ASSOCIATION OF MICROALBUMINURIA, SERUM LIPIDS AND INFLAMMATORY MARKERS IN A RURAL BLACK POPULATION IN THE LIMPOPO PROVINCE

By

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DISSERTATION

Submitted in fulfillment of the requirements for the degree of

MASTER SCIENCE (MSc)

In

MEDICAL SCIENCES

In the

DEPARTMENT OF PATHOLOGY AND MEDICAL SCIENCE,

FACULTY OF HEALTH SCIENCES

(SCHOOL OF HEALTH CARE SCIENCES)

At

UNIVERSITY OF LIMPOPO

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2018

DEDICATION

I dedicate this study to the following:

- My grandparents: **Lesiba Elias and Semoko Fransina Mahwai.**
- My uncles: **Alfred, Christopher, Noah and Charles**
- My aunts: **Linkie and Violet**.
- My parents: **Julia and Johannes**.
- The wonderful ladies in my life: **Hunadi, Mpho, Rorisang, Busisiwe and Tshitiso**.

DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Medical Sciences, Chemical Pathology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Magwai T (Mr)	11 April 2018
Surname, Initials (tittle)	Date

ACKNOWLEDGEMENTS

I would like to acknowledge the following:

- **GOD,** for leading me throughout.
- **Dr Perpetua (SEP) Modjadji,** my supervisor and **Prof Marianne Alberts** my co-supervisors, for their support, patience, wisdom and good supervision.
- **Mr. Solomon SR Choma** for assistance he afforded me during laboratory analysis and statistical analysis.
- **Dr Matshane L Maseloma** for the assistance in my laboratory work and throughout my study.
- My grandparents: **Lesiba Elias and Semoko Fransina Mahwai** for raising me.
- My uncles: **Alfred, Christopher, Noah and Charles** and aunts: **Linkie and Violet** for the support they gave me.
- My mother: **Julia Maphuti Magwai** for her love and support.
- My little sisters: **Mpho, Rorisang, Busisiwe and Tshitiso** for their love.
- **Hunadi Shibe Aphane** for the support, love and understanding.
- **VLIR:** for financial support for the study
- The participants of **Dikgale Health and Demographic Surveillance Site** for their participation in this study

ABSTRACT

Background:

Microalbuminuria (MA) is considered to be a strong and independent risk factor for cardiovascular disease (CVD), chronic kidney disease (CKD) and end-stage renal disease (ESRD). Cross sectional studies have indicated that microalbuminuria is also associated with cardiovascular risk factors such as dyslipidaemia and low grade inflammation. Hence, the aim of this study was to investigate the association of microalbuminuria with serum lipids [Total cholesterol (TC), Triglycerides (TG), High Density Lipoproteins Cholesterol (HDL-C), Low Density Lipoproteins Cholesterol (LDL-C), Lipoprotein a (Lp (a)] and inflammatory markers [C-reactive protein (CRP) and Interleukin-6 (IL-6)] in a rural black population.

Methods:

This is a cross-sectional study conducted in Dikgale Health and Demographic Surveillance System (HDSS) site and quantitative methods were used. The present study is part of a study titled "Prevention, control and integrated management of chronic diseases in a rural area, South Africa" conducted in the Department of Medical Sciences, University of Limpopo. In the above study blood samples were collected from 816 people aged 15 years and above. For the present study participants with HIV, macroalbuminuria, creatinine ≥170 μmol/land diabetes mellitus were excluded from the 816 people. Six hundred and two (602) participants fitted the inclusion criteria of the present study. Of the 602 participants 255 were men and 377 were women. From these participants, creatinine and albumin concentrations were measured in a morning spot urine sample and the albumin/creatinine ratio (ACR) was calculated. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using OMRON M5-I. Serum lipids (TC, TG, HDL-C, and LDL-C) and glucose were determined using ILAB 300 plus. Lp (a) and hs-CRP were determined using IMMAGE 800 Immunochemistry System. Insulin and IL-6 were determined using ACCESS 2 Chemistry System. Data was analysed using SPSS version 22.0. Statistical tests used included Student T-test, ANCOVA, ANOVA, linear regression and logistic regression.

Results:

The levels of serum lipids and inflammatory markers in this study were similar in participants with and without microalbuminuria. In a linear regression model TG was the only lipid

parameter found to be associated with microalbuminuria (p = 0.018). Inflammatory markers were not associated with microalbuminuria. In a logistic regression model CRP and HDL-C showed negative association with microalbuminuria in men while in women no association was found. However men with a high CRP and a high TG were found to be more likely to have microalbuminuria (p = 0.007).

Conclusion:

A linear positive association was observed between microalbuminuria and TG in men and in women. The OR of having microalbuminuria was lower in participants with a high CRP, low HDL-C or in women with a high glucose. Women with a low HDL-C had higher OR of having MA and men with a high CRP and a high TG were found to be more likely to have microalbuminuria.

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RESEARCH PAPER PRESENTATIONS:

ORAL PRESENTATION, Award: Best postgraduate presentation, 2nd Prize **Research day (Sep 2014), School of Health Sciences, University of Limpopo** Title: Association between microalbuminuria and serum lipids in a rural black population. **Magwai T**, Modjadji S.E.P, Alberts M and Choma S.S.R

TERMINOLOGY

Allele	Any alternative form of a gene that can occupy a particular chromosomal locus
Atherogenic	Conductive to or causing atherogenesis
Atherogenesis	The formation of atheromatous lesions in the arterial intima
Chemiluminescent	Luminescence produced by direct transformation of chemical energy into light energy
Chromosome	A structure in the nucleus of an animal cell containing a linear thread of DNA, which transmits genetic information and is associated with RNA and histones
Diabetes	A general term referring to disorders characterised by excessive urine excretion (polyuria), as in diabetes mellitus and diabetes insipidus
Dyslipidaemia	Presence of an abnormal concentration of lipids in circulating blood
Essential hypertension	Hypertension occurring without discoverable organic cause
Glomerulonephritis	Nephritis accompanied by inflammation of the capillary loops in the glomeruli of the kidney
Glycation	The non-enzymatic reaction of a sugar molecule with a protein or lipid
Glycerophosphate	Any salt of glycerophosphoric acid

Glycocalyx	The glycoprotein and polysaccharide covering that surrounds many cells
Hyperfiltration	An elevation in glomerular filtration rate, often as a sign of early insulin-dependent diabetes mellitus
Hypertension	Persistently high arterial blood pressure
Hypertrophy	Enlargement or overgrowth of an organ or part due to an increase in size of its constituent cells
Inflammation	A localized protective response elicited by injury or destruction of tissue, which serves to destroy, dilute or wall off both the injurious agent and injured tissue
Luminometer	A sensitive photometer used for measuring very low light levels (as those produced in a luminescent process)
Mesangial	Pertaining to the mesangium
Mesangium	The thin membrane which helps to support the capillary loops in a renal glomeruli
Metabolism	The sum of all the physical and chemical process by which living organisms. substances is produced and maintained and also the transformation by which energy is available for the uses of the organism
Morbidity	A diseased condition or state, the incidence or prevalence of a disease in a population
Mortality	State of being liable to die

Obesity	An increase in body weight beyond the limitation of skeletal and physical requirement, as a result of an excessive accumulation of fat in the body
Pentraxin	An evolutionary conserved family of proteins characterised by containing a pentraxin protein domain
Podocyte	A modified epithelial cell of the visceral layer of the renal glomerulus, having a small perikaryon and a number of primary and secondary foot like radiating process which interdigitate with those of other podocytes
Polymorphism	The quality or character of occurring in several different forms
Proliferation	The reproduction or multiplication of similar forms, especially of cells and morbid cysts
Quinoneimine	Group of dyes that are derivatives of para-quinone imine or para-quinonediimine, in which the hydrogen atom of an imine group is replaced by an aromatic residue containing an NH_2 or OH^- group

ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACR	Albumin creatinine ratio
Ang II	Angiotensin two
ANCOVA	Analysis of covariance
ANOVA	One-way analysis of variance
Apo	Apo lipoprotein
BH4	Tetrahydrobiopterin
BMI	Body mass index
CARDIA study	Coronary Artery Risk Development in Young Adults study
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CKD	Chronic kidney disease
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DCCT/EDIC	Diabetes Control and Complications Trail/Epidemiology of Diabetes Interventions and Complications
EDTA	Ethylene diamine tetra-acetic acid
EPIC-Norfolk	European Prospective Investigation into Cancer in Norfolk
eGFR	Estimated glomerular filtration rate

ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESRD	End stage renal disease
ET-1	Endothelin 1
GFR	Glomerular filtration rate
HDL	High density lipoprotein
HDSS	Health and Demographic Surveillance System
HDL-C	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HL	Hepatic lipase
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
Hs-CRP	High sensitivity C reactive protein
ICAM-1	Intercellular adhesion molecule-1
IDL	Intermediate density lipoprotein
IL-4	Interleukin 4
IL-10	Interleukin 10
IL-6	Interleukin 6
LCAT	Lecithin: Cholesterol Acyltransferase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
Lp (a)	Lipoprotein (a)

LPL	Lipoprotein lipase
LRP	Lipoprotein receptor-related protein
MA	Microalbuminuria
MREC	Medunsa Research Ethics Committee
NHANES	National Health and Nutrition Examination Survey
nm	Nanometre
NO	Nitric oxide
pg	Pico gram
RAAS	Renin-angiotensin-aldosterone system
RPM	Rotations per minute
SBP	Systolic blood pressure
SD	Standard deviation
SPSS	Statistical package for social sciences
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tissue necrosis factor alpha
UAE	Urinary albumin excretion
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
WHO	World Health Organisation

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CHAPTER ONE

1. INTRODUCTION

Cardiovascular diseases (CVDs) are the main non-communicable diseases (NCD) and are among the leading public health burdens worldwide (Lloyd-Jones et al., 2010). In Africa, CVDs are reported to account for 9.2 % of deaths in populations (Kadiri, 2005, Livesay, 2007, World Health, 2011). Approximately 195 people in South Africa died per day between 1997 and 2004 due to CVDs (Steyn and Fourie, 2007). In rural South African populations, the transition to an urban lifestyle contributes to the CVD epidemic (Kimani-Murage et al., 2010). Among cardiovascular risk factors are microalbuminuria (MA), abnormal serum lipids and low grade inflammation (Ibsen et al., 2005, Ridker, 2003, Yusuf et al., 2004).

Microalbuminuria (MA), defined as the appearance of small quantities of albumin in urine (Levey et al., 2003), is associated with chronic kidney disease (CKD) and end-stage renal disease (ESRD) (Hemmelgarn et al., 2010, James et al., 2010) and with cardiovascular disease (CVD) in diabetes, hypertension and also in seemingly healthy populations (Ärnlöv et al., 2005, de Zeeuw et al., 2006, Parving et al., 2006).

Microalbuminuria was found to be associated with increased levels of C reactive protein (CRP) (Sabanayagam et al., 2010, Xu et al., 2014) and IL-6 (Moubarak et al., 2012, Yu et al., 2010).However other studies have reported contradicting results wherein microalbuminuria was not associated with CRP (Palmieri et al., 2003, Perticone et al., 2007) and IL-6 (Ng et al., 2008, Zahran et al., 2012). The discrepancy in the results may be due to the difference in the method classification of MA or the study approach (Perticone et al., 2007).Several studies did not control for the effects of confounders like dyslipidaemia (Ridker, 2003) as dyslipidaemia is also a risk factor for CVD just like microalbuminuria (Ibsen et al., 2005) and low grade inflammation (Yusuf et al., 2004).

Microalbuminuria was found to be positively associated with serum total cholesterol (TC), triglycerides (TG) and low density lipoprotein cholesterol (LDL-C) and negatively associated with high density lipoprotein cholesterol (HDL-C) in hypertensive patients (Busari et al., 2010), diabetic patients (Retnakaran et al., 2006) and non-diabetic normotensive patients (Kim et al., 2001). However, contradicting results have been reported were no significant association was found between microalbuminuria and serum lipids (Muttur et al., 2010, Sheng et al., 2011).

This might be due to differences in sample size, method of diagnosing microalbuminuria (Sheng et al., 2011) or controlling for confounding like low grade inflammation. High levels of lipoprotein (a) [Lp (a)] have been reported in patients with MA (Siekmeier et al., 2008). In South Africa, the prevalence of hypertension has been reported to be 26.9% (Rayner and Becker, 2006).

To the best of our knowledge little information is available on inflammatory markers and serum lipids in South African black rural subjects with microalbuminuria. In addition, most studies on microalbuminuria, worldwide, focused on MA and inflammatory markers or on MA and serum lipids separately. As a result, there is a paucity of data on the association among these three cardiovascular risk factors. Hence, the present study, to determine the association of microalbuminuria, inflammatory markers and serum lipids in a black rural population.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Cardiovascular disease burden

World health Organization (WHO) has reported non communicable diseases (NCDs) to cause most deaths across the globe (Lloyd-Jones et al., 2010). In 2008, approximately 36 million deaths were reported to be due to NCD's (World Health, 2008). According to the estimates of the burden of diseases in South Africa by the WHO, 28 % of the total disease burden measured by disability adjusted life years in 2004 is due to non-communicable diseases (World Health, 2008). The most common NCDs are cardiovascular disease (CVDs), diabetes, cancer and chronic respiratory diseases (Al-Mawali, 2015). Cardiovascular disease (CVD) encompasses diseases affecting the heart or blood vessels, including heart failure and stroke (Al-Mawali, 2015, Manson and Bassuk, 2015).

Cardiovascular diseases (CVD) are the main non-communicable diseases (NCD), are the leading public health burden worldwide and account for 9.2 % of death in the African continent (Kadiri, 2005, Livesay, 2007, World Health, 2011). Cardiovascular diseases have been found to be the leading cause of death in people aged 45 years and above (Gaziano, 2008). It has been estimated that by the year 2020 approximately 40 % of all deaths worldwide will be due to cardiovascular diseases (Willerson and Ridker, 2004). Approximately 195people in South Africa died per day between 1997 and 2004 due to CVDs (Steyn and Fourie, 2007). Even in rural South African populations the transition to an urban lifestyle contributes to the CVD epidemic (Kimani-Murage et al., 2010).

It has been reported that microalbuminuria (Ibsen et al., 2005, Volpe et al., 2003), dyslipidaemia (Ridker, 2003) and low grade inflammation (Yusuf et al., 2004) are some of the cardiovascular risk factors and thus are thought to be associated with each other. Microalbuminuria (MA) has been associated with cardiovascular disease (CVD) in diabetes, hypertension and also in seemingly healthy populations (Ärnlöv et al., 2005, de Zeeuw et al., 2006, Parving et al., 2006). Furthermore microalbuminuria was found to be an independent predictor of all-cause mortality, cardiovascular mortality and cardiovascular morbidity in diabetic, hypertensive and in general populations (Bakker et al., 2005, Romundstad et al., 2003, Wachtell et al., 2003). Studies have reported that patients with both MA and high hs-CRP have a lower HDL-C, a greater BMI and active smoking than their counterparts but non-significant

difference in TG, LDL-C and TC between the two groups (Eliasson, 2003, Pedrinelli et al., 2004).

2.2. Microalbuminuria

Microalbuminuria (MA) is defined as the appearance of small quantities of albumin in urine (Levey et al., 2005). Microalbuminuria is a condition where the urinary excretion of albumin is above the normal range but below levels that can be detected clinically by standard screening test like the urine dipstick test (Incerti et al., 2005, Kilaru and Bakris, 1994). Microalbuminuria was first described in patients with diabetes mellitus in 1969 by Keen and colleagues (Keen et al., 1969, Keen et al., 2014). Later, Parving and colleagues (Parving et al., 1974) reported an association between microalbuminuria and essential hypertension in non-diabetic patients. Microalbuminuria is an early marker of chronic kidney disease (Diercks et al., 2002, Sarnak et al., 2003) and is associated with an increased likelihood of progression of chronic kidney disease (CKD) to more advanced stages or even to end-stage renal disease (ESRD) in large epidemiologic studies (Hemmelgarn et al., 2010, James et al., 2010). Studies have demonstrated microalbuminuria as an important and independent predictor of cardiovascular diseases (Ibsen et al., 2005, Volpe et al., 2003). Ridker, (Ridker, 2003) reported an association between low grade inflammation and increased risk for cardiovascular diseases. Dyslipidaemia is also recognised as a cardiovascular risk factor (Yusuf et al., 2004). Microalbuminuria, serum lipids and inflammatory markers are cardiovascular risk factors and are therefore thought to be associated.

Microalbuminuria can be diagnosed from a 24-hour urine sample with a urinary albumin excretion of 30–300 mg/24 hours (Abid et al., 2001, Mogensen, 1984) but compliance with the method is difficult and the method is time consuming (Pathania et al., 2013). Microalbuminuria can also be diagnosed using a random spot urine sample (Abid et al., 2001) to determine the urinary albumin creatinine ratio (ACR). An ACR of 2.5–25 mg/mmol in males and 3.5–35 mg/mmol in females indicate microalbuminuria (Chadban et al., 2010) (see table 2.1). The measurement of microalbuminuria by ACR takes into consideration the differences in muscle mass between females and males, as males have higher muscle mass and hence higher creatinine excretion than females (Mattix et al., 2002). The use of urinary albumin: creatinine ratio (ACR) for diagnosis of microalbuminuria has been supported by several studies (Brantsma et al., 2007, Gansevoort et al., 2006, Jafar et al., 2007).

Some urine dipstick tests such as Clinitek Micro-albumin and Chem-strip Micral-Test are available to detect small amounts of albumin but these strips may not provide precise quantitative measurements of albumin levels (Sarafidis et al., 2008). The tests are intended for screening as they are semi-quantitative (Toto, 2004). The levels of albumin in urine of microalbuminuria patients are too low to be detected by standard methods thus requiring sensitive methods (Incerti et al., 2005, Kilaru and Bakris, 1994). Urinary Albumin can be measured using commercially available immune-based techniques like immunoturbidimetry, immunonephelometry and enzyme-linked immunosorbent assay (ELISA) (Bianchi et al., 1999).

Table 2.1: Definition of microalbuminuria

Timed urine collection			Spot morning urine specimen			
Albuminuria level	24-hour albumin excretion (mg/day)	Overnight albumin excretion (µg/min)	UAC (mg/L)	Albumin-to-creatinine ratio		
				Gender	Mg/mmol	Mg/g
Normal	<30	<20	<20	Male	<2.5	<20
				Female	<3.5	<30
Microalbuminuria	30 – 300	20 – 200	20 – 200	Male	2.5 – 25	20 – 200
				Female	3.5 – 35	30 – 200
Gross proteinuria	>300	>200	>200	Male	>25	>200
				Female	>35	>200

UAC- urinary albumin concentration. Adopted from (Yuyun et al., 2005)

2.3. Prevalence of microalbuminuria

An international observational cross sectional study on 22282 patients, a sub-analysis of a survey of 26 countries worldwide assessed the urine microalbuminuria using a dipstick test reported a worldwide microalbuminuria prevalence of 58.3 %, with 51.7 % being newly diagnosed and 6.71 % being the know patients (Habbal et al., 2010). A higher prevalence of MA has been associated with older age, female gender, and non-Hispanic black ethnicity among U.S. adults (Coresh et al., 2005). The third National Health and Nutrition Examination Survey (NHANES) in the United States estimated that 6% of men and 10% of women had microalbuminuria (Jones et al., 2002). The NHANES also reported the prevalence of microalbuminuria to be 16% in hypertensive subjects, 28% in diabetes subjects and 5.1% in patients without diabetes, hypertension, CVD or increased serum creatinine (Jones et al., 2002). A cross sectional study on the National Health and Nutrition Examination Survey (NHANES 1988-1994 and NHANES 1999-2004) in the United States on a nationally representative sample of non-institutionalized adults aged 20 years and older reported the MA prevalence of 7.1% and 8.2% in the 1988 to 1994 and 1999 to 2004 periods respectively (Coresh et al., 2007). The European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study on 20 911 individuals aged between 40-70 years recruited between 1993 and 1997 showed that the prevalence of MA in the British population is 11.8% (Yuyun et al., 2004). The overall prevalence of MA in the adult Galician population was found to be 4.7%, 14.9% in diabetic patients, 8.1% in hypertensive patients and 19.4% in patients with hypertension and diabetes (Tome et al., 2012).

A study in the general population aged between 40 and 87 years in Takahata, Japan with diabetes and/or hypertension and a mean age of 60 years reported a 13.7% prevalence of microalbuminuria (Hao et al., 2007).The prevalence of MA was found to be 6.7% in a Chinese population with a mean age of 50 years living in the urban area of Shanghai (Li et al., 2004) and 8.8% in a Chinese population with a mean age of 44 years living in a city south of Shanghai (Chen et al., 2010). A study in Taiwan reported a prevalence of MA of 11.5% in a population with a mean age of 57 years(Lin et al., 2007). A study on 288 Type 2 diabetes patients with a mean age of 53.2 ± 9.9 years and mean duration of diabetes of 9.3 ± 6.3 years, attending the Yadz diabetes research centre, Iran, reported an overall MA prevalence of 14.2 % (Afkhami-Ardekani et al., 2008). A cross-sectional study carried out to assess the prevalence of diabetes mellitus complications in Al-Ain district, United Arab Emirates, reported a high prevalence of

microalbuminuria (60 %) in diabetic patients in the UAE (Al-Maskari et al., 2008). The prevalence of MA in Type 1 diabetic patients with mean disease duration of 3 years attending a diabetic clinic in Dar er Salaam, Tanzania, was found to be 12.1% from a cross sectional analysis of 244 patients with diabetes (Lutale et al., 2007). A prospective, cross sectional study of diabetic patients attending an outpatient diabetic clinic in Kumasi, Ghana, reported a microalbuminuria prevalence of 43% in diabetic patients (Eghan et al., 2007).

A study on 926 hypertensive and/or diabetic patients aged 35 years in the Park Health Centre area of Ankara, Turkey, reported a MA prevalence of 17.4% in hypertensive patients, 20.4% in diabetic patients and 22.1% in patients with both diabetes and hypertension (Col et al., 2004). A study on a sample representative of the general population of the Seychelles aged between 25 and 64 years, using a semi quantitative point of care analyser for MA determination and adjusting for urine creatinine reported an age-adjusted prevalence of MA of 11.4% (Pruijm et al., 2008). The study also reported that in the age group 25–64 years, the prevalence of MA was 5% in persons without diabetes and hypertension, 20% in persons with either condition and 41% in persons with both conditions (Pruijm et al., 2008).

In a hypertensive group aged 35 years and older the prevalence of MA in 334 participants without severe or malignant hypertension, diabetes or established renal disease Rayner and Becker, 2006). The prevalence of MA was 39.7% in diabetic patients attending the Diabetes Service at the Johannesburg Academic Hospital, South Africa (Kalk et al., 2010). A study in the United States of America reported that Black and Hispanic patients with hypertension were more likely to have both microalbuminuria and macroalbuminuria than Caucasians and Asians (Young et al., 2005). Reasons for the difference are socioeconomic status (Brancati et al., 1992), access to health care (Brancati et al., 1997, Brancati et al., 1992, Klag et al., 1997), glycaemic control (Harris et al., 1999), high prevalence of diabetes in minorities (Cowie and Harris, 1997, Harris, 2001, Harris et al., 1999), uncontrolled hypertension (Brancati et al., 1992) and possible biological and genetic differences (Young et al., 2005).

2.4. Endothelial dysfunction

The endothelium is a biological active inner layer of a blood vessel that serves as a site for vascular and organ function control (Durand and Gutterman, 2013, Verhamme and Hoylaerts, 2006). The endothelium senses different stimuli like mechanical and hormonal stimuli and in response it releases agents that regulate the vasomotor function and respond by releasing

vasodilatory mediators like nitric oxide (NO), prostacyclin, C type natriuretic peptide and vasoconstrictors like endothelin-1 (ET-1), angiotensin (Schiffrin, 2001, Verma and Anderson, 2002). The endothelial cells lining the endothelium release factors that are involved in the regulation of coagulation, platelet activation, vascular permeability and inflammation (Pober and Sessa, 2007). Structural and functional alteration of the endothelium result in endothelial dysfunction which is characterised by reduced bioavailability of vasodilators like NO and increased endothelial-derived contracting factors like endothelin-1 (Herrmann et al., 2008, Noguchi et al., 1997).

Endothelial dysfunction is a condition wherein the physiological property of the endothelium is lost and the endothelium shifts towards the pro-thrombotic, pro-inflammatory and vasoconstrictor state (Marasciulo et al., 2006, Mather et al., 2004). A reduction in the bioactivity of NO is the earliest and most important marker of endothelial dysfunction (Kielstein et al., 2003, Lüscher et al., 1992, Rubanyi and Vanhoutte, 1986).

Angiotensin II has been found to contribute to sclerosis of podocytes leading to excretion of large molecules like albumin (Matsusaka et al., 2010) by activation of angiotensin II type 1 receptors, which leads to increased production of inflammatory mediators like IL-6, generation of reactive oxygen species (ROS), induction of the receptors for oxidised low-density lipoprotein and of adhesion molecules, which mediate endothelial dysfunction locally in the kidney leading to microalbuminuria and systemically in the blood vessels leading to atherosclerosis, see figure 1 (Basi and Lewis, 2006, Warnholtz et al., 1999). Endothelial dysfunction may contribute to microalbuminuria by increasing glomerular pressure and membrane permeability or by influencing mesangial cell and podocytes function in a paracrine fashion (Deen et al., 2001, Haraldsson and Sörensson, 2004). Endothelial dysfunction was found to be associated with MA (Liu et al., 2014), dyslipidaemia (Engler et al., 2003) and obesity (Raitakari et al., 2004).

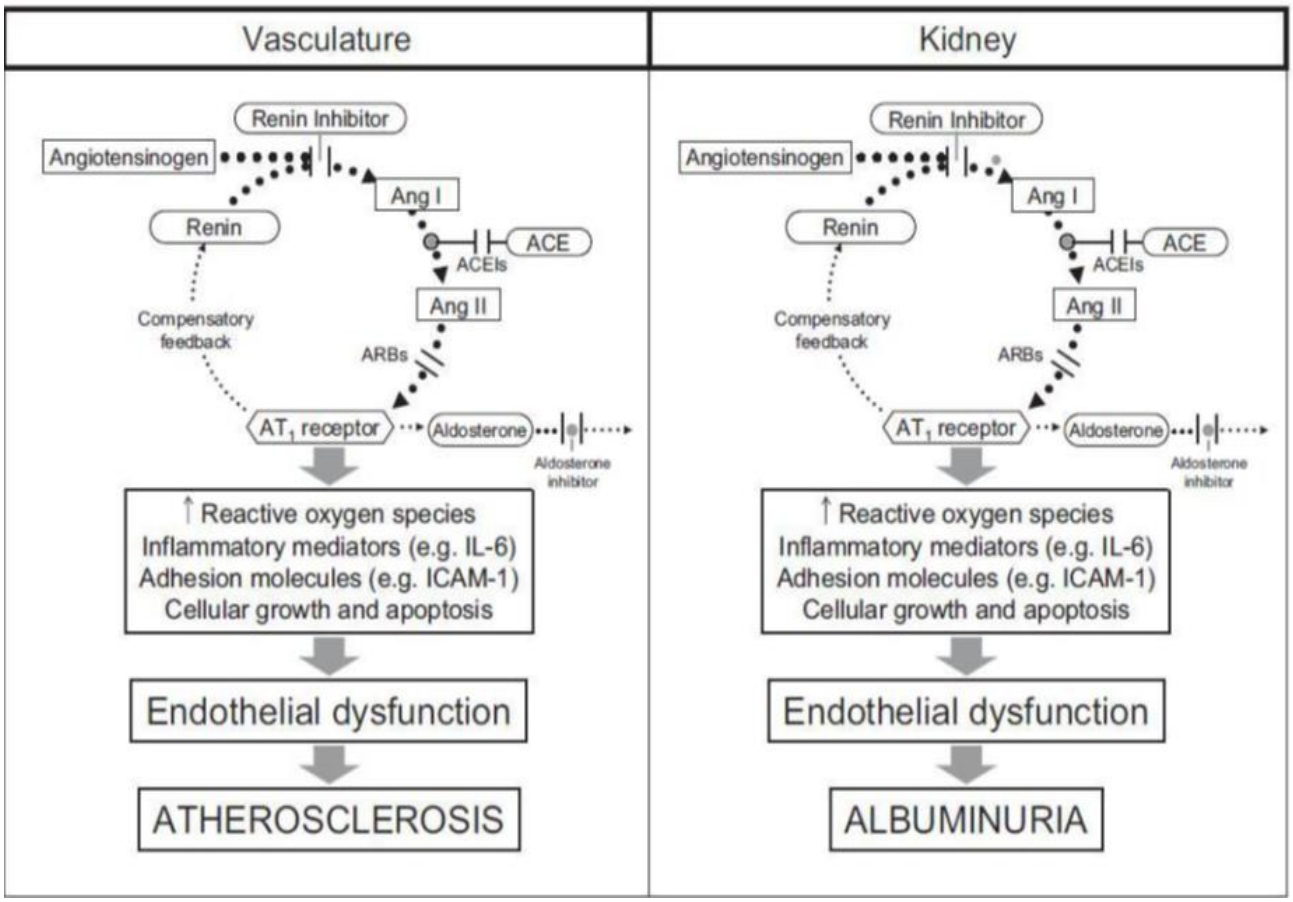

Figure 1: Simple illustration of mechanisms by which malfunction of RAAS could mediate albuminuria and atherosclerosis. Adapted from (Basi and Lewis, 2006)

2.4.1. Endothelin-1 and microalbuminuria

Endothelin-1 (ET-1) is the predominant isoform of the endothelin family (Hopfner and Gopalakrishnan, 1999). It is a potent vasoconstrictor peptide produced by endothelial and vascular smooth muscle cells, it is found in a variety of tissues and is thought to modulate vascular tone, cell proliferation and hormone production (Levey et al., 2003). Endothelin-1 is associated with severe intra-renal vasoconstriction, decreased glomerular infiltration rate, mesangial cell contraction and proliferation (Ferri et al., 1995, Marsen et al., 1994). Endothelin-1 was found to be independently associated with urinary albumin excretion (Zanatta et al., 2008).

2.5. Hypertension

Hypertension is defined as a blood pressure exceeding the 95th percentile for age and sex in adolescence while in adults it's a blood pressure of greater or equal to 140/90 mmHg

(Constantine et al., 2008). The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) created a new category of hypertension called ‘‘pre-hypertension” which is categorised by a blood pressure of 120⁄80 mm Hg to 139⁄89 mm Hg (Chobanian et al., 2003). Parving and colleagues (Parving et al., 1974) reported an association between microalbuminuria and essential hypertension in non-diabetic patients five years after Keen and colleagues (Keen et al., 1969) reported the first case of MA in diabetic patients. A study that was embedded into the SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) reported that arterial stiffness and ambulatory blood pressure are associated with low-grade albuminuria in non-diabetic African men with normal kidney function (Schutte et al., 2011). The study showed that in African men, night time systolic blood pressure, diastolic blood pressure and mean arterial pressure were positively correlated with ACR (Schutte et al., 2011). Hypertension has been proposed as one of the mechanism leading to the appearance of small quantities of albumin in urine and it is one of the extensively studied predictors of microalbuminuria (Moran et al., 2006).

Normally the constriction of afferent arterioles can protect the kidneys from the elevations in hydrostatic pressure that is caused by the hypertension (Ito et al., 1992). However, when hypertension become prolonged, it induces the impairment of the above mentioned auto regulatory process, thus causing hyper filtration and leakage of proteins like albumin in to urine (Mountokalakis, 1997). The hypothesis is supported by observation in an experimental animal study, where partial nephrectomy was found to lead to hyper filtration in the remaining nephrons in order to maintain the GFR, which in turn increases the intra-glomerular pressure and lead to the passage of protein into the urine (Metcalfe, 2007).

2.6. Insulin resistance

Insulin resistance is a condition in which a normal serum concentration fails to produce a normal metabolic response (Kahn, 1978, Sowers and Frohlich, 2004). Studies have reported that insulin resistance is a strong predictor in the development of MA (Hsu et al., 2011, Orchard et al., 2002). Hypertensive patients with MA were found to have high fasting insulin (Bianchi et al., 1994, Redon et al., 1997) and hyperinsulinemia was found to be related to MA in hypertensive patients (Redon et al., 1997). It has been suggested that insulin resistance proceeds and probably contribute to the development of MA (Orchard et al., 2002). The association between MA and insulin resistance is also supported by several clinical studies in

which reducing insulin resistance and hyperinsulinemia resulted in the reduction of urinary albumin excretion in patients with MA (Sarafidis, 2007, Sarafidis and Bakris, 2006).

2.7. Age

A cross-sectional hospital-based study conducted among adults with type 2 diabetes attending the Diabetes Clinic at the Kilimanjaro Christian Medical Centre (KCMC) between August 2010 and February 2011 where two or more positive results of albuminuria over six months' period was considered microalbuminuria reported a significant association between microalbuminuria and age (Ghosh et al., 2012). Other studies also have reported a significant association between age and microalbuminuria (Ruilope and Segura, 2006, Varghese et al., 2001). A cross sectional study conducted between 2005 and 2007on 300 type two diabetic patients attending the Yazd diabetic research centre with microalbuminuria diagnosed as a urine albumin to creatinine ration of between 30 and 300mg/g in two occasions over three months found no association between microalbuminuria and age (Afkhami-Ardekani et al., 2008). Another cross sectional study on 91 type 1 and 153 type 2 Muhimbili outpatient clinic at Tanzania where microalbuminuria was diagnosed as average albumin excretion rate of between 20 and 200 ug/min also found no significant association between MA and age (Lutale et al., 2007).

2.8. Obesity

Several studies reported no relationship between microalbuminuria and body mass index, waist circumference, waist hip ratio or visceral obesity (Hoffmann et al., 2001, Kim et al., 2001, Yesim et al., 2007). In studies that found an association between microalbuminuria and obesity the prevalence of microalbuminuria was found to be increased in obese participants as compared to the studies that found no association (Yesim et al., 2007). It has been suggested that microalbuminuria is associated with obesity in the presence of endothelial dysfunction and insulin resistance (Kim et al., 2001). Analysis from the Diabetes Control and Complications/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) diabetic population revealed that WHR, not body mass index (BMI), is associated with abnormal urinary albumin excretion (de Boer et al., 2007, Sibley et al., 2006) and the multinational observational study of hypertensive out-patients reported that obesity is associated with MA (Thoenes et al., 2009).

2.9. Genetic factors

Several studies have shown that a genetic background is a contributing factor in the development of MA in hypertensive patients (Chaves et al., 2001, Fernández-Llama et al., 1998, Yudkin, 1997). Microalbuminuria and hypertension were found to share some genetic determinants (Guo et al., 2005). The D allele of the polymorphism insertion/deletion of the angiotensin converting enzyme (ACE) gene has been reported to be associated with a high MA prevalence (Redon et al., 2000). Chaves and colleagues (Chaves et al., 2001) reported that the polymorphism in the 3' region of angiotensin 2 receptor type 1 (AT1) gene, 573 C >T, which is linked to 1166 A >C polymorphism, seemed to protect against blood pressure induced MA.

2.10. Low–grade inflammation

Low grade inflammation is defined as a 2- to 3- fold increase in plasma levels of cytokines and acute phase protein (Petersen and Pedersen, 2005). Low grade inflammation may underlie certain chronic disease like diabetes, dyslipidaemia, atherosclerosis and metabolic syndrome (Alexandraki et al., 2006, Iyer et al., 2010). Low grade inflammation has been reported to be associated with microalbuminuria and it is also thought to be a cause or consequence of endothelial dysfunction (Jager et al., 2000). Low grade inflammation is associated with an increase in the risk of developing cardiovascular disease (Ridker et al., 2003). In some studies IL-6 was found to cause the up regulation of the Ang II type 1 receptors and also the production of ROS due to Ang II (Klausen et al., 2005, Kuwahara et al., 2003, Mann, 2002). The release of renin due to inflammatory markers like IL-6 together with inflammation of the renal tubules and interstetium may be unfavourable to the glomerular haemodynamic response to renal dysfunction , thus causing an inflammatory reaction and the eventual development of MA (Ibsen et al., 2005, Luft, 2001, Szmitko et al., 2003).

Elevated inflammatory markers such as C- reactive protein (CRP), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) have been reported in subjects with microalbuminuria (Jager et al., 2002, Schram et al., 2005, Stehouwer et al., 2002). However, some studies reported that no significance difference was present in the levels of inflammatory markers such as CRP (Palmieri et al., 2003, Perticone et al., 2007) and IL-6 (Moriwaki et al., 2003) between subjects with and without MA. Markers of endothelial dysfunction such as endothelin-1 (ET-1), adhesion molecules [vascular cell adhesion molecule-1 (VCAM-1), endothelial-leukocyte adhesion molecule-1 (E-selectin), and intercellular adhesion molecule-1 (ICAM-1)] are

increased in subjects with microalbuminuria (Hocher et al., 2001, Lee et al., 1994, Nakamura et al., 2001, Sorokin and Kohan, 2003).

2.10.1. C reactive protein and microalbuminuria

C-reactive protein (CRP), an acute-phase reactant that belongs to the pentraxin family, is produced in the hepatocytes under the control of other inflammatory cytokines like interleukin-1 beta or interleukin-6 (Taylor et al., 1990). It is called C reactive protein because of its ability to precipitate the somatic C-polysaccharide of Streptococcus pneumonia (Pepys and Baltz, 1983). High sensitivity CRP allows for both detection of CRP levels within the normal range of <10 mg/L and mild elevations of CRP (Ridker et al., 1997, Ridker et al., 1998). The synthesis of CRP occurs primarily in the liver where it's produced by hepatocytes (Hurlimann et al., 1966). Extra hepatic synthesis of CRP is also possible in the neurons, atherosclerotic plaques, monocytes and lymphocytes (Jialal et al., 2004, Kuta and Baum, 1986). C reactive protein is produced in response to conditions such as inflammation, trauma and infection (Pepys and Hirschfield, 2003). Interleukin 6, interleukin 1, TNF-α and corticosteroid are the stimuli for the synthesis of CRP (Slade et al., 2003).

C-reactive protein is synthesized as part of the acute phase protein as a monomer and assembled in the endoplasmic reticulum to form a pentamer (Ganter et al., 1989, Yap et al., 1991). In the resting state CRP is retained in the endoplasmic reticulum by binding of carboxylesterases (Macintyre et al., 1994). Upon stimulated synthesis the binding in the endoplasmic reticulum weaken and CRP is released in to circulation (Macintyre et al., 1985). C- reactive protein (CRP) was found to promote production of pro-inflammatory cytokines thus leading to mesangial cell proliferation, matrix overproduction and increased vascular permeability causing MA (Pai et al., 2004, Verma et al., 2002).

An association has been reported between C-reactive protein (CRP) and MA in diabetic, non-diabetic (Festa et al., 2000, Marcovecchio et al., 2008, Xu et al., 2014), and hypertensive subjects (Assadi, 2008, Salles et al., 2007) and in the general population (Kshirsagar et al., 2008, Nakamura et al., 2004, Sabanayagam et al., 2010). The Oxford Regional Prospective Study found that in young type 1 diabetic patients, the CRP levels were raised after the development of microalbuminuria (Marcovecchio et al., 2008) and this may reflect a general state of inflammation. According to Feldt-Rasmussen, 2000 microalbuminuria is associated with subclinical inflammation and endothelial dysfunction (Feldt-Rasmussen, 2000). However,

in some studies CRP was found not to be associated with MA (Palmieri et al., 2003, Perticone et al., 2007) and it has been suggested that the association between CRP and MA may be due to a high blood pressure, since an association is seen in hypertensive subjects (Stuveling et al., 2004).

2.10.2. Tumour necrosis factor alpha (TNF-α) and microalbuminuria

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine produced mainly in macrophages and monocytes and is involved in systemic inflammation that induces a local inflammatory response by initiating a cascade of cytokines and increasing vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection (Luo et al., 2010). Tumour necrosis factor alpha (TNF-α) is usually not detected in an apparently healthy individual but increased levels are seen in inflammatory and infectious conditions (Robak et al., 1998). Some of the inhibitors of TNF-α synthesis are IL-6, IL-4, IL-10 and tumour growth factor beta (Dinarello, 2002).

Tumour necrosis factor alpha (TNF-α) is synthesized as a 26kDa pro-TNF protein mostly by activated macrophages and T lymphocytes. The pro-TNF is expressed on the plasma membrane and it can be cleaved in the extracellular domain by the matrix metalloproteinase resulting in the release of a 17 k DA soluble form. The remaining TNF-α on the membrane after cleavage is then released by TNF-α converting enzyme (ADAM-17) from the surface of the membrane in to circulation (Black et al., 1997). Tumour necrosis factor alpha (TNF-α) promotes local reactive oxygen species generation (Koike et al., 2007, McCarthy et al., 1998), increasing the permeability of albumin (McCarthy et al., 1998).

Serum levels of TNF-α were found to be higher in microalbuminuric and macroalbuminuric patients as compared to those with normoalbuminuria (Kalantarinia et al., 2003). Serum TNF-α levels were found to be independently and significantly associated with albumin: creatinine ratio (ACR) in diabetic patients (Ahmed et al., 2012, Navarro et al., 2006, Refat et al., 2010) and hypertensive patients (Yu et al., 2010). A study indicated that in newly diagnosed essentially hypertensive patients, UAE is independently associated with urinary but not plasma TNF-α levels (Navarro-González et al., 2008). Microalbuminuria was found not to be associated with serum TNF-α levels in patients with type II diabetes (Lampropoulou et al., 2014).

2.10.3. Interleukin-6 and microalbuminuria

Human interleukin 6 (IL-6) is composed of 184 amino acids and produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangium cells, and several tumour cells (Kishimoto, 1989). The gene for interleukin 6, located on chromosome 7, consist of approximately 5 kilo base long sequence with 5 exons (Sehgal et al., 1986). The 1.3 kilo base microsomal RNA in fibroblasts, transformed T cells and monocytes is translated and transcribed into a 212amino acid pro-peptide with a molecular weight of 26 KDa. The pro-peptide is then cleaved to release a 28 amino acid peptide and form a 184 amino acid peptide. The protein undergoes some glycosylation and phosphorylation to produce a mature IL-6 (Bauer et al., 1988, Gross et al., 1989, Helfgott et al., 1989, May et al., 1988).

The synthesis of IL-6 was found to be increased in low physical activity (Fischer, 2006), insulin resistance (Bruun et al., 2003) and CVD (Fisman et al., 2006). Interleukin 1 and TNF-α are some of the stimulators of IL-6 synthesis while dexamethasone inhibit IL-6 synthesis (Akira et al., 1993). Interleukin-6 (IL-6) can stimulate the synthesis and excretion of colloid enzyme and other extracellular enzyme produced by the matrix cells, which can lead to the degradation of negatively charged glycoprotein that is located at basement membrane and thus lead to the leakage of negatively charged proteins from plasma and whereby to result in proteinuria (Ritz and Stefanski, 1996).

Studies have reported that MA was found to be associated with increased concentrations of IL-6 (Moubarak et al., 2012, Yu et al., 2010). However, there are contradicting results were IL-6 was found to not to be an independent predictor of urinary albumin excretion (Ng et al., 2008, Zahran et al., 2012). Interleukin-6 was found to be negatively correlated with estimated glomerular filtration rate (Zahran et al., 2012). High serum and urinary concentrations of IL-6 are associated with greater albuminuria in patients with diabetic nephropathy (Navarro et al., 2006). A study in the USA in diabetic patients showed that diabetic patients with MA had significantly higher levels of IL-6 levels when compared with diabetic patients with normoalbuminuria (Vaidya et al., 2011). In another study serum levels of IL-6 were similar in Type 2 diabetic patients with and without MA (Moriwaki et al., 2003).

2.11. Serum lipids

There are different types of lipoproteins which circulate in the plasma but their structures are similar. The lipoproteins differ in size, density, electrophoretic mobility, composition and their functions (Breslow, 2000, Jonas, 2002). Lipoproteins are classified into five major categories based on their plasma density after ultracentrifugation and the classes are: chylomicrons (lightest and most buoyant), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Cholesterol and triglyceride are the most important serum lipids (Breslow, 2000). Cholesterol functions as a cell membrane component, as a precursor for steroid hormones, bile acids and vitamin D (Incardona and Roelink, 2000).

2.11.1. Metabolism of chylomicrons

The nascent chylomicrons are produced from fats and apolipoproteins and released into the circulation where they acquire free cholesterol, Apo C-II, and E from HDL in exchange for Apo A-I, A-II and phospholipid (Vaziri, 2003). The triglyceride core of the chylomicrons is hydrolysed by lipoprotein lipase leading to the release of free fatty acids and glycerol thus causing the chylomicrons to shrink and the phospholipids and Apo C are transferred to HDL. The fatty acids are taken up by adipose tissue to be re-esterified and stored as triglyceride or by muscles where they are used as a source of energy. The remnants are removed from circulation by remnant receptor on hepatocytes, see fig. 2-2 (Hui et al., 1986, Schneider, 2002).

2.11.2. Metabolism of VLDL and IDL

Very-low-density lipoprotein is important for the transport of endogenous lipids. It is formed by lipidation of Apo B by microsomal triglyceride transfer protein in the rough endoplasmic reticulum leading to the formation of a pre-VLDL particle which is later converted to a VLDL poor in triglyceride (Bostrom et al., 1988, Stillemark-Billton et al., 2005). The VLDL poor in triglyceride can be secreted from the cell as VLDL-2 or lipidated further to a more mature VLDL rich in triglyceride (Stillemark et al., 2000, Stillemark-Billton et al., 2005) and released from the liver in to circulation. In the circulation it acquires Apo C and E from HDL in exchange for Apo A-I, A-II and additional phospholipid. This leads to the conversion of VLDL to intermediate density lipoprotein which is then hydrolysed by hepatic lipase to a cholesterol

rich low density lipoprotein or removed by the liver through the LDL receptors or LDL-receptor related protein (Vaziri, 2003).

2.11.3. Metabolism of LDL

Low-density lipoprotein (LDL) particles, important for the transport of cholesterol and cholesterol esters in the human circulation, are implicated in the development of cardiovascular diseases, in particular atherosclerosis (Colvin and Parks, 1999, Linsel-Nitschke and Tall, 2005). The LDL particle binds to LDL receptors on the membrane of target cells specific for Apo B-100 on the particle surface, thus inducing translocation of the receptors leading to endocytosis formation of an endosome (Beisiegel, 1998, Rader, 2008). The endosome fuses with the lysosome in the cytoplasm which degrades the LDL particle to its primary components. The cholesterol forms the intracellular cholesterol pool (Beisiegel, 1998, Rader, 2008) and can be used for steroid synthesis, membrane synthesis or bile acid synthesis, storage or excretion in the liver (Brown and Goldstein, 1984).

2.11.4. Metabolism of HDL

Apolipoproteins A1 and A2 (main Apo protein of HDL particles) dissociate from the original particle in the extracellular space and fuse to form a nascent HDL which then incorporates Apo C and E phospholipid complex to form an HDL-3 particle poor in cholesterol, see fig. 2-2 (Yancey et al., 2003). The cholesterol poor HDL-3 particle then binds to HDL-binding protein leading to mobilization of intracellular cholesterol pool and translocation of free cholesterol to plasma membrane creating a concentration gradient leading to the esterification of the cholesterol by lecithin-cholesterol acyltransferase (LCAT) in the presence of Apo A1 to form cholesterol ester. (Fielding and Fielding, 2002, Jonas, 2002). The HDL particle dissociates from HDL-binding protein and return to circulation for transport to the liver. The HDL-2 donates part of its cholesterol ester to VLDL remnants in exchange for triglyceride in the presence of cholesterol ester transfer protein (CETP) and it also acquires Apo C and E from chylomicrons and VLDL, see fig. 2-2. The HDL binds reversibly to HDL receptor facilitating the unloading of cholesterol ester content into hepatocytes (Vaziri, 2003).

2.11.5. Metabolism of lipoprotein (a)

Lipoprotein (a) [Lp (a)] is a genetic variant of low-density lipoprotein (LDL) in which apolipoprotein B-100 is linked by a disulphide bond to apolipoprotein (a) [Apo (a)]

(Koschinsky and Marcovina, 2004) that is potentially atherogenic and a known risk factor of cardiovascular disease (Sun et al., 2002). Apolipoprotein (a) [Apo (a)] is structurally similar to plasminogen and renders Lp (a) uniquely atherogenic and thrombogenic (Boffa et al., 2004). Apolipoprotein (a) is synthesized in the liver and after its synthesis it binds to Apo B on the LDL particles via the disulphide bridge of Apo B and Apo (a) (Frank and Kostner, 1997, Kostner and Kostner, 2005). The Lys-rich epitopes on the LDL particle binds to Apo (a) thus forming a complex dissociable by ε-amino hexoic acid (ε-AHA). A disulphide is formed between Apo (a) and Apo B (Steyrer et al., 1994). There is considerable heterogeneity in the Lp (a) polymorphisms that exist in different populations (Scanu and Fless, 1990) that can be categorized in to six groups depending on the Apo (a) isoforms. The groups are therefore named as F, B, S1, S2, S3 and S5 (Saleh et al., 2010).

Black people were found to have the highest levels of Lp (a) than any other population, with median Lp (a) levels in blacks being approximately three times higher than in white population (Bianchi et al., 1994, Emerging Risk Factors et al., 2009, Howard et al., 1994) and Lp (a) is said to confer less risk in blacks than I Asian Indian/whites (Sharrett et al., 2001).

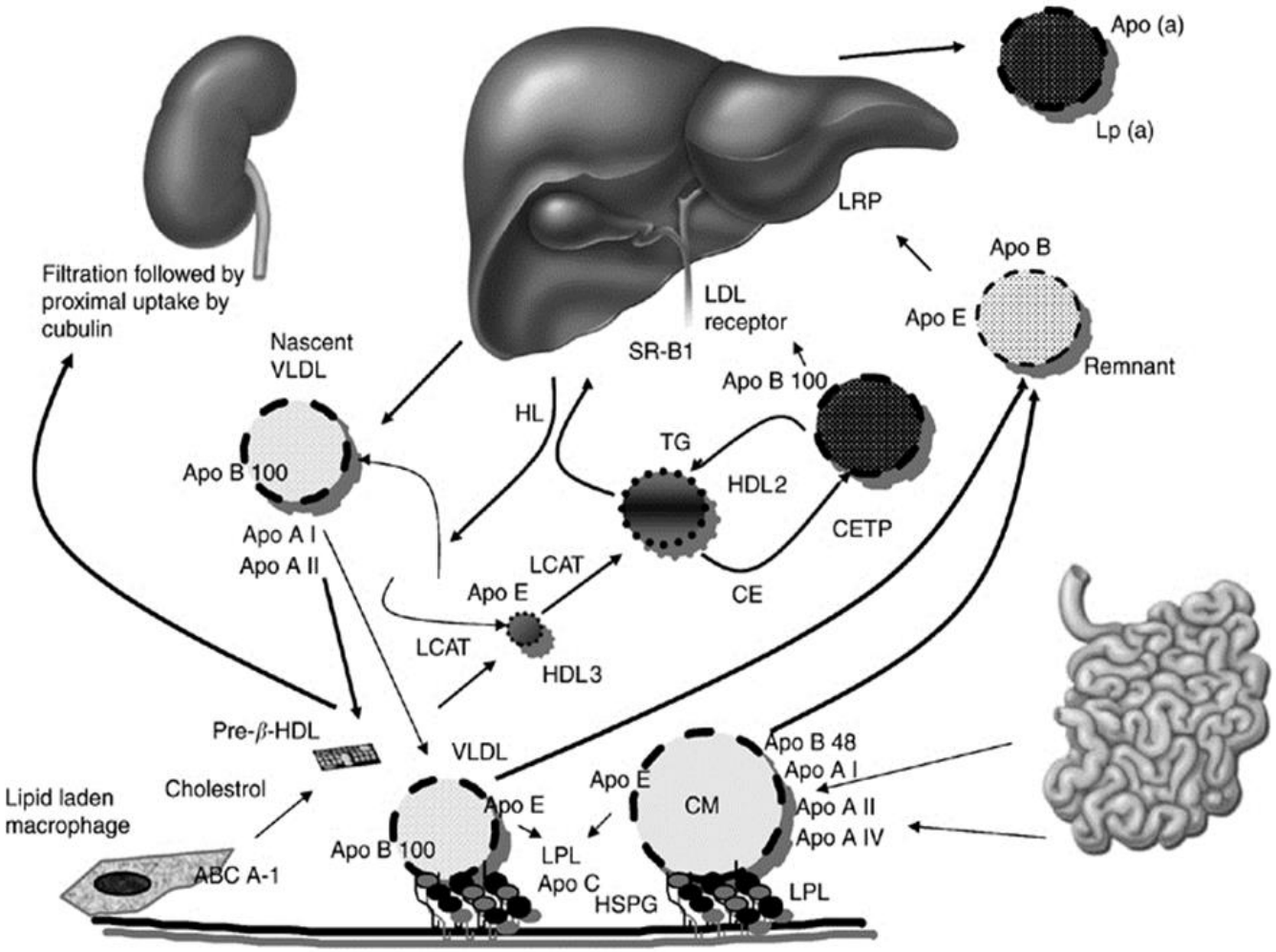

Figure 2: Summary of lipoprotein metabolism. LPL-lipoprotein lipase, LCAT- lecithin: cholesterol acyltransferase, LRP-lipoprotein receptor- related protein, HL-hepatic lipase, CE-cholesterol ester, CETP- cholesterol, ester transfer protein. Adopted from (Kaysen et al., 2006).

2.11.6. Association of serum lipids and microalbuminuria

Dyslipidaemia is commonly seen in diabetic patients with renal disease, which can contribute to the higher morbidity and mortality (Battisti et al., 2003, Tolonen et al., 2008). Dyslipidaemia was more common in patients with microalbuminuria than those without microalbuminuria and a low HDL-C and high TG were the most common types of dyslipidaemia (Busari et al., 2010). Protein loss via urine that is evident in microalbuminuria together with a consequent hypoalbuminemia can cause the up regulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA), a rate limiting enzyme in cholesterol synthesis, the up regulation of HMG-CoA leads to hypercholesterolemia (Vaziri et al., 2003). A low HDL-C in microalbuminuria and a poor maturation of HDL-3 to cholesterol-rich HDL-2 is because of the acquired lecithin-

cholesterol acyltransferase deficiency caused by abnormal urinary loss of the enzyme during microalbuminuria (Vaziri et al., 2001).

Studies reported that diabetic patients with MA had higher levels of TC, TG and LDL-C with lower HDL-C than those without MA (Khan et al., 2012, Wang et al., 2012). A cross sectional study on 62 type 2 diabetes patients attending the outpatient department of internal medicine at C.S.M medical university, Uttar Pradesh, India, aged between 40 and 70 years reported that diabetic patients with microalbuminuria had a higher TC, TG, LDL-C and a lower HDL-C as compared to those without microalbuminuria (Khan et al., 2012). Muttur and colleagues (Muttur et al., 2010) also found similar results where no significant association between microalbuminuria and serum lipids in type II diabetic patients. A prospective, cross sectional study of all diabetic patients attending an outpatient diabetic clinic at the Komfo Anokye Teaching Hospital diabetic centre in Kumasi, Ghana reported a high triglycerides level in patients with microalbuminuria while other serum lipids showed no significant difference (Eghan et al., 2007).

Serum triglyceride (Mattock et al., 2001, Retnakaran et al., 2006) was found to be a determinant of MA in patients with diabetes. A retrospective study on 1060 type 2 diabetes inpatients admitted to the Zhongda hospital of Southeast University, China, aged 18 years and older reported a higher TC, LDL-C, lower HDL-C but comparable levels of TG in patients with and without microalbuminuria (Wang et al., 2012). Serum TC, HDL-C, LDL-C and TG were found to be similar in insulin dependent diabetes mellitus patients with and without microalbuminuria (Rudberg and Persson, 1995). A cross sectional study on 3250 randomly selected type 1 diabetic patients from 31 diabetes clinic in 16 European countries between 1989 and 1990 reported microalbuminuria to be significantly associated with increased levels of TG, TC, TC/HDL-C and decreased HDL-C levels in women but only associated with increased TG levels in the total population (Mattock et al., 2001).

Some studies reported that hypertensive patients with MA had significantly higher mean serum TC, LDL cholesterol and atherogenic index and significantly lower mean serum HDL than their counterparts without MA (Busari et al., 2010, Jensen, 2000). Similarly levels of TG, TC and LDL-C were not different between hypertensive patients with microalbuminuria and those without microalbuminuria while HDL-C levels were lower in those with microalbuminuria (Poudel et al., 2012, Shoji et al., 2001). A prospective study on 96 adult Nigerians with newly diagnosed hypertension and their control at the cardiology unit of the university of Ilorin

teaching hospital, Ilorin, Nigeria reported that hypertensive patients with microalbuminuria had higher means of TC, LDL-C and atherogenic index and a lower HDL-C as compared to their counterparts, the study also reported microalbuminuria to be correlated positively with TC and LDL-C and negatively with HDL-C (Busari et al., 2010). Other studies found similar results where hypertensive patients with MA had significantly higher mean serum TC, LDL cholesterol and atherogenic index and significantly lower mean serum HDL than their counterparts without MA (Jensen, 2000) and in the hypertensive population microalbuminuria was positively associated with serum TC and LDL-C and negatively associated with serum HDL-C (Bigazzi et al., 1995, Jensen, 2000).

A hospital based cross sectional study conducted in Nepal between February 2008 and august 2010 found similar levels of TG, TC and LDL-C were not different between hypertensive patients with microalbuminuria and those without microalbuminuria while HDL-C levels were lower in those with microalbuminuria (Poudel et al., 2012). The study by Sheng and colleagues (Sheng et al., 2011) also reported contradicting results in hypertensive patients where microalbuminuria was not associated with HDL-C and TC has also been reported.

A study conducted in South Korea in 2001 reported that levels of TG and TC were similar in non-diabetic normotensive patients with and without microalbuminuria and that HDL-C levels were lower in non-diabetic normotensive patients with microalbuminuria as compared to those without microalbuminuria (Kim et al., 2001). Another cross sectional study based on follow up data of an ongoing longitudinal population study in Shanghai, reported no association between microalbuminuria and serum lipids in a general population (Sheng et al., 2011). Microalbuminuria was found not to be associated with HDL-C and TG in non-diabetic normotensive patients (Kim et al., 2001). A general health survey conducted during the 1995 to 1997 period in the Nord-Trondelag County, Norway, reported that microalbuminuria was not associated with microalbuminuria in non-diabetic normotensive participants (Romundstad et al., 2002). A cross sectional cohort study on participants of Groningen, Netherlands, reported that microalbuminuria was not associated with hyperlipidaemia in non-diabetic normotensive participants of Groningen, Netherlands (Hillege et al., 2001). A cross sectional study by Choi and colleagues (Choi et al., 2006) on adults living in the northern part of Seoul, South Korea, reported a significantly higher TG and LDL-C in subjects without diabetes and hypertension but the levels of HDL-C were not significantly different. A study conducted in South Korea in 2001 reported that levels of TG and TC were similar in non-diabetic normotensive patients

with and without microalbuminuria and that HDL-C levels were lower in non-diabetic normotensive patients with microalbuminuria as compared to those without microalbuminuria (Kim et al., 2001).

Lipoprotein (a) [Lp (a)] is a lipoprotein subclass that is potentially atherogenic and a known risk factor of cardiovascular disease (Sun et al., 2002). Higher levels of Lp (a) are reported with vascular complications and MA (Siekmeier et al., 2008). Studies reported that the levels of Lp (a) were similar in diabetic normoalbuminuric and microalbuminuric subjects (Rudberg and Persson, 1995, Tseng, 2009). Other studies however reported a higher Lp (a) in diabetes patients with MA as compared to those without MA and also reported an association between Lp (a) and albumin excretion rate (Hernandez et al., 1997, Kaur et al., 2012, Meca et al., 2007). Lowering serum Lp (a) can ameliorate microalbuminuria and postpone the occurrence of renal function failure in patients with type 2 diabetes (Zhou et al., 2007). Tseng, reported the lack of association between Lp (a) and MA in diabetic patients (Tseng, 2009).

CHAPTER THREE

3.1. PURPOSE OF STUDY

3.1.1. Aim

To determine the association of microalbuminuria with serum lipids and inflammatory markers in a rural black population in the Limpopo province.

3.1.2. Objectives

- To determine the association between MA and serum lipids in a black population
- To determine the association between MA and inflammatory markers in a black population
- To determine the association of MA, serum lipids and inflammatory markers in a black population.

3.1.3. Research question

What is the association amongst microalbuminuria, serum lipids and inflammatory markers in a rural black population of Limpopo province?

CHAPTER FOUR

4. METHODOLOGY

4.1. Study design

This was a cross-sectional study. Cross-sectional studies measure the prevalence of disease and are often called prevalence studies. In a cross-sectional study the measurements of exposure and effect are made at the same time (Bonita et al., 2006). In this study, data was collected at once from the population to determine the association of microalbuminuria, serum lipids and inflammatory markers and this qualifies the study to be a cross sectional study.

4.2. Study area

The study was conducted in Dikgale Health and Demographic Surveillance System (Dikgale HDSS) centre which is made up of 15 villages with total a population of approximately 35 000. The Dikgale HDSS is situated at about 40 km north-east of Polokwane, the capital city of Limpopo Province, and slightly closer to the University of Limpopo Turf loop campus. The site is located between 29.65º and 29.85ºE, and 23.65º and 23.90ºS. Figure 3 shows the location of Limpopo Province within South Africa and of Dikgale District within the Province (Alberts et al., 2015). Majority of the inhabitants of this area are northern Sotho-speaking people (Alberts et al., 1999).

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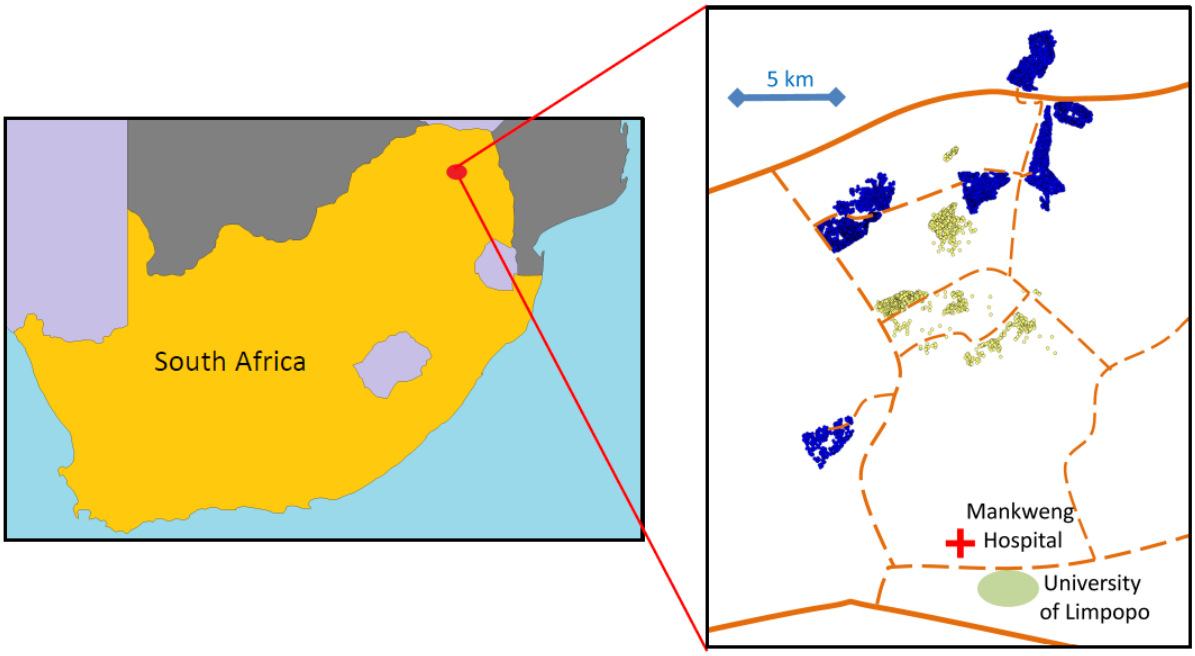

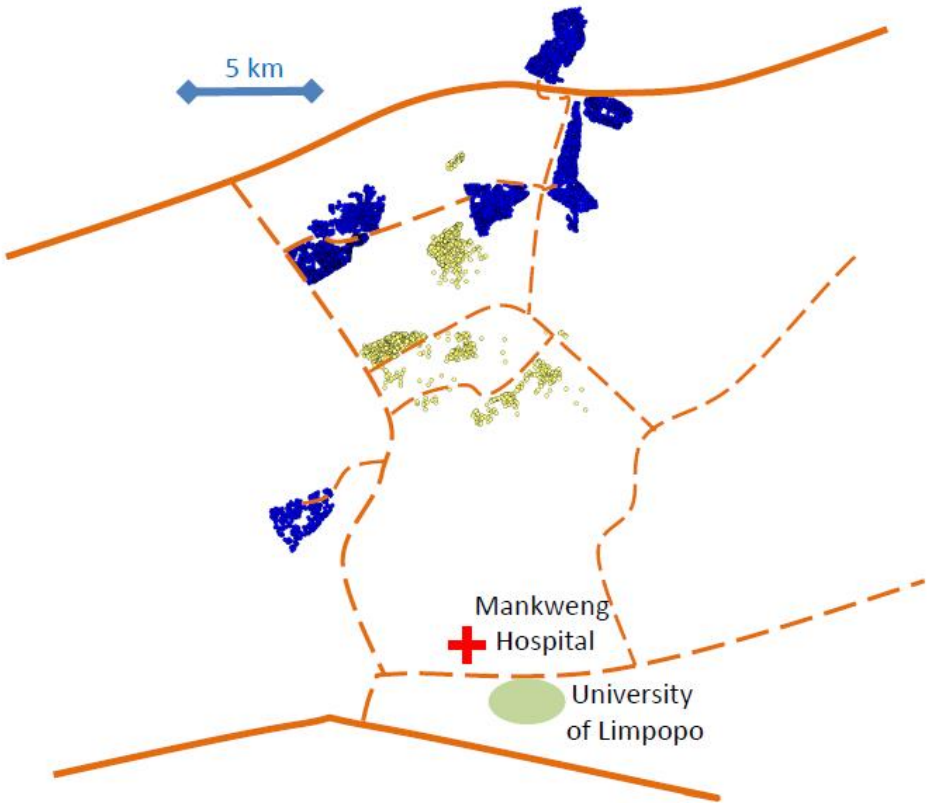

Figure 3: Maps of South Africa and Limpopo Province. Adopted from (Alberts et al., 2015).

4.3. Study population

The study is part of the project "Prevention, control and integrated management of chronic diseases in a rural area, South Africa" conducted at the Dikgale HDSS.As part of the larger study 2 981 participants in total were selected randomly to participate in the study from the DHSS database. Of the 2981 participants that were selected to participate, only 1 407 participants, of which 878 were women and 525 were men, completed the WHO STEP-wise questionnaire. For the collection of blood samples only 817 of the participants were available to donate a fasting blood sample. Some of the reasons for the low participation were leaving for work in the morning, unavailability after repeated visits, refusal to participate, death or migrated out of the study area (Maimela et al., 2016). The blood samples were centrifuged and biochemical parameters like serum lipids, urine albumin, urine and serum creatinine, glucose, insulin and hs-CRP were determined. An HIV test was done on participants who gave consent for the test. The results were captured in a database. The remaining blood samples were stored in cryo tubes at -80^{o}C for further use.

4.4. Sampling

Sampling is the selection of some part of a group or totality on the basis of which a judgement or inference about the group or totality is made (Websters, 1987). Sampling for the present study was done from the data of a project titled "Prevention, control and integrated management of chronic diseases in a rural area, South Africa".

For the present study all the 817 participants in larger study were included but after exclusion criteria 602 participants remained in the study. Subjects with an ACR of 2.5–25 mg/mmol in males and an ACR of 3.5–35 mg/mmol in females (Chadban et al., 2010) were considered microalbuminuric and subjects with an ACR of < 2.5 mg/mmol in males and an ACR of < 3.5 mg/mmol in females were considered normoalbuminuric. Subjects with microalbuminuria were regarded as the cases and subjects with normoalbuminuria were regarded as controls for the present study.

4.5. Exclusion criteria

To avoid interferences with results, participants who were HIV-positive (rapid HIV test was done) and on lipid lowering drugs (a questionnaire was administered) were excluded from the study. Also, participants with macroalbuminuria (ACR of >25 mg/mmol in males and > 35mg/mmol in females), diabetes (fasting glucose of ≥ 7.0 mmol/l and/or history of diabetes) and renal disease (creatinine of ≥170 μmol/l) were excluded from the study. Diabetes, HIV and lipid lowering drugs were excluded because they are known to interfere with serum lipids while macroalbuminuria and kidney disease were excluded because the focus of this study is microalbuminuria.

4.6. Data collection

4.6.1. Anthropometric and blood pressure measurements

In the "Prevention, control and integrated management of chronic diseases in a rural area, South Africa" project, anthropometry and blood pressure were collected according to the WHO standard procedures (consultation, 2004, World Health, 1995, World Health, 2000) as follows:

Body weight of the participants was measured using an OMRON B-400, a digital weight scale (OMRON UK). The participants were asked to remove their heavy clothing, and carefully step on to the weight scale. The participants were asked to look straight ahead, to stand very still,

and remain on the scale until the body weight number on the scale does not move. Weight was measured to the nearest 0.1 kilogram. Height was measured using a stadiometer. For the measurement of standing height, the participants were asked to stand with their back against the board. The back, scapulae and buttocks are in contact with the vertical board if possible, or whichever part of the body touches the board first. Participants were asked to stand up straight and look straight ahead. Height was measured to the nearest 0.1 metre.

From the body weight and height, the body mass index (BMI) of each participant was calculated using the following formula:

$$\text{BMI} = {Weight\ (Kg)} \Big/ {[Height\ (m)]^2}$$

Participants with the BMI of less than 18.50 kg/m^2 were considered as underweight, between 18.50 kg/m^2 and 24.99 kg/m^2 were considered as normal, between 25 and 29.9 kg/m^2 were considered as overweight and ≥ 30kg/m^2 were considered as obese (consultation, 2004, World Health, 1995, World Health, 2000).

Waist circumference was measured with the participants in a standing position, using a non-extendible and flexible anthropometric measuring tape. Waist circumference was taken at the midpoint between the lower costal rib and iliac crest, perpendicular to the long axis of the trunk (Marfell-Jones et al., 2006).

Blood pressure (mmHg) was measured using an OMRON M-5 (OMRON UK); the participants were asked to rest for five minutes without eating, drinking and smoking. There after the cuff was placed on the participant's arm and the blood pressure measured. Three measurements were taken but only the mean of the last two was considered. Participants with a systolic blood pressure (SBP) of ≥ 140 mmHg, diastolic blood pressure (DBP) of ≥ 90 mmHg and/or history of hypertension were considered as hypertensive (Crowther and Norris, 2012).

For the present study, information on anthropometry and blood pressure for the participants was extracted from the database.

4.6.2. Sample collection

In the project "Prevention, control and Management of chronic diseases in a rural area, South Africa" overnight fasting blood samples were collected from all participants by a registered

nurse. Blood was collected in silica coated vacutainer tubes, EDTA containing tubes and sodium fluoride tubes. Serum samples were collected as part of the larger study, during 2011 and 2012. The blood samples were centrifuged for separation of serum and plasma. Serum lipids, Hs-CRP, glucose, insulin, HIV status and creatinine were measured immediately after sample collection. The rest of the serum was stored in cryo tubes at -80^{o}C. For the present study, the stored sera were used for determination of Lp (a), TNF-α, IL-6 and ET-1. The determination of Lp (a), TNF-α, IL-6 and ET-1 was done in 2014. A spot urine sample was collected in a sterile urine jar from each participant for albumin and creatinine determination during the 2011/2012 period. Immediately after the samples were collected, they were analysed for urine albumin and creatinine.

4.7. Biochemical analysis

For the present study, results on serum lipids, urinary creatinine, glucose, Hs-CRP and urinary albumin were extracted from the database. In addition, available sera was used for the determination of Lp (a), TNF-α, IL-6 and ET-1 using IMMAGE immunochemistry system, ACCESS chemistry system and ELISA kits respectively. Serum lipids, urinary creatinine and glucose were determined using ILAB 300 plus chemistry system, high sensitivity C reactive protein (Hs-CRP) and urinary albumin were determined using IMMAGE immunochemistry system and the results were entered in to the database.

4.7.1. Determination of urine albumin

The IMMAGE Immunochemistry system (Beckman Coulter, USA) was used for the determination of urine albumin.

- **Principle**

Rate nephelometry analysis was used based on the following reaction.

Albumin + anti-Albumin antibodies → [Albumin-antibody (aggregates)]

Albumin in the sample reacts with the antibody from the reagent to form an albumin-antibody complex. The rate of increase in light scattered from particles suspended in a solution as a result of presence of complexes was measured and it was proportional to the concentration of albumin in the sample.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

All reagents were ready for use.

- **Procedure**

In the IMMAGE immunochemistry system (Beckman Coulter, USA), 21 µL of the sample, 21 µL of the antibody and 300 µL of the buffer were mixed and light scatter was measured.

- **Method performance**

According to the manufacture the method's CV is 5.0 % and its minimum detection limit is 0.22 to 7.15 mg/dL and there is no significant interference from lipaemia, bilirubin and haemoglobin. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Urine protein control level 1 and 2 was used for quality control. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range

4.7.2. Determination of creatinine (urinary and serum)

The I Lab 300 plus chemistry system (Instrumentation Laboratory, Italy) was used for the determination of creatinine.

- **Principle**

Fixed time analysis based on the following reaction

$$\text{Creatinine + picric acid} \xrightarrow[\text{Alkaline conditions}]{} \text{Coloured complex}$$

The creatinine in the sample reacts with picric acid under alkaline conditions to yield a coloured complex and the absorbance of the complex was measured at a wavelength of 510 nanometres. The absorbance of the complex was directly proportional to the concentration of creatinine in the sample.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

All reagents were ready for use.

- **Procedure**

In the ILAB 300 plus (Instrumentation Laboratory, Italy), 20 µL of the sample was mixed with 150 µL of reagent 1 and 150 µL of reagent 2 and incubated at 2-8 ^{o}C for 72 seconds. The absorbance was read at a wavelength of 510 nm.

- **Method performance**

According to the manufacture the method can't be interfered by lipaemia, haemoglobin and bilirubin. The method has a coefficient of variance of 3.1% and its minimum detection limit is 0.1 mg/dl (9 µmol/L).The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Sera Chem control level 1 (normal) and Sera Chem 2 (abnormal) was used for quality assurance. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range

4.7.3. Determination of glucose

The ILAB 300 plus chemistry system (Instrumentation Laboratory, Italy) was used for the determination of glucose.

- **Principle**

End point analysis based on the following reactions

$$\text{B-D-Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{red quinoniemine} + 4H_2O$$

Glucose in the sample was oxidised by glucose oxidase enzyme in the presence of oxygen and water to yield gluconic acid and hydrogen peroxide. Hydrogen peroxide then reacts with phenol and 4-aminoantipyrine in the presence of peroxidase to yield a red quinoneimine and four molecules of water. The increase in absorbance generated by the red dye is proportional

to the glucose concentration in the sample. The absorbance was measured at a wavelength of 510 nanometres.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

Using a pipette 12 ml of deionized or distilled water was added to one bottle of GLUC OX reagent and the bottle was inverted to mix the solution.

- **Procedure**

In the I Lab 300 plus (Instrumentation Laboratory, Italy), 4 µL of the sample was mixed with 360 µL of the GKUC OX reagent and incubated at 2 – 8 ºC for 512 seconds. The absorbance was read at a wavelength of 510 nm.

- **Method performance**

According to the manufacture the method can't be interfered by lipaemia, haemoglobin and bilirubin. The method has a coefficient of variance of 2.2% and its minimum detection limit is 0 mg/dl (0 µmol/L).The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Sera Chem control level 1 (normal) and Sera Chem 2 (abnormal) was used for quality assurance. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range.

4.7.4. Determination of high sensitivity C reactive protein

The IMMAGE immunochemistry system (Beckman Coulter, USA) was used for the determination of high sensitivity C reactive protein.

- **Principle**

Rate nephelometry analysis based on the following reaction

C-reactive protein + particle bound anti-CRP antibodies → [C-reactive protein-antibody complex]

A sample containing CRP will agglutinate when mixed with the latex reagent. The degree of light scatter is directly proportional to the CRP concentration in the sample.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

All reagents were ready for use.

- **Procedure**

In the IMMAGE immunochemistry system (Beckman Coulter, USA), 0.46 µL of the sample was mixed with 23 µL of the antibody, 300 µL of the buffer and 16.04 µL of the diluents and the light scatter was measured

- **Method performance**

According to the manufacture the method's CV is 3.8% and its minimum detection limit is 3mg/dL to 100 mg/dL and there is no significant interference from lipaemia, bilirubin and haemoglobin. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Quantex ASO-CRP-RF control I and ASO-CRP-RF control II was used for quality control. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range

4.7.5. Determination of tumour necrosis factor-α

Tumour necrosis factor-α was measured using the ELISA MAX Deluxe kit (Bio Legend, USA).

- **Principle**

Sandwich Enzyme-Linked Immunosorbent Assay based on the following reaction

$$\text{Well bound anti- TNF-}\alpha\text{ capture Ab + ET-1} \xrightarrow{\text{Wash}} \text{AB- TNF-}\alpha\text{ complex}$$

$$\text{Ab- TNF-}\alpha\text{ complex + enzyme linked detection Ab} \xrightarrow{\text{Wash}} \text{Ab- TNF-}\alpha\text{-Ab-Enzyme complex}$$

Wash

Ab- TNF-α-Ab-Enzyme complex + substrate ⟶ Ab- TNF-α-Ab-Enzyme complex + colour change

Bio Legend's ELISA MAX Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human TNF-α specific monoclonal antibody is first coated on a 96-well plate and the plate is sealed and incubated in 16 to 18 hours at 4 degrees Celsius. Serially diluted standards, of concentrations 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 0pg/ml, samples are added to the wells and the TNF-α antigen binds to the immobilized capture antibody. Next, a biotinylated anti-human TNF-α detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, producing a blue colour in proportion to the concentration of TNF-α present in the sample. Finally, the Stop Solution changes the reaction colour from blue to yellow, and the micro well absorbance is read at 450 nm with a micro plate reader. The expected minimum detectable concentration of TNF-α for this test is 2 pg/ml. There is no cross reactivity when this kit was used to analyse multiple human, mouse and rat recombinant proteins. Serum TNF-α was measured and almost all of the results were below the lowest standard which was set to be 0.001 pg/ml which was found to complicate our analysis. Due to most results being below 0.001 pg/ml, TNF-α was removed from the study.

- **Reagent preparation**

All reagents used were supplied by Bio Legend, USA. All reagents were brought to room temperature before use.

Phosphate-Buffered Saline (PBS) was prepared by dissolving 8.0 g of sodium chloride (NaCl), 1.16 g of disodium orthophosphate (Na_2HPO_4), 0.2 g of potassium dihydrophosphate (KH_2PO_4) and 0.2 g of potassium chloride (KCl) in deionized water to form 1 litre of PBS and corrected to a pH of 7.4. Wash buffer was prepared by mixing 999.5 ml of PBS with 0.45 ml of tween 20 to produce 1 litre of wash buffer.

Coating buffer (5X) was diluted to 1X using deionised water by diluting 2.4 mL 5X coating buffer in 9.6 mL deionized water for one full plate. The pre-titrated capture antibodies were diluted to 1:200 in 1X coating buffer by diluting 60 µL of capture antibody in 11.94 mL of 1X coating buffer for use on 12 strip plate. Assay diluent A was diluted from 5X to 1X with PBS

(pH 7.4) by mixing 10 mL of 5X assay diluent A with 40 mL of PBS to form 50 ml of 1X assay diluent.

Lyophilized standard were reconstituted with 0.2 mL of 1X assay diluent A to produce 55 ng/ml of stock standard recombinant human TNF-α and allowed to sit for 15 minutes at room temperature. A top standard of 500 pg/ml was prepared by adding 9.09 µl of the stock standard solution to 990.91 µl of 1X assay diluent A. the remaining stock standard solution was aliquoted into vials and stored at – 80^{o}C for future use.

A six two-fold serial dilutions of the 500 pg/mL top standard with 1X assay diluent A was performed in separate tubes. After diluting, the human TNF-α standard concentrations were 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL and 7.8 pg/mL, respectively. Assay Diluent A (1X) served as the zero standard (0 pg/mL).

Pre-titrated biotinylated detection antibody was diluted to 1:200 by diluting 60 µl of detection antibody in 11.94 ml of 1X assay diluent A. Avidin-HRP was diluted to 1:1000 by diluting 12 µl of avidin-HRP in 11.99 ml of 1X assay diluent A. TMB substrate solution was prepared by mixing equal volumes of substrate solution A and substrate solution B immediately prior to use.

- **Procedure**

One day prior to running the ELISA 100 µL diluted capture antibody where pipetted into all wells of a 96-well plate provided in the set. The plate was sealed and incubate overnight (16-18 hrs) at 4°C.

All standards and samples were run in duplicate. The plate was washed 4 times with 300 µL of wash buffer per well and the residuals were by firmly tapping plate upside down on a dry absorbent paper. All subsequent washes were performed similarly. To block non-specific binding and reduce background 200 µL 1X assay diluent were pipetted into each well with micropipette using sterile pipette tips. The plate was then sealed and incubated at room temperature for 1 hour with shaking at 400 rpm on a plate shaker. The plate was washed 4 times with wash buffer. Standards or samples (100 µL) were pipetted into their appropriate wells. The plate was sealed and incubated at room temperature for 2 hours with shaking at 400 rpm on a plate shaker.

After, the plate was washed 4 times with wash buffer. Diluted detection antibody (100 µL) was pipetted into each well; the plate sealed and incubated at room temperature for 1 hour with shaking at 400 rpm on a plate shaker. The plate was washed 4 times with wash buffer. Diluted avidin-horse radish peroxidase (100 µL) was pipetted into each well, the plate sealed and incubated at room temperature for 30 minutes with shaking at 400 rpm on a plate shaker. The plate was washed 5 times with wash buffer and the wells were soaked with the wash buffer for 30 seconds and allowed 1 minute for each wash.

Freshly mixed TMB substrate solution (100 µL) was pipetted into each well, the plate was incubated in the dark for 15 minutes. Positive wells turned blue in colour. The reaction was stopped by adding 100 µL of stop solution into each well. Positive wells turned from blue to yellow. The absorbance was read at 450 nm within 30 minutes.

- **Method performance**

According to the manufacturer the CV for this method ranges from 1.7% to 2.7%. The lowest detectable level of IL-6 was 2.0 pg/mL. Multiple human, mouse and rat recombinant did not affect the concentration of IL-6 assayed. A standard curve was analysed with each plate for calibration.

4.7.6. Determination of endothelin-1

Endothelial 1 was measured using the Quantikine ELISA kit supplied by R&D system, USA.

- **Principle**

Sandwich enzyme linked immunosorbent assay technique based on the following reaction

Wash

Well bound anti-ET-1 capture Ab + ET-1 ⟶ AB-ET-1 complex

Wash

Ab-ET-1 complex + enzyme linked detection ⟶ Ab Ab-ET-1-Ab-Enzyme complex

Wash

Ab-ET-1-Ab-Enzyme complex + Luminol substrate ⟶ Ab-ET-1-Ab-Enzyme complex + light

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ET-1 has been pre-coated onto a micro plate. Standards and samples are pipetted into the wells and any ET-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for ET-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of ET-1 bound in the initial step. A micro plate luminometer is used to measure the intensity of the light emitted. Endothelin-1 Quantikine ELISA kit control low, normal and high were assayed together with samples for quality assurance. Serum ET-1 was measured and almost all of the results were below the lowest standard which was set to be 0.001 pg/ml which was found to complicate our analysis. Due to most results being below 0.001 pg/ml. ET-1 was removed from the study.

- **Reagent preparation**

All reagents used were supplied by R&D system, USA. Wash buffer was diluted by mixing 20 mL of Wash buffer Concentrate supplied with deionized or distilled water to prepare 500 mL of Wash buffer solution.

Colour Reagents A and B were mixed together in equal volumes within 15 minutes of use to prepare the substrate solution.

Calibrator diluent (1X)was prepared by diluting 5 mL of calibrator diluent concentrate into 20 mL of deionized or distilled water to prepare 25 mL of calibrator Diluent (1X).

Endothelin-1 standard was reconstituted with 1.0 mL of deionized or distilled water, mixed gently and allowed to sit for 15 minutes to produces a 10X stock solution. Into the tube labelled 25 pg/mL 900 µL of calibrator diluent and 100 µL of the stock solution were added and vortex mixed. Into the remaining six tubes, 500 µL of calibrator diluent (1X) was pipetted using a sterile pipette tip. The first standard was vortex mixed and 500 µL of the standard (25 pg/mL) was transferred from the fist to the subsequent tube, the last step was repeated until the last standard where the 500 µL of the last standard was discarded leaving 500 µL of the standard in the tube. The calibrator diluent (1X) served as a zero standard (0 pg/mL).

- **Procedure**

All reagents and samples were brought to room temperature before use. All standards, samples, and controls be assayed in duplicate. A face mask and gloves were worn at all times to protect kit reagents

Assay diluent (150 µL) was added to each well. In all the wells 75 µL of Standards, controls or sample was pipetted using a micropipette with sterile pipette tips. The plate was covered with the adhesive strip provided and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker set at 400 rpm. The plate was washed four times using 400 µL wash buffer on an automated plate washer. Two hundred microlitres (200 µL) of Endothelin-1 Conjugate was pipetted to each well using sterile pipette tips, the plate covered with a new adhesive strip and incubated for 3 hours at room temperature on the shaker. The wash step was repeated. A substrate solution (200 µL) was pipetted to each well using a sterile pipette tip and the plate was incubated for 30 minutes at room temperature on the bench top away from light. Later 50 µL of Stop Solution was pipetted into each well the colour in the wells changed from blue to yellow. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

- **Method performance**

According to the manufacturer the CV for this method ranges from 1.9% to 4.0% for intra assay and 5.3 % to 7.6 % for inter assay precision. The lowest detectable level of ET-1 was 0.0875 pg/mL. Human big endothelin-1 and 2, endothelin-2, sarafotoxinS6b and S6c did not affect the concentration of IL-6 assayed. A standard curve was analysed in each plate for calibration.

- **Quality assurance**

Endothelin control high, control medium and control high were assayed with the sample ant their results corresponded with the ranges provided by the manufactures before the results were considered.

4.7.7. Determination of interleukin-6

Interleukin-6 was measured using the Access II Immunoassay Analyser (Beckman Coulter, USA).

- **Principle**

IL-6 + particle bound anti-IL-6 Ab + blocking reagent + ALP conjugate $\xrightarrow{\text{Wash}}$ IL-6-Ab complex

IL-6-antibody complex + luminol substrate → light

The Access IL-6 is a simultaneous one step immunoenzymatic (sandwich) assay. A sample is added to a reaction vessels along with the paramagnetic particles coated with a mouse monoclonal anti-human IL-6, blocking reagent and the alkaline phosphate conjugate. After a while unbound materials are washed away. Then the chemiluminescent substrate lumi-phos 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light produced is directly proportional to the concentration of IL-6 in the sample. The amount of the analyte in the sample is determined from a stored, multi-point calibration curve.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

All reagents were ready to use.

- **Procedure**

In the access system, 50 µL of antibodies is mixed with 100 µL of blockers and 100 µL of the sample. The solution is then mixed with 100 µL of the conjugate and incubated for 19.2 minutes. Unbound material is then washed away at the wash station with the addition of 150 µL of the wash buffer and the substrate is then added to generate light this is then measured.

- **Method performance**

According to the manufacturer the CV for this method ranges from 1.7% to 2.7%. The lowest detectable level of IL-6 was 0.5 pg/mL. Haemoglobin, bilirubin, triglycerides, protein (human serum albumin), heparin, acetaminophen, acetylsalicylic acid, ibuprofen, α-2-Macroglobulin, α-1-Antitrypsin and α-globulins do not affect the concentration of IL-6 assayed. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Access interleukin 6 quality control levels 1, 2 and 3 were assayed samples for quality assurance. Results were only considered when the results of the controls where within the ranges set by the manufactures.

4.7.8. Determination of insulin

Insulin was measured using the Access II Immunoassay Analyser (Beckman coulter).

- **Principle**

Simultaneous one-step immunoenzymatic ("sandwich") assay based on the following reaction

Insulin + anti-Insulin ALP conjugate + particle bound anti-insulin antibody $\xrightarrow{\text{Wash}}$ particle bound Ab-Insulin-conjugate complex

Particle bound Ab-Insulin-conjugate complex + Lumi-Phos 530 $\longrightarrow$ Light

A sample is added to a reaction vessel along with mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles coated with mouse monoclonal anti-insulin antibody. The serum or plasma insulin binds to the antibody on the solid phase, while the conjugate reacts with a different antigenic site on the insulin molecule. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Then, the chemiluminescent substrate Lumi-Phos 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is directly proportional to the concentration of insulin in the sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

All reagents are ready to use.

- **Procedure**

In the access system, 50 µL of paramagnetic particle is mixed with 50 µL of conjugate linked antibodies, 50 µL of monoclonal antibody, 80 µL of buffer and 20 µL of the sample. The solution is then incubated for 28.8 minutes. Unbound material is then washed away at the wash station with the addition of 250 µL of the wash buffer and the substrate is then added to generate light this is then measured.

- **Method performance**

According to the manufacturer the CV for this method ranges from 1.7% to 2.7%. The lowest detectable level of insulin is 0.03 µIU/mL (0.21 pmol/L). Samples containing up to 10 mg/dL (171 µmol/L) bilirubin and lipemic samples containing the equivalent of 1,800 mg/dLm (20.32 mmol/L) triglycerides do not affect the concentration of insulin assayed.

- **Quality assurance**

Insulin quality control levels 1 and 2 were assayed samples for quality assurance. Results were only considered when the results of the controls where within the ranges set by the manufactures.

4.7.9. Determination of total cholesterol

The ILAB 300 PLUS chemistry system (Instrumentation Laboratory, Italy) was used for the determination of total cholesterol.

- **Principle**

End point analysis based on the following reaction

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-en-3-ene} + H_2O_2$$

$$2H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{red quinoniemine} + 4H_2O$$

Cholesterol ester from the subject's serum reacts with water in the presence of cholesterol esterase in the cholesterol reagent to form fatty acids and cholesterol. Cholesterol then reacts with oxygen in the presence of cholesterol oxidase found in the cholesterol reagent to form superoxide and cholest-4-en-3-one, which then reacts with phenol from the reagent and two molecules of hydrogen peroxide to form four molecules of water and a red quinoneimine. The increase in absorbance generated by the red dye was proportional to the cholesterol concentration in serum at a wavelength of 510 nm.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

Cholesterol reagent is prepared by adding 23 mL of distilled water to one bottle of CHOL.

- **Procedure**

The determination of total cholesterol using I Lab 300 plus, Instrumentation Laboratory, is a fully automated procedure. In I Lab 300 plus, 4 µL of the sample was mixed with 360 µL of CHOL reagent and incubated at 2-8 ^{o}C for 350 seconds. The absorbance was measured at a wavelength of 510 nm.

- **Method performance**

According to the manufacture the method can't be interfered by lipaemia, haemoglobin and bilirubin. The method has a coefficient of variance of 1.6% and its minimum detection limit is 1 mg/dl. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Sera Chem control level 1 (normal) and Sera Chem 2 (abnormal) was used for quality assurance. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range.

4.7.10. Determination of high density lipoprotein cholesterol

The ILAB 300 PLUS chemistry system (Instrumentation Laboratory, USA) was used for the determination of high-density lipoprotein cholesterol.

- **Principle**

Anti-human beta lipoprotein antibodies + non-HDL cholesterol fractions → Ag-Ab complex

$$\text{HDL-cholesterol} \xrightarrow{\text{PEG-cholesterol oxidase}} H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{HSDA} \xrightarrow{\text{Peroxidase}} \text{purple/blue pigment}$$

Anti-human beta lipoprotein antibody in HDL reagent 1 binds all lipoprotein other than high density lipoprotein- cholesterol. The antibody-antigen complexes formed blocks the reaction in all the lipoprotein except in high density lipoprotein- cholesterol when HDL reagent 2 is added. Cholesterol esterase and cholesterol oxidase in HDL reagent 2 react with cholesterol only in the high density lipoprotein- cholesterol of the serum to produce hydrogen peroxide that yields a blue coloured complex upon oxidative compensation of the chromogen. The

concentration of the blue dye (absorbance) was proportional to concentration of high density lipoprotein- cholesterol in serum at a wavelength of 620 nm.

- **Reagent preparation.**

All reagents used were supplied by Beckman Coulter, USA.
All reagents were ready for use.

- **Procedure**

In the I Lab 300 plus (Instrumentation Laboratory, Italy), 3 µL of the sample was mixed with 270 µL of reagent 1 and incubated at 2 – 8 ^{o}C for 252 seconds. 90 µL of reagent 2 was added and incubated at 2 – 8 ^{o}C for 242 seconds. The absorbance was measured at a wavelength of 620 nm.

- **Method performance**

According to the manufacture the method's CV is 1.7% and its minimum detection limit is 0.03 mmol/l. The method isn't interfered by lipaemia, haemoglobin and bilirubin. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Sera Chem control level 1 (normal) and Sera Chem 2 (abnormal) will be used for quality assurance. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range.

4.7.11. Determination of triglycerides

The ILAB 300 plus chemistry system (Instrumentation Laboratory, Italy) was used for the determination of triglycerides.

- **Principle**

End point analysis based on the following reactions

$$\text{Triglycerides} \xrightarrow[\text{Lipoprotein lipase}]{} \text{Glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow[\text{Glycerol Lipase}]{} \text{Glycerol-3-phosphate + fatty acids}$$

Glycerol phosphate oxidase

Glycerol-3-phosphate + O_2 ⟶ Dihydroxyacetone phosphate + H_2O_2

Peroxidise

H2O2 + 4-chlorophenol + 4-aminoantipyrine ⟶ red quinoneimine +$2H_2O$

Triglyceride from serum was converted by lipoprotein lipase in the triglycerides reagent to fatty acids and glycerol. Glycerol then reacts with ATP from reagent in the presence of glycerol kinase to form fatty acids and glycerol-3-phosphate which was oxidised by glycerophosphate oxidase found in the reagent to hydrogen peroxide and dihydroxyacetone. Hydrogen peroxide reacts with 4-chloro-phenol in the reagent and 4-aminoantipyrine in the reagent in the presence of peroxidase found in the reagent to form two molecules of water and a red quinoneimine. The increase in absorbance due to the red dye is proportional to the triglycerides concentration in serum at a wavelength of 510 nm.

- **Reagent preparation.**

All reagents were supplied by Beckman Coulter, USA.
All reagent is ready for use.

- **Procedure**

In I Lab 300 plus (Instrumentation Laboratory, USA), 300 µL of triglyceride reagent was mixed with 3 µL of the sample and incubated at 2 – 8 ºC for 350 seconds. The absorbance was then measured at a wavelength of 510 nm.

- **Method performance**

According to the manufacture the method's CV is 2.3% and its minimum detection limit is 4 mg/dL. The method isn't interfered by haemoglobin of up to 100 mg/dL and bilirubin of up to 14 mg/dL. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Sera Chem control level 1 (normal) and Sera Chem 2 (abnormal) will be used for quality assurance. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range.

4.7.12. Determination of lipoprotein (a)

Lipoprotein (a) was measured using IMMAGE Immunochemistry System (Beckman Coulter USA).

- **Principle**

Rate nephelometry based on the following reaction

Lipoprotein (a) + particle bound anti-Lp (a) antibodies → [Lipoprotein (a)-antibody complex]

Lipoprotein (a) in the sample reacts with the antibody from the reagent to form a lipoprotein (a)-antibody complex. The rate of increase in light scattered from particles suspended in a solution as a result of presence of complexes is measured and it is proportional to the concentration of lipoprotein (a) in the sample.

- **Reagent preparation**

All reagents used were supplied by Beckman Coulter, USA.

All reagents are ready for use.

- **Procedure**

In IMMAGE immunochemistry system (Beckman Coulter, USA), 0.56 µL of the sample was mixed with 21 µL of antibodies, 300 µL of the buffer and 19.44 of the diluents and the light scatter was then measured

- **Method performance**

According to the manufacture the method's CV is 5.0 % and its minimum detection limit is 2.09 to 204 mg/dL and there was no significant interference from lipaemia, bilirubin and haemoglobin. The analyser calibrated and runs were performed only within the period of a valid calibration.

- **Quality assurance**

Virgil protein control level 1, 2, 3 and multi-level were run with samples for quality assurance. Results were only considered when the results of the controls where within the ranges set by the manufactures. Quality control samples were analysed in the beginning, middle and the end of each run and the run was only accepted if the QC was within the QC range

3.7.12. CALCULATIONS:

- **Calculation of ACR**

Albumin to creatinine ratio was calculated as:

$$ACR = \frac{urinaryalbumin}{urinarycreatinine}$$

- **Calculation of eGFR**

eGFR is estimated GFR calculated by the abbreviated Modification of Diet in Renal Disease (MDRD) equation:

$$eGFR = 186 \times \left(\frac{Creat\left[\frac{mmol}{l}\right]}{88.4^{-1.154}} \right) \times Age\ [years]^{-0.203} \times 0.742\ (for\ female) \times 1.210\ (for\ blacks)$$

Creat = serum creatinine

eGFR = estimated glomerular filtration rate

- **Calculation of LDL-C and lipid ratio**

Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula, if the triglyceride level is less than 4.5, LDL-C was not calculated in participants with a TG above 4.5. : $LDL - C\ = \ TC - \left(HDL - C\ + \frac{TG}{2.2}\right)$ (Friedewald et al., 1972).

The lipid ratios were calculated as follows:

$$TC{:}\ HDL - C\ ratio\ = \frac{TC}{HDL - C}$$

and apoB/apoA ratio:

$$apoAratio\ = \frac{ApoB}{ApoA}$$

Non-HDL-C was calculated as follows:

$$Non - HDL - C = \ TC - HDL - C$$

4.8. Data analysis

Statistical Package for Social Sciences (SPSS) version 22.0 was used for data analysis. Data that was normally distributed (Gaussian distribution) was expressed as mean (± standard deviation) and the abnormally distributed or skewed data was expressed as median (interquartile range) and was logarithmic-transformed for further analysis. An independent sample student t- test was used to compare means of anthropometric measures, inflammatory markers and serum lipids between microalbuminuria subjects those without microalbuminuria. An independent sample student t-test was also used to compare anthropometric measures, inflammatory markers and serum lipids between subjects with and without microalbuminuria in different genders. ANCOVA was used to compare means after adjusting or age. One-way ANOVA was used to compare means of anthropometric measures, inflammatory markers and serum lipids amongst quartiles of ACR.

Linear regression analysis was used to explore the linear relationship between microalbuminuria measured as a continuous variable (ACR) and its predictors (serum lipids and inflammatory markers). Multivariate model and backward linear regression analyses were used to determine the association of microalbuminuria with serum lipid levels and inflammatory markers. In a backward linear model all predictors were entered in to the model, the weakest predictors were removed and the regression re-calculated, the predictors were re-entered if their removal weakened the model or otherwise removed.

Binary logistic regression was used to determine the predictors (serum lipids and inflammatory markers) of microalbuminuria as a categorical variable (presence or absence of microalbuminuria). A simple and multivariate logistic regression analysis was used to determine the association of microalbuminuria, serum lipids and inflammatory markers in participants with microalbuminuria. A simple logistic regression was used to determine bi-variate relationship between microalbuminuria and predictors. Predictors with a P value of less or equal 0.250 were entered in to the first adjusted model and those with a weakest prediction (with a p value of greater than 0.250) were removed and the model ran again to get the last adjusted model. A p-value of less or equal to 0.05 was considered significant for all the statistical tests in this study.

4.9. RELIABILITY and VALIDITY

Reliability is the extent to which results are consistent over time and an accurate representation of the total population under study (Joppe, 2000). Reliability in this study was assured by running controls with any sample and only accepting results where the controls were within the ranges. Validity determines whether the research truly measures that which it was intended to measure or how truthful the research results are (Joppe, 2000). In this study validity was assured by setting an aim and objective before the study was approved and following them in data collection and analysis. For every variable controls were used to determine the inter and intra assay CV with controls from one run used for intra assay CV and those from different runs used for intra assay CV.

4.10. BIAS

Bias is a systematic error that can prejudice evaluation findings in some way (Steinbock, 1978) The study is part of a larger study titled "Prevention control and integrated management of chronic disease in a rural area, South Africa" and a random selection method was used to select the participants from the population. In this present study participants who were enrolled in the larger study and had their urinary albumin and creatinine results were included.

4.11. ETHICAL APPROVAL

The proposal was approved by the Medunsa Research and Ethics Committee (MREC) project number MREC/HS/102/2014: PG prior to commencing with the study. The current study is part of a larger study and consent from the larger study was used as the blood samples were taken from those stored from the larger study. The aim, objectives and the possible consequences of the study were explained to the subjects prior to their participation in the study and they were also made aware of the fact that they are free to choose to participate or not and those who agree to participate in the study gave a written consent. Anonymity is the state of lacking individuality, distinction, or recognisability (Froomkin, 1995). Confidentiality is a method of preserving authorised restriction on information access (McCallister et al., 2010). In this study, information identifying participants and confidential information will be kept away from the data file and kept here there is restricted access.

CHAPTER FIVE

5. RESULTS

Of the 687 participants recruited into the study, 85 participants had diabetes diagnosed by fasting blood glucose of ≥ 7 mmol/l and/or the history of diabetes and 320 participants had hypertension diagnosed by a systolic blood pressure of ≥ 140 mmHg, diastolic blood pressure of ≥ 90 mmHg and/or history of hypertension. The prevalence of microalbuminuria in the total population was found to be 36.5 %, 40.5 % in those with hypertension and 23.6 % were those without diabetes and hypertension.

Of the 85 participants with diabetes 36 participants had microalbuminuria. The 85 participants with diabetes were excluded from the study as diabetes interferes with serum lipid, leaving a total of 602 participants. Of the six hundred and two (602) participants recruited for this study, 251 were men and 377 were women. Participants had a mean age of 48.63 ± 20.89 years. Participants were later grouped into quartiles of ACR with the lowest quartile having a lower ACR and the last quartile having a higher ACR.

Of all the measured variables age, BMI, WC, SBP, DBP, glucose, TC, HDL-C, LDL-C, Apo A, Apo B, TC/HDL-C, Non-HDL-C, eGFR and serum creatinine were normally distributed and were reported as mean ± standard deviation. However, ACR, insulin, IL-6, hs-CRP, TG, Lp (a) and ApoB/ApoA were skewed (either positive or negative) and were reported as median (interquartile range) and logarithmic transformed for further analysis.

5.1. Descriptive statistics of the total population.

Results of anthropometric and biochemical parameters in participants with and without MA are presented in **Table 5.1**. Normally distributed data is presented as mean ± standard deviation while skewed data is expressed as median (Interquartile range). Participants with MA were older than those without MA (51.74 ± 21.56 vs. 46.90 ± 20.34, p-value= 0.006). After adjusting for age, means of BMI and WC did not differ between participants with and without MA. Participants with MA had a significantly higher SBP (134.92 ± 30.14) compared to participants without MA (128.01 ± 20.77) at a p-value= 0.017 after adjusting for age). However, the levels of insulin were significantly higher in the participants with MA compared to the participants without MA with a median (interquartile range) of 7.48 (3.51 – 15.50) vs. 5.85 (3.23 – 9.32) at a p-value=0.000 after adjusting for age. HOMA index levels were found in this study to be significantly higher in participants with microalbuminuria as compared to those with normoalbuminuria (1.63 ± 1.19 vs 2.14 ± 1.51) with a p value of 0.000 after adjusting for age.

Participants with MA had a lower eGFR as compared to those without MA (94.31 ± 25.19 vs. 99.03 ± 23.30, with a p-value= 0.021 before adjusting for age). Still after controlling for age there was no significant difference (P-value> 0.100). Participants with microalbuminuria had a higher serum creatinine as compared to those without microalbuminuria (84.66 ± 21.15vs. 81.00 ± 18.92) with a p value of 0.035 before adjusting for age but after controlling the effect of age there was no significant difference (P-value> 0.077).

Table 5.1: Descriptive statistics of the total population and by microalbuminuria status

Variables	Total population	Normoalbuminuria	Microalbuminuria	P value	Age adjusted P value
Male/Female	225/377	137/250	88/127		
Number	602	387	215		
Age (years)	48.63 ± 20.89	46.90 ± 20.34	51.74 ± 21.56	**0.006**	---
ACR (mg/mmol)	2.04 (1.05 – 4.71)	1.25 (0.77 – 1.97)	5.93 (4.36 – 9.73)	**0.000**	**0.000**
BMI (Kg/m^2)	25.77 ± 7.00	25.54 ± 6.47	26.45 ± 7.54	0.136	0.211
WC (cm)	85.14 ± 14.28	85.19 ± 14.81	85.05 ± 13.31	0.911	0.307
SBP (mmHg)	130.47 ± 24.73	128.01 ± 20.77	134.92 ± 30.14	**0.003**	**0.017**
DBP (mmHg)	81.81 ± 14.05	81.27 ± 13.39	82.77 ± 15.15	0.229	0.448
Glucose (mmol/l)	5.15 ± 0.84	5.16 ± 0.85	5.12 ± 0.82	0.517	0.4058
Insulin (µIU/L)	6.23 (3.33 – 11.85)	5.85 (3.23 – 9.32)	7.48 (3.51 – 15.50)	**0.000**	**0.000**
HOMA	1.81 ± 1.33	1.63 ± 1.19	2.14 ± 1.51	**0.000**	**0.000**
IL-6 (pg/ml)	3.09 (1.96 – 4.94)	3.00 (1.94 – 5.07)	3.14 (2.00 – 4.89)	0.495	0.433
Hs-CRP (mg/L)	1.50 (0.56 – 4.28)	1.60 (0.58 – 4.46)	1.34 (0.50 – 3.98)	0.270	0.151
TC (mmol/l)	4.45 ± 1.23	4.41 ± 1.29	4.53 ± 1.09	0.263	0.753
HDL-C (mmol/l)	1.34 ± 0.51	1.34 ± 0.58	1.34 ± 0.34	0.991	0.992
LDL-C (mmol/l)	2.54 ± 1.09	2.51 ± 1.17	2.58 ± 0.91	0.482	0.978
TG (mmol/l)	1.11 (0.73 – 1.68)	1.11 (0.72 – 1.57)	1.10 (0.74 – 1.82)	0.141	0.491
Lp (a) (mg/dl)	48.55 (25.80 – 79.70)	49.50 (23.40 – 80.40)	48.10 (26.00 – 79.70)	0.629	0.569
Apo A (mg/dl)	147.79 ± 31.92	146.06 ± 32.34	150.82 ± 31.01	0.084	0.138
Apo B (mg/dl)	85.01 ± 26.16	84.65 ± 27.08	85.63 ± 24.54	0.665	0.667
ApoB/ApoA	0.58 (0.45 – 0.72)	0.59 (0.45 – 0.73)	0.58 (0.46 – 0.67)	0.383	0.158
TC/HDL-C	3.52 ± 1.13	3.51 ± 1.14	3.54 ± 1.12	0.761	0.619
Non-HDL-C (mmol/l)	3.12 ± 1.19	3.08 ± 1.27	3.19 ± 1.04	0.289	0.860
eGFR (ml/min/1.73m^2)	97.34 ± 24.07	99.03 ± 23.30	94.31 ± 25.19	**0.021**	0.100
Creatinine (µmol/L)	82.31 ± 19.81	81.00 ± 18.92	84.66 ± 21.15	**0.035**	0.077

5.2. Results on anthropometric and biochemical parameters by gender

In **Table 5.2** anthropometric and biochemical parameters are presented by gender for those with and without MA. Normally distributed data is presented as mean ± standard deviation and skewed data expressed as median (Interquartile range). Women with MA were significantly older than those without MA (53.66 ± 19.90 vs. 48.25 ± 19.55p-value= 0.012). Participants with MA had a higher SBP than participants without MA (134.94 ± 30.60 vs. 125.28 ± 21.51 age adjusted p-value= 0.007).

Men with and without MA had similar levels of glucose and in women there was no significant difference in levels of glucose between participants with and without MA (5.05 ± 0.84 vs. 5.22 ± 0.85) but after adjusting for age the difference was found to be significant at a p-value= 0.043. Men with MA were found to have higher levels of insulin compared to men without MA but after adjusting for age the difference was non-significant. Insulin levels were significantly higher in women with MA as compared to women without MA [8.09 (4.62 – 15.60) vs. 6.11 (3.23 – 9.68), after adjusting for age (p-value= 0.000). HOMA index levels were found in this study to be significantly higher in men with microalbuminuria as compared to those with normoalbuminuria (1.56 ± 1.18 vs 2.02 ± 1.57) with a p value of 0.030 after adjusting for age. In women with microalbuminuria HOMA was higher compared to those with normoalbuminuria (1.67 ± 1.19 vs 2.22 ± 1.46) with a p value of 0.000 after adjusting for age. Before adjusting for age, the median hs-CRP were similar between men with and without MA but after adjusting for age the median hs-CRP was significantly higher in men without MA compared to men with MA [1.41 (0.48 – 2.06) vs. 0.92 (0.44 – 2.06) at p-value= 0.013].

Women with MA had a higher median TG as compared to women without [1.19 (0.82 – 1.88) vs. 1.06 (0.68 – 1.66), at p-value= 0.028] but after controlling for age the difference was not significant (P-value> 0.05). Women with MA were found to have a lower eGFR as compared to women without MA (96.17 ± 22.64 vs. 88.76 ± 24.47) with a p-value of 0.004 before adjusting for age and P= 0.028 after adjusting while in men no significant difference in eGFR was observed. The levels of creatinine were found to be comparable between men with and without MA after adjusting for age (P-value= 0.860) but the levels were higher in women with as compared to those without MA with a p-value= 0.034 after adjusting for age.

Women without MA had a higher BMI and a higher WC compared to men while women with MA had a higher BMI and WC compared to men. Systolic blood pressure (SBP) was higher in

women without MA as compared to men without MA. Women with MA had a significantly higher hs-CRP compared to men.

Women with MA had a higher TC, TC/HDL-C, Apo B, ApoB/ApoA and non-HDL-C but lower eGFR as compared to men with microalbuminuria. Women without MA had a higher Apo B and lower eGFR and creatinine compared to men without MA.

Table 5.2: Descriptive statistics of participants by gender

Variables	Male		P value	Age adjusted	Female		P value	Age adjusted
	Normoalbuminuria	**Microalbuminuria**		P value	**Normoalbuminuria**	**Microalbuminuria**		P value
Number	137	88			250	127		
ACR (mg/mmol)	1.12 (0.71 – 1.75)	5.09 (3.43 – 6.72)	**0.000**	**0.000**	1.36 (0.82 – 2.08) #	6.94 (4.83 – 10.73) #	**0.000**	**0.000**
Age (years)	44.45 ± 21.57	48.97 ± 23.59	0.141	---	48.25 ± 19.55	53.66 ± 19.90	**0.012**	---
BMI (Kg/m^2)	23.30 ± 4.85	24.03 ± 6.79	0.380	0.469	26.77 ± 6.91 #	27.66 ± 8.25 #	0.267	0.321
WC (cm)	80.18 ± 12.79	79.81 ± 11.09	0.825	0.430	87.90 ± 15.14 #	88.74 ± 13.55 #	0.599	0.862
SBP (mmHg)	132.98 ± 18.38	134.89 ± 29.64	0.591	0.987	125.28 ± 21.51 #	134.94 ± 30.60	**0.002**	**0.007**
DBP (mmHg)	81.80 ± 13.74	81.06 ± 15.61	0.718	0.554	80.98 ± 13.21	83.93 ± 14.78	0.051	0.144
Glucose (mmol/l)	5.07 ± 0.85	5.22 ± 0.79	0.186	0.215	5.22 ± 0.85	5.05 ± 0.84	0.067	0.043
Insulin (µIU/L)	5.36 (3.18 – 9.30)	5.65 (3.20 – 15.18)	0.042	0.239	6.11 (3.23 – 9.68)	8.09 (4.62 – 15.60)	**0.000**	**0.000**
HOMA	1.56 ± 1.18	2.02 ± 1.57	0.000	0.030	1.67 ± 1.19	2.22 ± 1.46	**0.012**	**0.000**
IL-6 (pg/ml)	2.94 (1.94 – 5.48)	3.25 (1.96 – 4.68)	0.511	0.637	3.09 (1.94 –4.68)	3.11 (2.02 – 4.98)	0.888	0.522
Hs-CRP (mg/L)	1.41 (0.48 – 2.06)	0.92 (0.44 – 2.06)	0.089	0.013	1.86 (0.68 – 5.33)	1.78 (0.58 – 5.42) *	0.915	0.467
TC (mmol/l)	4.33 ± 1.05	4.34 ± 1.09	0.903	0.865	4.64 ± 1.41	4.65 ± 1.09 *	0.161	0.616
HDL-C (mmol/l)	1.31 ± 0.32	1.37 ± 0.31	0.170	0.115	1.36 ± 0.68	1.32 ± 0.36	0.565	0.464
LDL-C (mmol/l)	2.42 ± 0.93	2.41 ± 0.94	0.946	0.734	2.57 ± 1.29	2.69 ± 0.87	0.293	0.754
TG (mmol/l)	1.21 (0.77 – 1.51)	0.95 (0.61 – 1.77)	0.685	0.064	1.06 (0.68 – 1.66)	1.19 (0.82 – 1.88)	0.028	0.233
Lp (a) (mg/dl)	48.60 (27.70 – 89.80)	53.20 (26.85 – 88.40)	0.939	0.766	50.90 (22.30 – 79.40)	43.70 (25.20 – 76.30)	0.462	0.656
Apo A (mg/dl)	147.47 ± 30.65	155.41 ± 32.82	0.070	0.061	145.28 ± 33.28	147.59 ± 29.37	0.514	0.852
Apo B (mg/dl)	80.16 ± 24.15	79.79 ± 21.28	0.909	0.628	87.90 ± 28.32 *	89.72 ± 25.90 *	0.400	0.999
ApoB/ApoA	0.56 (0.41 – 0.71)	0.51 (0.40 – 0.63)	0.407	0.508	0.60 (0.46 – 0.73)	0.60 (0.50 – 0.73) *	0.836	0.908
TC/HDL-C	3.44 ± 1.01	3.31 ± 1.14	0.395	0.189	3.54 ± 1.20	3.69 ± 1.08 *	0.272	0.669
Non-HDL-C(mmol/l)	3.01 ± 0.99	2.98 ± 1.07	0.810	0.550	3.3.12 ± 1.40	3.33 ± 0.99 *	0.110	0.546
eGFR (ml/min/1.73m^2)	104.24 ± 23.65	102.33 ± 24.15	0.558	0.696	96.17 ± 22.64 #	88.76 ± 24.47 #	**0.004**	**0.028**
Creat (µmol/L)	87.60 ± 21.44	87.49 ± 20.86	0.970	0.860	77.39 ± 16.34 #	82.71 ± 21.20	**0.007**	**0.034**

**-P value of less/equal than 0.05 and #-P value of less/equal than 0.001 vs. males*

5.3. Serum lipids and inflammatory markers by quartiles of ACR

In **Table 5.3** the results of anthropometric and biochemical parameters are presented in quartiles of ACR. Normally distributed data is presented as mean ± standard deviation and skewed data is expressed as median Interquartile range. ACR is expressed as mean (confidence interval).

The mean age increased from the first to the second quartile, decreased from the second to the third quartile and increased from the third to the last quartile ($p \leq 0.001$) The mean SBP increased from the first to the second quartile, decreased from the second to the third quartile and increased from the third to the last quartile of ACR ($p \leq 0.001$). The levels of insulin increased from the first quartile to the second quartile of ACR, decreased from the second to the third quartile of ACR and then increased from the third to the fourth quartile in this population ($p \leq 0.001$). The levels of HOMA index increased from the first quartile to the second quartile of ACR, decreased from the second to the third quartile of ACR and then increased from the third to the fourth quartile in this population ($p = 0.000$).

In this population serum TC, Apo A and TG levels were found to increase from the first to the second quartile, decrease from the second quartile to the third quartile and increase from the third to the fourth quartile of ACR (p 0.037, 0.02 and 0.013 respectively).

Table 5.3: Biochemical and anthropometric variables by ACR quartiles

Variables	1st quartile (0.075 – 1.054)	2nd quartile (1.057–2.043)	3rd quartile (2.049-4.732)	4th quartile (4.771-34.469)	P trend
Number	150	151	151	150	
Male/Female	62/88	56/95	57/94	50/100	
Age (years)	46.45 ± 19.62	48.34 ± 21.14	45.62 ± 20.79	54.29 ± 21.13	**0.001**
BMI (Kg/m^2)	25.58 ± 6.91	24.66 ± 5.71	25.87 ± 7.57	26.72 ± 7.79	0.087
WC (cm)	85.06 ± 14.70	84.89 ± 14.63	85.00 ± 14.70	85.74 ± 13.14	0.956
SBP (mmHg)	127.94 ± 19.15	129.96 ±22.10	124.84 ± 20.46	139.28 ± 32.19	**0.000**
DBP (mmHg)	81.16 ± 12.12	81.34 ± 14.51	81.28 ± 13.68	83.55 ± 15.64	0.397
Glucose (mmol/l)	5.20 ± 0.86	5.12 ± 0.90	5.15 ± 0.73	5.10 ± 0.87	0.753
Insulin (µIU/L)	5.73 (2.94 – 9.38)	6.20 (3.50 – 10.11)	5.31 (2.77 – 9.30)	9.16 (4.22 – 16.23)	**0.000**
HOMA	1.158 ± 1.15	1.78 ± 1.26	1.56 ± 1.19	2.32 ± 1.54	**0.000**
IL-6 (pg/ml)	3.23 (1.10 – 5.57)	2.12 (1.61 – 4.02)	2.59 (1.98 – 3.57)	3.29 (1.80 – 4.40)	0.256
Hs-CRP (mg/L)	1.68 (0.54 – 4.33)	1.81 (0.65 – 4.98)	1.14 (0.55 – 3.48)	1.55 (0.55 – 4.27)	0.505
TC (mmol/l)	4.34 ± 1.29	4.62 ± 1.33	4.28 ± 1.15	4.57 ± 1.10	**0.037**
HDL-C (mmol/l)	1.25 ± 0.34	1.34 ± .77	1.14 ± 0.76	1.43 ± 0.86	0.071
LDL-C (mmol/l)	2.52 ± 1.11	2.64 ± 119	2.40 ± 1.13	2.58 ± 0.88	0.303
TG (mmol/l)	1.19 (0.75 – 1.54)	1.2 (082 – 1.67)	0.94 (0.59 – 1.58)	1.15 (0.81 – 1.89)	**0.013**
Lp (a) (mg/dl)	45.90 (27.85 – 77.38)	44.70 (20.30 – 79.70)	56.00 (26.90 – 88.00)	52.10 (27.73 – 82.25)	0.781
Apo A (mg/dl)	142.13 ± 30.64	151.40 ± 30.94	145.31 ± 35.47	152.03 ± 29.60	**0.020**
Apo B (mg/dl)	85.17 ± 26.57	86.00 ± 27.95	81.25 ± 24.82	87.01 ± 24.56	0.261
ApoB/ApoA	0.63 (0.46 – 0.73)	0.57 (0.43 – 0.72)	0.58 (0.45 – 0.68)	0.58 (0.47 – 0.67)	0.386
TC/HDL-C	3.62 ± 1.18	3.55 ± 1.13	3.35 ± 1.14	3.55 ± 1.06	0.204
Non-HDL-C (mmol/l)	3.10 ± 1.19	3.25 ± 1.31	2.91 ± 1.19	3.23 ± 1.03	0.052
eGFR (ml/min/1.73m^2)	99.99 ± 23.00	98.75 ± 24.84	99.30 ± 22.35	91.24 ± 25.21	**0.005**
Creatinine (µmol/L)	80.90 ± 18.07	81.92 ± 21.09	80.95 ± 19.00	85.73 ± 20.77	0.112

5.4. Association of serum lipids and inflammatory markers with ACR

In **Table 5.4** the multivariate linear regression serum lipids and inflammatory markers with ACR are presented in men and in women.

Age was found not to be significantly associated with ACR in men and women with P values of 0.348 and 0.062 respectively. In men and in women ACR was not associated with BMI and

WC. Systolic blood pressure (SBP) was positively associated with ACR in women (beta value of 0.498 and p-value of 0.014).

Glucose and insulin showed no significant association with ACR in men and glucose was also not associated with ACR in women. Insulin levels were negatively associated with ACR in women (p-values of 0.041). The levels of TG were not associated with ACR in men while in women TG was positively associated with ACR (p value of 0.029).

Table 5.4: Association of serum lipids and inflammatory markers with ACR

Variable	**Males(225)**		**Females(377)**	
	ACR		**ACR**	
	Beta	**P value**	**Beta**	**P value**
Age (years)	0.195	0.348	-0.276	0.062
BMI (Kg/m^2)	-0.203	0.300	-0.320	0.053
WC (cm)	0.022	0.941	0.057	0.729
SBP (mmHg)	0.145	0.627	0.498	**0.014**
DBP (mmHg)	0.059	0.822	-0.109	0.468
Glucose (mmol/l)	0.190	0.293	-0.026	0.821
Insulin (µIU/L)	0.244	0.376	-0.301	**0.041**
IL-6 (pg/ml)	-0.073	0.674	0.060	0.632
Hs-CRP (mg/L)	0.009	0.962	0.039	0.757
TC (mmol/l)	0.081	0.934	-1.678	0.168
HDL-C (mmol/l)	-0.424	0.449	0.743	0.194
LDL-C (mmol/l)	0.215	0.771	1.200	0.180
TG (mmol/l)	0.377	0.219	0.844	**0.029**
Lp (a) (mg/dl)	0.197	0.266	-0.087	0.469
ApoB/ApoA	0.190	0.409	0.102	0.483
TC/HDL-C	-0.975	0.211	0.317	0.432

5.5. Backward linear regression of ACR with biochemical parameters

In **Table 5.5** the backward linear regression of serum lipids and inflammatory markers with ACR in men and in women is presented. The backward linear regression showed that ACR was associated with triglycerides in men (p-value = 0.018) and with BMI (p value = 0.025), SBP (p value = 0.004), insulin (p value = 0.033) and triglycerides (p value = 0.002) in women.

Table 5.5: Backward linear regression of ACR with biochemical parameters

Variable	Males (225) ACR		Females (377) ACR	
	Beta	**P value**	**Beta**	**P value**
Age (years)			-0.209	0.082
BMI (Kg/m^2)			-0.231	**0.025**
SBP (mmHg)			0.451	**0.004**
Insulin (µIU/L)	0.226	0.099	-0.284	**0.033**
TG (mmol/l)	0.372	**0.018**	0.354	**0.002**
Lp (a) (mg/dl)	0.252	0.075		
TC/HDL-C	-0.287	0.059		

5.6. Association of serum lipids and inflammatory markers with microalbuminuria

5.6.1. Predictors of microalbuminuria(Simple logistic regression)

In **Table 5.6 and 5.7** predictors of microalbuminuria were represented in an unadjusted regression model. The variables were categorized and participants in the normal group were considered as the reference group. The relationship between age and microalbuminuria was found to be significant in women with women aged more or equal 60 years old being 2.190 (P value = 0.004) more likely to have microalbuminuria as compared to women aged less or equal 39 years old. A significant association was found between microalbuminuria and BMI in men with the obese men being 2.740 (P value = 0.019) more likely to have microalbuminuria than those with normal weight. In women with hypertension were more likely to have microalbuminuria as compared to the normotensive women (p value of 0.001)

In men glucose and insulin were found not to be associated with MA in a crude model. Women with glucose of between 5.6 mmol/L and 7.0 mmol/L were less likely to have microalbuminuria than their counterparts with glucose of less than 5.6 (OR of 0.541 and P value of 0.014). Women in the last quartile of insulin (Insulin resistant) were 2.078 (P value of 0.044) more likely to have MA than those in the first quartile of insulin (Insulin sensitive).

Table 5.6: Predictors of microalbuminuria (Unadjusted model)

Variable		Number of MA participants	Number of MA men	Men		Number of MA women	Women	
				Odds ratio	P value		Odds ratio	P value
Age	≤ 39	67	35	1 (Ref)		32	1 (Ref)	
	40 - 59	54	17	1.122	0.755	37	1.105	0.724
	≥60	94	35	1.681	0.093	59	2.190	**0.004**
BMI	Normal weight	90	44	1 (Ref)		46	1 (Ref)	
	Overweigh	47	15	0.782	0.498	32	0.971	0.917
	Obese (≥ 30 kg/m^2)	60	17	2.740	**0.019**	43	1.320	0.289
WC	Normal	113	76	1 (Ref)		37	1 (Ref)	
	High (≥80-F& ≥94-M)	100	11	0.762	0.497	89	0.930	0.762
Hypertension	Normotensive	104	49	1 (Ref)		55	1 (Ref)	
	Hypertensive	111	38	0.828	0.492	73	2.030	**0.001**
Glucose	Normal	149	57	1 (Ref)		92	1(Ref)	
	High (≥ 5.6 mmol/l)	65	30	1.630	0.104	35	0.541	**0.014**
Insulin	First quartile	44	25	1 (Ref)		20	1 (Ref)	
	Last quartile	78	28	1.493	0.313	50	2.078	**0.044**
HOMA	Normal	132	55	1 (Ref)		77	1 (Ref)	
	High (2.5)	82	32	2.238	0.001	50	2.286	**0.007**
IL-6	Normal	63	29	1 (Ref)		34	1 (Ref)	
	High	12	6	0.989	0.986	6	1.372	0.661
Hs-CRP	Normal	145	71	1 (Ref)		74	1 (Ref)	
	High (≥ 3 mg/l)	70	16	0.617	0.153	54	1.230	0.352
TC	Normal	136	57	1 (Ref)		79	1 (Ref)	
	High (≥ 5.0 mmol/l)	71	25	1.387	0.296	46	1.096	0.689
HDL-C	Normal	142	49	1 (Ref)		93	1 (Ref)	
	Low (1.1 and 1.3 mmol/l)	61	31	0.557	0.098	30	0.976	0.915
LDL-C	Normal	95	40	1 (Ref)		55	1 (Ref)	
	High (≥ 3.0 mmol/l)	107	40	1.014	0.965	67	0.971	0.901

Table 5.7: Predictors of microalbuminuria (Unadjusted model) continued

TG	**Normal**	**140**	**58**	**1 (Ref)**		**82**	**1 (Ref)**	
	High (≥ 1.7 mmol/l)	65	22	1.473	0.250	43	1.597	0.058
Lp (a)	Normal	42	19	1 (Ref)		23	1 (Ref)	
	High (≥ 30 mg/dl)	94	36	0.912	0.795	58	0.976	0.931
ApoB/ApoA	Normal	194	84	1 (Ref)		110	1 (Ref)	
	High (0.86-F & 0.97-M)	15	1	0.368	0.375	14	0.727	0.345
TC/HDL-C	Normal	172	71	1 (Ref)		101	1 (Ref)	
	High	31	9	1.259	0.616	22	1.148	0.628

5.6.2. Multivariate regression analysis of predictors of microalbuminuria

In **Table 5.8** the multivariate logistic regression adjusted model of the association of MA with serum lipids and inflammatory markers in men is represented. The second model was considered because of the high classification accuracy as compared to the first model. The variables included in tables 5.7 are the remaining predictors in each model after the backward logistic regression model and removal of weak associations (p >0.250).

For the first adjusted model variables with a p-value of ≤ 0.250 on the unadjusted model were included. The first adjusted model had a classification accuracy of 68.6%, -2 Log Likelihood of 249.837, Chi square of 29.265, Hosmer and Lemeshow test of 57.3 % and Negelkerke R Square of 17.7 %. Men with a high CRP were less likely to have microalbuminuria with an odds ratio of 0.379, p value of 0.028 as compared to those with a normal CRP. The CRP result is unexpected with no explanation but this could be due to the method of diagnosis of microalbuminuria used in the present study using one result as opposed to three results over three months' period thus leading to miss diagnosis of some patients. In the first adjusted model, men with a high HOMA index were more likely to have microalbuminuria than those with a normal HOMA index with an OR of 2.430 and a p value of 0.028.

The second adjusted model had a classification accuracy of 67.1 %, -2 Log Likelihood of 250.180, Chi square of 28.922, Hosmer and Lemeshow test of 77.0 % and Negelkerke R Square of 17.5 %. In this model MA was not significantly associated with BMI and glucose in men. Men with a high CRP were found to be less likely to have MA than those with a normal CRP

with the odds ratio of 0.204 (p value of 0.004). In this model, men with a high HOMA index were more likely to have microalbuminuria than those with a normal HOMA index with an OR of 2.982 and a p value of 0.004. Men with both a high TG and a High CRP were found to be more likely to have microalbuminuria in this model with an odds ratio of 9.434 and a p value 0.007 as compared to men with both a normal TG and a normal CRP.

Table 5.8: Multivariate regression analysis of microalbuminuria with serum lipids and inflammatory markers in men

Variable		Adjusted model 1		Adjusted model 2	
		Odds ratio	P value	Odds ratio	P value
Age	≤ 39	1 (Ref)			
	40 – 59	1.213	0.696		
	≥60	1.384	0.436		
BMI	Normal weight	1 (Ref)		1 (Ref)	
	Overweight	0.467	0.080	0.514	0.109
	Obese	2.070	0.143	1.812	0.226
Glucose	Normal	1 (Ref)			
	High	1.196	0.634		
Hs-CRP	Normal	1 (Ref)		1 (Ref)	
	High	0.379	**0.028**	0.204	**0.004**
HDL-C	Normal	1 (Ref)			
	Low	0.882	0.719		
HOMA	Normal	1 (Ref)		1 (Ref)	
	High	2.430	**0.028**	2.982	**0.004**
TG	Normal	1 (Ref)			
	High	1.521	**0.295**		
Hs-CRP*TG	Normal CRP*Normal TG			1 (Ref)	
	High CRP*High TG			9.434	**0.007**
Classification accuracy		63.8 %		67.1 %	
Hosmer and Lemeshow test		75.4 %		77.0 %	
-2 Log Likelihood		257.383		250.180	
Chi Square		21.719		28.922	
Negelkerke R Square		13.4 %		17.5 %	

5.6.3. Multivariate regression analysis of microalbuminuria with serum lipids and inflammatory markers in women

Table 5.9 presents the multivariate logistic regression adjusted models of the association of MA with serum lipids and inflammatory markers in women. The second model was considered

because of the high classification accuracy as compared to the first model. The variables included in tables 5.8 are the remaining predictors in each model after the backward logistic regression model and removal of weak associations (p >0.250).

For the first adjusted model variables with a p-value of ≤ 0.250 on the unadjusted model were included. The first adjusted model had a classification accuracy of 70.1 %, -2 Log Likelihood of 426.429, Chi square of 36.743, Hosmer and Lemeshow test of 13.5 % and Negelkerke R Square of 13.4 %. In the first adjusted model in women, those aged more or equal 60 years were found to be 1.893 more likely to have microalbuminuria (P-value of 0.046) compared to those with an age of less or equal 39 years old. Women falling in the last quartile of insulin were more likely to have MA with an OR of 5.090 in this model (P-value of 0.033) in this model.

The second adjusted model had a classification accuracy of 72.5 %, -2 Log Likelihood of 407.273, Chi square of 39.382, Hosmer and Lemeshow test of 18.7 % and Negelkerke R Square of 14.8 %. Women with an age of more or equal 60 years were more likely to have MA with the odds ratio of 1.819 compared to women with an age of less or equal 39 years with a p value of 0.047.Women with a high glucose were found to be less likely to have MA compared to women with a normal glucose (odds ratio of 0.597 and P-value of 0.040) while those with in the last quartile of insulin were more likely to have MA with an OR of 2.905 compared to those in the first quartile of insulin in this model with a p-values = 0.003,.

Table 5.9: Multivariate regression analysis of microalbuminuria with serum lipids and inflammatory markers in women

Variable		Adjusted model 1		Adjusted model 2	
		Odds ratio	**P value**	**Odds ratio**	**P value**
Age	≤ 39	1 (Ref)		1 (Ref)	
	40 – 59	0.959	0.895	0.930	0.814
	≥60	1.893	**0.046**	1.819	**0.047**
Hypertension	Normotensives	1 (Ref)		1 (Ref)	
	Hypertensive	1.382	0.232	1.321	0.293
Glucose	Normal	1 (Ref)		1 (Ref)	
	High	0.640	0.086	0.597	**0.040**
Insulin	First quartile	1 (Ref)		1 (Ref)	
	Last quartile	5.090	**0.033**	2.905	**0.003**
TG	Normal	1 (Ref)			
	High	1.205	0.496		
HOMA	Normal	1 (Ref)			
	High	0.545	0.393		
Classification accuracy		70.1 %		72.5 %	
Hosmer and Lemeshow test		13.5 %		18.7 %	
-2 Log Likelihood		426.429		407.273	
Chi Square		36.743		39.382	
Negelkerke R Square		13.4%		14.8 %	

CHAPTER SIX

6. DISCUSSION

The aim of the current study was to assess the association of microalbuminuria with serum lipids and inflammatory markers in a rural black population. The levels of serum lipids and inflammatory markers were similar in participants with and without microalbuminuria. After dividing the participants by gender no difference was found and no trend was found in the serum lipids and inflammatory marker but the P trend for TG, TC and Apo A were significant though showing no real trend. In a linear regression model TG was the only lipid parameter found to have an association with ACR with an R-value of 0.372 (P value of 0.018) and 0.354 (P value of 0.002) for men and women respectively. None of the inflammatory markers were associated with microalbuminuria. Only CRP showed negative association with microalbuminuria in men with an OR of 0.204 (P value of 0.004) while in women no association was found.

The interactions of serum lipids and inflammatory markers were tested; CRP and TG interacted and contributed to the association. Men with a high CRP and a high TG were found to be more likely to have microalbuminuria, with an OR of 9.434 and a p value of 0.007. To the best of our knowledge this is the first study to report such an association between microalbuminuria and TG and Hs-CRP. The reason for this association is not known as only CRP and TG were collectively associated with microalbuminuria. The interaction could explain the association of microalbuminuria with chronic kidney disease (CKD) and cardiovascular disease (CVD) (Ibsen et al., 2005, Ridker et al., 2003, Yusuf et al., 2004). C reactive protein (CRP) has been reported to be increased in patients with CKD as compared to controls (Adejumo et al., 2016). Serum triglycerides levels have been acknowledged by the American Medical Association as important risk factors for CVD and death (Labreuche et al., 2010, Varbo et al., 2011). Furthermore low grade inflammation was found to be associated with the risk of developing CVD (Ridker, 2003) and CRP has been established as an important predictor of cardiovascular events in CKD patients (Abraham et al., 2009, Jalal et al., 2012).

The results of the present study show a significant association of the ACR levels with the levels of TG. The results are not in agreement with studies on hypertensive patients where no significant association was found between microalbuminuria and TG (Busari et al., 2010, Sheng et al., 2011) but in agreement with studies on diabetic patients where microalbuminuria

was positively associated with TG (Mattock et al., 2001, Retnakaran et al., 2006, Tseng, 2009). However, in the logistic regression model no significant association was found between microalbuminuria and TG. The result is in agreement with other studies which also found no association between microalbuminuria and TG (Ozkurt et al., 2007, Tseng, 2009). The reason for the finding of the association in a linear model and not in the logistic model could be that the linear regression model looks at the concentration of TG and relate it to that of ACR while the logistic model looks at TG as a predictor of microalbuminuria. Thus this means that the concentration of TG in this study may lead to an increase in ACR but can't predict microalbuminuria.

In the present study an association between microalbuminuria and HDL-C was found, with participants with a low HDL-C were found to be less likely to have microalbuminuria as compared to their counterparts with a normal HDL-C in men but not in women. The results imply that participants with a lower HDL-C were less likely to have microalbuminuria as compared to normal HDL-C contrary to what other studies have reported, which imply that a low HDL-C is associated with microalbuminuria (Busari et al., 2010, Mattock et al., 2001). This result is an unexpected with no explanation but this could be due to the method of diagnosis of microalbuminuria used in the present study using one result as opposed to three results over three months' period thus leading to a miss diagnosis of some patients. This could also be because most of the studies that have found the association were involving diabetes and in the current study diabetes patients were excluded.

In the present study the concentrations of serum lipids such as TC, TG, HDL-C and LDL-C were found to be similar in participants with and without microalbuminuria which is in agreement with results of other studies which found the concentrations of TC, TG and LDL-C were similar in hypertensive patients with and without microalbuminuria (Poudel et al., 2012, Shoji et al., 2001). However, the difference was in the levels of HDL-C which were increased in the hypertensive patients with microalbuminuria in the two studies but in the present study the levels were similar between those with and without microalbuminuria.

However, when looking at lipid levels by quartiles of ACR, the concentrations of serum lipids which showed no real trend but was still significant. This is in agreement with results of (Poudel et al., 2012, Shoji et al., 2001) similar concentration of serum lipids but decreased HDL-C in hypertensive patients with microalbuminuria. The present study showed that in men and in women concentrations of Lp (a), Apo A, Apo B, ratio of ApoB/ApoA, non-HDL-C and

TC/HDL-C were found to be similar in participants with and without microalbuminuria, the concentration of Lp (a), Apo B, ratio of ApoB/ApoA and TC/HDL-C were similar in all quartiles of ACR. This is in agreement with other studies which found no significant difference in the concentration of serum lipids (Kim et al., 2001, Poudel et al., 2012).

The hypothesis that microalbuminuria is associated independently with low grade inflammation was not supported in this study. Microalbuminuria was found not to be associated with the inflammatory markers (CRP and IL-6) and this is in agreement with other studies that found no association between microalbuminuria and inflammatory markers (Ng et al., 2008, Palmieri et al., 2003, Perticone et al., 2007, Zahran et al., 2012). However other studies have reported a positive association between microalbuminuria and inflammatory markers (CRP and IL-6) (Moubarak et al., 2012, Sabanayagam et al., 2010, Xu et al., 2014, Yu et al., 2010). The discrepancies of the results of the present study with other studies may be due to difference in the methods used to determine and diagnose microalbuminuria.

Women with and without microalbuminuria had similar concentrations of IL-6 and hs-CRP while men with microalbuminuria had a lower Hs-CRP concentration as compared to their counterparts without microalbuminuria but similar concentration of IL-6. Studies have reported similar concentrations of CRP (Palmieri et al., 2003, Perticone et al., 2007) and IL-6 (Moriwaki et al., 2003) and this is in agreement with the results of the present study where the concentration were similar in those with and without microalbuminuria and in quartiles of ACR. However, men with microalbuminuria showed a low hs-CRP than those with microalbuminuria. A study by Lokoski and colleagues (Lakoski et al., 2006) reported a high CRP in women across all ethnicity and also proposed that a gender specific cut-off be determined for determining CV risk. The reason for the low CRP in men and not in women could be the difference in the concentrations.

The results of the present study found that women aged more or equal 60 years old were found to have a 1.891 (p value of 0.047) OR in women of having microalbuminuria and the results are in agreement with other studies that reported age to be associated with microalbuminuria (Konta et al., 2006, Yuyun et al., 2005, Yuyun et al., 2004). The results support the hypothesis that an increasing age is associated with microalbuminuria. These findings are also supported by findings of Cores hand colleagues (Coresh et al., 2005) that old age is associated with microalbuminuria. Measures of obesity like BMI and WC were similar in participants with and without microalbuminuria, body mass index and WC were similar among quartiles of ACR

and the results of the present study are in agreement with other studies (Yuyun et al., 2004, Perticone et al., 2007, Poudel et al., 2012).

In a study by Thoenes and colleagues (Thoenes et al., 2009) an inverse relationship between microalbuminuria and BMI was reported and this is in consistent with the results of the present study which reported an inverse association between ACR and BMI after a backward linear model in women but not in men. Of the two measures of obesity (BMI and WC) none was found to predict microalbuminuria in men or women. Other studies reported no significant association of microalbuminuria with BMI, WC and WHR (Hoffmann et al., 2001, Yersin et al., 2000). However, analyses from the DCCT/EDIC population in diabetic men and women showed WHR to be associated with an abnormal urinary albumin excretion rate (de Boer et al., 2007, Sibley et al., 2006). The results of the present study show that in this population older participants are more likely to have microalbuminuria; as such they should be screened for microalbuminuria. However, a high WC was found to be protective of microalbuminuria.

In the total population participants with microalbuminuria had a higher SBP as compared to their counterparts without microalbuminuria, levels of SBP were found to increase with the increase in quartiles of ACR and no significant difference of SBP was found in men with and without microalbuminuria while women with microalbuminuria showed a higher SBP as compared to women without microalbuminuria. The results of the present study are supported by other studies which found SBP to be increased in participants with microalbuminuria (Busari et al., 2010, Ozkurt et al., 2007, Yuyun et al., 2004). The results of the present study are different to those of Ozkurt and colleagues (Ozkurt et al., 2007). Ozkurt and colleagues reported that men with microalbuminuria had a higher SBP as compared to those without microalbuminuria (Ozkurt et al., 2007). The difference between the current study and the study by Ozkurt and colleagues (Ozkurt et al., 2007) is the sample size, the method used to diagnose microalbuminuria as in the study by Ozkurt and colleagues, a urinary albumin excretion rate was used (Ozkurt et al., 2007) as compared to the creatinine adjusted and gender specific ACR used in the present study as in men the muscle mass could contribute more to the ACR than in women.

In the total population participants with microalbuminuria and those without microalbuminuria had comparable levels of DBP; the levels of DBP were similar in all quartiles of ACR. The levels of DBP were comparable in both genders with and without microalbuminuria. The results of the present study are sin agreement with results from other studies which found only

SBP to be increased in microalbuminuria while DBP remains similar (Ghosh et al., 2012, Ozkurt et al., 2007, Tseng, 2009, Wang et al., 2012). However, the results are different from those of Poudel and colleagues (Poudel et al., 2012) where both SBP and DBP were increased in microalbuminuria patient but Poudel and colleagues (Poudel et al., 2012) did not use gender specific cut off of ACR for diagnosis of microalbuminuria.

Among participants with hypertension, the prevalence of microalbuminuria in the present study was 40.5 %. Compared to what other researchers reported in South Africa, a lower prevalence of 19.5% has been reported in Gugulethu, Western Cape (Okpechi et al., 2007). The discrepancy of the findings may be due to the different method of definition of microalbuminuria because in the present study, gender-specific cut offs were used while the study from Gugulethu did not. Also, an HIV test was not performed so only those on treatment of HIV were excluded in a Gugulethu study while in our present study HIV test was performed to exclude HIV positive participants. Further than that, the sample size between our study and a Gugulethu study differs. In other African countries such as Nigeria, the prevalence of MA was similar to the prevalence in present study (Salako et al., 2007) whereas in Seychelles a lower prevalence of 11.2% was reported. The difference in the prevalence may be due to not using gender-specific cut-offs and using a semi quantitative method for MA determination in the Seychelles study (Pruijm et al., 2008). When hypertension becomes prolonged, it induces impairment of the above auto regulatory process, thus causing hyperfiltration and leakage of proteins like albumin in to urine causing microalbuminuria (Metcalfe, 2007, Mountokalakis, 1997).

LIMITATIONS OF THE STUDY

In the present study only one measurement was taken for diagnosis of microalbuminuria while at least three positive results a month apart are required before a definite diagnosis can be made. Serum TNF-α and ET-1 were measured and almost all of the results were below the lowest standards. This limited the information available with regards to inflammatory status of the individuals. The study is also limited by the small sample size.

CHAPTER SEVEN

7. SUMMARY, RECOMMENDATIONS and CONCLUSION

7.1. SUMMARY

The levels of serum lipids and inflammatory markers were found in this study to be similar in participants with and without microalbuminuria. In a linear regression model TG was the only lipid parameter found to be associated with microalbuminuria and the inflammatory markers were not associated with microalbuminuria. In a logistic regression model only CRP and HDL-C showed negative association with microalbuminuria in men while in women no association was found. However, the interactions of serum lipids and inflammatory markers were tested and CRP and TG interacted and contributed to the association. Men with a high CRP and a high TG were found to be more likely to have microalbuminuria.

7.2. RECOMMENDATIONS

Further studies are required that will include a bigger sample size, including more inflammatory markers to assess the association of micoalbuminuria, serum lipids and inflammatory markers in a rural black population. There is also a need for studies that will determine the association of microalbuminuria, serum lipids and inflammatory markers but diagnosing microalbuminuria with at least three times in three to three months as stipulated.

7.3. CONCLUSION

A linear positive association was observed between microalbuminuria and TG in men and in women. The OR of having microalbuminuria was less in men with a high CRP, low HDL-C or in women with a high glucose. Women with a low HDL-C had higher OR of having MA and men with a high CRP and TG had even higher OR of having MA

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APPENDICES

APPENDIX 1: SCATTER PLOTS

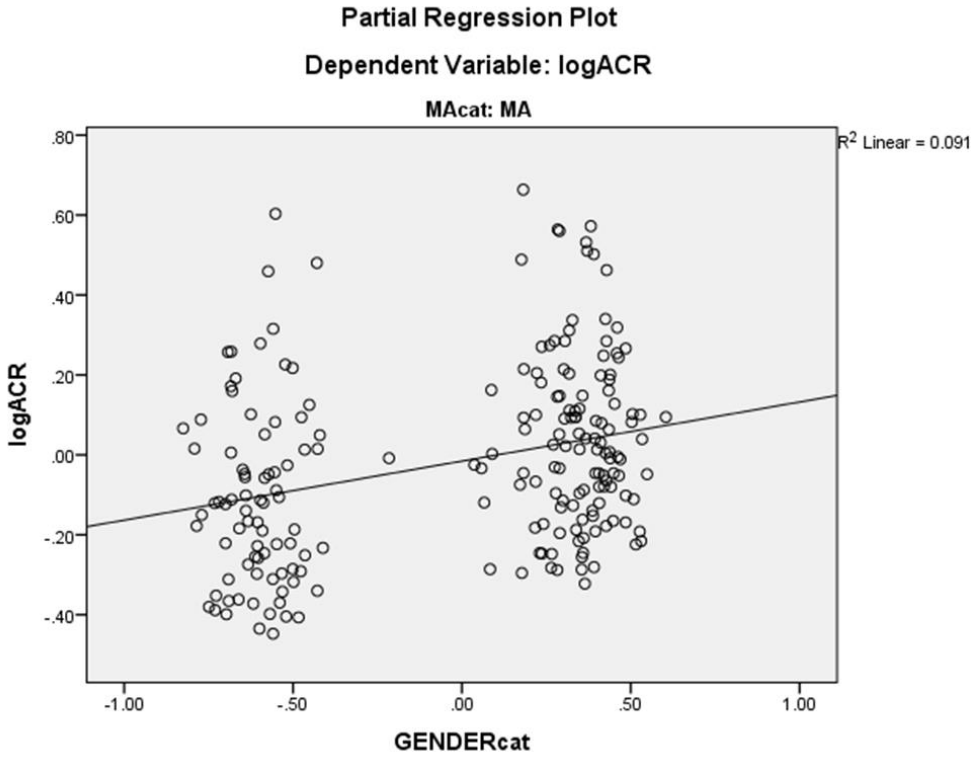

Figure 4: Association between ACR and gender in all participants with microalbuminuria.

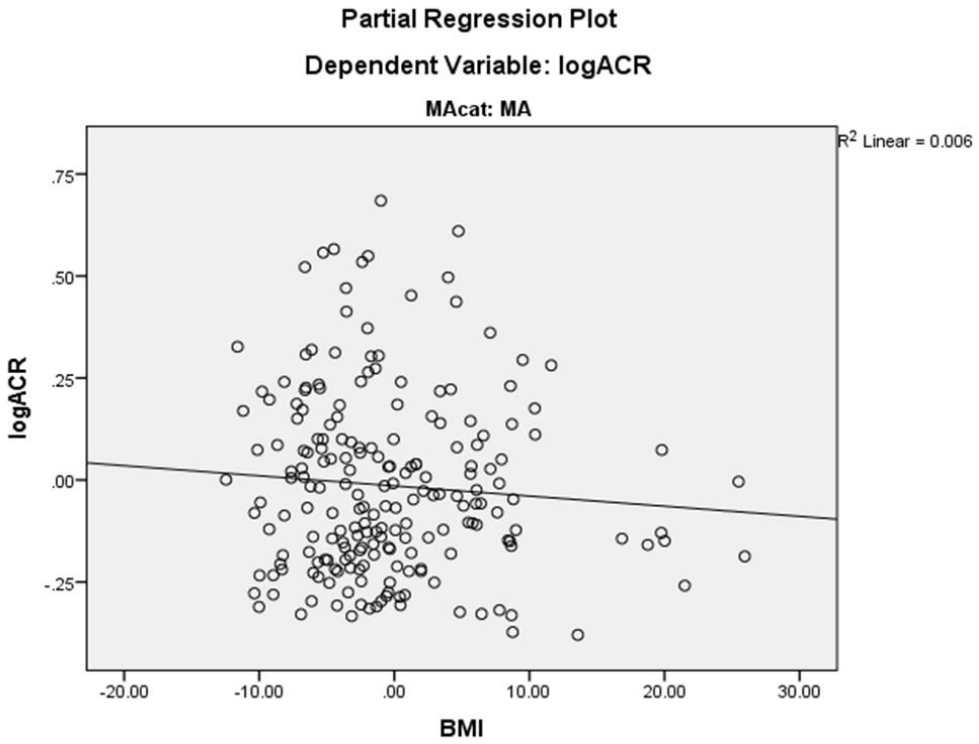

Figure 5: Association between ACR and BMI in all participants with microalbuminuria

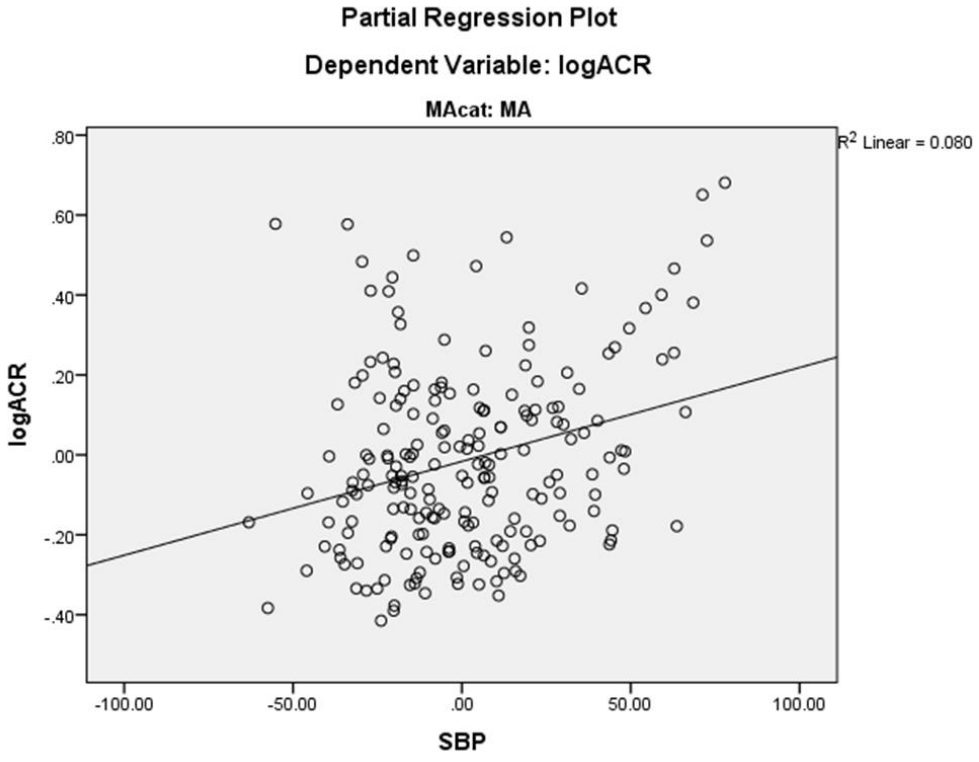

Figure 6: Association between ACR and SBP in all participants with microalbuminuria.

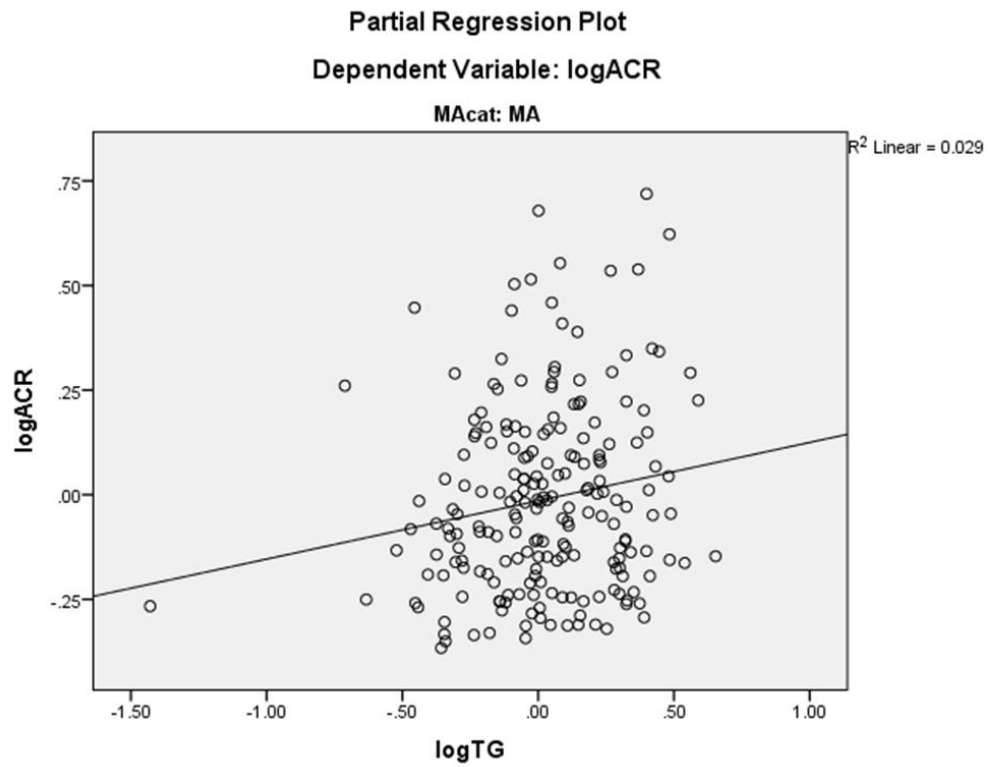

Figure 7: Association between ACR and TG in all participants with microalbuminuria

Gender was not associated with ACR in participants with microalbuminuria with a p value of 0.002 and beta value of 0.262. In this population, a negative linear association between microalbuminuria and BMI with a beta value of -0.190 and a p value of 0.025 implying that an increase in BMI leads to a decrease in ACR in participants with microalbuminuria, see figure 5. An increase in the levels of SBP in all participants with microalbuminuria leads to an increase in the levels of ACR (beta value of 0.213 and p value of 0.013) and this is represented by figure 6. In participants with microalbuminuria (see figure 7) a positive linear association was found between TG levels and ACR levels (beta value of 0.243 and p value of 0.005).

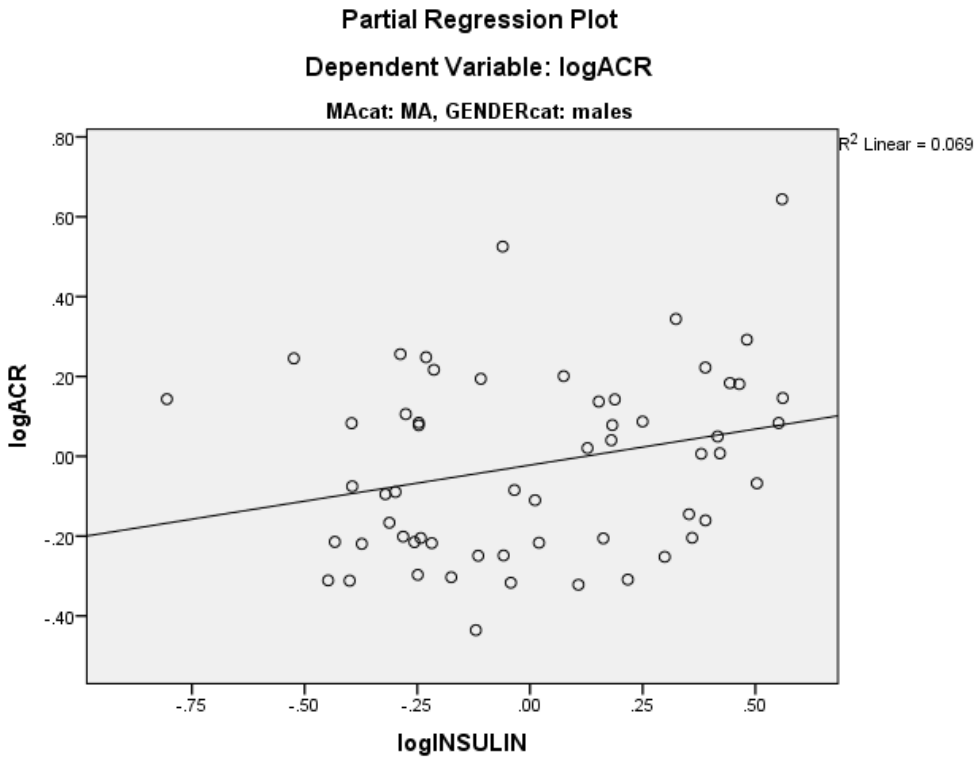

Figure 8: Association between ACR and insulin in men with microalbuminuria

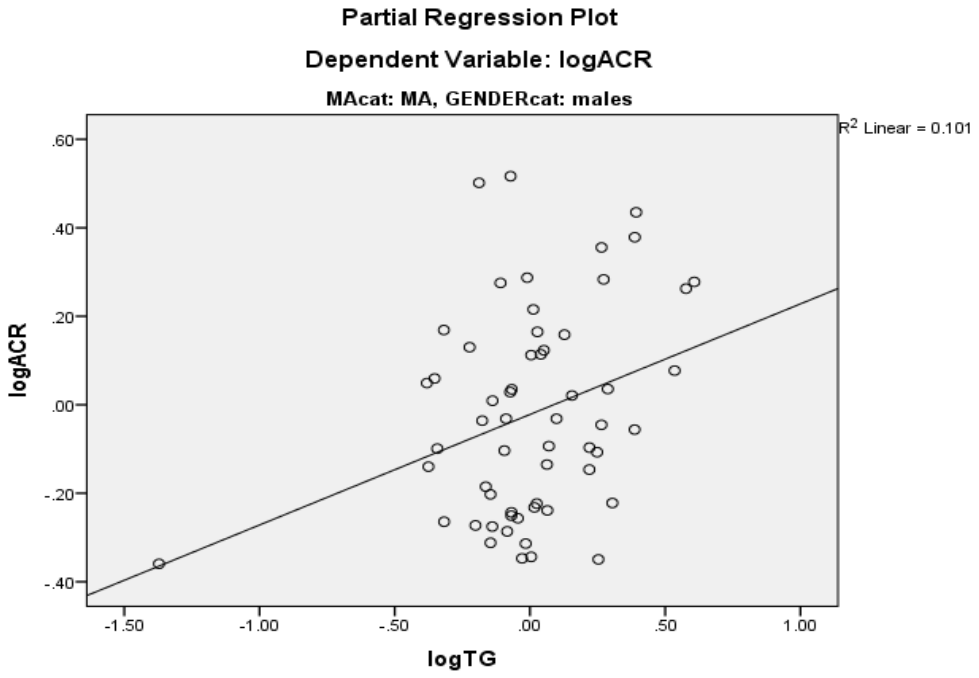

Figure 9: Association between ACR and TG in men with microalbuminuria

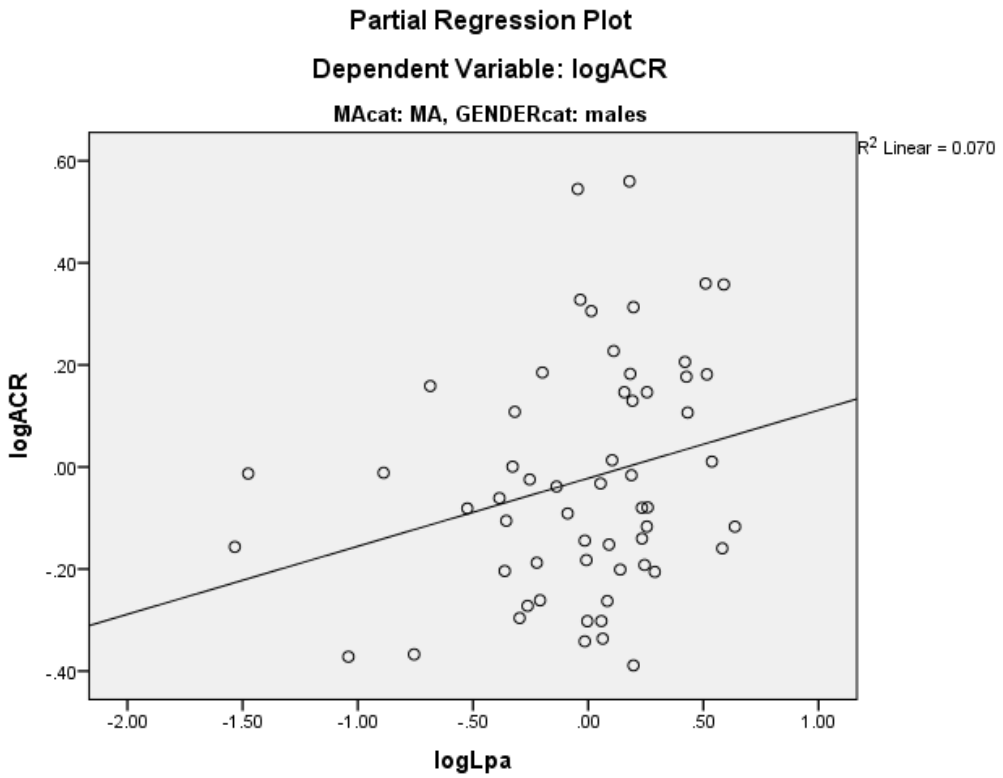

Figure 10: Association between ACR and Lp (a) in men with microalbuminuria

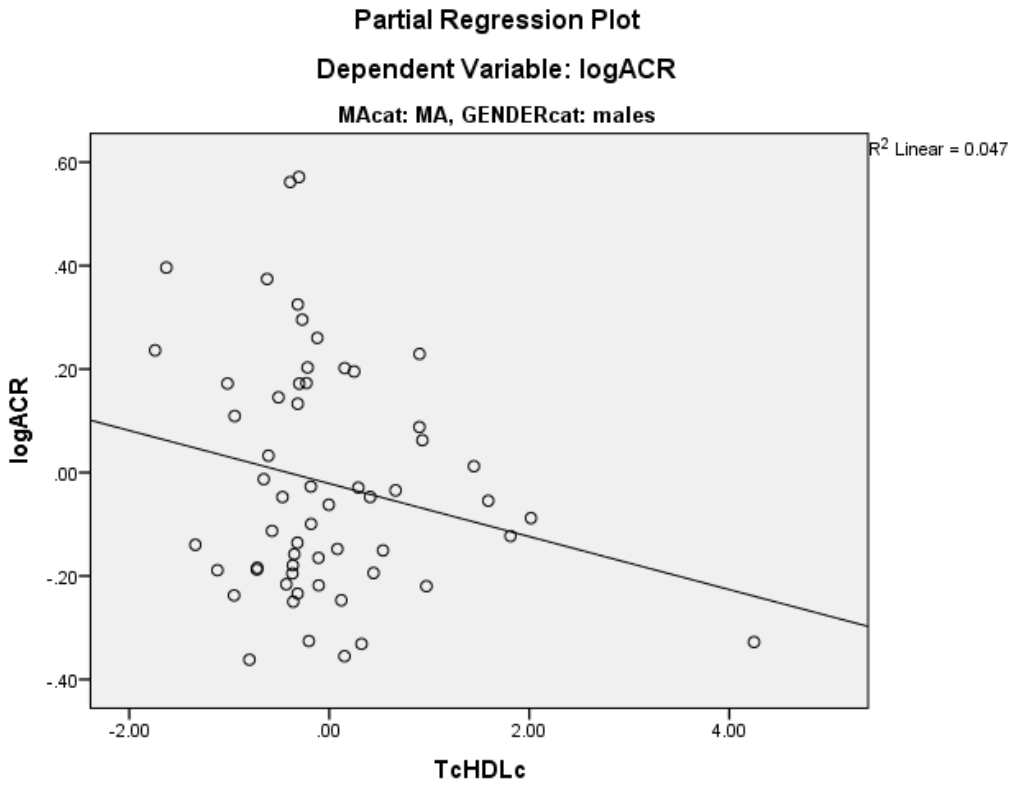

Figure 11: Association between ACR and TC/HDL-C in men with microalbuminuria

In men a significant linear positive association was found between the levels TG and the levels of ACR in those with microalbuminuria with a beta value of 0.372 and a p value of 0.018 (see figure 9). In men the levels of insulin, Lp (a) and TC/HDL-C showed no significant association with ACR in those with microalbuminuria, figure 8, 10 and 11 represents the scatter plots for association between ACR and Insulin, Lp (a) and TC/HDL-C respectively.

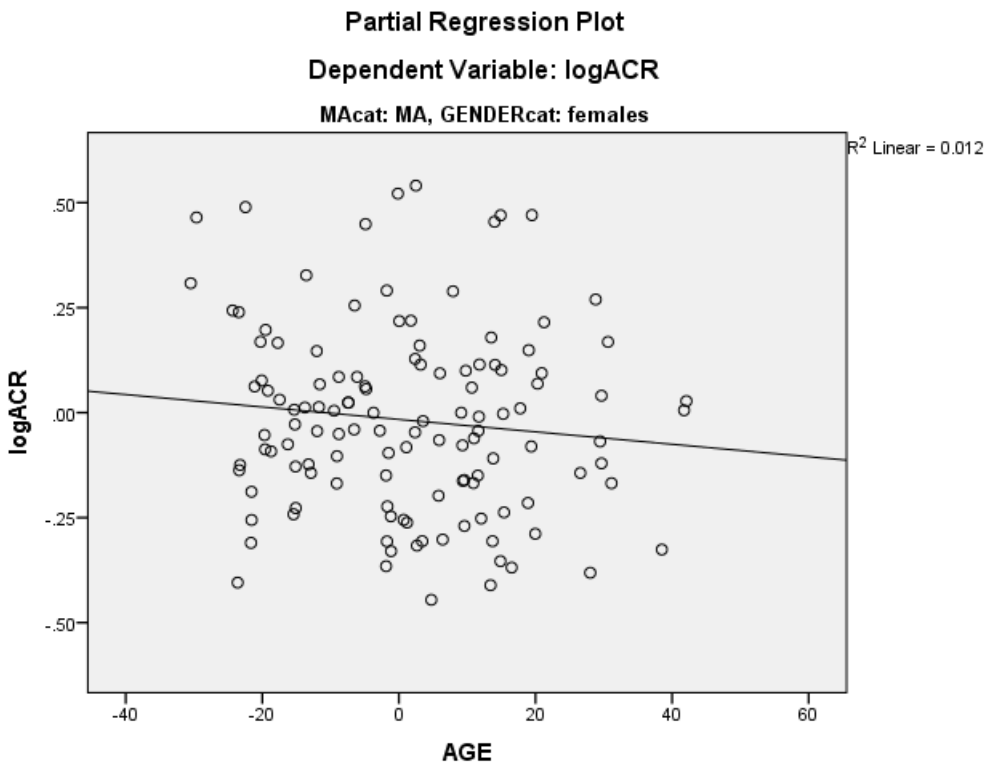

Figure 12: Association between ACR and age in women with microalbuminuria

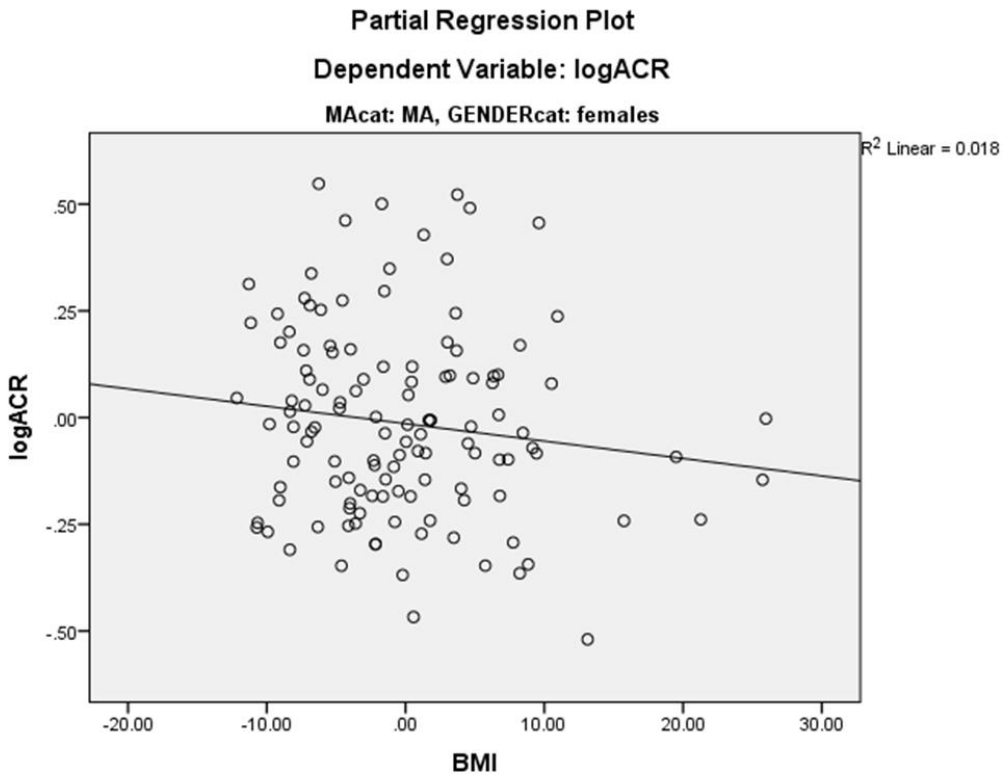

Figure 13: Association between ACR and BMI in women with microalbuminuria

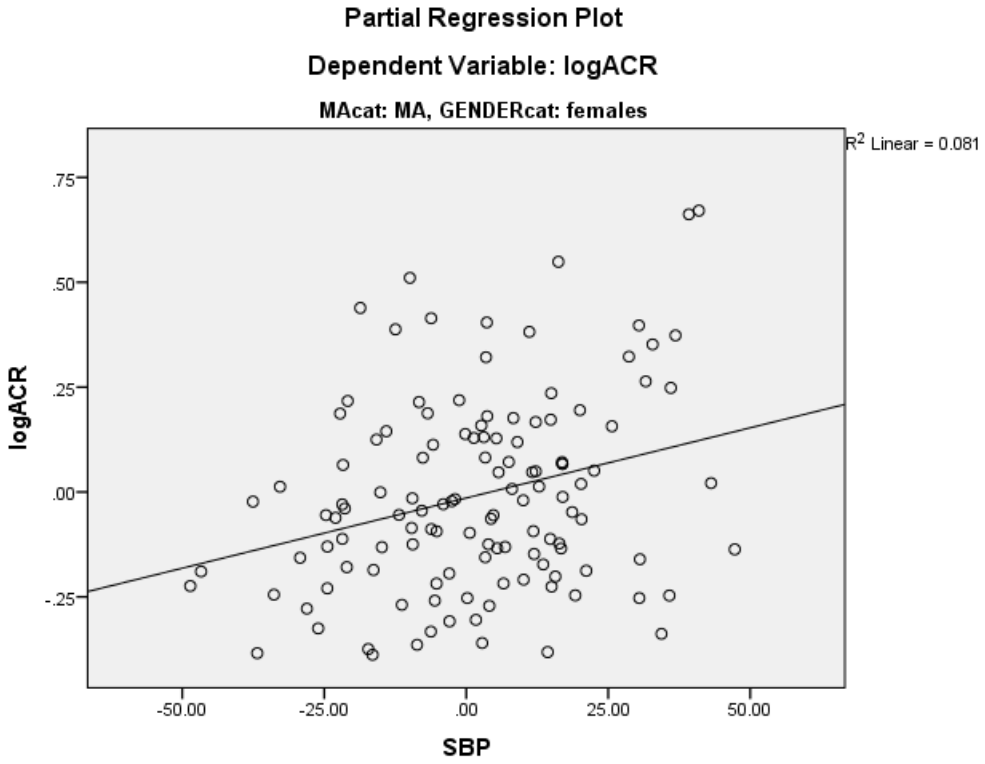

Figure 14: Association between ACR and SBP in women with microalbuminuria

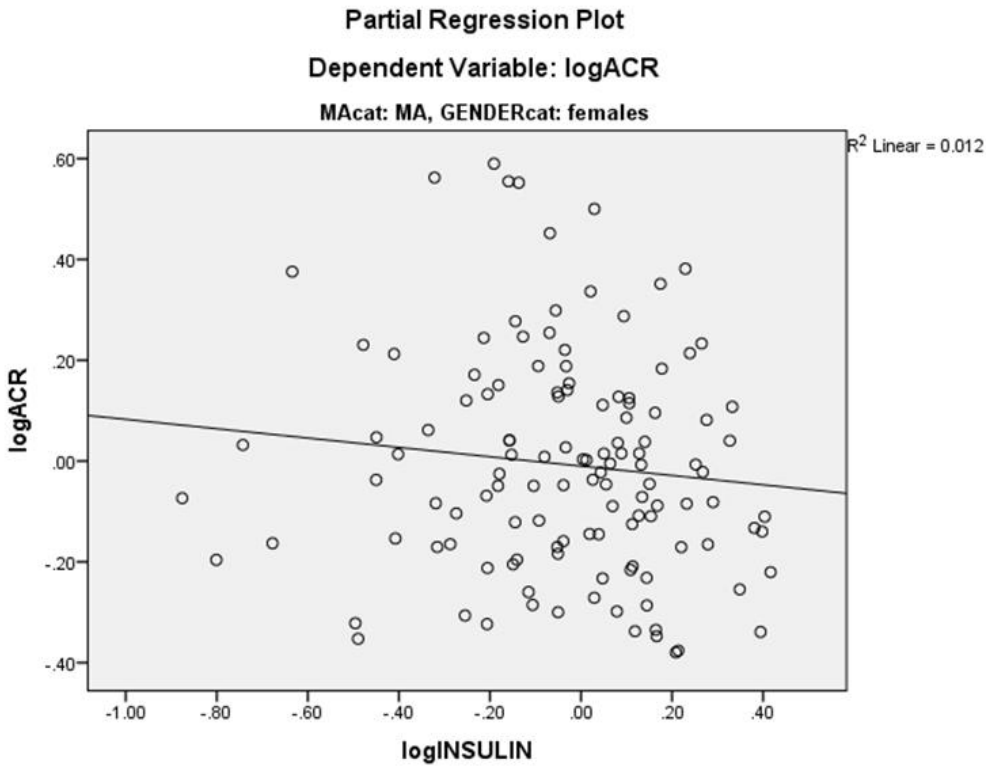

Figure 15: Association between ACR and insulin in women with microalbuminuria

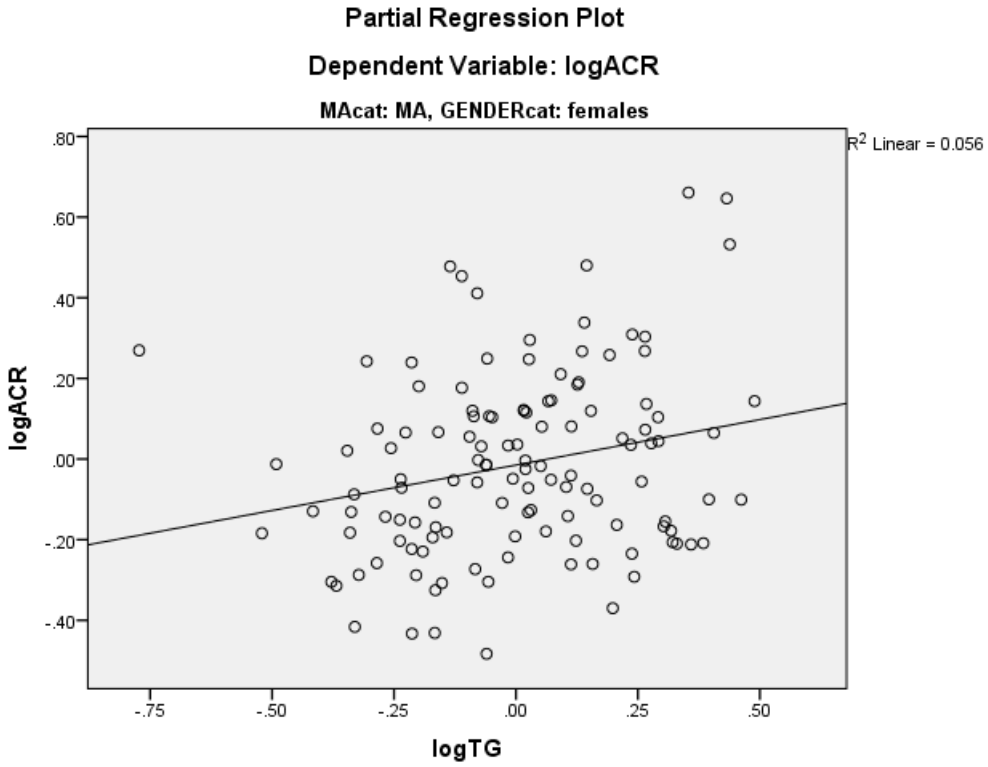

Figure 16: Association between ACR and TG in women with microalbuminuria

APPENDIX 2: ETHICAL APPROVAL CERTICICATE

UNIVERSITY OF LIMPOPO
Medunsa Campus

MEDUNSA RESEARCH & ETHICS COMMITTEE

CLEARANCE CERTIFICATE

MEETING:	**03/2014**
PROJECT NUMBER:	**MREC/HS/102/2014: PG**
PROJECT:	
Title:	Microalbuminuria, serum lipids and inflammatory markers in a rural black population in the Limpopo province
Researcher:	Mr T Magwai
Supervisor:	Dr SEP Modjadji
Co-supervisor:	Prof M Alberts
Department:	Medical Sciences, Public Health & Health Promotion
Degree:	MSc Medical Science (Chemical Pathology)

DECISION OF THE COMMITTEE:

MREC approved the project.

DATE: 03 April 2014

UNIVERSITY OF LIMPOPO
Medunsa Campus
2014 -04- 03
MEDUNSA RESEARCH ETHICS COMMITTEE
MREC CHAIRPERSON

PROF GA OGUNBANJO
CHAIRPERSON MREC

The Medunsa Research Ethics Committee (MREC) for Health Research is registered with the US Department of Health and Human Services as an International Organisation **(IORG0004319)**, as an Institutional Review Board **(IRB00005122)**, and functions under a Federal Wide Assurance **(FWA00009419)**
Expiry date: **11 October 2016**

Note:
i) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee.**
ii) **The budget for the research will be considered separately from the protocol.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

Finding Solutions for Africa

PROUDLY SOUTH AFRICAN

APPENDIX 3: CONSENT FORM

UNIVERSITY OF LIMPOPO (Medunsa Campus) ENGLISH CONSENT FORM

Statement concerning participation in Research Project

Name of Project: Microalbuminuria, serum lipids and inflammatory markers in a rural black population in the Limpopo province

I have heard the aims and objectives of the proposed study and was provided the opportunity to ask questions and given adequate time to rethink the issue. The aim and objectives of the study are sufficiently clear to me. I have not been pressurized to participate in any way.

I am aware that this material may be used in scientific publications which will be electronically available throughout the world. I consent to this provided that my name is not revealed.

I understand that participation in this Project is completely voluntary and that I may withdraw from it at any time and without supplying reasons. This will have no influence on the regular treatment that holds for my condition neither will it influence the care that I receive from my regular doctor.

I know that this Project has been approved by the Medunsa Research Ethics Committee (MREC), University of Limpopo (Medunsa Campus) / Dr George Mukhari Hospital (project number MREC/HS/102/2014: PG). I am fully aware that the results of this Project will be used for scientific purposes and may be published. I agree to this, provided my privacy is guaranteed.

I hereby give consent to participate in this Project*.

....

Name of patient/volunteer Signature of patient or guardian

....

Place. Date Witness

Statement by the Researcher

I provided verbal and/or written* information regarding this Project

I agree to answer any future questions concerning the Project as best as I amiable.

I will adhere to the approved protocol.

....…… .....

Name of Researcher Signature Date Place

UNIVERSITY OF LIMPOPO (Medunsa Campus) SEPEDI CONSENT FORM

Setatamente mabapi le go tšeakaroloka go Protšekeya Dinyakišišo.

Leina la Protšeke: Microalbuminuria, serum lipids and inflammatory markers in a rural black population in the Limpopo province

Ke kwele kaga tshedimošo mabapi le maikemišetšo le morero wa dinyakišišotšeo di šišintšwego gomme ke ile ka fiwa monyetlawa go botšiša dipotšišo gomme ka fiwa nako yeo e lekanego gore ke naganišiše kaga taba ye. Ketloga ke kwešiša maikemišetšo le morero wa di nyakišišo tše ga botse. Ga se kagapeletšwa go kgatha tema ka tsela efe go baefe.

Ke a kwešiša gore go kgatha tema Protšekeng ke ga boithaopo gomme nkatlogela go kgatha tema nakong efe goba efe ntle le gore kefe mabaka. Se seka se be le khuetšo efe goba efe go kalafo yaka mehlaya maemo a ka gape e ka se huetše le ge e ka batlhokomelo yeo ke e humanago go ngaka yaka ya kamehla.

Ke a tseba gore Protšeketše di dumele tšweke Medunsa Research Ethics Committee (MREC), Yunibesithiya Limpopo (Khamphase ya Medunsa)/ Dr George Mukhari Hospital (nomoro ya projeke ke MREC/HS/102/2014:PG. Ke tseba gabotse gore dipoelo tša Teko/ Dinyakišišo/ Protšeketše di tla di rišeša merero ya saense gomme di ka phatlalatšwa. Kedumelelana le se, ge fela bose phiri bjaka bokatiišetšwa.

Mo ke fa tumelelo ya go kgatha tema Protšekeng.

....

Leina la molwetši/ moithaopi Mosaeno wa molwetši goba mohlokomedi.

....

Lefelo. Letšatšikgwedi. Tlhatse

_____

Setatamente ka Monyakišiši

Ke fana ka tshedimošo ka molomo le/goba yeo e ngwadilwego mabapi le Protšeke ye.

Ke dumela go araba dipotšišo dife goba dife tša ka moso mabapi le Protšeke ka bokgoni ka moo nka kgonago kagona.

Ke tlalatela melao yeo e dumeletšwego.

....…… ……..

Leina la Monyakišiši Mosaeno Letšatšikgwedi Lefelo