division of Nephrology. There will not be any conflict of interest on my part.

Source of additional information

All the study participants will be under the care of Dr. Muzamil Hassan who will be reachable 24 hours everyday on this number; 083-547-4789. Additional information can be obtained from the Chairperson of the University of Witwatersrand Human Research Ethics Committee, Professor Peter Cleaton-Jones on; 011-717-2301.

Part C

Informed Consent: General

I hereby confirm that I have been informed by the study investigator Dr. Muzamil Hassan, about the nature, conduct, benefits and possible risks of the study. I have also received, read and understood the above written participant information sheet regarding the study. I am aware that the results of the study will be anonymously processed in a study report and that I may, at any stage without prejudice withdraw my consent and participation in the study. I had sufficient opportunity to ask questions and of my own free will have declared myself prepared to participate in the study.

PARTICIPANT (Printed name):

.....	
Signature	Date

Witness:.....		
(Study nurse)Printed name	Signature	Date

I, Dr. Muzamil Hassan, herewith confirm that the above participant has been fully informed about the nature and conduct of the above study.

STUDY INVESTIGATOR

.....		
Printed name	Signature	Date

Part D

Informed Consent: Genetic study

I hereby confirm that I have been informed by the study investigator Dr. Muzamil Hassan, about the nature, conduct, benefits and possible risks of the study procedure. I have read and fully understood this form, and I consent to genetic testing. I am aware that the results of the study will be anonymously processed in a study report and that I may, at any stage without prejudice withdraw my consent and participation in the study. I had sufficient opportunity to ask questions and of my own free will have declared myself prepared to participate in the study.

PARTICIPANT (Printed name):.....

.....	
Signature	Date

Witness:		
(Study nurse)Printed name	Signature	Date

I, Dr. Muzamil Hassan, herewith confirm that the above participant has been fully informed about the nature and conduct of the above study.

STUDY INVESTIGATOR

.....		
Printed name	Signature	Date

APPENDIX 4

DATA COLLECTION SHEET

TITLE: THE ROLE OF CIRCULATING ENDOTOXAEMIA AS A PRO-INFLAMMATORY MEDIATOR OF ATHEROSCLEROSIS IN CHRONIC KIDNEY DISEASE PATIENTS.

STUDY NUMBER: M130127

INVESTIGATOR: DR MUZAMIL OLAMIDE HASSAN

1. GENERAL INFORMATION

PARTICIPANT'S NUMBER	
ENROLLMENT DATE	
CONTACT NUMBER	

2. DEMOGRAPHICS AND ANTHROPOMETRIC PARAMETERS

AGE (YEARS)	
SEX	☐ MALE ☐ FEMALE
RACE	☐ BLACK ☐ WHITE ☐ COLOURED ☐ INDIANS
WEIGHT(KG)	
HEIGHT (CM)	
BODY MASS INDEX (KG/M ²)	
WAIST CIRCUMFERENCE (CM)	
HIP CIRCUMFERENCE(CM)	

3. MEDICAL HISTORY

ALCOHOL INTAKE	☐ NON-DRINKER ☐ MODERATE DRINKER ☐ HEAVY DRINKER
TOBACCO	☐ SMOKER ☐ FORMER SMOKER ☐ NON-SMOKER
MEDICATIONS	☐ ANTIHYPERTENSIVES ☐ STATINS ☐ CA-PO4 BINDERS ☐ ACEIs/ARBs ☐ β -BLOCKERS ☐ ASPIRIN
TYPE OF DIALYSIS	☐ HD ☐ PD ☐ NON DIALYSIS-CKD ☐ CONTROL
CAUSE OF CKD	

4. INTRADIALYSIS VITAL SIGNS

	PREDIALYSIS	POSTDIALYSIS
TEMPERATURE		
BLOOD PRESSURE		
MEAN ARTERIAL PRESSURE		
PULSE PRESSURE		

5. ROUTINE INVESTIGATIONS

TESTS	1 ST MONTH	2 ND MONTH	3 RD MONTH
HAEMOGLOBIN			
SERUM ALBUMIN			
CHOLESTEROL			
HDL-CHOLESTEROL			
LDL-CHOLESTEROL			
TRIGLYCERIDES			
CREATININE			
UREA			
CALCIUM			
PHOSPHATE			
PTH HORMONE			

6. SPECIAL ASSAYS

	1 ST ASSAY	2 ND ASSAY
Hs-CRP		
ENDOTOXIN		
LPS BINDING PROTEIN		
HUMAN CD14		
INTERLEUKIN – 6		
INTERLEUKIN – 8		
MCP – 1		
OXIDISED LDL		
LDL/OXIDISED LDL RATIO		
TGF- β 1		
TGF- β 2		
TGF- β 3		

7. ECHOCARDIOGRAPHY

LEFT ATRIUM (END SYSTOLE)	
INTERVENTRICULAR SEPTAL THICKNESS (IVSd)	
LEFT VENTRICULAR END DIASTOLIC DIAMETER (LVIDd)	
LEFT VENTRICULAR POSTERIOR WALL THICKNESS (LVPWd)	
LEFT VENTRICULAR END SYSTOLIC DIAMETER (LVIDs)	
EJECTION FRACTION (EF)	
REGIONAL WALL MOTION ABNORMALITIES	
AORTIC ANNULUS	
SINUS VALSALVA	
SINO-TUBULAR JUNCTION	
AORTIC MAXIMUM DIAMETER (SYSTOLE)	
AORTIC MAXIMUM DIAMETER (DIASTOLE)	
LEFT VENTRICULAR MASS	
LEFT VENTRICULAR MASS INDEX	
E/A	
E/E'	
DECELERATION TIME	
DIASTOLIC DYSFUNCTION (GRADE)	
MITRAL REGURGITATION	
MITRAL STENOSIS	
AORTIC REGURGITATION	
AORTIC STENOSIS	
PERICARDIAL EFFUSION	
TRICUSPID REGURGITATION	
RIGHT ATRIAL PRESURE (RAP)	
TR MAX	
PULMONARY ARTERY PRESSURE (RAP+TR MAX)	
INFERIOR VENA CAVA DIAMETER (IVCD)	
IVCD (INSPIRATION)	
IVCD (EXPIRATION)	
COLLAPSIBILITY INDEX	

8. CAROTID DOPPLER USS

	RIGHT	LEFT
CAROTID INTIMAL MEDIA THICKNESS		
NO OF PLAQUES		

9. BIOIMPEDANCE ANALYSIS

	1 ST MEASUREMENT	2 ND MEASUREMENT	3 RD MEASUREMENT
TBW			
ECW			
ICW			
E/I			
OVERHYDRATION			
OH/ECW (%)			
LTI			
FTI			
LTM, rel. LTM			
FAT, rel. FAT			
ATM			
BCM			
NH WEIGHT			

10. CYTOKINE GENOTYPING

POSITIONS		1H/3H/ 5H/7H/ 9H/11H	1G/3G/ 5G/7G/ 9G/11G	1F/3F/ 5F/7F/ 9F/11F	1E/3E/ 5E/7E/ 9E/11E	1D/3D/ 5D/7D/ 9D/11D	1C/3C/ 5C/7C/ 9C/11C	1B/3B/ 5B/7B/ 9B/11B	1A/3A/ 5A/7A/ 9A/11A	2H/4H/ 6H/8H/ 10H/12H	2G/4G/ 6G/8G/ 10G/12G	2F/4F/ 6F/8F/ 10F/12F	2E/4E/ 6E/8E/ 10E/12E	2D/4D/ 6D/8D/ 10D/12D	2C/4C/ 6C/8C/ 10C/12C	2B/4B/ 6B/8B/ 10B/12B	2A/4A/ 6A/8A/ 10A/12A
RESULTS (mark positive locations)																	
PRODUCT SIZE (bp)		750	125	125	175	175	125	125	300	300	300	250	250	175	175	250	250
SPECIFICITY		Neg Ctrl	TNF- α promoter -308A	TNF- α promoter -308G	TGF- β 1 codon 10T	TGF- β 1 codon 10C	TGF- β 1 codon 25C	TGF- β 1 codon 25G	IL-10 promoter -1082A,-819T	IL-10 promoter -1052G,-819C	IL-10 promoter -1052A,-819C	IL-10 promoter -819T,-592A	IL-10 promoter -819C,-592C	IL-6 promoter -174C	IL-6 promoter -174G	IFN- γ intron 1 +874T	IFN- γ intron 1 +874A
TNF- α	G/G (low)																
	G/A (high)																
	A/A (high)																
TGF- β 1	T/T G/G (high)																
	T/C G/G (high)																
	T/C G/C (intermediate)																
	C/C G/G (intermediate)																
	T/T G/C (intermediate)																
	C/C G/C (low)																
	C/C C/C (low)																
IL-10	T/T C/C (low)																
	T/C C/C (low)																
	GCC/GCC (high)																
	GCC/ACC (intermediate)																
	GCC/ATA (intermediate)																
IL-6	ACC/ACC (low)																
	ACC/ATA (low)																
	ATA/ATA (low)																
	G/G (high)																
IFN- γ	G/C (high)																
	C/C (low)																
	T/T (high)																
IFN- γ	T/A (intermediate)																
	A/A (low)																

APPENDIX 5

Limulus Amebocyte Lysate (LAL) QCL-1000™ (LONZA)

Principle

Proenzyme $\longrightarrow$ Enzyme

Substrate + H₂O $\longrightarrow$ Peptide + pNA

Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the release of pNA from the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA. The free pNA is measured photometrically at 405–410 nm after the reaction is stopped with stop reagent. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1–1.0 EU/ml range. The concentration of endotoxin in a sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standard.

Sample Collection and Preparation

Careful technique must be used to avoid microbial or endotoxin contamination. All materials coming in contact with the sample or test reagents must be endotoxin-free. Clean glassware and materials may be rendered endotoxin-free by heating at 250°C for 30 minutes. Appropriate precautions should be taken to protect depyrogenated materials from subsequent environmental contamination. Most sterile, individually wrapped, plastic pipettes and pipette tips are endotoxin-free. However, these materials should be tested before regular use. It may be necessary to adjust the pH of the sample to within the range 6.0–8.0 using endotoxin-free sodium hydroxide or hydrochloric acid. Always measure the pH of an aliquot of the bulk sample to avoid contamination by the pH electrode. Do not adjust unbuffered solutions. Samples to be tested must be stored in such a way that all bacteriological activity is stopped or the endotoxin level may increase with time. For example, store samples at 2–

8°C for less than 24 hours and frozen for periods greater than 24 hours. It is the responsibility of the end-user to validate the proper container and storage conditions for their samples. If the container of diluent used to rehydrate the reagents has been opened previously or was not supplied by Lonza, the diluent alone must be tested for endotoxin contamination.

Reagent Preparation

Allow reagents to equilibrate to room temperature prior to use. In each series of determinations, four standard endotoxin solutions should be used. The table below suggests a dilution scheme for the construction of these standards from the endotoxin supplied in the kit. Alternative dilution schemes can be used as well as other endotoxins not supplied in this kit. The initial dilution from the endotoxin stock is 1/X, where X equals the concentration of the endotoxin vial. This yields an endotoxin solution containing 1.0 EU/ml. For example, if the potency is 23 EU/ml, the initial dilution is 1/23 or 0.1 ml of endotoxin stock into 2.2 ml of LAL reagent water.

Note: Plastic tubes are not recommended for making endotoxin dilutions.

Endotoxin Concentration [EU/ml]	Endotoxin Stock Solution	Endotoxin Std. Solution 1.0 EU/ml	LAL Reagent Water
1.0	0.1 ml	—	(X-1)/10 ml
0.5	—	0.5 ml	0.5 ml
0.25	—	0.5 ml	1.5 ml
0.1	—	0.1 ml	0.9 ml

X = endotoxin concentration of the vial

1. Prepare a solution containing 1.0 EU/ml endotoxin by diluting 0.1 ml of the endotoxin stock solution with (X-1)/10 ml of LAL reagent water in a suitable container, where X equals the endotoxin concentration of the vial. Label this container as 1.0 EU/ml. This

solution should be vigorously vortexed for at least 1 minute before proceeding. For example, if $X = 23$ EU/ml, then dilute 0.1 ml of the endotoxin stock solution with 2.2 ml, $(23-1)/10$, LAL reagent water.

- 2. Transfer 0.5 ml of this 1.0 EU/ml solution into 0.5 ml of LAL reagent water in a suitable container and label 0.5 EU/ml. This solution should be vigorously vortexed for at least 1 minute before use.*
- 3. Transfer 0.5 ml of the 1.0 EU/ml solution into 1.5 ml of LAL reagent water in a suitable container and label 0.25 EU/ml. This solution should be vigorously vortexed for at least 1 minute before use.*
- 4. Transfer 0.1 ml of the 1.0 EU/ml solution into 0.9 ml of LAL reagent water in a suitable container and label 0.1 EU/ml. This solution should be vigorously vortexed for at least 1 minute prior to use.*

Test Procedure

The addition of all reagents in the LAL assay must be consistent. All tubes or microplate wells must be treated in exactly the same manner in order to determine the proper endotoxin concentration. It is suggested that, in a series of tests, reagents should be pipetted in the same order from tube to tube or well to well, and at the same rate. The table below outlines the test procedure:

	Sample	Blank
Test sample or standard at 20–25°C	50 µl	—
LAL Reagent Water	—	50 µl
LAL	50 µl	50 µl
Mix and incubate at 37°C ± 1°C	10 min.	10 min.
Substrate solution at 37°C ± 1°C	100 µl	100 µl
Mix and incubate at 37°C ± 1°C	6 min.	6 min.
Stop reagent	100 µl	100 µl
Mix immediately	—	—

Test Tube Method

- Carefully dispense 50 µl of sample or standard into the appropriate endotoxin-free reaction tube in a 37°C ± 1°C block or waterbath. Each series of determinations must include a blank plus the four endotoxin standards run in duplicate. The blank tubes contain 50 µl of LAL reagent water instead of sample. All reagent additions and incubation times are identical.*
- At time $T = 0$, add 50 µl of LAL to the reaction vessel. Begin timing as LAL is added to the first reaction vessel. It is important to be consistent in the order of reagent addition from vessel to vessel and in the rate of pipetting. Thorough mixing of the two solutions is essential, but do not vortex.*
- At $T = 10$ minutes, add 100 µl of substrate solution (pre-warmed to 37°C ± 1°C). Pipette the substrate in the same order as in Step 2. Maintain a consistent pipetting rate. Assure thorough mixing of solutions.*
- At $T = 16$ minutes, add 100 µl of stop reagent. Maintain the same pipetting order and rate as in Steps 2 and 3. Mix well.*
- Read the absorbance of each reaction tube at 405–410 nm using distilled water to adjust the photometer to zero absorbance.*

Microplate Method

1. *Pre-equilibrate the microplate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the heating block adapter.*

Note: Do not use a cabinet-style incubator to run this test.

2. *While leaving the microplate at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, carefully dispense 50 μl of sample or standard into the appropriate microplate well. Each series of determinations must include a blank plus the four endotoxin standards run in duplicate. The blank wells contain 50 μl of LAL reagent water instead of sample. All reagent additions and incubation times are identical.*
3. *At time $T = 0$, add 50 μl of LAL to the first microplate well, or first column of microplate wells if using a multi-channel pipette and reagent reservoir. Begin timing as the LAL is added. It is important to be consistent in the order of reagent addition from well to well or row to row, and in the rate of pipetting. Once the LAL has been dispensed into all microplate wells containing samples or standards, briefly remove the microplate from the heating block adapter and repeatedly tap the side of the plate to facilitate mixing. Return the plate to the heating block adapter and replace cover.*
4. *At $T = 10$ minutes, add 100 μl of substrate solution (pre-warmed to $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$). Pipette the substrate solution in the same manner as in Step 3. Maintain a consistent pipetting rate. Once the substrate solution has been dispensed into all microplate wells, briefly remove the microplate from the heating block adapter and repeatedly tap the side of the plate to facilitate mixing. Return the plate to the heating block adapter and replace cover.*
5. *At $T = 16$ minutes, add 100 μl of stop reagent. Maintain the same pipetting order as in Steps 3 and 4. Once the stop reagent has been dispensed into all microplate wells, remove the plate and repeatedly tap the side of the plate.*

6. Read the absorbance of each microplate well at 405–410 nm (using distilled water to adjust the photometer to zero absorbance, if necessary).

Note: The performance characteristics of certain microplate readers are optimal with sample volumes less than 300 μ l. The final reaction volume per well can be reduced by adding only 50 μ l of the above suggested stop reagents without adversely affecting the test results.

Calculation of Endotoxin Concentration

Under the standard conditions, the absorbance at 405–410 nm is linear in the concentration range of 0.1 to 1.0 EU/ml endotoxin. There are several methods to determine the endotoxin concentration of samples. Subtract the mean absorbance of the blank from the mean absorbance value of the standards and samples to calculate mean Δ absorbance.

A. Graphic Method

Plot the mean Δ absorbance for the four standards on the y-axis vs. the corresponding endotoxin concentration in EU/ml on the x-axis. Draw a best fit straight line between these points and determine endotoxin concentrations of samples graphically.

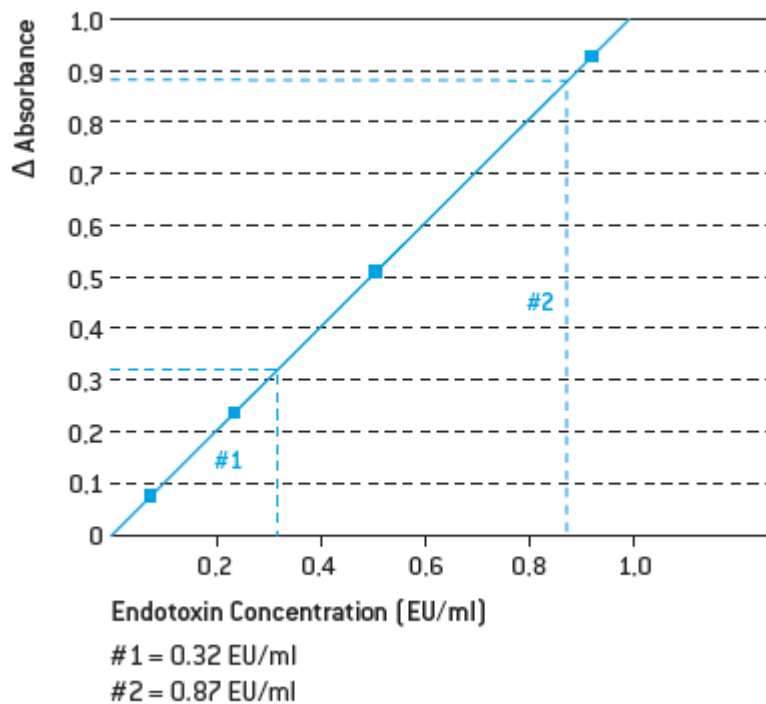
B. Calculator or Spreadsheet Method

A calculator or spreadsheet equipped with linear regression capability can be used. Enter the mean Δ absorbance and the corresponding concentrations of the four standards. Determine the corresponding endotoxin concentration of the samples from their absorbance by linear regression.

Example Data

Tube Well	Sample	Absorbance at 405 nm	Mean Absorbance	Mean Δ Absorbance
1	LAL Reagent	0.080	0.082	—
2	Water (Blank)	0.084		
3	0.1 EU/ml	0.160	0.170	0.088
4	Standard	0.180		
5	0.25 EU/ml	0.309	0.317	0.235
6	Standard	0.325		
7	0.5 EU/ml	0.570	0.564	0.482
8	Standard	0.557		
9	1.0 EU/ml	1.052	1.032	0.950
10	Standard	1.012		
11	Product	0.372	0.382	0.300
12	#1	0.392		
13	Product	0.916	0.914	0.832
14	#2	0.912		

A. Graphic Method



B. Calculator Method

$$\text{Slope} = \left(\frac{S_y}{S_x} \right) r$$

$$Y\text{-intercept} = \sum y / N - (\sum x / N \times \text{slope})$$

$$r = \frac{N \sum xy - (\sum x)(\sum y)}{N(N-1) S_x S_y}$$

$$\text{Endotoxin concentration} = \frac{\Delta \text{Abs.} - (y\text{-intercept})}{\text{slope}}$$

x = Endotoxin concentration in EU/ml.

y = Mean Δ Absorbance Value.

N = Number of standards used.

Σx = Summation of concentration of standards used in EU/ml.

Σy = Summation of Mean Δ Absorbance Values.

Σxy = Summation of the standard concentrations times Mean Δ Absorbance Value

$$S_x = \text{Standard deviation of } x = \sqrt{\frac{N\Sigma x^2 - (\Sigma x)^2}{N(N-1)}}$$

$$S_y = \text{Standard deviation of } y = \sqrt{\frac{N\Sigma y^2 - (\Sigma y)^2}{N(N-1)}}$$

Calculations using Example Data: (Page 19)

$$N = 4$$

$$\Sigma x = 1.85 = (0.100 + 0.250 + 0.500 + 1.00)$$

$$\Sigma y = 1.76 = (0.088 + 0.235 + 0.482 + 0.950)$$

$$\Sigma xy = 1.26 = (0.100 \times 0.088) + (0.250 \times 0.235) + (0.500 \times 0.482) + (1.00 \times 0.950)$$

$$S_x = 0.394$$

$$S_y = 0.378$$

$$r = \frac{4(1.26) - (1.85)(1.76)}{4(4-1)(0.394)(0.378)} = 1.00$$

$$\text{Slope} = \frac{0.378}{0.394} \times 1.00 = 0.959$$

$$Y\text{-intercept} = \frac{1.76}{4} - \left[\frac{1.85}{4} \times 0.959 \right]$$

$$Y\text{-intercept} = 0.440 - (0.463 \times 0.959) = -0.004$$

$$\begin{aligned}
 \text{Product \#1} \\
 \text{Endotoxin Conc. EU/ml} &= \frac{0.300 - (-0.004)}{0.959} \\
 &= \frac{0.304}{0.959} \\
 &= 0.317 \text{ EU/ml}
 \end{aligned}$$

$$\begin{aligned}
 \text{Product \#2} \\
 \text{Endotoxin Conc. EU/ml} &= \frac{0.832 - (-0.004)}{0.959} \\
 &= \frac{0.836}{0.959} \\
 &= 0.872 \text{ EU/ml}
 \end{aligned}$$

Note: If the concentration of endotoxin in the test sample is greater than 1.0 EU/ml, dilute sample 5-fold in LAL reagent water and retest. Calculate the concentration of the diluted sample and multiply by 5 to determine the original endotoxin concentration.

Product Inhibition

Product inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower, final Δ absorbance, indicating lower levels of endotoxin than what may actually be present in the test sample. The lack of product inhibition should be determined for each specific sample, either undiluted or at an appropriate dilution. To verify the lack of product inhibition, an aliquot of test sample (or a dilution of test sample) is spiked with a known amount of endotoxin (e.g. 0.4 EU/ml). The spiked solution is assayed along with the unspiked samples and their respective endotoxin concentrations are determined. The difference between these two calculated endotoxin values should equal the known concentration of the spike $\pm 25\%$. A spiked aliquot of the test sample (or dilution) may be prepared as follows:

- 1. Prepare a 1.0 EU/ml endotoxin solution in the test sample (or dilution) by diluting the endotoxin stock solution 1/X, where X is the endotoxin concentration of stock in EU/ml. Use the test sample (or dilution) as the diluent. This solution should be*

vigorously vortexed for one minute before proceeding. For example, if the concentration of the endotoxin stock solution is 24 EU/ml, the initial dilution is 1/24 or 0.1 ml of endotoxin stock solution into 2.3 ml of test sample (or dilution).

2. To prepare a 0.4 EU/ml endotoxin solution in test sample (or dilution), dilute the 1.0 EU/ml solution 1/2.5 using the test sample (or dilution) as the diluent. This can be done by combining 1.0 ml of the 1.0 EU/ml solution in test sample (or dilution) with 1.5 ml of test sample (or dilution). This solution should be vigorously vortexed for 1 minute prior to use. If the test sample (or dilution) is found inhibitory to the LAL reaction the sample may require further dilution until the inhibition is overcome.

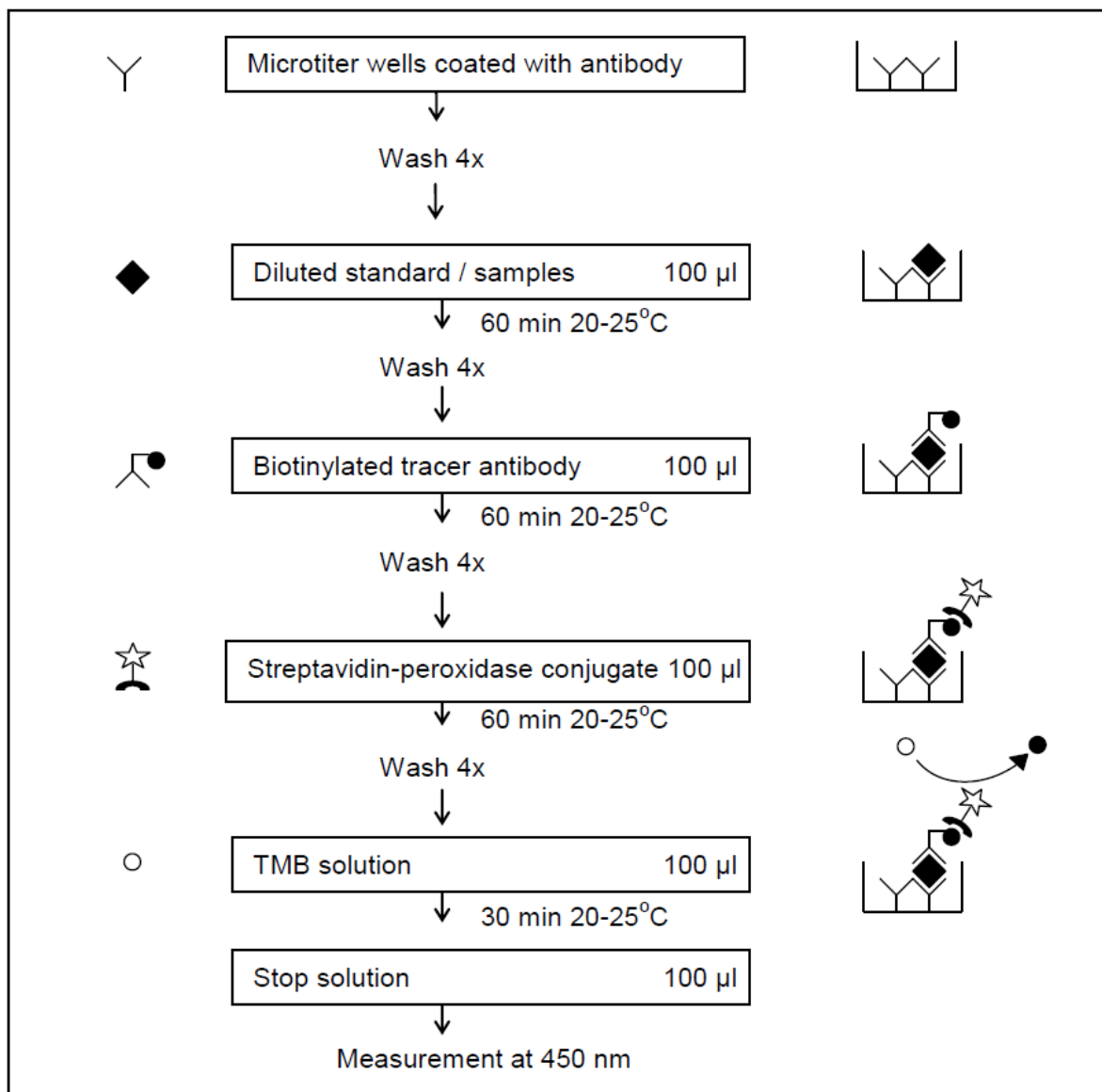
Example: Determination of a Non-Inhibitory Dilution

Calculated Endotoxin Concentration (EU/ml)			
Sample Dilution	Unspiked	Spiked	Difference
1/10	0.18	0.28	0.10 Inhibitory
1/20	0.11	0.36	0.25 Inhibitory
1/40	<0.1	0.44	0.44 Non-inhibitory

APPENDIX 6

HK315 HUMAN LBP ELISA kit (Hycult biotech)

PROTOCOL OVERVIEW



- The human LBP ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 3½ hours.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.

- *Samples and standards are incubated in microtiter wells coated with antibodies recognizing human LBP.*
- *Biotinylated tracer antibody will bind to captured human LBP.*
- *Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.*
- *Streptavidin-peroxidase conjugate will react with the substrate, tetramethylbenzidine (TMB).*
- *The enzyme reaction is stopped by the addition of oxalic acid.*
- *The absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human LBP standards (log).*
- *The human LBP concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.*

SAMPLE PREPARATION

Collection and handling

Serum or plasma

Collect blood using normal aseptic techniques. Blood samples should be kept on ice. If serum is used, separate serum from blood after clotting at room temperature within 1 hour by centrifugation (1500xg at 4°C for 15 min). Transfer the serum to a fresh polypropylene tube. If plasma is used, separate plasma from blood within 20 minutes after blood sampling by centrifugation (1500xg at 4°C for 15 min). Transfer the plasma to a fresh polypropylene tube.

Most reliable results are obtained if EDTA plasma is used.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of human LBP. Use samples within 24 hours after thawing. Avoid

multiple freeze-thaw cycles which may cause loss of human LBP activity and give erroneous results.

Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Serum or plasma samples

Human LBP can be measured accurately if serum or plasma samples are diluted at least 1000x with supplied wash/dilution buffer in polypropylene tubes.

Note that most reliable results are obtained with EDTA plasma.

Remark regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline. The recovery of human LBP from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human LBP.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

Guideline for dilution of samples

Please see the table below for recommended sample dilutions. Volumes are based on a total volume of at least 230 µl of diluted sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend to use at least 10 µl of sample.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of Wash/Dilution buffer required
1.	10x	Not necessary	25 µl (sample)	225 µl
2.	20x	Not necessary	15 µl (sample)	285 µl
3.	50x	Not necessary	10 µl (sample)	490 µl
4.	100x	Not necessary	10 µl (sample)	990 µl
5.	500x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	490 µl
6.	1000x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	990 µl
7.	2000x	Recommended: 20x (see nr.2)	10 µl (pre-dilution)	990 µl
8.	5000x	Recommended: 50x (see nr.3)	10 µl (pre-dilution)	990 µl

REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use.

Wash/dilution buffer

Dilute 20x concentrated wash/dilution buffer A by mixing 60 ml with 540 ml distilled or de-ionized water. Dilute 40x concentrated wash/dilution buffer B by mixing 30 ml with 570 ml distilled or de-ionized water. Finally combine both solutions equally and mix well. The wash/dilution buffer is sufficient for 2 x 96 tests.

In case less tests are required, prepare the required volume by dilution of 1 part 20x concentrated wash/dilution buffer A with 9 parts of distilled or de-ionized water and 1 part 40x concentrated wash/dilution buffer B with 19 parts of distilled or de-ionized water. Finally combine both solutions equally and mix well.

Concentrated wash/dilution buffer may contain crystals. In case crystals do not disappear at room temperature within 1 hour, concentrated wash/dilution buffer can be warmed up to 37°C. Do not shake.

Standard solution

The standard is reconstituted by pipetting the amount of wash/dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure 1. Prepare each human LBP standard in polypropylene tubes by serial dilution of the reconstituted standard

with wash/dilution buffer as shown in Figure 1. After reconstitution the standard must be used within 1 hour and the standard cannot be stored for repeated use.

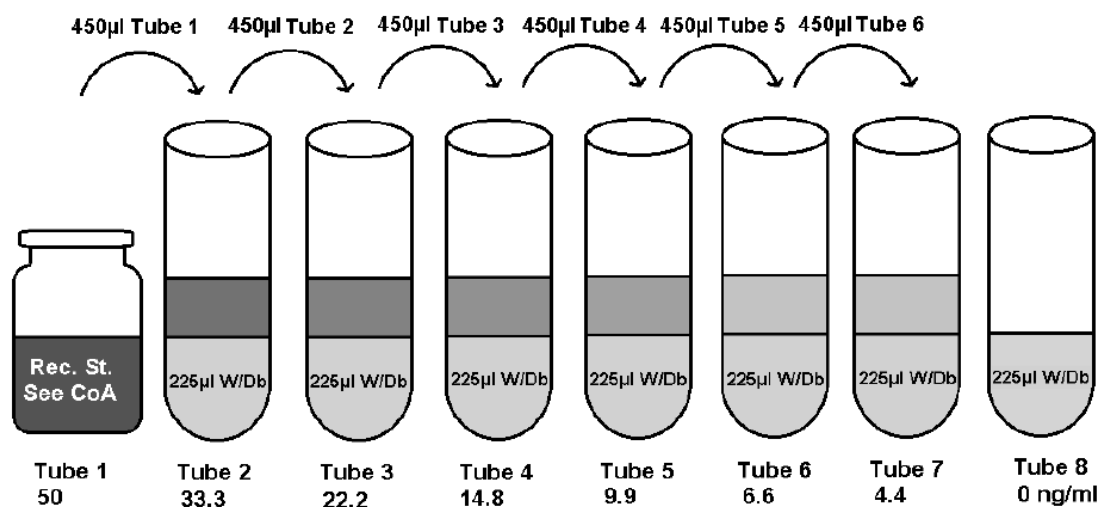


Figure 1

Tracer solution

The tracer is reconstituted by pipetting 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml tracer with 11 ml wash/dilution buffer, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 11 parts of wash/dilution buffer.

Streptavidin-peroxidase solution

It is advised to spin down streptavidin-peroxidase tubes before use. Prepare the streptavidin-peroxidase solution by mixing 0.25 ml of the 100x streptavidin-peroxidase solution with 24.75 ml wash/dilution buffer, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of streptavidin-peroxidase solution by diluting 1 part of the 100x streptavidin-peroxidase solution with 99 parts of wash/dilution buffer.

ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.
2. Wash the plates 4 times with wash/dilution buffer using a plate washer or as follows:
 - a. Carefully remove cover, avoid splashing.
 - b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
 - c. Add 200 µl of wash/dilution buffer to each well, wait 20 seconds, empty the plate as described in 2b.
 - d. Repeat the washing procedure 2b/2c three times.
 - e. Empty the plate and gently tap on thick layer of tissues.
3. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.
4. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
5. Incubate the strips or plate for 1 hour at room temperature.
6. Repeat the wash procedure described in step 2.
7. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
8. Cover the tray and incubate the tray for 1 hour at room temperature.
9. Repeat the wash procedure described in step 2.
10. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 3. Do not touch the side or bottom of the wells.
11. Cover the tray and incubate the tray for 1 hour at room temperature.
12. Repeat the wash procedure described in step 2.

13. Add 100 μl of TMB substrate to each well, using the same pipetting order as applied in step 3. Do not touch the side or bottom of the wells.

14. Cover the tray and incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the micro well strips to direct sunlight. Covering the plate with aluminium foil is recommended.

15. Stop the reaction by adding 100 μl of stop solution with the same sequence and timing as used in step 13. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.

16. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.3.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

- *Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.*

APPENDIX 7

Bio-plex ProTM Cytokine, Chemokine, and Growth Factors Assays (IL-6, IL-8, MCP-1)

Principle

Technology

The Bip-Plex suspension array system is built around the three core elements of xMAP technology.

- Fluorescently dyed microspheres (also called beads), each with a distinct colour code or special address to permit discrimination of individuals tests within a multiplex suspension. This allows simultaneous detection of more than 100 different types of molecules in a single well of a 96-well microplate.*
- A dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads.*
- A high-speed digital processor that efficiently manages the fluorescence data.*

Assay Format

Bio-plex ProTM Cytokine, Chemokine, and Growth Factors Assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA (Figure 1). Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator, or reporter.

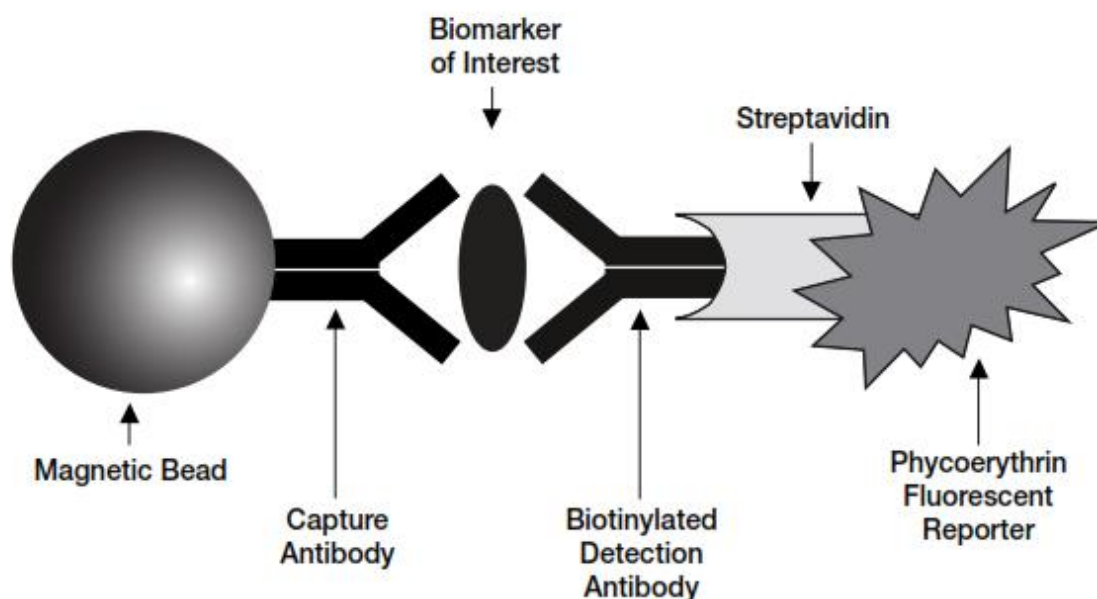


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader for example, a red (635nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532nm) laser excites PE to generate a reporter signal which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages data output and Bio-Plex Manager™ software presents data as Median Fluorescence Intensity (MFI) as well as concentration (pg/mL). the concentration of analyte bound to each bead is proportional to the median fluorescence intensity (MFI) of reporter signal.

Assay Protocol

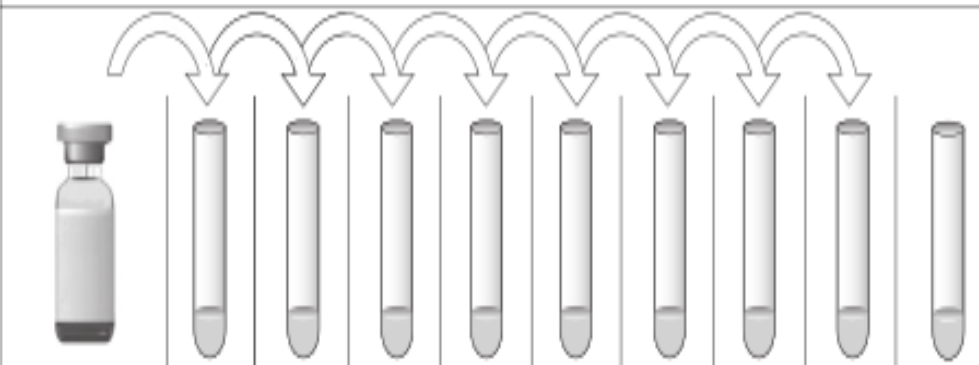
Initial Preparation

1. Plan the plate layout
2. Start up/warm up the Bio-Plex® system (30mins)

- Bring assay buffer, wash buffer, and sample diluent to room temperature (RT). Keep other items on ice until needed.
 - Begin to thaw frozen samples.
3. Prime wash station for flat bottom plate or set vacuum manifold to -1 to -3 $\square \square$ Hg for filter plate.
 4. Calibrate the Bio-Plex system by following the prompts within Bio-Plex Manager™ software. This can be done now or during an assay incubation step.
 5. Reconstitute a single vial of standards in 500 μ l of a diluent similar to the final sample type or matrix. Vortex for 5 sec and incubate on ice for 30 min.

Sample Type	Diluent for Standards	Add BSA
Serum and plasma	Standard diluent	None
Culture media, with serum	Culture media	None
Culture media, serum-free	Culture media	To 0.5% final (w/v)

6. Prepare a fourfold standard dilution series and blank as shown. Vortex for 5 sec between liquid transfers.

	128	50	50	50	50	50	50	50		Transfer Volume, μ l
										
Reconstituted Standard	72	150	150	150	150	150	150	150	150	Diluent, μ l
	S1	S2	S3	S4	S5	S6	S7	S8	Blank	

Note: Change tips between each dilution.

7. After thawing samples, prepare as shown below.

Assay	Serum and Plasma		Culture Supernatant and Other Fluids		Cell and Tissue Lysates	
	Dilution	Diluent	Dilution	Diluent	Dilution	Diluent
Human, mouse, and rat cytokines	1:4	Bio-Plex sample diluent	User optimized	Cell culture medium or buffer similar to sample*	User optimized (1:2 of lysates at 200–900 µg/ml protein)	Bio-Plex sample diluent
Human ICAM-1/VCAM-1	1:100	Bio-Plex sample, standard diluent	User optimized			
Mouse ICAM-1	1:200	Bio-Plex serum-based diluent	User optimized			

* If samples are serum-free, add BSA to 0.5% final w/v.

8. Vortex the 10x or 20x coupled beads for 30 sec and dilute to 1x in Bio-Plex assay buffer as shown. Protect from light.

Human and Mouse Cytokine Group I and II Assays.

# of Wells	10x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	575	5,175	5,750

Mouse Cytokine Group III and Rat Cytokine Group I Assays.

# of Wells	20x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	288	5,472	5,760

Running the Assay

1. Prewet filter plate with 100 µl Bio-Plex assay buffer (skip for flat bottom).
2. Vortex the diluted (1x) beads for 10-20 sec. Add 50 µl to each well of the assay wash plate.
3. Wash the plate two times with 100 µl Bio-Plex wash buffer.
4. Vortex samples, standards, blanks. Add 50 µl to each well.
5. Cover the plate with sealing tape and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm at RT. See table for incubation time.

Incubation times for sample, detection Ab, and SA-PE.

Assay	Sample	Detection Ab	SA-PE
Bio-Plex Pro human and mouse cytokine (groups I and II)	30 min	30 min	10 min
Bio-Plex Pro mouse cytokine (group III)	1 hr	30 min	10 min
Bio-Plex Pro rat cytokine (group I)	1 hr	30 min	10 min

6. With 10 min left in incubation, vortex the 10x or 20x detection Abs for 5 sec and quick-spin to collect liquid. Dilute to 1x as shown below.

# of Wells	Detection Ab, μ l		Detection Ab Diluent, μ l	Total Volume, μ l
	10x	20x		
96	300	—	2,700	3,000
96	—	150	2,850	3,000

7. Wash the plate three times with 100 μ l wash buffer.
8. Vortex the diluted (1x) detection antibody. Add 25 μ l to each well.
9. Repeat step 5. See table for incubation time. Meanwhile, prepare Bio-Plex manager™ software protocol; enter standard S1 values from the assay kit.
10. With 10 min left in the incubation, vortex the 100x SA-PE for 5 sec, and quick-spin to collect liquid. Dilute to 1x as shown and protect from light.

# of Wells	100x SA-PE, μ l	Assay Buffer, μ l	Total Volume, μ l
96	60	5,940	6,000

11. Wash the plate three times with 100 μ l of wash buffer.
12. Vortex the diluted (1x) SA-PE. Add 50 μ l to each well.
13. Repeat step 5. See table for incubation time.
14. Wash the plate three times with 100 μ l of wash buffer.
15. Resuspend beads in 125 μ l assay buffer. Cover plate as in step 5 and shake the plate at 850 ± 50 rpm for 30 sec.

16. Remove sealing tape and read the plate using the setting below.

Assay	Low PMT, RP1	High PMT, RP1
Bio-Plex Pro human cytokine (group I and II)	•	—
Bio-Plex Pro mouse cytokine (group I, II, and III)	•	—
Bio-Plex Pro rat cytokine (group I)	—	•

Bead regions for Bio-Plex Pro cytokine assays

Human Cytokines				Mouse Cytokines				Rat Cytokines	
Group I	Bead Region	Group II	Bead Region	Group I	Bead Region	Group II	Bead Region	Group I	Bead Region
IL-1 β	39	IL-1 α	63	IL-1 α	53	IL-15	42	IL-1 α	21
IL-1ra	25	IL-2R α	13	IL-1 β	19	IL-18*	20	IL-1 β	28
IL-2	38	IL-3	64	IL-2	36	Basic FGF	25	IL-2	22
IL-4	52	IL-12p40**	28	IL-3	18	LIF	45	IL-4	33
IL-5	33	IL-16	27	IL-4	39	M-CSF	26	IL-5	52
IL-6	19	IL-18*	42	IL-5	52	MIG	44	IL-6	56
IL-7	74	CTACK	72	IL-6	38	MIP-2	27	IL-7	38
IL-8	54	GRO- α	61	IL-9	33	PDGF-BB	35	IL-10	19
IL-9	77	HGF	62	IL-10	56	VEGF	47	IL-12p40**	76
IL-10	56	IFN- α 2	20	IL-12p40	76	Group III		IL-12p70	78
IL-12p70	75	LIF	29	IL-12p70	78			IL-13	15
IL-13	51	MCP-3	26	IL-13	37			IL-17A	72
IL-15	73	M-CSF	67	IL-17	72	CD40L	30	IL-18*	20
IL-17	76	MIF	35	Eotaxin	74	IL-17F	28	EPO	14
Eotaxin	43	MIG	14	G-CSF	54	IL-21	14	G-CSF	54
Basic FGF	44	β -NGF	46	GM-CSF	73	IL-22	15	GM-CSF	37
G-CSF	57	SCF	65	IFN- γ	34	IL-23p19	61	GRO/KC	57
GM-CSF	34	SCGF- β	78	KC	57	IL-31	29	IFN- γ	34
IFN- γ	21	SDF-1 α	22	MCP-1(MCAF)	51	IL-33	13	M-CSF	26
IP-10	48	TNF- β	30	MIP-1 α	77	MIP-3 α	12	MCP-1	51
MCP-1	53	TRAIL	66	MIP-1 β	75	Group III Singleplexes		MIP-1 α	77
MIP-1 α	55	Group II Singleplexes		RANTES	55			MIP-2***	27
MIP-1 β	18			TNF- α	21			MIP-3 α	36
PDGF-BB	47					ICAM-1	22	RANTES	55
RANTES	37	ICAM-1	12			IL-25 (IL-17E)	67	TNF- α	43
TNF- α	36	V-CAM1	15			IL-27p28	43	VEGF	47
VEGF	45								

APPENDIX 8

Luminex® Performance Assay Human Biomarker Base Kit A (Human CD14) and Human Obesity Base Kit (Human CRP) – R&D systems

PRINCIPLE OF THE ASSAY

Luminex® Performance Assay multiplex kits are designed for use with a Luminex 100™, Luminex 200™, or Bio-Rad® Bio-Plex® dual laser, flow-based sorting and detection platforms manufactured by Luminex Corporation.

Analyte-specific antibodies are pre-coated onto colour-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. A final wash removes unbound Streptavidin-PE and the microparticles are resuspended in buffer and read using a Luminex or Bio-Plex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin derived signal, which is in direct proportion to the amount of analyte bound.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifuging for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma is not validated for use in this assay.

Icteric samples are not suitable for use in this assay.

SAMPLE PREPARATION

Use polypropylene tubes.

Serum and plasma samples require a 3-fold dilution when assaying BAFF/BLyS, MIP-3 α , BLC/BCA-1, CD27, and IL-2 R α . A suggested 3-fold dilution is 50 μ L of sample + 100 μ L of Calibrator Diluent RD6-60.

When assaying CD14, gp130, IL-6 R α , and TNF RII, serum and plasma samples must be further diluted 16.7-fold to a final 50-fold dilution. A suggested 50-fold dilution is 10 μ L of the 3-fold diluted sample + 157 μ L of Calibrator Diluent RD6-60.

Cell culture supernate samples require a 2-fold dilution. A suggested 2-fold dilution is 75 μ L of sample + 75 μ L of Calibrator Diluent RD6-60 (1:2).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

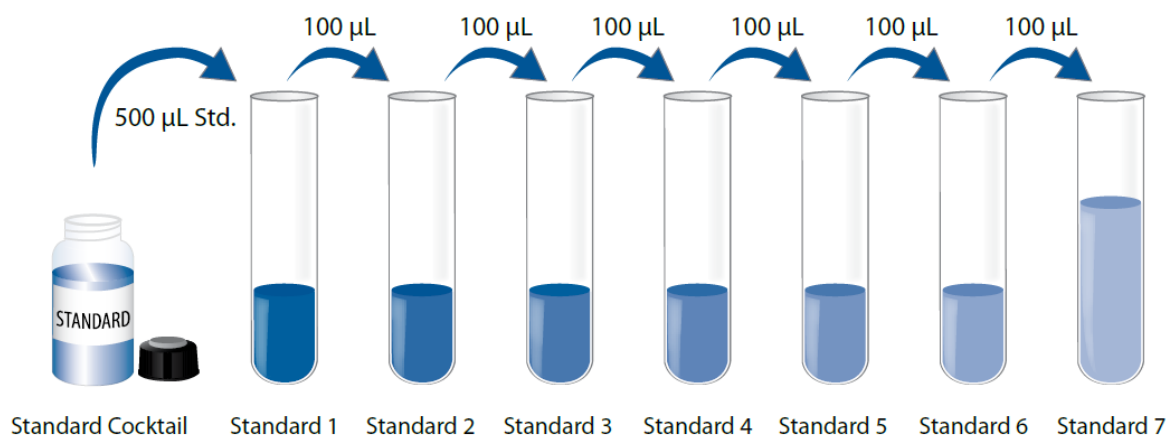
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD6-60 (1:2) - For cell culture supernate samples. Add 20 mL of Calibrator Diluent RD6-60 to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD6-60 (1:2).

Standard - Reconstitute Standard Cocktail with Calibrator Diluent RD6-60 (1:2) (for cell culture supernate samples) or Calibrator Diluent RD6-60 (for serum/plasma samples). Refer

to the Standard Value Card for the reconstitution volume. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 μL of the reconstituted Standard Cocktail into the working standard 1 tube. Pipette 200 μL of the appropriate Calibrator Diluent into the remaining tubes. Use working standard 1 to produce a 3-fold dilution series (below). See analyte specific datasheets for details. Mix each tube thoroughly before the next transfer. Working standard 1 serves as the high standard. The appropriate Calibrator Diluent serves as the blank. Refer to the Standard Value card for the assigned values of working standard 1.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge each Microparticle Concentrate vial for 30 seconds at $1000 \times g$ prior to removing the cap.
2. Gently vortex the vials to resuspend the microparticles, taking precautions not to invert the vials.
3. Dilute the Microparticle Concentrates in the mixing bottle provided. The volume of the Microparticle Concentrate listed in the table below is for each analyte (e.g. if measuring a full plate of IL-6 Ra and gp130, add 50 μL of IL-6 Ra Microparticle Concentrate and 50 μL of gp130 Microparticle Concentrate to 5 mL of Diluent RD2-5 and, if measuring a full plate

of CRP and IL-6, add 50 μL of CRP Microparticle Concentrate and 50 μL of IL-6 Microparticle Concentrate to 5 mL of Microparticle Diluent).

Number of Wells Used	Microparticle Concentrate	+	Diluent RD2-5
96	50.0 μL	+	5.00 mL
72	37.5 μL	+	3.75 mL
48	25.0 μL	+	2.50 mL
24	12.5 μL	+	1.25 mL

Note: Protect microparticles from light during handling. Diluted microparticles cannot be stored.

Prepare microparticles within 30 minutes of use.

DILUTED BIOTIN ANTIBODY COCKTAIL PREPARATION

1. Centrifuge each Biotin Antibody Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials, taking precautions not to invert the vials.
3. Add 50 μL of each Biotin Antibody Concentrate to one vial of Diluent RD2-5. Mix gently.

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil.

Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the 100X Streptavidin-PE to a 1X concentration by adding 55 μL of Streptavidin-PE to 5.5 mL of Wash Buffer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the Luminex analyzer to avoid puncturing the membrane.

Refer to the instrument manual.

- a) Assign the bead region for each analyte being measured (see tables below)

Bead region for Human Biomarker Base Kit A and Human Obesity Base Kit

Analyte	Catalog Number	Microparticle Region
BAFF/BLyS/TNFSF13B	LBA124	13
CCL20/MIP-3α	LBA360	19
CD14	LBA383	59
CD27/TNFRSF7	LBA382	26
CXCL13/BLC/BCA-1	LBA801	22
gp130	LBA228	60
IL-2 Ra	LBA223	32
IL-6 Ra	LBA227	79
TNF RII/TNFRSF1B	LUCA726	50

Analyte	Catalog Number	Microparticle Region
Adiponectin/Acrp30	LOB1065	02
CCL2/MCP-1	LUH279	78
C-Reactive Protein (CRP)	LOB1707	08
Complement Factor D/Adipsin	LOB1824	11
IL-6	LUH206	32
IL-10	LUH217	50
Leptin	LUB398	31
Resistin*	LOB1359	12
Serpin E1/PAI-1	LOB1786	10
TNF-α	LUH210	77

b) 50 events/bead

c) Minimum events: 0

d) Flow rate: 60μL/minute (fast)

e) Sample size: 50 μL

f) Doublet Discriminator gates at approximately 7500 and 15,500

g) Collect Median Fluorescence Intensity (MFI)

Note: For the Bio-Rad Bio-Plex analyzer set the gates at 4300 and 10,000. The CAL2 setting for the Bio-Rad Bio-Plex analyzer should be set at the low RP1 target value.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Note: Protect microparticles and Streptavidin-PE from light at all times.

- 1. Prepare all reagents, working standards, and samples as directed in the previous sections.*
- 2. Pre-wet the filter-bottomed microplate by filling each well with 100 μ L of Wash Buffer.*

Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.

Note: After each final wash cycle and subsequent reagent addition, blot the bottom of the microplate with a paper towel to prevent wicking.

- 3. Add 50 μ L of Standard or sample* per well.*
- 4. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 50 μ L of the Microparticle Cocktail to each well of the microplate. Securely cover with a foil plate sealer. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.*
- 5. Using a vacuum manifold device designed to accommodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 μ L) and removing the liquid again. All of the liquid must be removed through the filter at the bottom of the plate to avoid any loss of microparticles. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.*
- 6. Add 50 μ L of diluted Biotin Antibody Cocktail to each well. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.*
- 7. Repeat the wash as in step 5.*

8. Add 50 μ L of diluted Streptavidin-PE to each well. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.

9. Repeat the wash as in step 5.

10. Resuspend the microparticles by adding 100 μ L of Wash Buffer to each well. Incubate for 2 minutes on the shaker set at 500 ± 50 rpm.

11. Read within 90 minutes using a Luminex or Bio-Rad analyzer.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

CALIBRATION

This assay is calibrated against highly purified recombinant human biomarkers produced at R&D Systems.

PLATE LAYOUT

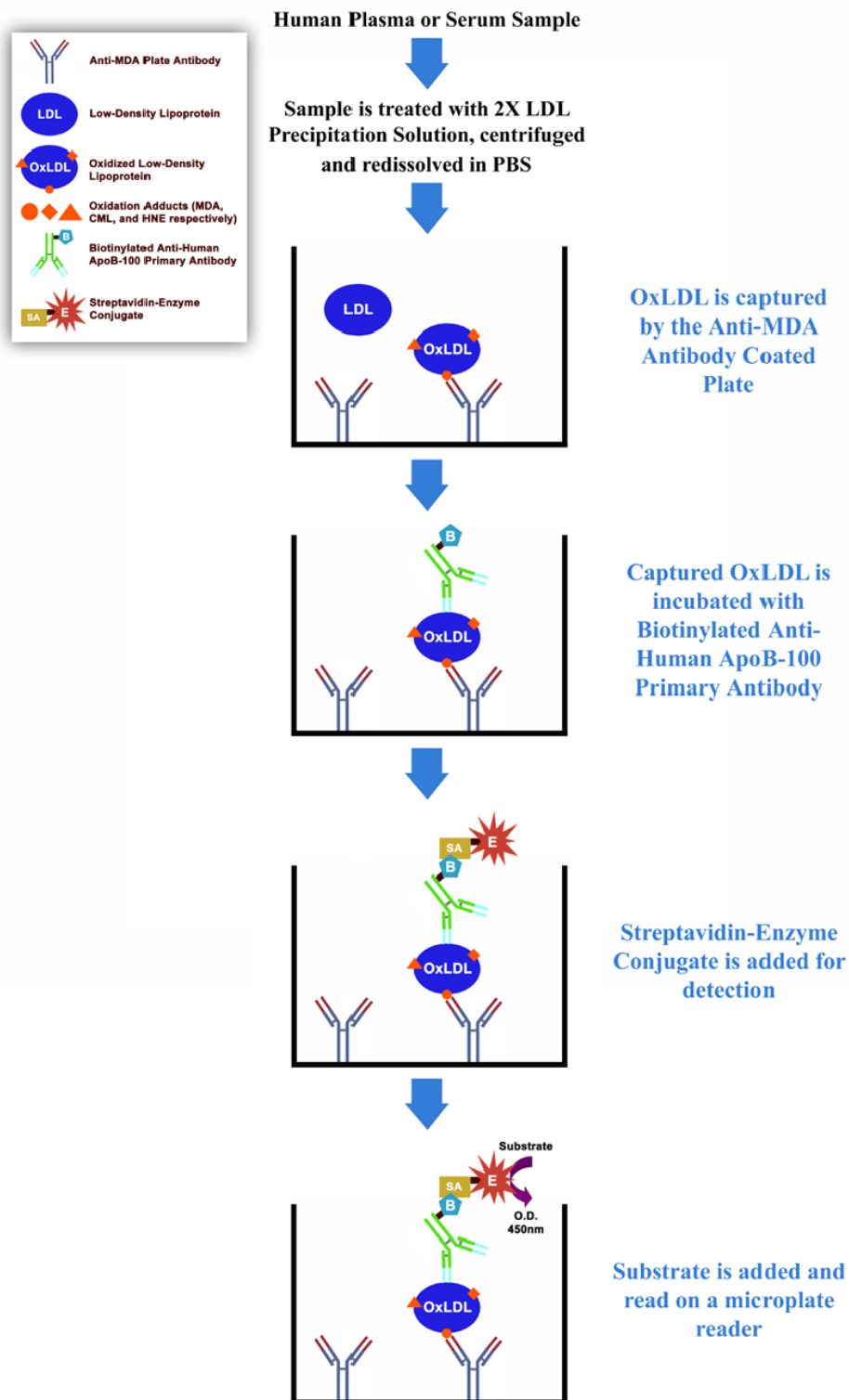
Use this plate layout to record standards and samples assayed.

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

APPENDIX 9

OxiSelect™ Human Oxidized LDL ELISA Kit (MDA-LDL Quantitation) – CELL BIOLABS, INC.

Assay Principle



Preparation of Reagents

- *1X Wash Buffer: Dilute the 10X Wash Buffer Concentrate to 1X with deionized water. Stir to homogeneity.*
- *Blocking Reagent: Immediately before use dilute the Blocking Reagent 1:100 with PBS. Do not store diluted solutions.*
- *Biotinylated Anti-Human ApoB-100 Antibody and Streptavidin-Enzyme Conjugate: Immediately before use dilute the Anti-ApoB-100 antibody 1:1000 and Streptavidin-Enzyme Conjugate 1:1000 with Assay Diluent. Do not store diluted solutions.*

Preparation of OxLDL Standard

Prepare a dilution series of OxLDL Standards in the concentration range of 0 to 1 $\mu\text{g/mL}$ in Assay Diluent (Table 1).

Standard Tubes	0.5 mg/mL OxLDL Standard (μL)	Assay Diluent (μL)	Final OxLDL Standard (ng/mL)
1	2	998	1000
2	250 of Tube #1	250	500
3	250 of Tube #2	250	250
4	250 of Tube #3	250	125
5	250 of Tube #4	250	62.5
6	250 of Tube #5	250	31.25
7	250 of Tube #6	250	15.63
8	0	250	0

Table 1. Preparation of OxLDL Standards

Preparation of Samples

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

- *Plasma: Collect blood with heparin or EDTA and centrifuge for 10 minutes at 1000 x g at 4°C. Remove 200 μL of plasma and add 200 μL of LDL Precipitation Solution, mixing well. Incubate at room temperature for 5 minutes (precipitation will occur). Centrifuge for 20 minutes at 2000 x g (pellet should be visible). Carefully aspirate the supernatant and collect*

the pellet. Resuspend and dissolve the pellet in 1.6 mL of PBS, vortexing well. Further dilute the sample 1:50 to 1:200 in Assay Diluent before running the ELISA. Assay immediately and do not store solutions.

• Serum: Harvest serum and centrifuge for 10 minutes at 1000 x g at 4°C. Remove 200 µL of serum and add 200 µL of LDL Precipitation Solution, mixing well. Incubate at room temperature for 5 minutes (precipitation will occur). Centrifuge for 20 minutes at 2000 x g (pellet should be visible). Carefully aspirate the supernatant and collect the pellet. Resuspend and dissolve the pellet in 1.6 mL of PBS, vortexing well. Further dilute the sample 1:50 to 1:200 in Assay Diluent before running the ELISA. Assay immediately and do not store solutions.

Assay Protocol

- 1. For plasma and serum samples, refer to the above Sample Preparation Section. These samples require LDL Precipitation Solution treatment immediately prior to running the assay.*
- 2. Add 100 µL of OxLDL standard or unknown sample to the Anti-MDA Antibody Coated Plate. Each OxLDL standard, blank and unknown sample should be assayed in duplicate.*
- 3. Cover with a plate cover and incubate at room temperature for 2 hours on an orbital shaker.*
- 4. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.*
- 5. Add 100 µL of diluted Blocking Reagent to each well. Cover with a plate cover and incubate at room temperature for 1 hour on an orbital shaker.*

6. Wash microwell strips 5 times with 250 μ L 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
 7. Add 100 μ L of the diluted Biotinylated Anti-Human ApoB-100 antibody to each well. Incubate at room temperature for 1 hour on an orbital shaker.
 8. Wash the strip wells 5 times according to step 6 above.
 9. Add 100 μ L of the diluted Streptavidin-Enzyme Conjugate to each well. Incubate at room temperature for 1 hour on an orbital shaker.
 10. Wash the strip wells 5 times according to step 6 above. Proceed immediately to the next step.
 11. Warm Substrate Solution to room temperature. Add 100 μ L of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 5-20 minutes.
- Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.*
12. Stop the enzyme reaction by adding 100 μ L of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
 13. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figures demonstrate typical results with the Human Oxidized LDL ELISA Kit. One should use the data below for reference only. This data should not be used to interpret actual results.

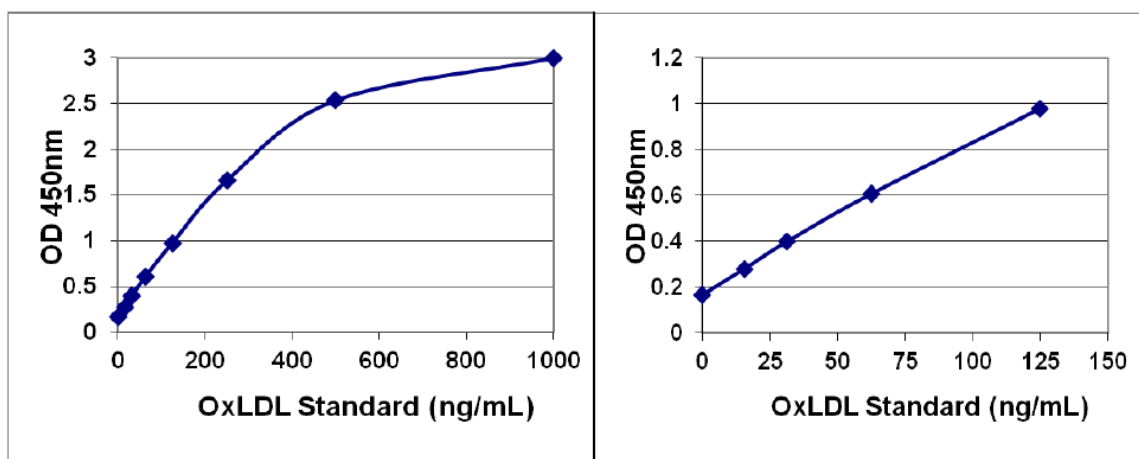


Figure 1: Human OxLDL Standard Curve.

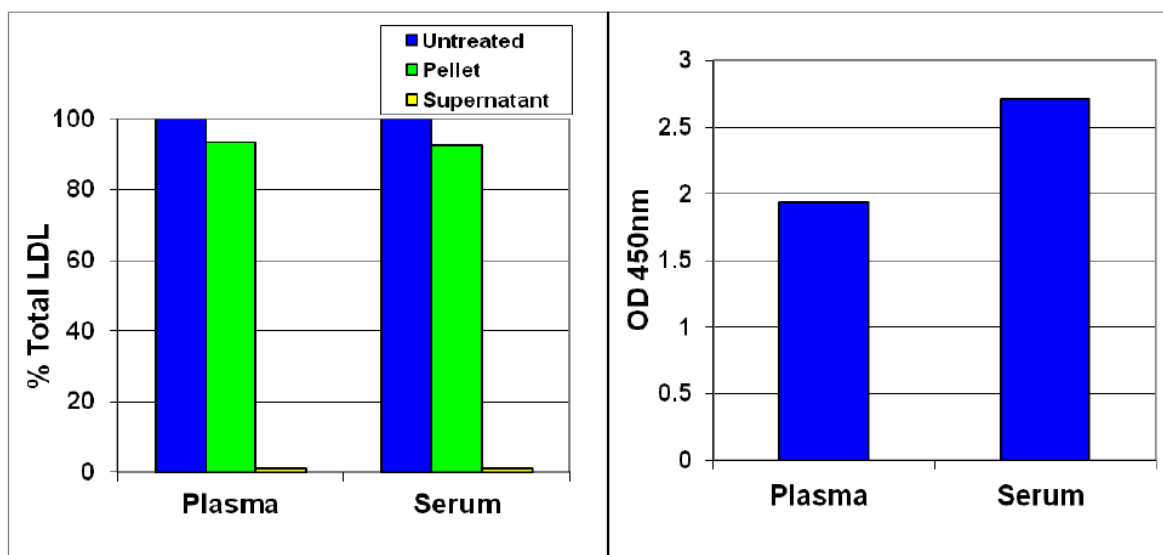


Figure 2: Quantitation of OxLDL in Serum and Plasma Samples. Left: LDL Recovery After Precipitation Solution. Serum and plasma samples were treated with LDL Precipitation Solution according to the Sample Preparation protocol. LDL recovery was determined by Human ApoB-100 ELISA (STA-368). **Right: OxLDL Determination of Serum and Plasma Samples.** Serum and plasma samples were treated with LDL Precipitation Solution according to the Sample Preparation Section. Precipitated LDL pellets were resuspended in 1.6 mL of PBS before further diluting 1:160 in Assay Diluent. Samples were tested according to the Assay Protocol.

APPENDIX 10

BCM - Body Composition Monitor (Fresenius Medical Care)



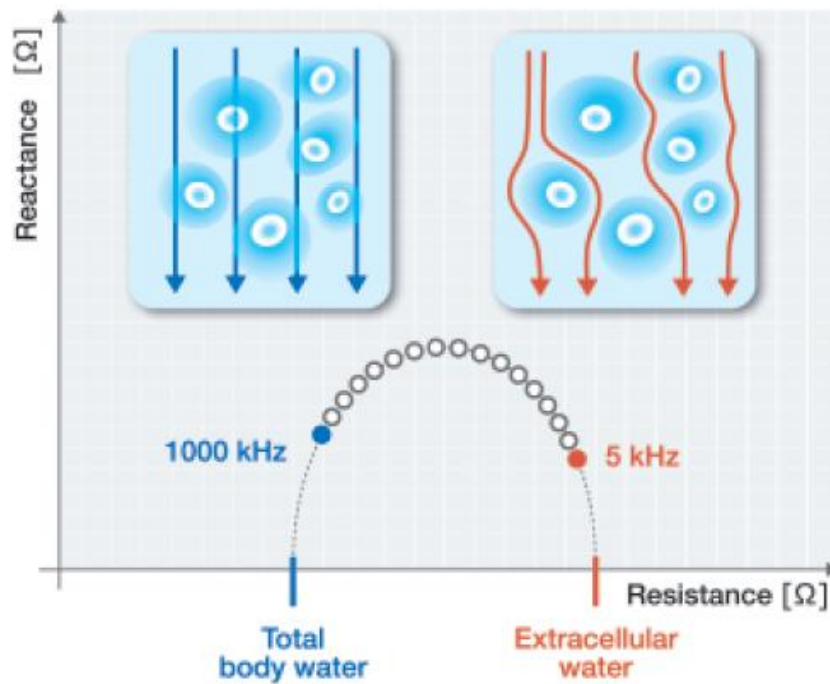
Principle and technology

The BCM - Body Composition Monitor employs the latest bioimpedance spectroscopy techniques. It measures at 50 frequencies over a range from 5 to 1000 kHz to determine the electrical resistances of the total body water (TBW) and the extracellular water (ECW).

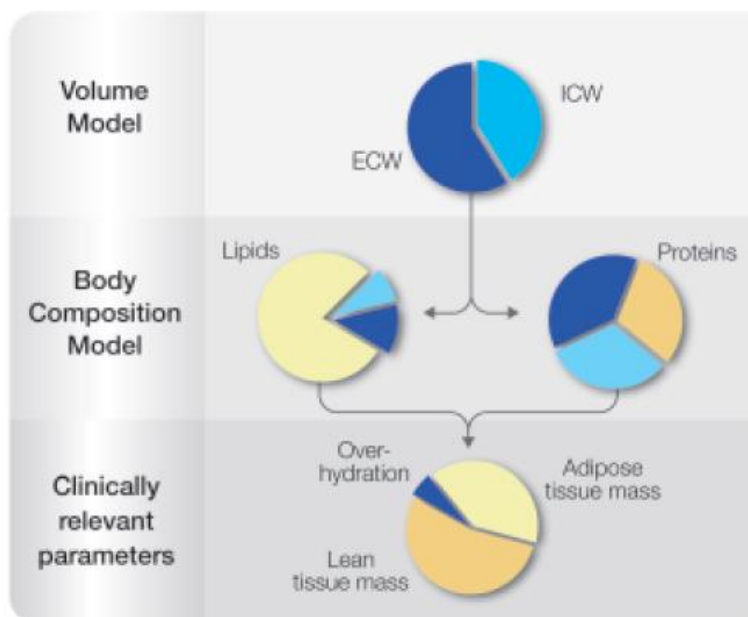
While high-frequency current passes through the total body water, low-frequency current cannot penetrate cell membranes and thus flows exclusively through the extracellular water.

To obtain the clinically relevant output parameters, two advanced physiological models are used in the BCM - Body Composition Monitor:

- A volume model describing electrical conductance in a cell suspension enabling the total body water and extracellular water as well as the intracellular water (ICW) to be calculated.*
- A body composition model calculating the three principal body compartments overhydration, lean tissue and adipose tissue from ECW and TBW information.*



The Cole-Cole plot displays the raw data of the measurement separating ECW from TBW.



The BCM - Body Composition Monitoring device takes three steps to display the final output parameters: overhydration, adipose tissue mass and lean tissue mass.

How is the BCM – Body Composition Monitor validated?

All output parameters have been validated against the gold standard reference methods in various studies involving more than 500 patients and healthy controls.

These reference methods include:

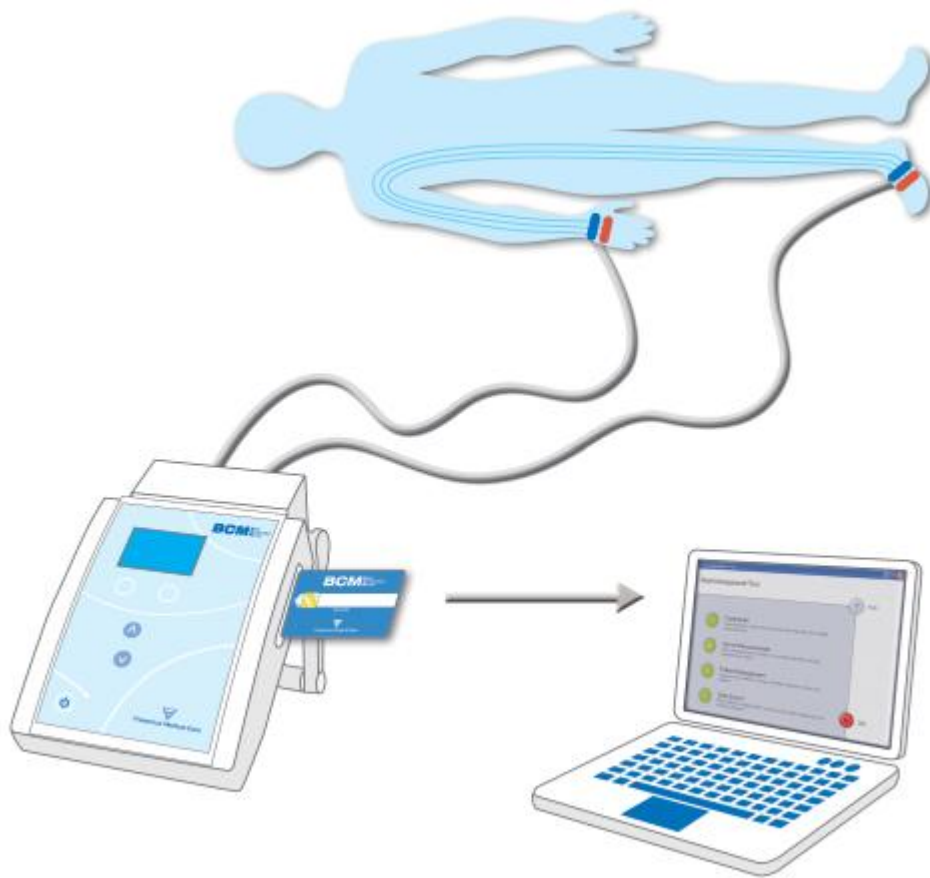
- Extracellular water – bromide dilution*
- Intracellular water – total body potassium (TBK)*
- Total Body Water – deuterium dilution*
- Lean Tissue Mass – Dual Energy X-ray Absorptiometry (DEXA)*
- Adipose Tissue Mass – 4-compartment modelling, DEXA, air displacement plethysmography and under water weighing*
- Body Cell Mass – magnet resonance tomography, TBK*
- Overhydration – by expert clinical assessment*

Handling

The BCM – Body Composition Monitor is designed for application in a variety of clinical settings. On treatment days, measurements are best performed before the start of treatment.

Handling is very straight forward involving the following steps:

- Electrodes are attached to one hand and one foot with the patient in a supine position.*
- Patient cable is connected.*
- Measurement is initiated and results are displayed within 2 minutes.*
- Results are stored on the PatientCard.*
- Data can be transferred via PatientCard to a personal computer for further analysis with the Fluid Management Tool (FMT).*



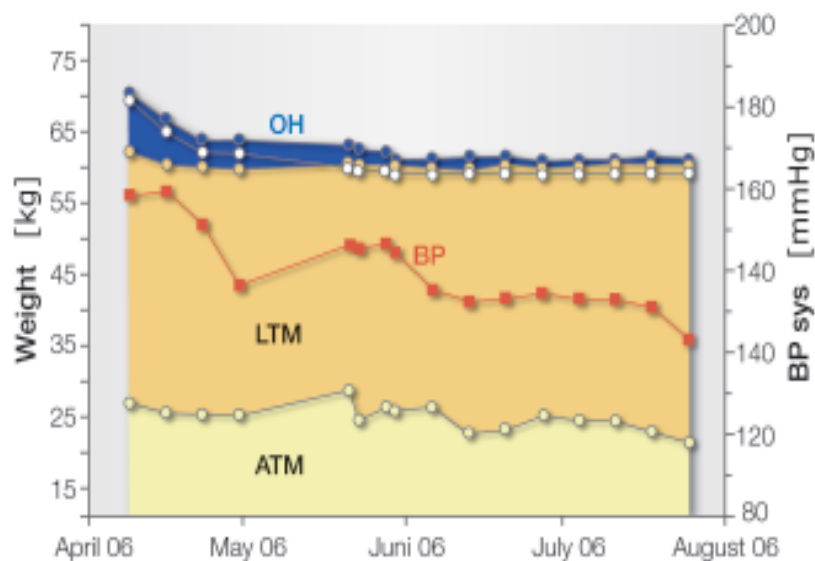
Patient analysis & management

The Fluid Management Tool (FMT) operates in conjunction with the BCM – Body Composition Monitor and can be easily installed on a personal computer.

This software provides a quick overview of the fluid status and body composition of the patient - including reference ranges for dialysis patients and the healthy population.

Body Composition Plot

The FMT displays the development of the three primary compartments adipose tissue mass (ATM), lean tissue mass (LTM) and overhydration (OH) over time. In addition, the systolic blood pressure (BP sys) is displayed which allows the influence of overhydration on blood pressure to be identified. Consequently, it is easy to observe changes in body composition and how these impact overhydration.



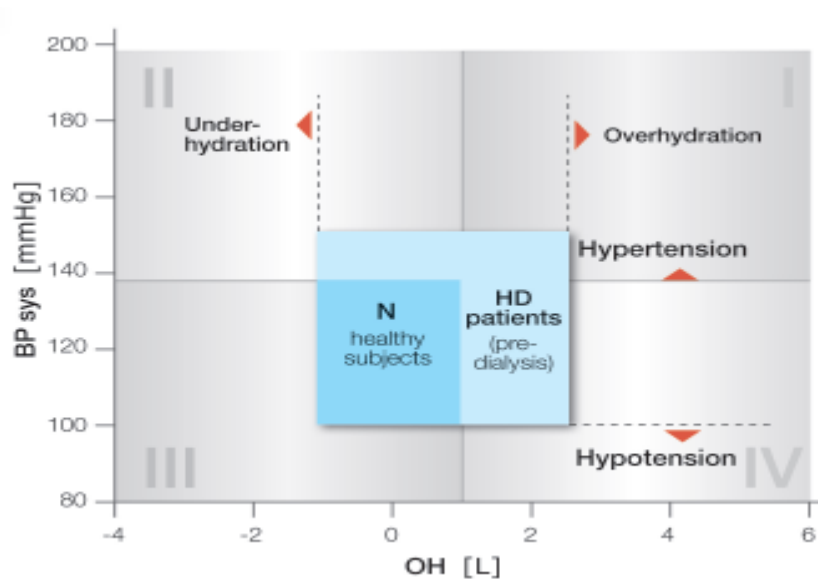
Body composition and blood pressure variation over time.

Hydration Reference Plot

It is known that overhydration often leads to hypertension. However, in many patients this relationship can be heavily influenced by underlying comorbidities. For example, in some patients, hypertension may be dependent on vascular disorders⁶ while on the other hand, there are patients who exhibit apparently normal or low blood pressure despite gross overhydration.

The hydration reference plot depicts the association between overhydration status and systolic blood pressure on one graph. The easiest way to identify abnormal conditions is to compare patient results with a reference population.

The reference region N has been defined from measurements in healthy subjects allowing direct comparison of patient parameters. A second reference region indicates the typical range of well managed patients measured pre-dialysis and with a maximum weight gain of 2.5 L. Both reference ranges are adjusted for age and sex. The position of the patient in the plot allows a first interpretation of the patient's condition at the time of the measurement and aids the physician to possible actions the patient might benefit from.



Overhydration (OH) and systolic blood pressure (BP sys) are combined in one graph – the Hydration Reference Plot (HRP).

Technical data	
Key parameters	Unit
Overhydration (OH) (pre-/postdialytic)	[L]
Lean tissue index (LTI)	[kg/m ²]
Fat tissue index (FTI)	[kg/m ²]
Total body water (TBW) (Urea distribution volume V)	[L]
Extracellular water (ECW)	[L]
Intracellular water (ICW)	[L]
ECW / ICW	-
Lean tissue mass	[kg] and [%]
Fat mass	[kg]
Adipose tissue mass	[kg] and [%]
Body Cell Mass	[kg]

APPENDIX 11

Bio-Plex Pro™ TGF-β Assays

Principle

Technology

The Bio-Plex suspension array system is built around the three core elements of xMAP technology.

- Fluorescently dyed microspheres (also called beads), each with a distinct colour code or special address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of more than 100 different types of molecules in a single well of a 96-well microplate.*
- A dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads.*
- A high-speed digital processor that efficiently manages the fluorescence data.*

Assay Format

Bio-plex Pro™ Cytokine, Chemokine, and Growth Factors Assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA (Figure 1). Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator, or reporter.

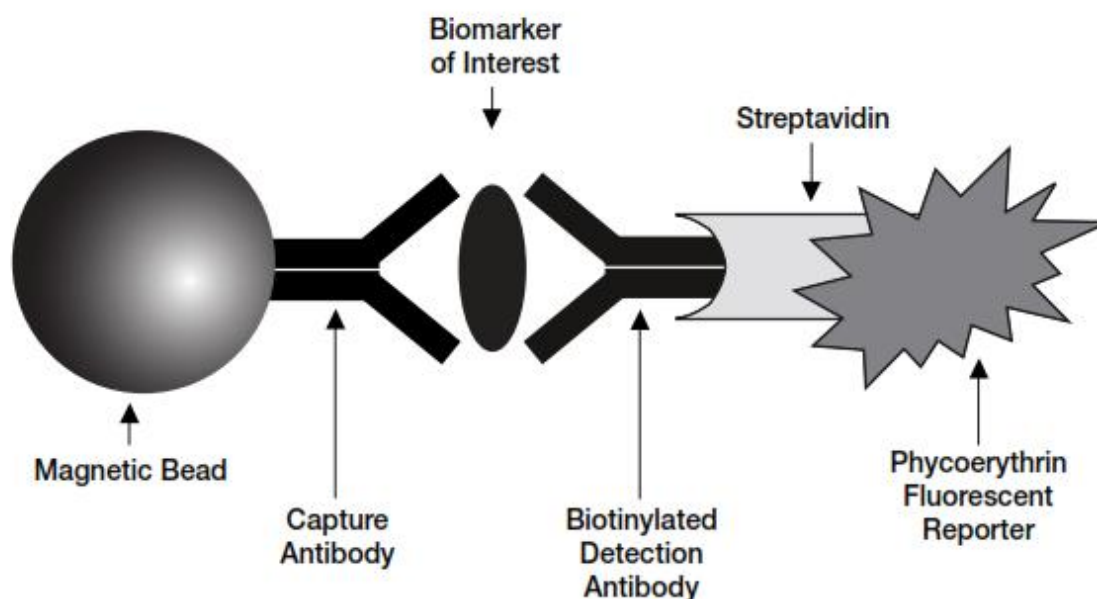


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader for example, a red (635nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532nm) laser excites PE to generate a reporter signal which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages data output and Bio-Plex Manager™ software presents data as Median Fluorescence Intensity (MFI) as well as concentration (pg/mL). the concentration of analyte bound to each bead is proportional to the median fluorescence intensity (MFI) of reporter signal.

Assay Protocol

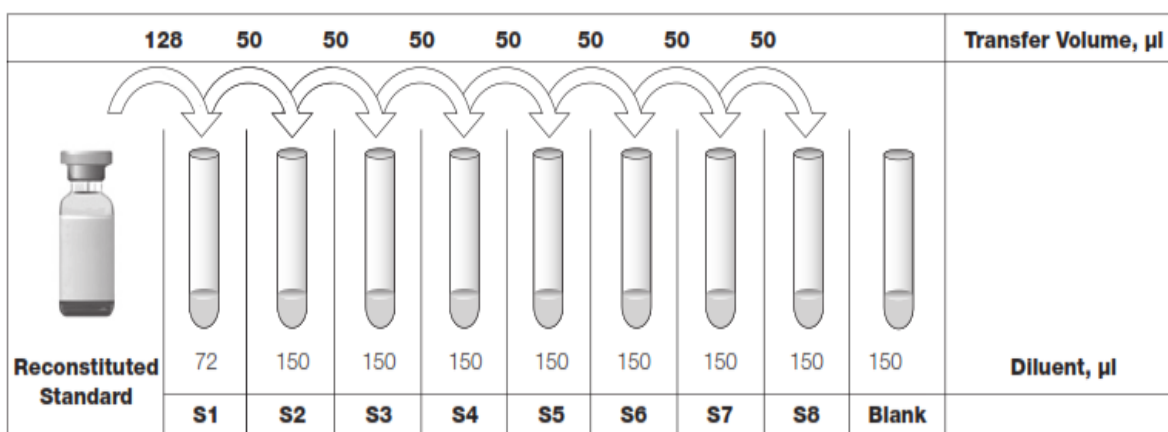
Initial Preparation

1. Plan the plate layout
2. Start up/warm up the Bio-Plex® system (30mins)

- Bring assay buffer, wash buffer, and sample diluent to room temperature (RT). Keep other items on ice until needed.
 - Begin to thaw frozen samples.
3. Prime wash station for flat bottom plate or set vacuum manifold to -1 to -3 mm Hg for filter plate.
 4. Follow the prompt in Bio-Plex Manager™ software to calibrate the system. This can be done now or during an incubation step
 5. Mix 1 volume of Bio-Plex standard diluent with 3 volumes of Bio-Plex sample diluent (each supplied in the kit). The resulting solution is used for reconstitution and subsequent dilution of standards. This results in a serum-matrix based diluent that mimics the matrix in 1:16 diluted serum and plasma samples. For samples in serum-free media and other biological fluids, use a diluent that most closely matches the sample matrix. Add carrier protein such as BSA at a final concentration of 0.5% (w/v).
 6. Reconstitute a single vial of standards in 500 µl of a diluent similar to the final sample type or matrix as shown below. Vortex for 5 sec and incubate on ice for 30min.

Sample Type	Diluent for Standard	Add BSA
Serum and plasma	Standard/sample diluent mix (1:3)	None
Culture media, with serum	Culture media	None
Culture media, serum-free	Culture media	To 0.5% final (w/v)
Lavage, lysate, other fluids	Sample diluent	To 0.5% final (w/v)

7. Prepare a fourfold standard dilution series and blank as shown. Vortex for 5 sec between liquid transfers.

	128	50	50	50	50	50	50	50		Transfer Volume, μ l
	72	150	150	150	150	150	150	150		
Reconstituted Standard	S1	S2	S3	S4	S5	S6	S7	S8	Blank	Diluent, μl

8. After thawing samples, activate by adding 1 volume of 1 N HCl to 5 volumes of samples. Vortex, incubate at RT for 10min. neutralize by adding the same volume of base (1.2 N NaOH/0.5M HEPES). After treatment, dilute samples as shown below.

Sample Type	Diluent	Add BSA	Sample Dilution
Serum and plasma	Sample diluent	None	1:16 final*
Culture media, with serum	Culture media	None	User optimized
Culture media, serum-free	Culture media	To 0.5% final	User optimized
Lavage, lysate, other fluids	Sample diluent	To 0.5% final	User optimized

* For example, activate 25 μ l sample, neutralize, and bring to a final volume of 400 μ l.

9. Vortex the 20x coupled beads for 30 sec and dilute to 1x in Bio-Plex assay buffer as shown. Protect from light.

# of Wells	20x Beads, μ l	Assay Buffer, μ l	Total Volume, μ l
96	288	5,472	5,760

Running the Assay

1. Prewet filter plate with 100 μ l Bio-Plex assay buffer (skip for flat bottom).
2. Vortex the diluted (1x) beads for 10-20 sec. Add 50 μ l to each well of the assay wash plate.
3. Wash the plate two times with 100 μ l Bio-Plex wash buffer.

4. Vortex samples, standards, blanks. Add 50 μ l to each well.
5. Cover the plate with sealing tape and protect from light with aluminum foil. Incubate on shaker at 850 ± 50 rpm at RT. See table for incubation time.

Note: 850 rpm provides equivalent performance to recommended shaker setting in previous manuals (1,100 rpm for 30 sec, 300 rpm for incubation).

6. With 10min left in the incubation, vortex the 20x detection antibodies for 5 sec and quick-spin to collect liquid. Dilute to 1x in detection antibody diluent as shown below.

# of Wells	20x Detection Ab, μ l	Detection Ab Diluent, μ l	Total Volume, μ l
96	150	2,850	3,000

7. Wash the plate three times with 100 μ l wash buffer.
8. Vortex the diluted (1x) detection antibody. Add 25 μ l to each well.
9. Cover and incubate at 850 ± 50 rpm, as in step 5, for 1 hr at RT. Meanwhile, prepare Bio-Plex manager[™] software protocol; enter standard S1 values from the assay kit.
10. With 10 min left in the incubation, vortex the 100x SA-PE for 5 sec, and quick-spin to collect liquid, and dilute to 1x as shown and protect from light.

# of Wells	100x SA-PE, μ l	Assay Buffer, μ l	Total Volume, μ l
96	60	5,940	6,000

11. Wash the plate three times with 100 μ l of wash buffer.
12. Vortex the diluted (1x) SA-PE. Add 50 μ l to each well.
13. Cover and incubate at 850 ± 50 rpm, as in step 5 and for 30 min at RT.
14. Wash the plate three times with 100 μ l of wash buffer.

15. Resuspend beads in 125µl assay buffer. Cover and shake at 850 ± 50 rpm, as in step 5, for 30 sec.

16. Remove sealing tape and read the plate using the setting below.

Instrument	RP1 (PMT)	DD Gates	Bead Events
Bio-Plex 100, 200*	Low	5,000 (low), 25,000 (high)	50
Bio-Plex 3D*	Standard	Select MagPlex beads	50
Bio-Plex® MAGPIX™*	N/A, use default instrument settings		Default

* Or similar Luminex-based system.

Table 14. TGF-β assay bead regions.

Analyte	Bead Region
TGF-β1	13
TGF-β2	72
TGF-β3	66

APPENDIX 12

Cytokine genotyping tray



21001 Kittridge Street, Canoga Park, CA 91303-2801 Tel: (818) 702-0042 Fax: (818) 702-6904

www.onelambda.com

PRODUCT INSERT

CYTOKINE GENOTYPING TRAY

For Research Use Only. Not for use in diagnostic procedures.

Catalog Number	Description	Cytokines	Number of Tests	Number of D-Mix Tubes	Tray Seals	Number of PCR Reactions per Test	D-mix Volume μ l per Test	DNA Volume μ l per Test	Taq Polymerase Volume μ l per Test
CYTGEN	Cytokine Genotyping Tray	TGF- β 1, TNF- α , IL-6, IL-10, IFN- γ	24	24	6	16	180	19	1

INTENDED USE

A test assay for genotyping of the following cytokine factors:

- Transforming growth factor - β 1 (TGF- β 1)
- Tumor necrosis factor - α (TNF- α)
- Interleukin - 6 (IL-6)
- Interleukin - 10 (IL-10)
- Interferon - γ (IFN- γ)

These four factors have been selected based on published investigative interest in their possible roles in transplant rejection.

SUMMARY AND EXPLANATION

Cytokines are crucial to cellular communication and, thus, may be key elements in turning on and off the immune response. Studies have linked the genotypes TNF- α , TGF- β 1, IL-6, and IL-10 with the expression levels of these cytokines. Furthermore, these cytokine genotypes may be relevant to an organ transplant patient's immunological response to the graft. This cytokine genotyping tray will identify those genotypes whose expression levels have been observed to be associated with transplant rejection.

PRINCIPLE(S)

The PCR-SSP methodology is based on the principle that completely matched oligonucleotide primers are more efficiently used in amplifying a target sequence than a mismatched oligonucleotide primer by recombinant Taq polymerase. Primer pairs are designed to have perfect matches only with a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly matched primer pairs result in the amplification of target sequences (i.e., a positive result), while mismatched primer pairs do not result in amplification (i.e., a negative result).

After the PCR process, the amplified DNA fragments are separated by agarose gel electrophoresis and are visualized by staining with ethidium bromide and exposure to ultraviolet light. Interpretation of PCR-SSP results is based on the presence or absence of a specific amplified DNA fragment. Since amplification during the PCR reaction may be adversely affected by various factors (pipetting errors, poor DNA quality, presence of inhibitors, etc.) an internal control primer pair is included in every PCR reaction. The control primer pair amplifies a conserved region of the Human β -globin gene, which is present in all DNA samples and is used to verify the integrity of the PCR reaction. In the presence of a positive typing band (specific amplification of a cytokine allele), the product of the internal control primer may be weak or absent due to the difference in concentration and melting temperatures between the specific primer pairs and the internal control primer pair. The amplified DNA fragments of the specific cytokine primer pairs are smaller than the product of the internal control primer pair, but larger than the diffuse, unincorporated primer band. Thus, a positive reaction for a specific cytokine allele or allele group is visualized on the gel as an amplified DNA fragment between the internal control product band and the unincorporated primer band.

REAGENTS

A. Identification

The Cytokine Genotyping Tray provides sequence-specific oligonucleotide primers for amplification of selected TNF- α , TGF- β 1, IFN- γ , IL-6, and IL-10 alleles and the human β -globin gene by the polymerase chain reaction (PCR). These alleles are known to be associated with the expression level of these factors.¹⁻¹⁰ Pre-optimized primers are presented (dried) in different wells of a 96-well 0.2 ml thin-walled tube tray for PCR and are ready for the addition of DNA samples, recombinant Taq polymerase, and specially formulated dNTP-buffer mix (D-mix). Each tray includes a negative control reaction tube that detects the presence of the internal control product generated by the tray. The internal control PCR product, amplified from the human β -globin gene is the most likely contaminating PCR product due to its amplification in every well. The amount of primer is adjusted for optimal

amplification of 100 ng of sample DNA when used in conjunction with the D-mix, the prescribed amount of recombinant Taq polymerase, and the PCR reaction profile detailed below. See the provided worksheet for specific alleles which can be amplified by each primer set under the specified PCR conditions of the assay. For lot specific primer site locations, please refer to the worksheet.

B. Warning or Caution

Note: There are no specific safety concerns for materials supplied by One Lambda. Other warnings pertain to materials not provided by One Lambda. In all cases the manufacturer's MSDS should be consulted for specific warnings and guidelines.

1. For Research Use Only. Not for use in diagnostic procedures.
2. Carcinogen Warning: The ethidium bromide used for staining of DNA is a potential carcinogen. Always wear gloves when handling stained gels.
3. Biohazard Warning: All blood products should be treated as potentially infectious.
4. Caution: Wear UV-blocking eye protection, and do not view UV light source directly when viewing or photographing gels.
5. Pipettes used for Post-PCR manipulations should not be used for Pre-PCR manipulations.

C. Instructions for Use

See "Directions For Use."

D. Storage Instructions

Store reagents at temperature indicated on package. Use before printed expiration date.

E. Purification or Treatment Required for Use

1. Any primer sets with cracks in the tubes or on the lip, which may affect complete sealing of the reaction tubes to prevent evaporative losses, should be considered unusable.
2. If salts have precipitated out of the solution in the D-mix aliquots during shipping or storage, re-dissolve by extended vortexing at room temperature (20 - 25°C).
3. D-mix aliquots, upon thawing at room temperature (20 - 25°C), should be pink to light purple in color. Any D-mix aliquot without the specified coloration should be considered unusable.

F. Instability Indications

1. Any primer sets with cracks in the tubes or on the lip, which may affect complete sealing of the reaction tubes to prevent evaporative losses, should be considered unusable.
2. If salts have precipitated out of the solution in the D-mix aliquots during shipping or storage, re-dissolve by extended vortexing at room temperature (20 - 25°C).
3. D-mix aliquots, upon thawing at room temperature (20 - 25°C), should be pink to light purple in color. Any D-mix aliquot without the specified coloration should be considered unusable.

INSTRUMENT REQUIREMENTS

A. Programming the Perkin Elmer 9600 or 9700 Thermocycler.

The following program is designed to program 96-Well GeneAmp® PCR System 9600, 9700 or Veriti™ 96-Well Thermal Cycler (Applied Biosystems). Set ramp speed to 9600 for 96-Well GeneAmp® PCR System 9700. Set ramp speed to 9600 Emulation Mode for Veriti™ 96-Well Thermal Cycler. Set reaction volume to 10 µl volume. Program the thermal cycler before starting the "Step-by-Step procedure" below.

One Lambda PCR Program (OLI-1)

# of Cycles	Step	Temp. (°C)	Time (sec.)
1	1	96	130
	2	63	60
9	1	96	10
	2	63	60
20	1	96	10
	2	59	50
	3	72	30
End	1	4	—

For specific thermal cycler information, refer to the manufacturer's user manual.

B. 2.5% Agarose Gel Preparation

(for Gel System, OLI Cat. #MGS108)

- a. To set up:
 - Slide the locking pin on the base to the open position.
 - Insert the gel box into the base matching the color-coded sides to assure proper orientation.
 - Lock the gel box into the base by sliding the locking pin into the locked position.
 - Use the leveling bubble and the three height adjustable legs to level the base.
- b. Orient and fully insert the 14 gel combs into the gel comb holder.
- c. To 100 ml of 1x Tris Borate EDTA buffer (1xTBE) with 0.5 µg/ml ethidium bromide (in a 500 ml glass bottle), add 2.5 g electrophoresis grade agarose. Heat until a homogeneous solution is formed.
- d. Add 30 ml of the gel solution to the gel box. Make sure the agarose covers the entire surface evenly by tilting the gel box back and forth immediately after the gel solution is added. Quickly place the gel comb holder on the filled gel box by matching the color coding. Allow to set for 15 minutes.
- e. Remove the gel combs by lifting the gel comb holder while holding the base. Add 10 ml of 1x TBE containing 0.5 µg/ml ethidium bromide evenly across the gel to fill every well.

C. Gel Electrophoresis

(for Gel System, OLI Cat. #MGS108)

- a. After completing the PCR Reaction:
 - Orient the DNA primer set tray and gel box with the negative control well in the upper left hand corner.
 - Gently remove the tray seal without splashing the samples.
- b. Transfer each PCR reaction (10 µl) in sequence to the 2.5% agarose gel. Make sure to transfer all samples in the proper sequence. (No addition of electrophoresis dye is necessary.) Use of an 8- or 12- channel Pipetman® is recommended.
Note: The order of samples (to match the worksheet) is from left to right, top to bottom.
- c. Cover the gel box with the gel box cover by matching the color-coded sides. Electrophorese the samples at 140 - 150 volts until the red tracking dye has migrated about 0.5 cm into the gel (approximately 3 - 5 minutes, depending on the agarose used). Remove the cover.
- d. Slide the locking pin on the base to the open position and remove the gel box. Transfer the gel box to a UV transilluminator. Photograph the completed gel.
- e. Orient the photograph with the negative control reaction in the upper left corner, and mark the corresponding positive allele groups on the worksheet provided with the tray.

SPECIMEN COLLECTION AND PREPARATION

1. DNA can be purified from human leukocytes by any preferred method.
2. The DNA sample to be used for PCR-SSP analysis should be re-suspended in sterile water or in 10 mM Tris-HCl, pH 8.0 - 9.0 at a concentration of 25 - 200 ng/µl with the A260/A280 ratio of 1.65 - 1.80.
3. **Samples should not be re-suspended in solutions containing chelating agents, such as EDTA, above 0.5 mM in concentration.**
4. DNA samples may be used immediately after isolation or stored at -20°C or below for extended periods of time (over 1 year) with no adverse effects on results.
5. DNA samples should be shipped at 4°C or below to preserve their integrity during transport.

PROCEDURE

A. Materials Provided

- Four 96-well microtiter® trays with dried primers in each reaction well
- 24 tubes D-mix (180 µl each)
- 6 tray seals

B. Materials Required, But Not Provided

- Pipetting devices, such as: Gilson® P-20, Gilson® P200 Pipetman®
- Disposable pipette tips
- Vortex mixer
- Microcentrifuge
- PCR tray microtube storage rack and cover (Robbins Scientific, Cat. #1044-39-5)
- Pressure pad (OLI Cat. #SSPPAD) *Note: The pressure pad is good for a maximum of 300 PCR runs. Please order a new pad if the surface of the pad that contacts the tray seal is no longer smooth.*
- for use with tray seal

- 96-Well GeneAmp® PCR System 9600, 9700 or Veriti™ 96-Well Thermal Cycler (Applied Biosystems). Set ramp speed to 9600 for 96-Well GeneAmp® PCR System 9700. Set ramp speed to 9600 Emulation Mode for Veriti™ 96-Well Thermal Cycler.
- Hot plate or microwave oven for heating agarose solutions
- Electrophoresis apparatus/power supply (150V minimum capacity)
- UV transilluminator (Example: Fotodyne FOTO/UV®21)
- Photographic or image documentation system

Recombinant Taq polymerase (Perkin-Elmer AmpliTaq® DNA Polymerase—5 units/μl)

- C. Step-by-step procedure.
See “Directions For Use.”

DIRECTIONS FOR USE

A. Sample Preparation

1. Purify genomic DNA from leukocyte sample by method of choice. Final DNA concentration should be 25 - 200ng/μl (100ng/μl is optimal) with the A260/A280 ratio between 1.65-1.80.
2. For specific information on sample preparation and storage, see **Specimen Collection and Preparation** above.
3. Perform PCR on the purified DNA sample using Cytokine Genotyping Tray, or store DNA sample at -20°C or below until ready to type.

B. Reagent/Equipment Preparation

4. Program a thermal cycler to run the One Lambda PCR program. (See “Instrument Requirements” above.)
 5. Have available: recombinant Taq polymerase (5 units/μl). Store at -20°C.
 6. Prepare electrophoresis gel (2.5% agarose) using the Gel System (OLI Cat. #MGS108) or with at least 96 sample wells (space rows of wells at least 1 cm apart).
- C. **Instructions for Pipetting Taq Polymerase:** To avoid waste, please follow the simple instructions listed below for pipetting Taq polymerase.

Note: Taq polymerase is very viscous and special care must be taken in the aliquoting process. Failure to follow the steps described below may result in reagent loss.

1. Pipette slowly, using a calibrated Gilson® Pipetman®. (A P10 Pipetman® is recommended for increased accuracy.)
2. The pipette tip should just pass the surface layer of the liquid.

Warning: Do not immerse the pipette tip in the liquid.

3. Carefully wipe excess liquid from pipette tip on the rim of the vial.

D. Stepwise Procedure

1. Remove from the indicated storage temperature: the volume (tube) of the D-Mix for the Cytokine Genotyping primer set tray, the primer set tray(s), and the appropriate number of DNA samples. Thaw at room temperature (20 - 25°C).

Note: You may cut tray into the number of tests needed for a single work session. Immediately return the un-used portion of tray to the appropriate storage temperature.

•Vortex DNA samples to mix.

•Place the primer set tray in a PCR Tray Microtube Storage Rack (Robbins Scientific, Cat. #1044-39-5) and remove the tray label.

2. Remove recombinant Taq polymerase from -20°C, and keep on ice until ready to use.
3. Using a Pipetman® (or equivalent), add 1 μl of DNA diluent to the negative control reaction tube on the primer set tray.
- E. Using a Pipetman®, add recombinant Taq polymerase (5 units/μl) to the D-mix tube. (See chart at the beginning of this document for amount.)
4. Cap tube and vortex for 5 seconds. Pulse-spin the D-mix tube in a microcentrifuge to bring all liquid down from sides of the tube.
5. Using a P20 Pipetman®, pipette 9 μl of the D-mix to the negative control reaction tube.
6. Using a Pipetman®, add the DNA sample to the D-mix tube. (See chart at the beginning of this document for amount.)
7. Cap tube and vortex for 5 seconds. Pulse-spin the D-mix tube in a microcentrifuge.
8. Using a P20 Pipetman® or an electronic Pipetman®, aliquot 10 μl of the sample-reaction mixture from the D-mix tube into each reaction tube, **except the negative control reaction tube**, of the primer set tray.

Important: Be sure to apply the sample above the primers (dried at the bottom of each reaction tube) to avoid cross-contamination between tubes. Touch the inside wall of the tube with the pipette tip to allow the sample to slide down to the bottom of the tube. Check that all samples have dropped to the bottom of each tube. If not, tap the tray gently on the bench top so that all samples settle at the bottom of the tube before you begin PCR.

9. Cover the reaction tubes with the tray seal provided. Check that all reaction tubes are completely covered by the tray seal to prevent evaporative loss during PCR.
10. Place the primer set tray in the thermal cycler.
11. Place a pressure pad on top of the tray before closing the thermal cycler.

LIMITATIONS OF THE PROCEDURE

1. PCR-SSP is a dynamic process requiring highly controlled conditions to ensure discriminatory amplification. The procedures provided in this product must be strictly followed.
2. The extracted sample DNA provides the template for the specific amplification process, and, thus, must have its concentration and purity within the ranges specified in the procedure.
3. All instruments (e.g., PCR thermal cycler, pipetting devices) must be calibrated according to the manufacturer's recommendations.
4. This assay does not diagnose any clinical condition nor level of risk. It detects only genotypes of selected cytokine genes.

EXPECTED VALUES

Gel Interpretation*

	Positive Reaction	Negative Reaction	Non- amplification
Well			
Internal Control Band			
Positive Typing Band			
Primer Band			

*The internal control band and broad unincorporated primer band serve as size markers. Any visible band between the two size markers should be considered positive typing bands.

SPECIFIC PERFORMANCE CHARACTERISTICS

Not applicable; product for research use only.

BIBLIOGRAPHY

1. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene. *Eur J Immunogenetics* 1997; 24, 1-8.
2. Awad MR, Turner DM, Sinnott PJ, Hutchinson IV. Polymorphism in the TGF- β 1 gene. *Eur. J Immunogenetics* 1997; 24, 45.
3. Turner DM, Sankaran D, Grant SCD, Yonan N, Sinnott PJ, Dyer PA, Hutchinson IV. The effect of polymorphism in the TNF- α and IL-10 genes on heart transplant rejection. *Int J Heart Lung Transplant* 1997; 16, 108.
4. Turner DM, Grant SCD, Yonan N, Sheldon S, Dyer PA, Sinnott PJ, Hutchinson IV. Cytokine genotypes and heart transplant rejection. *Transplantation* 1997; 64, 776-778.
5. Sankaran D, Turner DM, Johnson RW, Dyer PA, Sinnott PJ, Hutchinson IV. Interleukin-10 and tumour necrosis factor alpha gene polymorphisms predict renal transplant outcome. *Eur J Immunogenetics* 1997, 24, 65.
6. Sankaran D, Ashraf S, Askderakis A, Roberts ISD, Short C, Dyer PA, Sinnott PJ, Hutchinson IV. Recipient tumour necrosis factor- α and interleukin 10 gene polymorphisms predict acute graft rejection following renal transplantation. *Trans. Immunol.* (in press)
7. Pravica V, Perrey C, Plevris J, Bathgate A, Hayes PC, Sinnott PJ, Hutchinson IV. Elevated frequency of TNF- α -308 A allele in patients requiring liver transplantation. *Immunology* 1997; 92, Suppl 1, 13.3.
8. Awad MR, El-Gamel A, Simm E, Hasleton P, Yonan N, Deiraniya AK, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-beta 1 gene: association with TGF- β production, fibrotic lung disease and graft fibrosis after lung transplantation. *Transplantation* 1998. 66(8):1014-20.
9. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo, P. The Effect of Novel Polymorphisms in the Interleukin-6 (IL-6) Gene on IL-6 Transcription and Plasma IL-6 Levels, and an Association with System-Onset Juvenile Chronic Arthritis. *J. Clin. Invest.* 1998. 102:1369-1376.
10. Hutchinson, IV et al. Genetic Regulation of Cytokine Synthesis: Consequences for Acute and Chronic Organ Allograft Rejection. *Graft* 1998. 1, 15-21.

TRADEMARKS USED IN THIS DOCUMENT

®FMC SeaKem is a registered trademark of FMC Corporation.

®Fotodyne FOTO/UV 21 is a registered trademark of Fotodyne Incorporated.®GeneAmp is a registered trademark of Applied Biosystems.

®Gilson and Pipetman are registered trademarks of Rainin Instrument Co., Inc.

™ARMS is a trademark of Zeneca Limited.

™Veriti is a trademark of Applied Biosystems.

REVISION HISTORY

Revision	Date	Revision Description
4	2005/02	Clarify intended use; update references and template.
5	2009/02	Added the Veriti™ Thermal Cycler in the Instrument Requirements Section; Separated "Thermal Cycler" into two words.
6	2009/12	A typographical error was corrected in the Summary and Explanation Section; a redundant statement was also removed from the Summary and Explanation Section.

APPENDIX 13

Copyright license

WOLTERS KLUWER HEALTH, INC. LICENSE TERMS AND CONDITIONS

Mar 30, 2015

This Agreement between muzamil o hassan ("You") and Wolters Kluwer Health, Inc. ("Wolters Kluwer Health, Inc.") consists of your license details and the terms and conditions provided by Wolters Kluwer Health, Inc. and Copyright Clearance Center.

License Number	3576350976860
License date	Feb 26, 2015
Licensed Content Publisher	Wolters Kluwer Health
Licensed Content Publication	Circulation
Licensed Content Title	Atherosclerotic Vascular Disease Conference: Writing Group III: Pathophysiology
Licensed Content Author	David P. Faxon, Valentin Fuster, Peter Libby, Joshua A. Beckman, William R. Hiatt, Robert W. Thompson, James N. Topper, Brian H. Annex, John H. Rundback, Rosalind P. Fabunmi, Rose Marie Robertson, Joseph Loscalzo
Licensed Content Date	Jun 1, 2004
Licensed Content Volume Number	109
Licensed Content Issue Number	21
Type of Use	Dissertation/Thesis
Requestor type	Individual
Portion	Figures/table/illustration
Number of figures/tables/illustrations	1
Figures/tables/illustrations used	Figure 1
Author of this Wolters Kluwer article	No
Title of your thesis / dissertation	The role of circulating endotoxaemia as a proinflammatory mediator of atherosclerosis in chronic kidney disease patients
Expected completion date	Mar 2015
Estimated size(pages)	250
Requestor Location	muzamil o hassan renal unit department of medicine, oauthc ile-ife, Nigeria +234

WOLTERS KLUWER HEALTH, INC. LICENSE TERMS AND CONDITIONS

Mar 30, 2015

This Agreement between muzamil o hassan ("You") and Wolters Kluwer Health, Inc. ("Wolters Kluwer Health, Inc.") consists of your license details and the terms and conditions provided by Wolters Kluwer Health, Inc. and Copyright Clearance Center.

License Number	3576341351250
License date	Feb 26, 2015
Licensed Content Publisher	Wolters Kluwer Health
Licensed Content Publication	ATVB
Licensed Content Title	Potential Role of Endotoxin as a Proinflammatory Mediator of Atherosclerosis
Licensed Content Author	Lynn L. Stoll, Gerene M. Denning, Neal L. Weintraub
Licensed Content Date	Oct 7, 2004
Licensed Content Volume Number	24
Licensed Content Issue Number	12
Type of Use	Dissertation/Thesis
Requestor type	Individual
Portion	Figures/table/illustration
Number of figures/tables/illustrations	1
Figures/tables/illustrations used	Proposed model of proatherogenic interactions between endotoxin (LPS) and vascular cells
Author of this Wolters Kluwer article	No
Title of your thesis / dissertation	The role of circulating endotoxaemia as a proinflammatory mediator of atherosclerosis in chronic kidney disease patients
Expected completion date	Mar 2015
Estimated size(pages)	250
Requestor Location	muzamil o hassan renal unit department of medicine, oauthc ile-ife, Nigeria +234 Attn: muzamil o hassan
Billing Type	Invoice
Billing Address	muzamil o hassan renal unit department of medicine, oauthc ile-ife, Nigeria +234

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Mar 30, 2015

This Agreement between muzamil o hassan ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3576350785361
License date	Feb 26, 2015
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Preventive Cardiology
Licensed Content Title	Subclinical Atherosclerosis: Evolving Role of Carotid Intima-Media Thickness
Licensed Content Author	Farouk Mookadam, Sherif E. Moustafa, Steven J. Lester, Tahlil Warsame
Licensed Content Date	Apr 29, 2010
Pages	12
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 1
Will you be translating?	No
Title of your thesis / dissertation	The role of circulating endotoxaemia as a proinflammatory mediator of atherosclerosis in chronic kidney disease patients
Expected completion date	Mar 2015
Expected size (number of pages)	250
Requestor Location	muzamil o hassan renal unit department of medicine, oauthc ile-ife, Nigeria +234 Attn: muzamil o hassan
Billing Type	Invoice
Billing Address	muzamil o hassan renal unit department of medicine, oauthc ile-ife, Nigeria +234