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Assessment of Adiponectin and Endothelial Nitric Oxide Synthase Levels in Black Zambian Patients with Hypertension at the University Teaching Hospital, Lusaka

A Dissertation Submitted to the University of Zambia, in Partial Fulfilment of the Requirements for the Master of Science Degree in Pathology (Chemical Pathology)

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Declaration

This work or dissertation in its present form has not been submitted or accepted previously for the award of a degree or diploma in this or any tertiary institution, and is not being submitted for a degree or diploma in any tertiary institution or for another degree or diploma at this institution. I declare that this dissertation contains my own work except where specifically acknowledged. I further declare that i followed all the applicable ethical guidelines in the conduct of the research. This dissertation has been prepared in accordance with the Master of Science in Pathology (Chemical Pathology), University of Zambia.

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Certificate of Approval

The University of Zambia approves this dissertation on **Assessment of Adiponectin and Endothelial Nitric Oxide Synthase Levels in Black Zambian Patients with Hypertension at the University Teaching Hospital, Lusaka.**

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Abstract

Context - A number of biomarkers are implicated in the pathophysiology of hypertension. Low adiponectin levels have been associated with hypertension and this might be due to its effects on endothelial nitric oxide synthase levels.

Aims - In this study we aimed to assess plasma adiponectin and endothelial nitric oxide synthase in patients with hypertension.

Methods and Results - An analytical cross-sectional study was undertaken in black Zambians. Plasma adiponectin and endothelial nitric oxide synthase levels were assessed in hypertensive and non-hypertensive participants using ELISA. Our results showed that there was no difference in adiponectin levels between hypertensive and non-hypertensive participants ($p=0.291$). Our results showed that there was no difference in eNOS levels between hypertensive and non-hypertensive participants ($p=0.197$). Plasma adiponectin showed no correlation with eNOS in hypertension ($r=0.036$, $p=0.825$).

Conclusion - The study showed that there was no difference in mean adiponectin concentration and mean eNOS concentration between hypertensive patients and non-hypertensive participants. There was no relationship between adiponectin and eNOS levels on their influence on hypertension.

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List of Abbreviations

- AM – Ante Meridiem
- BMI – Body Mass Index
- BP – Blood Pressure
- BREC – Biomedical Research Ethics Committee
- CI – Confidence Interval
- DNA – Deoxyribonucleic Acid
- DRGS – Directorate of Graduate Studies
- ELISA – Enzyme-Linked Immunosorbent Assay
- eNOS – Endothelial Nitric Oxide Synthase
- HRP – Horseradish Peroxidase
- HTN- Hypertension
- IBM – International Business Machines
- ICF – Informed Consent Form
- kDa – Kilodalton
- MEPI- Medical Education Partnership Initiative
- ml- millilitre
- MSc – Master of Science
- ng- nanogram
- NO – Nitric Oxide
- OD – Optical Density
- PM – Post Meridiem
- RPM – Revolutions Per Minute
- SD – Standard Deviation

- SEM-Standard Error Mean
- SPSS – Statistical Package For Social Sciences
- TMB - 3,3',5,5'-tetramethylbenzidine
- UNZA – University Of Zambia
- UNZABREC – University Of Zambia Biomedical Research Ethics Committee
- UTH – University Teaching Hospital
- vs- Versus
- WHO – World Health Organization

1.0 Introduction

1.1 Background

Hypertension is a common disease that increases the risk for cardiovascular disease, and it is also a component of the metabolic syndrome (Yoshia *et al*, 2004; Karki *et al*, 2013). There have been various definitions of the metabolic syndrome since 1998 (Grundny *et al*, 2005; Reaven, 2006). The International Diabetes Federation (IDF) and American Heart Association (AHA) definition in 2009 was as follows: any three (or more) of the following factors constitute a diagnosis of metabolic syndrome. 1. Increased waist circumference: ethnicity specific-for example, Caucasian men ≥ 94 cm and women ≥ 80 cm; South Asian men ≥ 90 cm and women ≥ 80 cm 2. If body mass index is over 30 kg/m^2 , central obesity can be assumed and waist circumference does not need to be measured 3. Raised triglycerides: i) $>150 \text{ mg/dL}$ (1.7 mmol/L) ii) or specific treatment for this lipid abnormality 4. Reduced high density lipoprotein-cholesterol: i) $<40 \text{ mg/dL}$ (1.03 mmol/L) in men ii) $<50 \text{ mg/dL}$ (1.29 mmol/L) women iii) or specific treatment for this lipid abnormality 5. Raised blood pressure: i) Systolic $\geq 130 \text{ mmHg}$ ii) Diastolic $\geq 85 \text{ mmHg}$ iii) or treatment of previously diagnosed hypertension 6. Raised fasting plasma glucose: i) fasting plasma glucose $\geq 100 \text{ mg/dL}$ (5.6 mmol/L) ii) most people with type 2 diabetes will have metabolic syndrome based on these criteria (Alberti *et al*, 2009). Hypertension causes significant morbidity and mortality worldwide. Unfortunately, despite recent advances in understanding and treating hypertension, its prevalence continues to rise (Dickson and Sigmund, 2006). Hypertension is the term used to describe high blood pressure. Blood pressure is a measurement of the force against the walls of the arteries as the heart pumps blood through the body. When the pressure is too high, the heart has to work extra harder to meet the requirements of the metabolising tissues and this could lead to organ dysfunction and several illnesses such as heart failure, stroke, heart attack, or renal damage (MedlinePlus Medical Encyclopaedia; A.D.A.M. Medical Encyclopaedia).

Hypertension, just like other cardiovascular diseases is associated with identifiable behavioural and biological risk factors. The major risk factors include race, obesity, diabetes mellitus, age, sex, alcoholism, sedentary lifestyle, diet (including salt intake), and family history of hypertension. Some of these risk factors for hypertension are modifiable through lifestyle interventions or at least their effects ameliorated by lifestyle modifications and medical management (Goma *et al*, 2011).

According to a study conducted among adults in urban Lusaka, Zambia, the prevalence for hypertension was found to be 34.8% (38.0% of males and 33.3% of females). (Goma *et al*, 2011). This relates to Malawi, where the age-standardised prevalence of hypertension (BP \geq 140/90 mmHg) was 33.2%. Hypertension was more frequent in males than in females (36.9% versus 29.9%), alcohol drinkers than non-alcohol drinkers (40.9% versus 31.6%), overweight than non-overweight (41.5% versus 30.7%) and increased with increasing age (21.4% in 25-34 years old versus 59.2% in 55-64 years old). (Kelias *et al*, 2012). Findings from a blood pressure survey recently conducted in an urban environment of Zimbabwe revealed a hypertension prevalence of 35% in women and 24% in men (Mufunda *et al*, 2006). Prior to 1990, population data suggested that hypertension prevalence was decreasing; however, recent data suggest that is again on the rise. In 1999-2002, 28.6% of the U.S population had hypertension. Hypertension prevalence has also been increasing in other countries, and more than 25% of the adult population in the world are suffering from essential hypertension and this is expected to rise to 30% in the next 15 years. Two thirds of those individuals reside in developing countries (Zhao and Philip, 2008). Incidence rates of hypertension range between 3% and 18%, depending on the age, gender, ethnicity, and the body size of the population studied. Despite advances in hypertension treatment, control rates continue to be suboptimal. Only about one third of all hypertensive patients are controlled in the United States (Hajjar *et al*, 2006).

Some researchers have made good progress in shedding more light on the molecular mechanisms which lead to progression to hypertension. There is a possible role of angiotensinogen gene, and other candidate genes; such as the angiotensin II receptor type 1 gene (Soubrier *et al*, 2006; Vasilios *et al*, 2010). Also, mutations in the endothelial nitric oxide synthase gene may be associated with abnormal nitric oxide (NO) production and cardiovascular diseases (Hoffman *et al*, 2005; Hai-Xiang *et al*, 2012).

During the last one and half decade, evidence has accumulated, demonstrating that adipose tissue is an important endocrine organ involved in metabolism. Adipose tissue can exert its effects through several mechanisms, the most important of which is the secretion of bioactive mediators from adipocytes and other cells. These bioactive substances, collectively termed adipokines include: leptin, adiponectin, resistin, tumour necrosis factor alpha, interleukin-6 and others, and they have been implicated in the pathophysiology of insulin resistance,

hyperlipidaemia, obesity, inflammation, atherosclerosis and the metabolic syndrome (Jyh *et al*, 2007). Adiponectin differs from all other adipocytokines except leptin in being secreted exclusively by adipocytes, the details of all the factors regulating its synthesis, secretion and clearance remain incomplete (Finucane *et al*, 2009). Adiponectin is 244 amino acid protein (Sattar *et al*, 2009). Hypoadiponectinaemia has been associated with hypertension and this might be due to its effects on endothelial nitric oxide synthase levels (Giuseppe and Matteo, 2007).

This research therefore had aimed to determine the relationship between adiponectin and endothelial nitric oxide synthase levels in patients with hypertension at University Teaching Hospital (UTH), Lusaka.

1.2 Statement of the Problem

The impact of ethnic and social differences on hypertension as well as suboptimal treatment for hypertensive patients underscores need for understanding molecular pathophysiologic mechanisms involved in the complex pathogenesis of hypertension. This study had also been necessitated by the continued rise in the prevalence of hypertension (Goma *et al*, 2011). Hypoadiponectinaemia has been associated with hypertension but the mechanisms are not clear. Hypoadiponectinaemia has been hypothesised to lower the levels of endothelial nitric oxide synthase which produces nitric oxide necessary for vasodilatation and its absence or decrease culminates in hypertension (Giuseppe and Matteo, 2007). In Zambia and most African countries, no studies on hypoadiponectinaemia and endothelial nitric oxide synthase levels in hypertensive patients had been reported. It led us to the question: was there a correlation between adiponectin and endothelial nitric oxide synthase levels in hypertension among black Zambians?

1.3 Justification of the Study

Effective treatment and management of hypertension requires a deep understanding of the disease pathogenesis and pathophysiology itself. In Zambia, there is need for more studies that focus on the molecular mechanisms of hypertension. Despite all that we have known over many years on hypertension, it is generally accepted that our understanding of hypertensive disease process remains incomplete and more pathways need to be elucidated on the pathogenesis and pathophysiology of hypertension.

Despite advances in hypertension treatment, control rates continue to be suboptimal. Thus strokes and heart attacks continue to be the major cause of morbidity and mortality among individuals with hypertension.

One of the reasons for the suboptimal control rates of hypertension might be that the important pathophysiological mechanisms involved in the development of hypertension have not been elucidated in addition to poor adherence to medication. Owing to this, this research was conducted to provide information on other pathogenetic mechanisms involved in the development of hypertension.

This study was aimed at providing evidence-based information that adiponectin and endothelial nitric oxide synthase levels are important in the pathogenesis of hypertension. Thus with such proven information, therapy may target the correct mechanism or mechanisms involved in the disease process. The study also described the probable relationship between adiponectin and endothelial nitric oxide synthase levels in hypertensive individuals in Zambia.

1.4.0 Literature Review

1.4.1 Hypertension

Hypertension is an increasing important medical and public health issue worldwide. More than 90% of hypertensive individuals suffer from essential hypertension. The factors linked to essential hypertension are age, obesity, smoking, and stress (Juliet *et al*, 2007; Shubhangi *et al*, 2009). Hypertension is estimated to account for more than 5.4% of deaths worldwide. Cardiovascular disease has become number one cause of death in the developing world. (Katharine *et al*, 2004; Thomas, 2007).

Hypertension affects about 23% of the world population and more than 25% of the adult population. This is expected to rise to 30% by 2030 (Zhao and Philip, 2008; Shubhangi *et al*, 2009). In 2012, it was reported that 1 in 3 adults has raised blood pressure and essential hypertension accounts for more than 90%. It was also reported that hypertension causes around half of all deaths from stroke and heart disease (WHO, 2012).

In Zambia, the prevalence of hypertension was 34.8% (38.0% of males and 33.3% of females) in 2011. Essential hypertension accounted for more than 90% of these statistics. The prevalence of hypertension in sub-saharan Africa ranges from 6% to 48%. For instance, the prevalence of hypertension in South Africa is 25.5% for females and 21.6% for males, Uganda 22%, Eritrea 16% and Zimbabwe has a prevalence of 41% (Goma *et al*, 2011).

Adiponectin interacts with eNOS by stimulating and increasing the levels of eNOS which produces NO an endothelium-derived relaxation factor. Thus hypoadiponectinaemia (a low level of adiponectin which is a protein produced by the adipose tissue and secreted into plasma) results in reduced eNOS levels and low NO leading to endothelial dysfunction and subsequent hypertension (Han *et al*, 2007).

1.4.2 Biology of Adiponectin

Hypoadiponectinaemia has been cited as an independent risk factor for hypertension (Yoshio *et al*, 2004; Paolo *et al*, 2013). Adiponectin is the most abundant gene product in adipose tissue and accounts for 0.01% of total plasma protein (Paul *et al*, 2007). This is a 30-kDa molecule that has been identified independently by four groups in 1995 and 1996 with different experimental methods (Takashi *et al*, 2006). Adiponectin is produced abundantly by adipose tissue and circulates at relatively high concentrations (micrograms per millilitre) (Jyh *et al*, 2007). In contrast to other adipokines (for example, leptin, tumour necrosis factor alpha and interleukin-6) and, although it is secreted by adipocytes, adiponectin is lower in obese subjects than in non-obese subjects (Jyh *et al*, 2007). In addition to its role in glucose metabolism, adiponectin has anti-atherogenic, anti-inflammatory and angiogenic properties (Jyh *et al*, 2007).

Since its discovery in the mid-1990, the adipocyte-produced peptide adiponectin has attracted the interest of many researchers as a tool for investigating the function of the adipose tissue and its clinical implications (Giuseppe and Matteo, 2007). On the epidemiological point of view, many studies suggest that abdominal adiposity is more closely associated with hypertension than total adiposity (Doll *et al*, 2002).

Hypoadiponectinaemia has been associated with increased plasma free-fatty acids and hepatic fat content and have been linked to the development of insulin resistance, which might, in

turn, represent a fertile ground for the development of hypertension (Giuseppe and Matteo, 2007).

Also association between obesity and hypertension has been recognized for many decades, and an almost linear relation appears between body mass index and systolic and diastolic blood pressure, at least over a body mass index range from 16 to 31 kilograms per metre square (Chiang *et al*, 1969; Jones *et al*, 1994).

Despite Chow *et al* suggesting for the first time the hypothesis that low adiponectin levels may play an important role in the pathogenesis of human hypertension, the challenge now is to establish the pathophysiological mechanisms whereby the expansion of adipose tissue and the associated hypoadiponectinaemia induce hypertension (Giuseppe and Matteo, 2007; Chow *et al*, 2007).

1.4.3 Molecular Biology and role of eNOS in hypertension

Nitric oxide (produced by eNOS) is a small (30 kDa) gaseous and lipophilic molecule, Key properties that enables it to function as a juxtacrine mediator of cell signaling processes that modulated blood vessel relaxation and help in maintaining normal blood pressure (Kerwin *et al*, 1995).

The endothelium, a single-layered continuous cell sheet lining the luminal vessel wall, separates the intravascular (blood) from the interstitial compartment and the vascular smooth muscle. Based on cell count (6×10^{13}), mass (1.5 kg), and surface area (1000m²) the endothelium is an autonomous organ (Thomas *et al*, 2008). Though for a long time regarded as a passive barrier for blood cells and macrosolutes, this view completely changed with the advent of endothelial autacoids like prostacyclin (PGI₂) (Moncada *et al*, 1978; Thomas *et al*, 2008), and nitric oxide (NO) (Palmer *et al*, 1987), as well as with the discovery of integrins and other surface signals (Stupack and Cheresh, 2004).

It is now evident that the endothelium is not only at the cross-bridges of communication between blood and tissue of surrounding cells by a plethora of signalling routes. One of the prominent communication lines is established by the so-called L-arginine-NO-cyclic guanosine monophosphate (GMP) pathway (Busse and Fleming, 1998). This signalling cascade starts with endothelial nitric oxide synthase (eNOS), which generates NO and L-

citrulline from L-arginine and oxygen gas in response to receptor-dependent agonists (bradykinin, acetylcholine, Adenosine triphosphate and physicochemical stimuli like stretch) (Fleming and Busse, 2003).

NO produced in the endothelial cell diffuses to the lumen where it is captured by erythrocytes and transferred into muscle cells where it induces relaxation, eliciting vasodilation. In this mechanism, guanosine 3',5'-cyclic monophosphate (cGMP), formed from guanosine 5'-triphosphate by the action of guanylate cyclase, is activated by NO. cGMP modulates the myosin light chain (MLC) phosphatase positively and MLC kinase negatively, resulting in the dephosphorylation of MLC with subsequent muscle relaxation (Ignarro, 2002). Therefore, low eNOS causes low NO leading to impaired vascular relaxation and elevation in blood pressure.

1.4.4 Pathogenesis of Hypoadiponectinaemia/Low eNOS

Hypoadiponectinaemia can be primary when it results from genetic defects or secondary when it results from positive energy balance, visceral fat accumulation and metabolic syndrome. One of the proposed hypothetical pathways leading from hypoadiponectinaemia to Hypertension is that hypoadiponectinaemia increases free-fatty acids and hepatic fat content which lead to insulin resistance and in turn hypertension (Giuseppe and Matteo, 2007). Hypoadiponectinaemia also can lead to hypertension through endothelial damage and the activation of the inflammatory cascade. Moreover, adiponectin attenuates growth factor-induced proliferation of vascular smooth muscle cells and this may lead to hypertension through the development of vascular hypertrophy and stiffness. The other important hypothetical pathway leading from hypoadiponectinemia to hypertension is that hypoadiponectinaemia decreases levels of endothelial nitric oxide synthase which is responsible for the production of endothelium-derived vascular relaxing factor nitric oxide. This ultimately results in endothelial dysfunction and subsequent hypertension (Mahmud and Freely, 2005; Han *et al*, 2007; Giuseppe and Matteo, 2007).

Nitric oxide is synthesized from L-arginine by the endothelial isoform of nitric oxide synthase. The gene encoding endothelial nitric oxide synthase is located in the long arm of chromosome 7 and contains 26 exons spanning 21 kilobytes of genomic DNA (Rodrigo *et al*, 2009). Both human and animal studies have shown onset of hypertension because of loss of nitric oxide, the endothelium-derived relaxing factor (Pascal *et al*, 2010). Therefore the

hypothetical pathway which state that hypoadiponectinaemia decreases endothelial nitric oxide needed to be further investigated because it directly interferes with substances or factors which are responsible for the relaxation state of the blood vessels resulting in hypertension.

No study had been done in Zambia to investigate hypoadiponectinaemia and endothelial nitric oxide levels in patients with hypertension. This study therefore sought to assess adiponectin and eNOS levels in hypertension and determine its role in development of hypertension in Zambia.

1.5 Research Question

Is hypoadiponectinaemia associated with decreased plasma endothelial nitric oxide synthase levels which can contribute to the development of hypertension in black Zambians?

2.0 Objectives

2.1.0 General Objective

To correlate plasma levels of adiponectin and endothelial nitric oxide synthase levels in black Zambian patients with hypertension.

2.2.0 Specific Objectives

- 2.2.1 To determine the mean difference in plasma adiponectin concentration among black Zambian patients with hypertension and non-hypertensive black Zambian patients.
- 2.2.2 To determine the mean difference in plasma endothelial nitric oxide synthase concentrations among black Zambian patients with hypertension and non-hypertensive black Zambian patients.
- 2.2.3 To describe the pattern of plasma adiponectin with plasma endothelial nitric oxide synthase concentration in hypertensive patients.

3.0 Methodology

3.1 Study Design and Site

An analytical cross-sectional study was conducted involving blood samples of hypertensive patients (cases). It was a laboratory based study using blood samples of 41 hypertensive patients who had their blood drawn by the nurse for routine laboratory tests. A control population of 41 normotensives matched for age and sex were also involved. The study was conducted at the University Teaching Hospital (UTH) clinic 5, in Lusaka, Zambia. Clinic 5 is a specialist clinic consisting of diabetic and cardiovascular patients who have been referred to UTH for further management.

3.2 Target Population

All adults aged between 18-77 years old with hypertension that reported to clinic 5 at UTH.

3.3 Study Population

All individuals that met the inclusion criteria were enrolled into the study. The healthy normotensives were those that did not have clinically or laboratory diagnosed hypertension that came to the UTH for medical checkups at high cost. All study participants gave informed consent to participate in the study as participation in this study was voluntary. The study sample and the control group were matched for age (categories) and sex in order to minimise bias.

3.4 Sample Size

A sample size of **126** participants (**63 patients and 63 controls**) had been calculated using the formula for determination of sample size for comparative research studies between two groups as given below;

$$N = \frac{4\sigma^2(z_{crit} + z_{pwr})^2}{D^2},$$

$$N = \frac{4 \times 10^2 (1.960 + 0.842)^2}{5^2}$$

$$N = \frac{4 \times 100 (2.802)^2}{25}$$

$$N = \frac{400(7.851204)}{25}$$

$$N = \frac{3140.4816}{25}$$

$$N = 125.619264$$

$$N \approx \underline{\underline{126}}$$

Where; N is the total sample size (the sum of both comparison groups), σ is 10; the assumed SD of each group (assumed to be equal for both groups), the Z_{crit} value is 1.960 as given in tables for Standard Normal Deviate (Z_{crit}) corresponding to the desired significance criterion of 0.05 or 95% confidence interval (CI), the Z_{pwr} value is 0.842 as given in Standard Normal Deviate (Z_{pwr}) tables corresponding to 80% statistical power, and D is the minimum expected difference between the two means which has been estimated at 5. Both Z_{crit} and Z_{pwr} are cut-off points along the X axis of a standard normal distribution that demarcate probabilities matching the specified significance criterion and statistical power, respectively. The two groups that make up N are assumed to be equal in number, also that the outcome variable of a comparative study is a continuous value for which means are compared and it is assumed that the two-tailed statistical analysis will be used (Eng, 2003). However, only **82** participants (**41 patients and 41 controls**) were enrolled due to inadequate reagents, limited by cost.

3.5 Sampling Methods

Systematic sampling in which consecutive blood samples of individuals with hypertension reporting to clinic 5 at UTH and found to meet the inclusion criteria (given below) were included into the study sample. At least 4mls of blood collected by the nurse for routine laboratory tests from each participant was put in 4mls lithium heparized vacutainers. The plasma was separated from the blood cellular components and stored at a temperature of -80°C for analysis on a later date. The control group were selected by means of frequency matching of the same proportional characteristics (age and sex) as the study sample. The age categories were defined as follows: 1 (15-34), 2 (35-54), 3 (55 and above).

3.6.0 Case Definition

1. Hypertensive individuals were considered to be:
 - (a) Those with BP \geq 140/90 mmHg and/or taking antihypertensive medication.
2. Normotensive individuals were considered to be:
 - (a) Those with no previous diagnosis of or treatment for hypertension and with Bp <140/90 mmHg.

3.6.1 Inclusion Criteria of Cases

1. Those individuals that had been diagnosed with hypertension within the last ten years and/or taking anti-hypertensive drugs.
2. Individuals between 18 and 77 years old.
3. Those that had read (or read to) and understood the rationale of participating in the research, thereafter giving written personal consent without undue duress.

3.6.2 Exclusion Criteria of Cases

1. Non-consenting individuals.
- ❖ The information above was obtained from the patient files and from the interviews administered to participants using a questionnaire.

3.6.3 Inclusion Criteria of Study Controls

1. Individuals without hypertension.
2. Individuals were not taking anti-hypertensive medication.
3. Individuals between 18 and 77 years according to the criteria above.
4. Individuals who consented to take part in the study.

3.6.4 Exclusion Criteria of Study Controls

1. Those who had hypertension.
2. Individuals were taking anti-hypertensive medication.
3. Those under 18 or above 77 years old according to the criteria above.

4. Individuals who did not consent to take part in the study.

3.7.0 Data Collection

3.7.1 Clinical Data and Demographic Data Collection

Participants (study controls) were recruited from healthy individuals who came for medical checkups at high cost in UTH during normal clinic hours from 07:00 AM to 12:00 PM, from Monday to Friday. As the participants were seen by the clinician they were informed and explained to about the study by the clinician who also provided the participants with the study information sheet. If a patient autonomously agreed to be part of the study, they were required to sign the consent form and assigned a serial number. Thereafter information on the patients' demographic data and medical history was collected and compiled using a questionnaire by UTH nurses and the researcher. The demographic data included the participants' age, sex, occupation and marital status. The medical history data included the specific date (month and year) in which the participant was diagnosed with hypertension. The patients file were also reviewed to find any further relevant data to the research and also to confirm the accuracy of information provided by the participant and recorded in the questionnaire.

3.7.2 Specimen Collection

Blood samples were collected from the conscious participants via venipuncture from the antecubital vein. 4mls of blood was collected in a 5ml syringe using a 21G bore size needles. The collected blood sample was then transferred into a 4ml blood volume lithium heparin anticoagulated vacutainers that was numbered with the unique participants' assigned serial number as recorded on the questionnaire. The blood specimen was transported to the clinical chemistry laboratory at UTH within 1 hour after collection for processing and analysis.

3.7.3 Specimen Preparation and Storage

In the laboratory, each specimen serial number was recorded onto a compilation summary sheet. Thereafter the blood specimen was centrifuged at 3000 revolutions per minute (3000 rpm) in order to separate the plasma (supernatant) from the blood cellular components (sediment). Only supernatant (plasma) was then meticulously collected from the lithium heparin vacutainers using pipettes and transferred to 2ml plastic cryovial containers with

sealable screw caps which was stored in a freezer at -80°C until the specimens were analysed in a batch.

3.8.0 Quality Control

To ensure reliable results, quality control was performed on all the analytical instruments and analysers to be used for any purpose during specimen analysis according to the UTH quality control guidelines. Quality control included equipment calibration and analytical control runs on every analysis before each test analysis.

3.9.0 Specimen Analysis

3.9.1. Adiponectin ELISA Test Protocol

Plasma adiponectin concentration was determined using the Novus[®] Human Adiponectin ELISA Kit; an enzyme-linked immunosorbent assay for the quantitative detection of human Adiponectin in cell culture supernatant, serum and plasma (citrate, heparin) according to the manufacturer's protocol given below. This assay employed an antibody specific for human Adiponectin coated on a 96-well plate.

3.9.1.1. Preparation of Reagents

All kit components and samples were brought to room temperature before use. The WASH BUFFER concentrate (20 \times) was diluted with 950 ml of distilled water. The diluted WASH BUFFER concentrate (20 \times) was mixed gently to avoid foaming. The ASSAY BUFFER concentrate (20 \times) was diluted with 95 ml of distilled water and mixed gently to avoid foaming. A 1:100 dilution of the concentrated BIOTIN-CONJUGATE solution with Assay Buffer (1 \times) was made in a clean plastic tube. A 1:400 dilution of the concentrated STREPTAVIDIN-HRP solution with Assay Buffer (1 \times) was made in a clean plastic tube. Human Adiponectin standard was reconstituted by addition of distilled water.

3.9.1.2. Test Protocol

Samples were prediluted before starting with the test procedure. Plasma was diluted 1:500 with Assay Buffer (1 \times) according to the following scheme: Dilution 1: 10 μl sample + 90 μl Assay Buffer (1 \times) and Dilution 2: 10 μl of dilution 1 + 490 μl Assay Buffer (1 \times). The number of microwell strips required to test the desired number of samples was determined

plus appropriate number of wells needed for running blanks and standards. Each sample, standard, and blank was assayed. Extra microwell strips were removed from holder and stored in foil bag with the desiccant provided at 2-8°C sealed tightly. The microwell strips were washed twice with approximately 400 µl WASH BUFFER per well with thorough aspiration of microwell contents between washes. The WASH BUFFER was allowed to sit in the wells for about 10-15 seconds before aspiration. Care was taken not to scratch the surface of the microwells. After the last wash step, microwell strips were placed upside down on a wet absorbent paper for not longer than 15 minutes. The microwell strips were used immediately after washing. Wells were not allowed to dry. 100 µl of Assay Buffer (1×) was added to all standard wells. 100 µl of prepared standard was pipette into well A1 and A2 in the supplied microtiter plate. The contents of wells A1 and A2 were mixed by repeated aspiration and ejection (concentration of standard 1, S1=50.0 ng/ml, and transferred 100 µl to wells B1 and B2 respectively. Care was taken not to scratch the inner surface of the microwells. This procedure was continued 5 times, creating two rows of human Adiponectin standard dilutions ranging from 50.0 to 0.78 ng/ml. 100 µl of Assay Buffer (1×) was added to the blank wells. 50 µl of Assay Buffer was added to the sample wells. 50 µl of Biotin-Conjugate was added to all wells. The microplate was then covered with an adhesive film and incubated at room temperature for 2 hours. The adhesive film was removed and wells emptied. Microwell strips were washed 6 times. Then 100 µl of diluted STREPTAVIDIN-HRP was added to all wells, including the blank wells. The microplate was covered with an adhesive film and incubated at room temperature for 1 hour. The adhesive film was removed the wells were emptied. The microwell were washed 6 times. Thereafter, 100 µl of TMB SUBSTRATE SOLUTION to all wells. The microwell strips were incubated at room temperature for 30 minutes. Direct exposure to intense light was avoided. Colour development on the plate was monitored and the substrate reaction stopped before positive wells were no longer recordable. The stop solution was added when the highest standard had reached a dark blue colour. The enzyme reaction was stopped by quickly pipetting 100 µl of STOP SOLUTION into each well. The STOP SOLUTION was spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results were read immediately after the STOP SOLUTION was added. Absorbance of each microwell were read on a spectro-photometer using 450 nm wavelength.

3.9.1.3. Data Processing

A standard curve was plotted relating the intensity of the colour (O.D.) to the concentration of standards. A standard curve was generated using Microsoft Excel 2011 for Mac. The human Adiponectin concentration in each sample was interpolated from this standard curve.

Figure 1: Adiponectin Calibration Curve

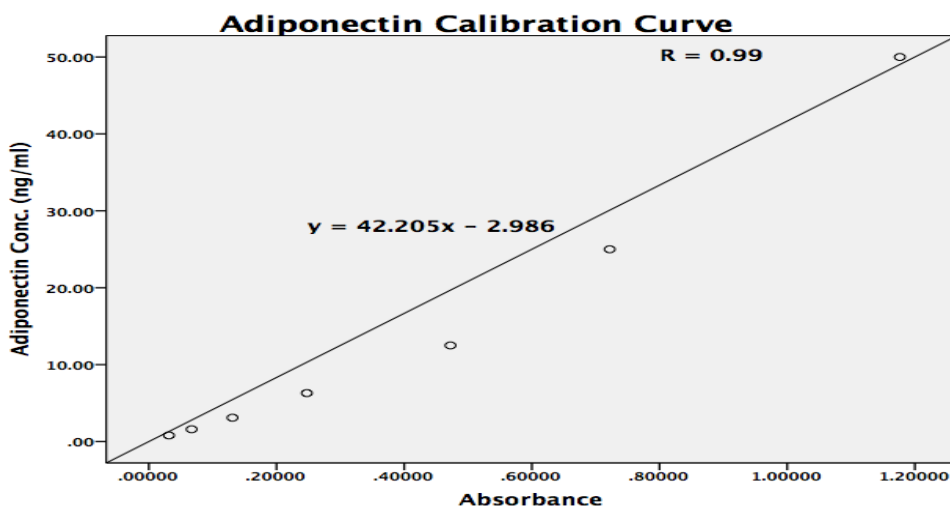


Fig.1: Adiponectin calibration curve plotted from standard absorbances (O.D) against concentrations. The regression equation was used to calculate sample concentration from their respective O.Ds.

To determine the concentration of circulating human Adiponectin for each sample, first the absorbance value on the abscissa was found and a vertical line was extended to the standard curve. At the point of intersection, a horizontal line was extended to the ordinate and the corresponding human Adiponectin was read.

3.9.1.4. Sensitivity

The limit of detection of human Adiponectin defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.01 ng/ml (mean of 6 independent assays).

3.9.1.5. Intra-assay Reproducibility

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 8 serum samples containing different concentrations of human

Adiponectin. 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 4.2%.

3.9.1.6. Inter-assay Reproducibility

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 8 serum samples containing different concentrations of human Adiponectin. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 3.1%.

3.9.1.7. Specificity

The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human Adiponectin positive sample. There was no cross reactivity or interference detected.

3.9.2. Endothelial Nitric Oxide Synthase ELISA Test Protocol

Plasma endothelial nitric oxide synthase was determined using the NeoBioLab® Human Endothelial Nitric Oxide Synthase 3 ELISA Kit; a sandwich immunoassay for the quantitative measurement of human endothelial nitric oxide synthase in cell culture fluid, body fluid, tissue homogenate, serum or blood plasma.

3.9.2.1. Reagent Preparation

All kit components and samples were brought to room temperature before use. The microtiter plate was brought to room temperature before opening. The WASH SOLUTION concentrate (100×) was diluted with 990 mL of distilled water.

3.9.2.2. Assay Procedure

50 µL of SAMPLE or STANDARD was added to the appropriate wells in the supplied microtiter plate. Wells had been pre-blocked and no additional blocking steps were required. The plate was then incubated for 1 hour at room temperature. After incubation, the wells were washed 3-5 times with 300-400 µL 1X WASH SOLUTION per well. Final wash was emptied and 100 µL of CONJUGATE added per well and mixed well. Then the plate was covered and incubated for 1 hour at 37°C in a humid chamber. After incubation, each well was washed 5 times with 1X WASH SOLUTION. After the last wash, the plate was inverted

and blotted dry by tapping on absorbent paper to completely remove the liquid. 50 μ L of SUBSTRATE A was added to each well followed by addition of 50 μ L SUBSTRATE B. The plate was Covered and incubated for 10-15 minutes at room temperature away from direct sunlight. 50 μ L of STOP SOLUTION was added to each well and mixed well. The optical density (O.D.) was read immediately at 450 nm. The mean blank value was subtracted from each SAMPLE or STANDARD value and the mean was calculated for duplicate wells. Standard curve was constructed in Microsoft Excel 2011 for Mac as given below. The concentration of all the samples was calculated using the equation $y=4.379x-3.66$.

Figure 2: eNOS-3 Calibration Curve

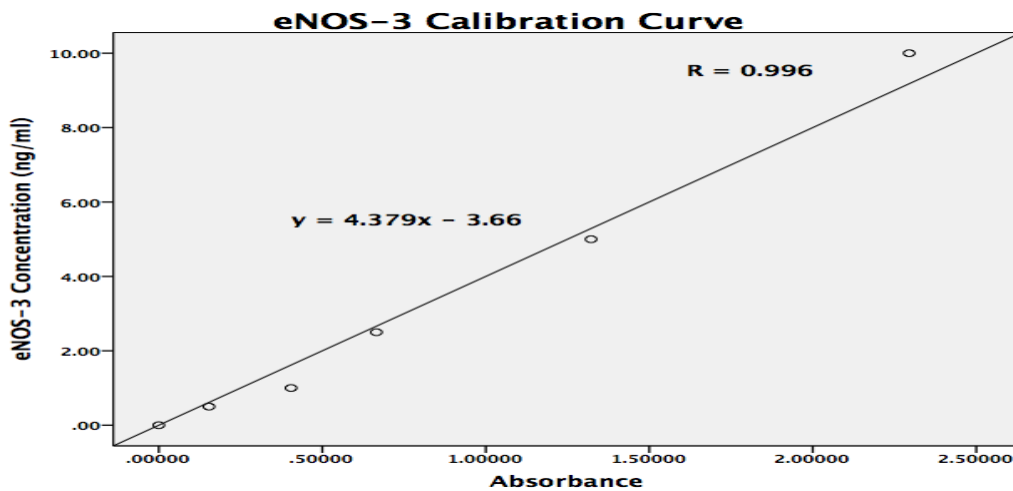


Fig.2: eNOS-3 Calibration Curve plotted from standard concentrations against standard absorbances (O.Ds). The regression equation was used to calculate sample concentrations from their respective O.Ds.

3.9.2.3. Sensitivity and Specificity

The sensitivity of this assay is approximately 0.1 ng/ml. This assay has high sensitivity and excellent specificity for the detection of eNOS-3. No significant cross-reactivity or interference between eNOS-3 and any homologous proteins assayed has been observed. Species cross-reactivity has not been specifically determined.

Both ELISA tests (Adiponectin and eNOS-3) were performed in the KS-HHV8 DIAGNOSTIC AND RESEARCH LABORATORY in the UTH. ELISA plates for plasma Adiponectin and eNOS-3 were read on the microwell reader available in the KS-HHV8 DIAGNOSTIC AND RESEARCH LABORATORY in the UTH.

3.10 Ethical Considerations and Permissions

Patient information and results were confidential and access to this information was restricted to the researcher, supervisor and clinicians only. Patient information was viewed by the approved study personnel only. The questionnaire captured the participants' file number, which was assigned a serial number hence specimen tubes were identified by serial numbers only. The file number was obtained for the purpose of returning the results.

The study participants (cases and controls) were provided with an information sheet and given a thorough explanation of intent and rationale of the research after which the patient gave written informed consent without duress, thus insured a true meeting of minds between the researcher and the patient. All this was done in private on a one to one basis to avoid undue influence that may affect or substitute the patient's will for that of any other persons.

Venipuncture may have been associated with the small risk of bleeding and infection. Those risks were minimised by skilful aseptic blood collection techniques and haemostatic procedures after blood collection.

The research proposal was submitted to the University of Zambia Biomedical Research Ethics Committee (UNZABREC) for approval.

Permission to conduct the study was sought from the: UTH medical superintendent, the consultants in the Department of Medicine, and the Directorate of Research and Graduate Studies (DRGS) through the Assistant Dean, Postgraduate.

Permission to use equipment and facilities in the clinical chemistry laboratory in the UTH was sought from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital, and permission to use the ELISA equipment and laboratory facilities at the UTH KS-HHV8 DIAGNOSTIC AND RESEARCH LABORATORY was sought from the KS-HHV8 DIAGNOSTIC AND RESEARCH LABORATORY Manager at the UTH.

4.0 Data Processing and Statistical Analysis

Data was analysed with IBM SPSS Statistical version 22 for Mac and Microsoft Excel 2011 for Mac and results summarised onto tables and graphs. Data were expressed as mean \pm SEM. The unpaired student *t*-test was used to compare mean values of plasma adiponectin concentration and eNOS concentration between the two groups (Hypertensive vs. Non-hypertensive group), and any other possible confounder. If data was not normally distributed, a non-parametric test (Mann Whitney U test) was used. All statistical tests were performed at 5% significance level or 95% confidence interval with p-value of <0.05 to determine statistical significance.

Bivariate linear regression and correlation coefficients were used to assess correlation between plasma adiponectin and eNOS in hypertension. The Bivariate linear regression data on adiponectin vs. eNOS was plotted and presented on scatter graphs.

5.0 Results

5.1 Clinical characteristics of Study Participants

74 (90.2%) of study participants were female while 8 (9.8%) were male. Hypertensive participants were older (53.2 ± 1.8) than non-hypertensive participants (47.5 ± 1.5) with statistical significance, $t(82)=2.439$, $p=0.017$ (Table 1.0). Systolic blood pressure was higher in hypertensive subjects (150.7 ± 4.3) than in healthy non-hypertensive subjects (113.7 ± 1.1) with statistical significance, $t(82)=8.336$, $p<0.001$ (Table 1.0). Diastolic blood pressure was higher in hypertensive participants (90.2 ± 2.2) than in non-hypertensive subjects (72.0 ± 1.1) with statistical significance, $t(82)=7.430$, $p<0.001$ (Table 1.0). Hypertensive subjects had statistically significant higher BMI (30.3 ± 1.0) than non-hypertensive subjects (25.7 ± 0.7), $t(82)=3.735$, $p<0.001$ (Table 1.0).

Table 1.0 Clinical Characteristics of Study Participants

Characteristic	Non-Hypertensive (n=41)	Hypertensive (n=41)	p-value
Age (years)	47.5 ± 1.5	53.2 ± 1.8	0.017
Systolic BP (mmHg)	113.7 ± 1.1	150.7 ± 4.3	<0.001
Diastolic BP (mmHg)	72.0 ± 1.1	90.2 ± 2.2	<0.001
BMI (kg/m^2)	25.7 ± 0.7	30.3 ± 1.0	<0.001

Table 1.0: Showing that hypertensive participants were older than non-hypertensives, hypertensive participants had higher BMI than non-hypertensives.

5.2 Adiponectin and eNOS Concentration Mean Difference

This study found that the mean adiponectin concentration in the hypertensive group was (11.5 ± 1.9 ng/mL) and in the non-hypertensive participants (11.0 ± 1.2 ng/mL), $t(78)=0.248$, $p=0.291$ (Fig. 3A), which was not statistically significant. eNOS concentration in the hypertensive participants was (1.5 ± 0.2 ng/mL) and (1.2 ± 0.1 ng/mL), in the non-hypertensive group, $t(80)=1.352$, $p=0.197$ (Fig. 3B), there was also no statistically significant difference.

Figure 3. Adiponectin and eNOS Mean Difference

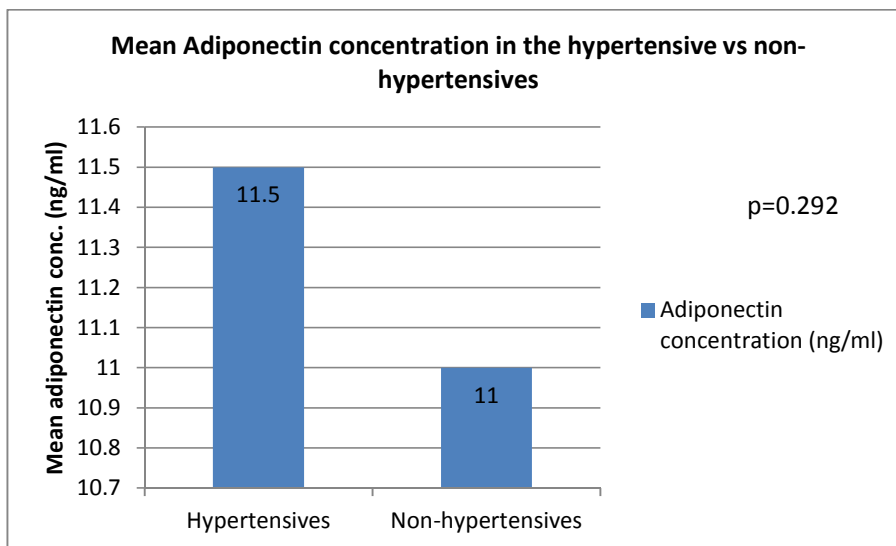


Fig 3A: The figure showed that there was no difference in mean adiponectin concentration between the hypertensive and the non-hypertensive group.

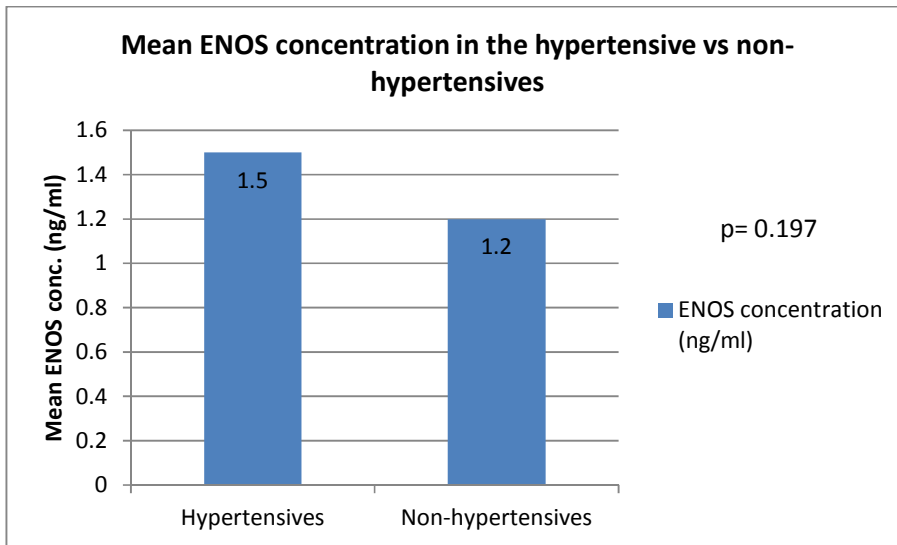


Fig 3B: The figure showed that there was no difference in mean eNOS concentration between the hypertensive and the non-hypertensive group.

5.3 Correlation of Adiponectin vs eNOS

Bivariate linear regression analysis of adiponectin vs eNOS showed a weak positive correlation in hypertensive participants without statistical significance ($r=0.036$, $p=0.825$) (Fig. 4A). Adiponectin vs eNOS also showed a weak positive correlation in healthy non-hypertensive participants ($r=0.135$, $p=0.406$) (Fig. 4B).

Figure 4. Adiponectin: eNOS Linear Correlation

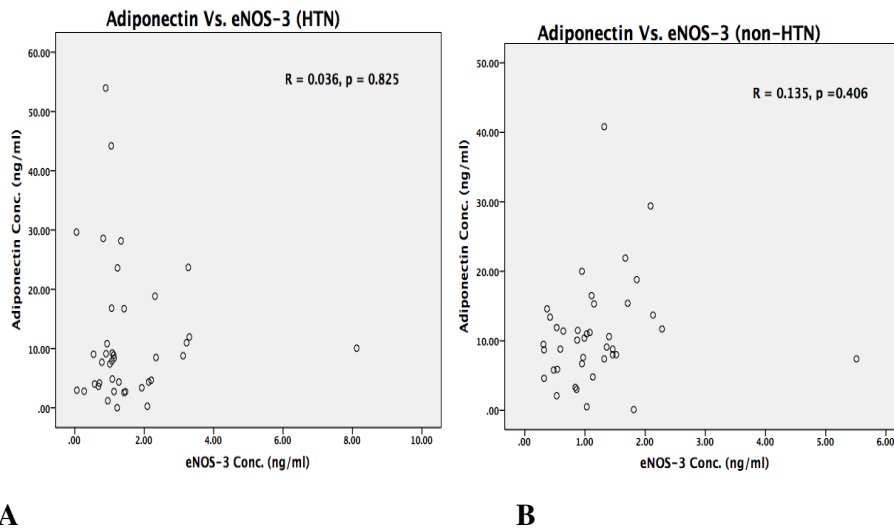


Fig 4A and 4B: The figures were showing that in both the hypertensives and non-hypertensives, there was no relationship between adiponectin and ENOS. They were independent of each other in terms of their influence on hypertension.

Figure 5: Relationship of Age group vs Mean Adiponectin concentration

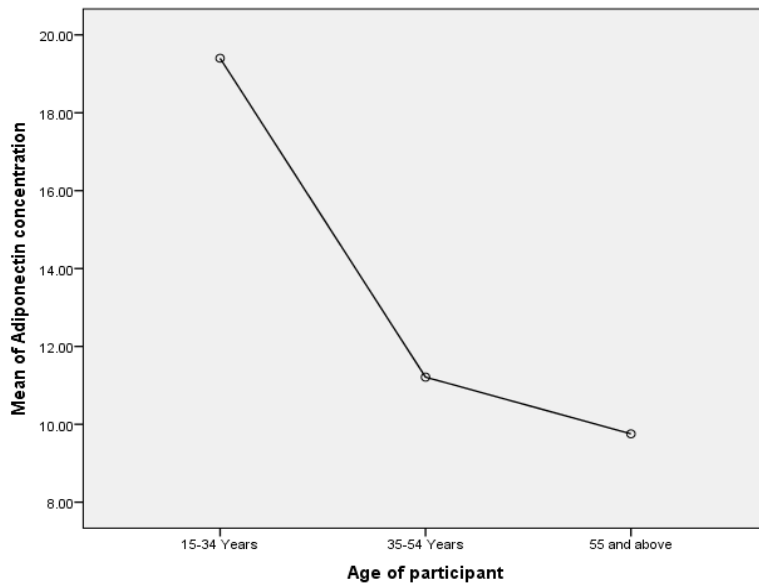


Fig.5: The figure showed that adiponectin levels were decreasing with increase in age.

Figure 6: relationship of BMI categories vs Mean Adiponectin concentration

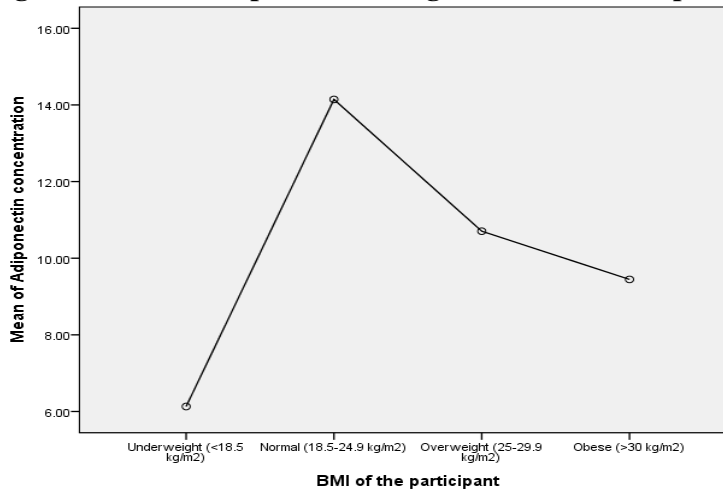


Fig.6: The figure is showing that adiponectin was low in the underweight, reached its peak in the normal and was decreased in the overweight and the obese.

Figure 7: Relationship between Age group vs Mean eNOS concentration

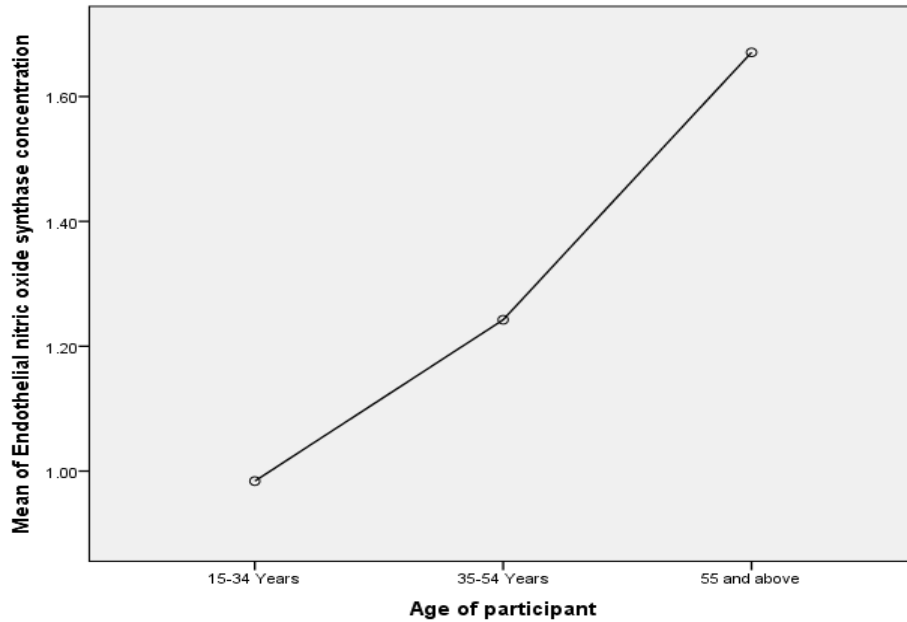


Fig.7: The figure showed that eNOS was increasing with increase in age.

Figure 8: relationship of BMI categories vs Mean eNOS concentration

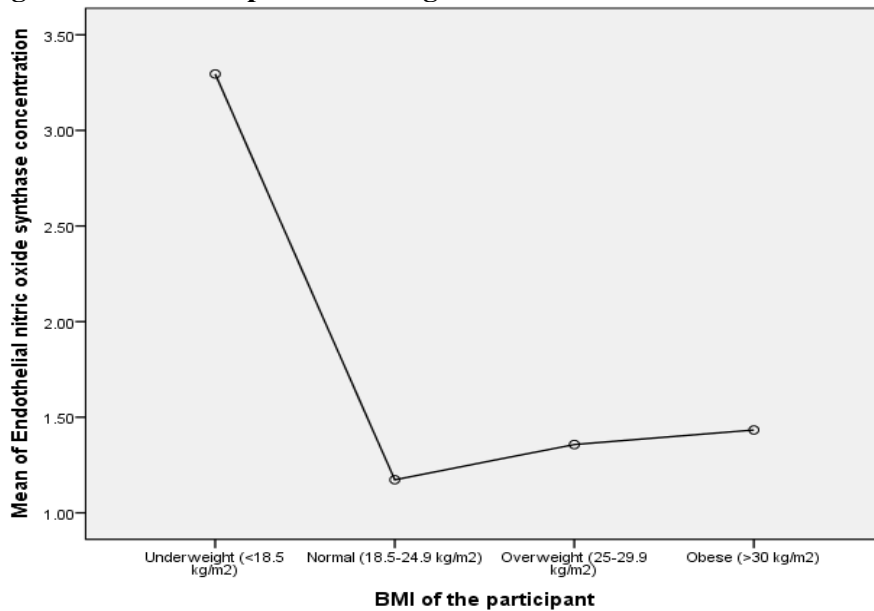


Fig.8: The figure showed that eNOS was reducing with an increase in BMI

6.0 Discussion

Adiponectin stimulates nitric oxide production in endothelial cells through AMPK-dependent and AMPK-independent phosphorylation of endothelial nitric oxide synthase (Cheng *et al*, 2007). Thus, hypoadiponectinaemia (a low level of adiponectin which is a protein produced by the adipose tissue and secreted into plasma) can result in decreased levels of endothelial nitric oxide synthase which produces the endothelium-derived vascular relaxing factor nitric oxide. This results in endothelial dysfunction and hypertension (Mahmud and Freely, 2005; Han *et al*, 2007; Giuseppe and Matteo, 2007).

Our study found that there was no difference in mean adiponectin levels between the hypertensive group and the non-hypertensive group. However, these results are in contrast to what we expected to find. One of the reasons for the non-significant p-value could be the small number of study participants involved in this study. These results are in contrast to findings by Adamczak *et al*, (2003) who reported that hypertensive patients had significantly lower concentrations of plasma adiponectin compared with normotensive healthy subjects. Yoshio *et al*, (2004) also reported a significantly lower lower concentrations of plasma adiponectin in patients with hypertension compared with normotensive healthy subjects. A study supporting our findings was done by Jung *et al*, (2009), who reported a non-significant difference in the adiponectin concentrations between the hypertensive group and the normotensive healthy group.

A number of reasons might have led to increased levels of adiponectin in the hypertensive group. This study was conducted in essential hypertensive patients on anti-hypertensive treatment. Anti-hypertensive drugs lead to an increase in adiponectin levels. Thus, anti-hypertensive therapy might have confounded these study findings. A modest but significant increase in plasma adiponectin levels has been observed after treatment with angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) in patients with hypertension (Furuhashi *et al*, 2003; Kon *et al*, 2005). Japanese adults with essential hypertension had significant increases in plasma adiponectin concentrations following three (3) months of losartan treatment (Watanabe *et al*, 2006). Blocking the renin-angiotensin system (RAS) was associated with an increase in plasma adiponectin levels. Furthermore, angiotensin II (AngII) infusion decreased plasma adiponectin concentrations and adipose tissue adiponectin expression (Hattori *et al*, 2005). Olmesartan, an angiotensin II type-1 receptor blocker, reversed obesity-induced hypoadiponectinaemia (Kurata *et al*, 2006).

However, the precise molecular mechanisms by which renin-angiotensin system inhibition stimulated adiponectin production remained unclear.

The other mechanism which could have affected the study findings was renal impairment. Essential hypertension is an important cause of renal dysfunction which impairs renal excretion of adiponectin in essential hypertensive (Takeshi *et al*, 2005). And since renal disease was only identified by written questionnaire, participants with renal dysfunction which was not medically confirmed might have confounded the study findings. Other life style interventions like exercise, controlled diet including salt intake also affect adiponectin concentration levels. Exercise was another way that elevated adiponectin levels, possibly by improving oxidative capacity. Overweight males exhibited higher level of adiponectin after 10 week aerobic training programme (Kriketos *et al*, 2004). Some dietary factors, such as soy protein (Nagasawa *et al*, 2002), fish oils (Flachs *et al*, 2006) and linoleic acid (Vivian, 2009) also tended to increase adiponectin levels in blood.

Overall, study participant's age group versus mean adiponectin concentration showed that adiponectin decreased with increasing age although this was not statistically significant. This was in contrast to findings of a study conducted by Takeshi *et al*, (2005) who reported increase in adiponectin concentration with increasing age. The decrease in adiponectin clearance in the kidney may have been the cause of high levels of adiponectin in the elderly. It should however be noted that the study by Takeshi *et al*, (2005) was a cross-sectional one. Therefore, more prospective studies may be necessary to explain the relationship between age and adiponectin concentration.

The results of this study showed that the mean adiponectin concentration was low in those who were underweight, and that it was high in those with normal weight, while adiponectin decreased in the overweight and obese even though this was not statistically significant. The initial clinical study of adiponectin measured its plasma levels in obese subjects (Yoshihisa *et al*, 2006). It showed that, obese subjects had significantly lower plasma adiponectin concentrations than non-obese subjects. (Arita *et al*, 1999). These findings on BMI and adiponectin were in agreement with findings by Phillips *et al*, (2008) who described how obesity was associated to hypoadiponectinaemia, probably through inhibition of gene transcription and a reduced release of the factor (adiponectin). A negative correlation between obesity and circulating adiponectin had been well established, and adiponectin

concentrations increased concomitantly with weight loss towards normal weight (Ursula and Axel, 2004).

Our study found that there was no difference in mean endothelial nitric oxide synthase concentration between the hypertensive group and the non-hypertensive group. However, we expected to find higher concentrations of endothelial nitric oxide synthase in the normotensive healthy subjects than in the hypertensive group. In a study previously done, most of the patients with hypertension had low levels of ENOS (Heller, 1996), which was the enzyme for synthesis of NO in vascular cells (Auggard, 1994; Bredt and Synder, 1994; Henrich, 1991; Murod, 1999). These findings were in contrast to those by Chandra *et al*, (2003) who reported that hypertensive patients had lower NO concentration levels than normotensive healthy subjects, which was an estimate of the activity of endothelial nitric oxide synthase. Arora *et al*, (2009) reported that nitrite levels, a metabolite of NO was lower in the hypertensive group than the non-hypertensive group. The non-significant differences for endothelial nitric oxide synthase might have been due to small sample size involved in this study.

A number of factors affect the concentration of endothelial nitric oxide synthase in plasma. One of the factors is drugs. ACE inhibitors and AT1-receptor antagonists increase NO production not only in nephrogenic but also in essential hypertension (Slaninka-Miceska *et al*, 2003; Yavuz *et al*, 2003). The long term loss of effectiveness was inapparent. The short acting beta-blockers inhibit NO production and decrease insulin sensitivity, while long-lasting beta-blockers and notably hybride beta-blockers carvedilol (Kalinowski *et al*, 2003) or nebivolol (Fratta Pasini *et al*, 2005) increase NO production. Calcium antagonists like dihydropyridines a benzodiazepines increased it, while phenylalkylamines do not influence NO production (Ding and Vaziri, 2000). Thus, one or a combination of the drugs above might have confounded the findings on the plasma concentrations of endothelial nitric oxide synthase, as they might have been administered to some hypertensive patients.

Our study also revealed that the mean of endothelial nitric oxide synthase concentration was increasing with age but it was not statistically significant. These findings were similar to those by Alusik *et al*, (2008) in which a group of apparently healthy elderly individuals aged over 80 had shown to have increased plasma levels of nitrates compared with controls: however, the difference was not statistically significant. In addition to dietary and renal

factors, the increased levels in the study by Alusik *et al* may have been due to enhanced NO production by inducible nitric oxide synthase in asymptomatic inflammation. Our study showed that the mean of endothelial nitric oxide synthase concentration was high in those who were underweight and those with normal weight. It was low in those who were overweight and obese which was not statistically significant.

However, adiponectin and endothelial nitric oxide synthase concentrations showed a weak positive non-significant correlation in individuals with essential hypertension. There was also non-statistically significant weak positive correlation between circulating plasma adiponectin and endothelial nitric oxide synthase concentration in the non-hypertensive group.

Race and ethnicity have an important influence in the biological markers and the study findings from the western world mainly carried out in the Caucasians should not be generalised and applied to the indigenous black Africans (Jeffrey *et al*, 2004). To the best of our knowledge, this is the first study in Zambia and probably in Africa which has assessed the levels of adiponectin and endothelial nitric oxide synthase and their probable relationship in hypertensive black Zambians.

The mean adiponectin in our Zambian hypertensive patients was lower than those reported in other places mainly the western countries for example (Adamczak *et al*, 2003) and (Yoshio *et al*, 2004). The mean adiponectin in our Zambian normotensive subjects was lower than the range of adiponectin reported in literature carried out in white subjects (Arita *et al*, 2002). This difference in mean adiponectin might explain why hypertension is more common in blacks than in whites (Oscar *et al*, 2000). Our results on the levels of endothelial nitric oxide synthase could not be compared to other studies due to scarcity of data as most studies concentrated on the activity rather than the concentration of eNOS.

6.1. Conclusion

The study showed that there was no difference in mean adiponectin concentration and mean eNOS concentration between hypertensive patients and non-hypertensive participants. There was no relationship between adiponectin and eNOS levels on their influence on hypertension.

6.2. Implications and Recommendations

Treatment options for hypertension remain suboptimal. In order to develop new, better and more effective treatment for hypertension, there is need for increased understanding of the pathogenetic mechanisms involved in the development of hypertension so that therapy may target the correct mechanism or mechanisms involved in the disease process. Findings of this study indicate that more research needs to be carried out on adiponectin and endothelial nitric oxide synthase as important biomarkers in the pathogenesis of hypertension that could provide diagnostic, prognostic and therapeutic advantages by providing information on the metabolic state of hypertensive individuals.

6.3. Limitations/Weaknesses and Assumptions

- The study was limited to hypertension. Other components of the metabolic syndrome like insulin resistance and hyperlipidaemia were not looked at. The pathophysiology of hypoadiponectinaemia has also been implicated in these conditions.
- Failure to clearly distinguish between primary and secondary hypertension by way of tests especially renal function tests.
- Drug history was not taken.
- Also measuring the activity of nitric oxide would have added significant meaning as to how the endothelial nitric oxide synthase was functioning.

6.4. Future Direction

With regard to above considerations, more studies need to be conducted with larger sample sizes. The relationship between adiponectin and hypertension need to be further elucidated.

Some more studies are needed to focus on the relationship between adiponectin and endothelial nitric oxide synthase levels in hypertension.

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8.0. Appendices

Appendix 1.0: Exclusion Criteria Explained

- Non-consenting individuals were not included into the study because participation in this study was voluntary.

Appendix 2.0: Table 2: Hypertensive Participant's Raw Data

NO.	SEX	AGE(years)	BP READING (mm/Hg)	WEIGHT (Kg)	HEIGHT (cm)	BMI (kg/m ²)	ADIPONECTIN LEVEL (ng/ml)	ENOS-3 LEVEL (ng/ml)
01	M	42	140/90	92	1.72	31.1	2.71	1.46
02	F	50	110/70	87	1.65	32.0	4.19	0.71
03	F	50	180/100	108	1.64	40.2	0.26	2.09
04	F	47	160/90	56	1.6	21.9	53.95	0.89
05	F	50	160/90	78	1.57	31.6	0.01	1.22
06	F	50	150/90	88	1.53	37.6	4.36	1.27
07	F	42	180/90	72	1.61	27.8	9.04	0.54
08	F	69	180/110	95	1.6	37.1	2.59	1.42
09	F	58	140/90	136	1.63	51.2	7.40	1.01
10	F	54	140/90	78	1.53	33.3	9.25	1.08
11	F	25	120/70	71	1.67	25.5	44.20	1.05
12	M	67	160/90	70	1.7	24.2	9.13	0.90
13	F	55	180/100	98	1.55	40.8	23.60	1.23
14	F	58	160/90	86	1.6	33.6	4.65	2.20
15	F	62	150/100	85	1.58	34.0	4.36	2.13
16	F	62	200/130	96	1.6	37.5	23.69	3.27
17	F	56	140/90	72.5	1.6	28.3	3.39	1.93
18	F	37	169/100	87	1.71	29.8	4.02	0.57
19	F	57	100/60	62	1.64	23.1	28.58	0.82
20	F	70	150/100	71	1.52	30.7	2.75	1.13
21	F	42	180/100	85	1.63	32.0	16.81	1.06
22	F	54	240/120	68	1.53	29.0	10.06	8.12
23	F	35	120/70	71	1.52	30.7	8.49	2.34
24	F	37	130/90	61	1.64	22.7	8.92	1.12
25	F	49	130/60	63	1.6	24.6	2.80	0.27
26	F	50	150/90	84	1.61	32.4	28.16	1.33
27	F	44	120/70	42	1.55	17.5	4.86	1.08
28	M	59	180/100	86	1.63	32.4	8.37	1.13
29	F	62	140/90	80	1.51	35.1	11.95	3.30
30	F	35	130/90	54	1.48	24.7	2.97	0.06
31	F	42	140/80	75	1.56	30.8	10.82	0.93
32	F	63	130/80	79	1.54	33.3	3.64	0.68
33	F	44	130/90	70	1.55	29.1	7.90	1.07
34	F	54	160/100	90	1.6	35.2	29.64	0.05
35	F	72	130/90	67	1.70	23.0	1.19	0.95
36	F	50	160/100	51	1.49	23.0	18.83	2.31
37	F	77	130/90	65	1.61	25.0	16.72	1.42
38	F	51	190/100	108	1.7	37.4	7.69	0.78
39	M	66	160/90	58	1.63	21.8	10.98	3.22
40	F	59	150/90	75	1.65	27.5	8.79	3.12
41	M	76	110/70	59	1.63	22.2	**	1.50

**=adiponectin result did not come out.

Appendix 3.0: Table 3: Non-hypertensive Participant's Raw Data

NO.	SEX	AGE(years)	BP READING (mm/Hg)	WEIGHT (Kg)	HEIGHT (cm)	BMI (kg/m ²)	ADIPONECTIN LEVEL (ng/ml)	ENOS-3 LEVEL (ng/ml)
01	F	46	130/80	62	1.6	24.0	29.40	2.09
02	F	58	120/80	72	1.6	28.0	5.80	0.48
03	F	36	120/70	78	1.63	29.4	9.50	0.31
04	M	64	110/70	68	1.78	21.5	21.90	1.67
05	M	55	120/80	75	1.82	22.6	3.30	0.84
06	F	45	110/80	91	1.5	40.4	11.70	2.28
07	M	64	110/70	48	1.65	17.6	7.40	5.51
08	F	33	110/80	62	1.71	21.2	20.0	0.95
09	F	37	110/70	57	1.6	22.3	13.70	2.13
10	F	29	110/60	68	1.61	26.2	2.10	0.53
11	F	33	110/70	68	1.59	26.9	18.80	1.86
12	F	38	120/70	58	1.58	23.2	7.60	0.97
13	F	45	100/60	70	1.6	27.3	15.40	1.71
14	F	44	100/60	72	1.62	27.4	11.50	0.88
15	F	31	120/70	56	1.7	19.4	11.90	0.53
16	F	51	110/70	71	1.57	28.8	8.80	0.59
17	F	36	110/70	67	1.6	26.2	10.40	0.99
18	F	35	120/80	58	1.66	21.0	8.00	1.52
19	F	35	120/80	76	1.71	26.0	11.40	0.64
20	F	44	110/60	69	1.59	27.3	11.20	1.08
21	F	55	130/60	62	1.6	24.2	16.50	1.11
22	F	51	120/80	78	1.56	32.0	7.40	1.32
23	F	53	110/70	69	1.61	26.6	13.40	0.42
24	F	53	110/80	58	1.71	19.8	4.60	0.32
25	F	55	130/80	56	1.59	22.0	8.70	0.32
26	F	47	110/70	68	1.6	26.6	5.90	0.54
27	F	60	110/80	59	1.6	23.0	8.00	1.46
28	F	45	120/80	56	1.6	21.9	15.30	1.15
29	M	46	110/70	72	1.82	21.7	11.00	1.03
30	F	45	110/70	80	1.63	30.0	8.80	1.18
31	F	54	110/80	69	1.59	27.3	4.80	1.18
32	F	44	110/70	58	1.6	22.7	6.70	1.18
33	F	51	110/70	77	1.58	30.8	10.60	1.18
34	F	62	110/80	64	1.63	24.1	3.00	1.18
35	F	52	110/70	68	1.54	28.7	14.60	1.18
36	F	57	120/60	83	1.58	33.2	0.50	1.18
37	M	48	110/70	80	1.72	27.0	0.10	1.18
38	F	50	110/70	58	1.56	23.8	40.80	1.18
39	F	48	110/70	82	1.63	30.8	10.10	1.18
40	F	53	120/60	68	1.58	27.2	9.10	1.18
41	F	59	110/80	59	1.6	23.0	**	1.18

**=adiponectin result did not come out.