MICRORNA PROFILING IN SOUTH AFRICAN INDIVIDUALS WITH HYPERTENSION AND ABSOLUTE BLOOD PRESSURE LEVELS

by

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DECLARATION

I, *Don Makwakiwe Matshazi*, hereby declare that the contents of this thesis reflect my own work and I have not, in part or its entirety, submitted it to any other university for academic examination. The opinions expressed in the thesis are my own and are not necessarily those of Cape Peninsula University of Technology.

02 November 2020

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Signature **Date**

STUDY SUMMARY

Background

Hypertension (HPT) is a complex, genetic and environmental condition characterised by sustained high blood pressure and a late presentation of symptoms. It is the single most important modifiable risk factor for cardiovascular diseases (CVDs) and its prevalence and the occurrence of related complications continue to rise in Africa, whilst its magnitude, underlying risk factors and mechanisms remain less understood. Epigenetics offers an avenue to understand the underlying mechanisms, essential in identifying individuals at increased risk of developing HPT, which in turn would facilitate the development of appropriate management strategies. Therefore, we aimed to investigate the microRNA (miRNA) profile in individuals with HPT using next generation sequencing (NGS) for miRNA sequencing as well as quantitative reverse transcription polymerase chain reaction (RT-qPCR). Furthermore, we investigated the functional pathways linked to dysregulated miRNAs in HPT.

Methodology

Total RNA was extracted from the whole blood of 573 normotensive, 304 screen-detected hypertensive and 579 known hypertensive male and female participants. Next generation sequencing (NGS) was conducted to screen for differentially expressed miRNAs in a cohort of 48 age-matched normotensive (n=12), screen-detected hypertensive (n=16) and known hypertensive (n=20) females. Findings of the two most differentially expressed miRNAs (miR-30a-5p and miR-1299) in NGS were validated using RT-qPCR in a cohort combining 48 participants from the original NGS investigation and an independent sample of 263 male and female normotensives, screen-detected hypertensives and known hypertensives. Furthermore, in order to identify dysregulated pathways in HPT, Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis was conducted. Using RT-qPCR, the difference in expression of five miRNAs (182-5p, 30a-5p, 30e-3p and 1299 as shown by miRNA sequencing) and miR-126-3p, and their relationship with HPT in 1456 normotensive and hypertensive male and female participants was investigated. The utility of novel miRNAs dysregulated during HPT in predicting clinical blood pressure (BP) parameters was also investigated in normotensive and hypertensive participants.

Results

Amongst the dysregulated miRNAs were miR126-3p, 30a-5p, 182-5p, 1299 and 30e-3p and the two novel miRNAs, miR-novel-chr1_36178 and miR-novel-chr15_18383. The dysregulation of these miRNAs was associated with various biological pathways including platelet activation, calcium signalling and vascular smooth muscle contraction pathways. A significantly higher expression of miR-126-3p and miR-182-5p in hypertensives (both screen-detected and known hypertension) was observed, although the expression of miR-30a-5p, 30e-3p and 1299 was not significantly different in screen-detected hypertensives (not on therapy) when compared to the normotensives. There was a significant association between the expression of miR-126-3p, 182-5p and 30a-5p with screen-detected and known hypertension, even after adjustment for age, sex, body mass index (BMI), glycated haemoglobin (HbA1c), triglycerides (TG) and total cholesterol (TC). MiR-novel-chr1_36178 expression significantly differed by sex and only at greater levels of expression was it associated with systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP). Additionally, at higher levels of expression of the novel miRNA, there was an increased likelihood of a diagnosis of screen-detected HPT being made.

Conclusion

Our findings provide evidence of miRNA dysregulation in HPT in an African population and suggest that these dysregulated miRNAs, through their effects on BP regulation pathways, are involved in the pathogenesis of HPT. Owing to their differential expression according to HPT status and correlation of their expression with clinical BP parameters, these miRNAs may be targeted for diagnostic, prognostic and therapeutic purposes in HPT.

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Last, but certainly not least, the Almighty, for without Him, I would not be where I am today.

DEDICATION

This thesis is dedicated to my dearly departed friend, Ms Hilory Munhuweyi. Even in your absence, you continue to inspire in me, a desire to never stop pushing forward and achieving things we both never dreamt possible. I know you are now in a better place my friend. Until we meet again, continue to rest in peace.

Education is the passport to the future, for tomorrow belongs to those who prepare for it today - Malcolm X

LIST OF ABBREVIATIONS AND ACRONYMS

ABPM	Ambulatory Blood Pressure Measurement
ACE	Angiotensin Converting Enzyme
Ago	Argonaute
AGTR1	Angiotensin II Type1 Receptor
Ang	Angiotensin
ANOVA	Analysis of Variance
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
cGMP	cyclic Guanosine Monophosphate
CKD	Chronic Kidney Disease
CRP	C-reactive Protein
CVD	Cardiovascular Disease
DASH	Dietary Approach to Stopping Hypertension
DBP	Diastolic Blood Pressure
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
ENaC	Endothelial Sodium Channel
eNOS	endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
FGF	Fibroblast Growth Factor
Gamma-GT	Gamma glutamyltransferase
GO	Gene Ontology
HbA1c	Glycated Haemoglobin

HCMV	Human Cytomegalovirus
HDL	High Density Lipoprotein
HIC	High Income Country
HPT	Hypertension
IFG	Impaired Fasting Glucose
ISH	International Society of Hypertension
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LDL	Low Density Lipoprotein
LMIC	Low-to-Medium Income Country
MAP	Mean Arterial Pressure
MBLN1	Muscleblind-like Splicing Regulator 1
MESH	Medical Subject Headings
miRISC	MicroRNA-induced Silencing Complex
miRNA	MicroRNA
mmHg	millimetres of mercury
mRNA	messenger RNA
NCD	Non-communicable Diseases
NDoH	National Department of Health
NGS	Next Generation Sequencing
NO	Nitric Oxide
OR	Odds Ratio
PAH	Pulmonary Arterial Hypertension
PBMC	Peripheral Blood Mononuclear Cell
PGI_2	Prostacyclin
PI	Phosphatidylinositide

RAAS	Renin-Angiotensin-Aldosterone System
RHD	Rheumatic Heart Disease
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SADHS	South African Demographic and Health Survey
SBP	Systolic Blood Pressure
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Product and Service Solutions
STAT	Signal Transducers and Activators of Transcription
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TG	Triglycerides
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VEGF	Vascular Endothelial Growth Factor
VMH	Vascular and Metabolic Health
VSMC	Vascular Smooth Muscle Cell
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

Hypertension (HPT) has been well-established as a modifiable risk factor for cardiovascular (Fuchs & Whelton, 2020) and renal disease (Ji *et al*., 2019) and continues to be a leading risk factor for the burden of disease (Lim *et al*., 2012). In 2010, according to the World Health Organisation (W.H.O), close to a third of the world's adult population (1.38 billion people) was reported to be hypertensive (Mills *et al*., 2016), compared to 594 million people forty years prior. The ever increasing global burden of HPT is highlighted by its current contribution to over 10 million deaths annually (Stanaway *et al*., 2018). More recently, there appears to be a deviation in the HPT picture, as while it has been traditionally a problem for high-income countries (HIC), the past few decades have borne witness to a shift in the burden to low-to-medium income countries (LMICs), (Zhou *et al*., 2017).

The period between 2000 and 2010 has seen the prevalence of HPT decrease by 2.6% in HICs, whilst increasing by 7.7% in LMICs (Mills *et al*., 2016). This is particularly worrying for Africa as the growing HPT pandemic, and its related cardiovascular complications, are added burdens on healthcare facilities which are already being stretched to their limits by infectious diseases like HIV/AIDS, Ebola, tuberculosis, malaria and more recently, the coronavirus disease of 2019 (COVID-19).

Whilst early identification, treatment and ultimately blood pressure (BP) control are important in avoiding clinically worse outcomes, up to 54% of hypertensive individuals are unaware of their condition (Peltzer & Phaswana-Mafuya, 2013; Babiker *et al*., 2013). As such, HPT usually goes uncontrolled for a long time, leading to cardiac and renal disease, end-stage organ damage, stroke or death. Multifaceted approaches that facilitate exploration of alternative ways in which the incidence of HPT can be detected and monitored, and its progression predicted, are essential as they may be useful in the avoidance or reversal of potentially deleterious HPT-related effects, without the need for pharmatherapeutic intervention. This is especially relevant as simple solutions like lifestyle and behavioural modifications have seen clinically relevant drops in high BP to normal levels (Svetkey *et al*., 2005).

The pathogenesis of HPT involves the complex interplay of environmental and genetic factors (Biino *et al*., 2013). Whilst obesity (Leggio *et al*., 2017), chronic stress (Spruill, 2010), a high-salt diet (He *et al*., 2013) and physical inactivity (Diaz & Shimbo, 2013) play significant roles in HPT, the focus on the pathophysiology of HPT has more recently included epigenetics. Mechanisms which involve heritable changes in the expression of genes without a change in the underlying deoxyribonucleic acid (DNA) sequence are also key role players in the development of HPT. These include DNA methylation and histone modifications and their contributions to the development of HPT have been well established (Stoll *et al*., 2018; Gonzalez-Jaramillo *et al*., 2019). In the past decade, microRNAs (miRNAs), which are non-coding, post-transcriptional regulators of gene expression, have been subjects of considerable research interest, particularly in disease. Their tissue specificity and stability in circulation (Gilad *et al*., 2008) has enabled them to be studied in various pathologies, including HPT. Since miRNA dysregulation has been associated with the development of diseases (Li & Kowdley, 2012; Silambarasan *et al*., 2016; Nemecz *et al*., 2016), they have been targeted as potential diagnostic and prognostic biomarkers of cancer disease (Xu *et al*., 2020) with considerable success (Fesler *et al*., 2018).

Various studies have analysed miRNA expression profiles in HPT (Huang *et al*., 2016; Yildirim *et al*., 2019; Kaneto *et al*., 2017; Özkan *et al*., 2019). However, a majority of the work has been done in high income countries, with disparate socio-demographic landscapes to sub-Saharan Africa. Worth noting is the crucial involvement of environmental factors in addition to genetic elements in the development of HPT (Biino *et al*., 2013). It is plausible that the expression and effects of miRNAs within the same disease state may be different for individuals in different geographical locations, owing to the difference in environmental conditions.

Whilst clear evidence of the importance of miRNAs in disease exists, this avenue of research has been largely neglected in sub-Saharan Africa. A Medical Subject Headings (MESH) search on PubMed of miRNA-related work done in Africa using the terms “microRNAs” and “Africa” returned only 80 publications. The bulk of these publications were on work conducted in North Africa (Egypt, Tunisia and Algeria), with a particular focus on cancer, and no publications focusing on HPT. The search returned only 13 publications when the criteria was narrowed down

to work done in South Africa. This demonstrates a paucity of data on the possible roles played by miRNAs in the pathogenesis of HPT in African settings. As such, a study to determine miRNA expression profiles in sub-Saharan African individuals is crucially important as it may shed light on how these molecules may be linked to the development of HPT and its related complications within this specific setting. This is also of particular importance in communities where a high prevalence of Type 2 Diabetes Mellitus (T2DM) has been previously reported (Erasmus *et al*., 2012), as up to 75% of T2DM patients lose their life as a result of cardiovascular complications mostly related to HPT (Campbell *et al*., 2011).

The clinical measurement of BP using automated monitors and sphygmomanometers is a non-invasive and cost-effective technique. However, there is evidence that recommendations for BP measurement are rarely followed in clinical practice (Sebo *et al*., 2014). In addition, there is anxiety that comes with being in a clinical setting, which may lead to the "white coat effect" and thus inaccurate measurements of BP. As a result, home blood pressure monitoring and self-reporting of BP has become a favourable alternative. However, there is low adherence to established BP measurement guidelines when individuals measure their BP (Wagner *et al*., 2013). In addition, the majority of self BP measurement devices available for purchase have not been independently validated for clinical accuracy (Sharman *et al*., 2020) and as a result, a lot of the BP measurements are not a true reflection of the situation. This is particularly important, as small inaccuracies in BP measurement may lead to patient misdiagnoses (both false positive and negative), inaccurate community HPT prevalence and control figures, of which these are important in determining the allocation of resources to stricken communities to curb HPT, as reported by Campbell *et al*., 2020. The aforementioned challenges favour the exploration of miRNAs as possible biomarkers for HPT diagnosis. Additionally, once identified, these miRNA biomarkers may also become possible targets for the development of therapeutic options.

The broad aim of the study was to profile the expression patterns of miRNAs in the whole blood of normotensive and hypertensive South African men and women. Various specific objectives were set to achieve the study aim and these include:

- Total RNA extraction from the whole blood of normotensive and hypertensive participants.
- Screening for differentially expressed miRNAs in hypertensives and normotensives using next generation sequencing (NGS) in a cohort of 48 participants.
- Validating the NGS findings in larger, independent samples of normotensive and hypertensive participants using quantitative reverse transcription polymerase chain reaction (RT-qPCR).
- Relating microRNA expression to BP parameters like systolic and diastolic BP.
- Identifying biological pathways and processes linked to miRNAs that are dysregulated in HPT.

In doing the aforementioned, the study generated data that could potentially shed light on the roles played by miRNAs in the pathophysiology of HPT and contribute to the understanding of genes and biological pathways which may form a basis for prognostic and early-diagnostic strategies. In addition, these investigations could provide insights into prevention, management and/or the development of alternative HPT treatment strategies. To our knowledge, no other study has investigated and compared miRNA expression profiles in normotensive and hypertensive South African individuals and findings from this study aim to fill that knowledge gap.

CHAPTER 2: LITERATURE REVIEW

2.1 The heart and blood pressure

The myocardium of the heart is responsible for pumping blood to all body tissues in order to provide cells with oxygen and nutritional requirements whilst collecting waste substances for excretion. However, as the heart contracts and relaxes, it exerts a force or pressure on the walls of the vessels responsible for blood transportation. The pressure on the arterial walls as the heart muscle contracts to force blood out from its chambers into circulation is termed systolic blood pressure (SBP) whilst diastolic blood pressure (DBP) refers to the pressure on the arterial walls as the heart muscle relaxes and the heart's chambers fill up with blood for the next SBP-DBP cycle. It is essential that these two measures of pressure on the arterial walls are kept below a certain threshold if pathologies related to abnormally high BPs are to be avoided. Whilst various bodily regulatory systems are in place to maintain the BP within physiologically optimal levels, sometimes the BP may rise due to unknown factors and as such, it is essential that individuals are aware of their BP status at regular intervals to institute corrective measures in the event of any haemodynamic dysregulation. If possible, it is advised that individuals of African descent over the age of 18 years be screened annually for HPT as they are considered high risk individuals with respect to HPT. However, for low risk patients (normal BP measurement) and in the absence of other risk factors, BP measurements can be conducted once in three years (Unger *et al*., 2020).

2.2 Blood pressure measurement

According to the hypertension practice guidelines recently released by the International Society of Hypertension (ISH), there are key points to note before and during BP measurement as shown in Figure 1 (Unger *et al*., 2020). Strict adherence to these guidelines is critically important as deviations from these guidelines can lead to either overestimation or underestimation of BP measurements. Even minute inaccuracies in the BP measurements are accompanied by negative downstream effects such as incorrect patient diagnosis and distortions in the population's prevalence and control of HPT (Campbell *et al*., 2020).

It is very important that the prevalence of HPT in a population is accurately measured as this may be important in informing budget allocations towards implementation of policies aimed at addressing HPT-related concerns in that population. In summary, the ISH guidelines require that three BP readings be taken on the upper arm of an individual at rest, with a validated electronic or manual device with a cuff of appropriate size at one minute intervals. The average of the last two BP readings represents an individual's BP reading (Unger *et al*., 2020).

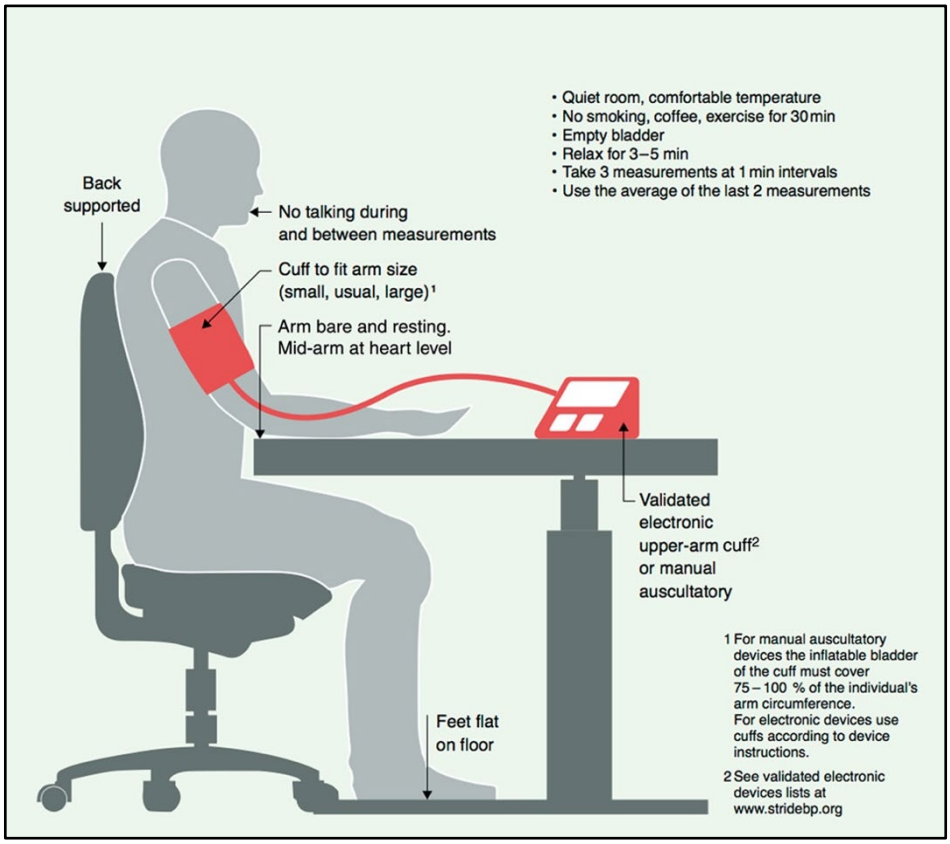

Figure 1. *ISH guidelines on the correct procedures for blood pressure measurement*

The image above was used with permission from the publishers, Wolters Kluwer Health, Inc. See license details in Appendix C (Unger et al., 2020)

Blood pressure measurements are expressed as two numerical figures, with the top and bottom figures representing systolic and diastolic measurements respectively. BP readings are expressed as millimetres of mercury (mmHg) and normal adults at rest should have a SBP within the 100-139 mmHg range and a diastolic blood pressure should be within the 60-89 mmHg range. Various thresholds denoting normal BP have been set by different bodies within the field of HPT. However, these are continuously reviewed and adjusted as new evidence on the beneficial effects of lowering

the thresholds for diagnosis and therapeutic interventions becomes available. Whilst the majority of HPT bodies define hypertension as measurements of SBP ≥140 mmHg and/or DBP ≥90 mmHg (Unger *et al*., 2020), the American College of Cardiology/American Heart Association deviates from the established criteria and defines HPT as SBP ≥130 mmHg and/or DBP ≥80mmHg (Flack & Adekola, 2020), to facilitate earlier interventions as well as trying to curb the development of complications that may take place before the blood pressure rises above the 140/90 mmHg threshold.

For a diagnosis of HPT to be made, it is advised that SBP and DBP measurements are taken over the course of 2-3 visits to the clinical setting, at 1-4 week intervals. If the SBP and DBP measurements remain elevated above a set BP threshold, a diagnosis of HPT can be made. A diagnosis of HPT can also be made on a single visit, but only when the SBP ≥180 mmHg and DBP is ≥110 mmHg (Unger *et al*., 2020). The most accurate method for diagnosing HPT is ambulatory blood pressure measurement (ABPM). This involves 24-hour blood pressure monitoring, with readings taken every 15-30 minutes during the day and 15-60 minutes at night. Despite its obvious benefits, major drawbacks to this method include the high monetary investment involved in the process as well as the lack of BP monitoring devices that have been independently validated for clinical accuracy. As such, ABPM is not readily available to physicians and their patients (Hinderliter *et al*., 2018), meaning that office and clinical measurement of BP for diagnosis of HPT remains the mostly used technique, despite the concerns raised earlier about non-adherence to established procedural guidelines in these settings.

2.3 The Silent Killer

As per the World Health Organisation, HPT is described as the persistent elevation of blood pressure in the arteries[1]. "Silent killer" is a common reference to HPT in literature, owing to the lack of symptoms in the developmental stages of the condition. Over 50% of hypertensive

[1] https://www.who.int/health-topics/hypertension/#tab=tab_1

individuals are unaware of their condition until complications related to organ dysfunction or damage start to manifest (Babiker *et al*., 2013). In a study on Tanzanian people living on a fishing island, a staggering 73% of participants were unaware of their hypertensive status (Muhamedhussein *et al*., 2016).

Hypertension is a complex, polygenic, multifactorial disease with both genetic and environmental (lifestyle related) factors, and its development is a major risk factor for adverse clinical outcomes. The condition exists in two forms, namely primary (essential) and secondary HPT. The former, more common in adulthood, describes sustained blood pressure increases above set thresholds without a known cause and accounts for 90-95% of HPT cases, whilst the latter, more common in younger people, describes forms of HPT with known causes and accounts for 5-10% of hypertension cases (Charles *et al*., 2017). The causes of secondary HPT include chronic kidney disease, primary aldosteronism, sleep apnoea, renal artery stenosis and use of some prescription medications (Puar *et al*., 2016).

Exhibiting high BP for a brief period of time does not come with any symptoms or complications. However, prolonged high BP can pose life-threatening consequences and has been implicated as a risk factor for serious conditions including stroke, heart failure, vascular disease, chronic kidney disease (CKD) and coronary artery disease (CAD) (Babiker *et al*., 2013). Hypertension is one of the most important modifiable risk factors for cardiovascular disease (CVD) and the burden of CVDs has been well described (Jones Buie *et al*., 2016). Currently, CVDs are a leading cause of morbidity and mortality in both the developed and even more so the developing parts of the world (Fuster *et al*., 2011).

2.4 Blood pressure regulation and pathogenesis of hypertension

A subject of intense study to this day, the pathogenesis of HPT is multifactorial and notoriously complex. Various mechanisms have been put forward to explain the development of HPT. BP regulation is under the control of various processes in the body and the kidney is a vital role player, through its hormone-driven renin-aldosterone-angiotensin system (RAAS).

The RAAS is one of the many regulatory systems in the body and is comprised of hormones essential for regulation of blood pressure and maintenance of body fluid balance, through the

combined effects of the adrenal glands and renal, cardiovascular and central nervous systems. The RAAS regulates BP by maintaining tight control of the volume of blood. A drop in the BP is responded to by juxtaglomerular cell secretion of renin to facilitate the conversion of angiotensinogen to angiotensin I (Ang I). The vascular endothelium releases angiotensin converting enzyme (ACE) to cleave Ang I into angiotensin II (Ang II), which is responsible for the constriction of blood vessels and reabsorption of sodium, leading to resistance to blood flow as well as enhanced sodium and fluid retention by the kidneys. Ang II also causes the release of aldosterone by the adrenal cortex and vasopressin by the posterior pituitary gland to facilitate fluid and sodium retention by the kidneys. The net effect of all this is a BP increase to normal levels as determined by the BP regulatory mechanisms (Nemecz *et al*., 2016). Altered regulation of the RAAS as a result of single nucleotide polymorphisms (SNPs) in the genes of angiotensin II type1 receptor – AGTR1 have been associated with a risk of HPT (Parchwani *et al*., 2018), although these findings continue to be a subject of debate (Mottl *et al*., 2008; Ji *et al*., 2017).

Endothelial cells (ECs) also play a vital role in BP regulation through the action of vasodilatory substances like nitric oxide (NO), prostacyclin (PGI_2) and the vasoconstrictor, endothelin-1 (ET-1). Damage to the ECs is characterised by platelet activation, a reduction in vasodilation, pro-inflammatory and thrombotic phenotypes, producing an environment favouring the release of Ang II and ET-1. The release of Ang II leads to the generation of reactive oxygen species (ROS) that contribute to oxidative stress in the wall of the vasculature and hampering of NO bioavailability, leading to increased peripheral vascular resistance (Savoia *et al*., 2011). All this culminates in the impaired BP regulation and may lead to the development of HPT.

2.5 Risk factors for hypertension

Although the aetiology of HPT is unknown in up to 95% of hypertensives, there are various genetic and environmental factors associated with the condition. They include age, a family history of the condition, obesity, racial background, gender, lack of adequate physical activity, smoking and high dietary sodium intake and are known to exacerbate the effects of HPT (Mayosi *et al*., 2009; Rossier *et al*., 2017), although some studies have reported a lack of association between smoking and HPT (Ntuli *et al*., 2015).

As people grow older, their arteries stiffen, particularly the large arteries, culminating in increases in the BP due to vascular resistance. According to the South African Demographic and Health Survey (SADHS) of 2016, 84% of female participants in the study above the age of 65 years were hypertensive in comparison to only 17% in participants aged between 15-24 years (National Department of Health (NDoH) *et al*., 2016). The trend of increased HPT prevalence with advanced age has been widely reported (Ntuli *et al*., 2015). Whilst the development of HPT in the elderly population is an inevitable occurrence, there exists an increasing relationship between the SBP and DBP with age. The Framingham Heart Study demonstrated a gradual increase in the SBPs of 30-84 year old participants followed up over the course of three decades. However, the increase in the DBP of these individuals ceased after turning 50 years old, followed by a gradual decrease for the next three decades of life. The study also showed the positive association between rising blood pressure and cardiovascular risk (Pinto, 2007). Another interesting link is that which exists between gender and HPT. Under the age of 60 years, the prevalence of HPT is generally higher in males than in females, due to various behavioural and biological factors (Everett & Zajacova, 2015). However, there is a shift in the HPT prevalence trend later in life as women tend to be more prone to HPT than men. In a Korean cohort of males and females, the prevalence of HPT was higher in males below 60 years. However, for individuals above 60 years of age, HPT was more prevalent in females (Choi *et al*., 2017). As a result of these observations, questions are being asked to consider the possibility of gender specific HPT guidelines (Gillis & Sullivan, 2016).

An essential nutritional requirement of the body, sodium is an important component for the optimal functioning of the muscular and nervous systems and plays a key role in the maintenance of fluid balance in the body (Ha, 2014). When present in excess in the body, sodium has potentially deleterious effects on the cardiovascular system and as such, kidneys excrete the unwanted sodium, as excess dietary sodium has been linked to increased arterial BP. The benefits of lowering dietary sodium intake on BP have been well demonstrated in the Dietary Approach to Stopping Hypertension (DASH)-Sodium trial and other animal and human studies on the effects of high sodium intake on BP (Sacks *et al*., 2001; Ha, 2014).

A lack of adequate physical activity, genetic predispositions, psychological stress, indulgence in high fat diets and inflammation contribute to a weight gain that may translate to the development of obesity (Jiang *et al*., 2016). Both general and central obesity are risk factors for HPT, although on its own, central obesity does not pose as much risk for CVD as it does when accompanied by HPT. There are various mechanisms through which central obesity leads to the development of HPT and these include promotion of insulin resistance, activation of the RAAS, promoting endothelial dysfunction and increased sodium reabsorption (Narkiewicz, 2006).

Disparities in the prevalence of HPT based on race have been previously described, with high HPT prevalence rates reported in African-American populations and individuals of African descent (Spence & Rayner, 2018). Whilst the prevalence of HPT continues to grow in sub-Saharan Africa, there are many HPT issues that are unique to the region, such as the salt-sensitivity, which is common in black Africans (Lindhorst *et al*., 2007). As a result of this, consumption of salt has a comparatively greater effect on the BP of Africans, as more salt is retained after a high sodium intake. Furthermore, the high prevalence of HPT amongst African populations has been linked to salt and water retention. For example, African hypertensives have been reported to possess the Liddle phenotype, a hereditary disorder characterised by overactivity of the renal tubular epithelial sodium channel (ENaC) as a result of mutations in the *SCNN1B* and *SCNN1G* genes. The consequence is the development of HPT due to the retention of sodium and water (Spence & Rayner, 2018).

The aforementioned factors all play significant roles in the development of HPT. The effect of one risk factor may vary from population to population or indeed within a population due to the diverse nature of human beings. However, for individuals at risk of HPT due to modifiable factors, alterations in behaviour may be the difference between BP control or development of HPT and susceptibility to worse clinical outcomes of uncontrolled HPT.

2.6 The global burden of hypertension

As the trend of epidemiological transition continues to grow in developing countries, healthcare systems are faced with a two-pronged challenge from both communicable and non-communicable diseases (NCDs). As shown in Figure 2, in 2015, raised blood pressure (SBP ≥ 140mmHg and/or

DBP ≥90 mmHg) was most prevalent on the African continent, which is predominantly made up of LMICs[2], although some regions in Europe and Asia showed a similar trend. Kearney *et al*., 2005 estimated that as much as 1.6 billion people would be hypertensive by the year 2025 and the current overall global trend in the prevalence of HPT suggests the prediction will not be far off the mark in five years' time. It is quite daunting however, that of the more than 1.3 billion currently hypertensive people worldwide, close to 75% of those are in the LMICs. Perhaps unsurprisingly, these high HPT prevalence figures in LMICs are also accompanied by a lack of awareness of HPT and treatment. Additionally, for those that are on treatment, the degree to which they have control on their HPT is also poor (Mills *et al*., 2016).

Hypertension is a well-known leading risk factor for CVD and in 2016, over 70% of deaths globally were attributed to NCDs, of which close to half of those (18 million) had a CVD link (Egan *et al*., 2019). To this day, HPT and associated CVDs remain a leading cause of death around the world, accompanied by substantial financial investments into their treatment and management across the globe (Gheorghe *et al*., 2018).

[2] https://www.who.int/images/default-source/maps/global_bloodpressureprevalence_2015_bothsexes.png?sfvrsn=65e7d3d3_0

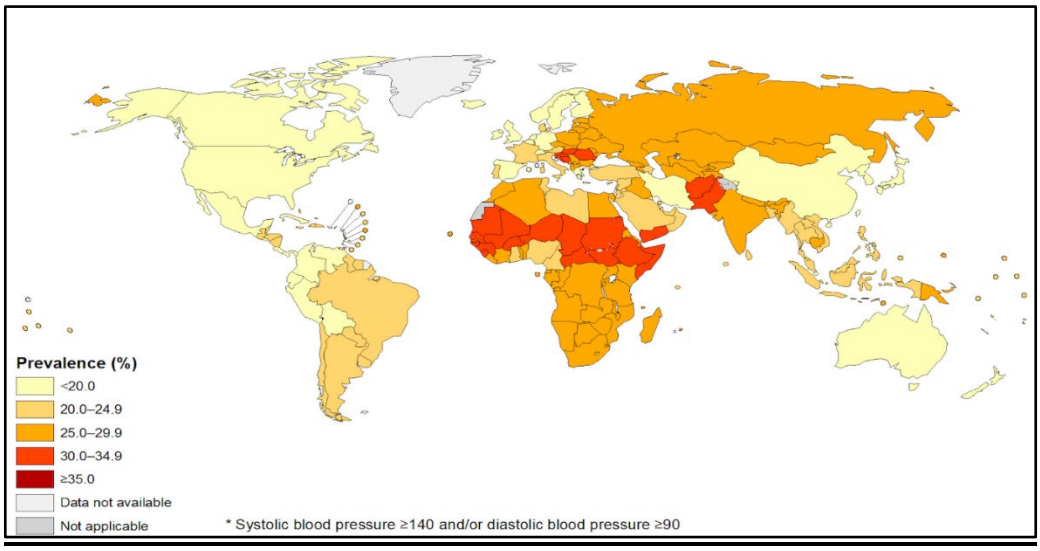

Figure 2. Global prevalence of raised blood pressure in males and females above the age of 18 as of 2015

2.6.1 Disease burden in South Africa

Four years after the 1994 South African democratic national elections, the country released its first SADHS. The objective of the report was to provide up-to-date evidence on various aspects of the population's health and BP was one of the anthropometric indicators assessed in the adult population. In the SADHS report, a diagnosis of HPT was made with reference to Hypertension Society of Southern Africa's guidelines where HPT was defined as a BP reading equal to or above 160/95 mmHg. When adjusted according to international guidelines for HPT diagnosis, the population prevalence of HPT in South Africa as of 1998 was 21% (Steyn *et al*., 2001). Whilst the 2003 SADHS reports a drop in the prevalence of HPT, serious problems with BP measurements were reported and the drop was described to be more likely a reflection of poor data quality than an actual drop. A brainchild of the World Health Organisation, the SAGE longitudinal study, an initiative to collect data on global ageing and adults' health (adults of age 50 years and above) was initiated in Mexico, China, India, Russia, Ghana and South Africa. Whilst a high prevalence of HPT was recorded in all six countries, the highest prevalence of HPT (78%) was recorded in the South African cohort (Lloyd-Sherlock *et al*., 2014; Peltzer & Phaswana-Mafuya, 2013), a worrying indication of the vast numbers of ageing South Africans at CVD risk. Close to two decades after the first SADHS, a nationally representative sample of 12132 individuals participated in the SADHS of 2016, the latest instalment of attempts by the government to provide

updated estimates of basic demographic and health indicators in the country. The document reported a HPT prevalence in male and female South Africans above the age of 15 years of 44% and 46% respectively; more than double the prevalence figures reported 18 years prior. Whilst some men and women were aware of their hypertensive status and on corrective therapy, shockingly, at least 80% of them did not have their HPT under control (National Department of Health (NDoH) *et al*., 2016).

In a study conducted in a highly populated rural area in the Limpopo province of South Africa, the prevalence of HPT in the more than 1200 study participants was just over 41% (Ntuli *et al*., 2015), comparatively similar to the SADHS 2016 findings. Whilst there has been an overall decrease in BP globally since 1980, that trend has not extended to the African population. The continent has instead experienced an increase in the mean SBP of African men and women (Danaei *et al*., 2011). Another study in a South African mixed-ancestry population demonstrated an increase in the mean SBP and DBP over a period of seven years, culminating in an increase in the prevalence of HPT (Davids *et al*., 2019). It is evident that the HPT epidemic has been well established on the African continent, particularly sub-Saharan Africa and if not properly addressed, its contribution towards loss of preventable lives will continue, whilst making a significant dent on the continent's already stretched fiscus through implementation of management and therapeutic strategies for HPT.

2.7 Epigenetics in hypertension

Undoubtedly a significant scientific landmark in human genetic studies, amongst other things, the Human Genome Project provided a platform on which over three billion nucleotide base pairs making up the entire human genome could be fully exploited to understand its contribution towards human diseases. However, the human genome alone was not enough to explain the pathogeneses of various diseases afflicting humans. As such, the epigenome, representing changes in the phenotype without changes to the underlying DNA sequence, became a significant field of study in human disease as it offered a route to link the genotype to the phenotype (Stoll *et al*., 2018). Various epigenetic mechanisms like DNA methylation, histone modification and non-coding RNAs like miRNAs have been implicated in the development of diseases, including HPT and as such have been targets for further research (Matsha *et al*., 2016; Wise & Charchar, 2016; Stoll *et*

al., 2018; Kazmi *et al*., 2020; Nemecz *et al*., 2016). Overall, the epigenome, through gene expression or repression depending on the DNA methylation patterns or modifications to histone proteins, has a significant role to play in the development of disease, including HPT and associated CVDs.

2.7.1 DNA methylation and histone modifications

In the promoter regions of genes usually lies short sequences of DNA where cytosine and guanine nucleotides, separated by a phosphodiester bond, occur more frequently. The locations on DNA in which these sequences exist are called 'CpG' islands, and it is here where methylation of DNA usually happens when DNA methyltransferases transfer a methyl group from *S*-adenosyl-L-methionine to the carbon at position 5 of the cytosine ring, forming 5-methyl-cytosine (Stoll *et al*., 2018). This results in the suppression of gene expression and silencing of the gene. Methylation of genes relevant to the regulation of blood pressure has been associated with HPT. Angiotensin converting enzyme 2 promotes vasodilation through degradation of the vasoconstrictor, Ang II, thus helping lower the BP. Fan *et al*., 2017 showed that methylation in the promoter regions of the *ACE2* gene was associated with an increased risk of essential HPT. In another study, an association between DBP and DNA methylation was demonstrated in a cohort of Europeans and Asians, although no such association with SBP and HPT was observed (Kazmi *et al*., 2020). However, Rangel and colleagues were able to show the existence of an inverse relationship between SBP and DNA methylation in their study (Rangel *et al*., 2014). SCNN1A is a subunit of the ENaC channel responsible for maintaining the BP and extracellular blood volume and when hypermethylation occurs at the CpG islands in the promoter regions of the *SCNN1A* gene, there is an increased risk of HPT development (Mao *et al*., 2016).

Two copies each of four histone proteins (H2A, H2B, H3 and H4) are wrapped around DNA to form the nucleosome, which is the building block for chromatin (Stoll *et al*., 2018). Depending on the nature of histone tail modifications (acetylation, methylation, deamination, phosphorylation) and the identity of the histone protein, the way chromatin is folded varies, thus influencing its accessibility to regulatory components and ultimately gene expression (Gamen *et al*., 2016). High

acetylation and methylation of histone 3 in the whole blood of Asian participants was associated with reduced SBP and DBP (Kresovich *et al*., 2017).

2.7.2 MicroRNAs and their biogenesis

Besides the implication of the previously described DNA methylation and histone modification in the development of HPT, another epigenetic factor has been a subject of recent research in disease, including HPT. The third class of epigenetic elements in question is miRNAs, which are a group of small, endogenous, non-coding ribonucleic acid (RNA) sequences ranging in length from 17 to 25 nucleotides (Shin & Chu, 2014). They are a vital component of the epigenetic machinery responsible for gene expression regulation, thus influencing a vast number of biological systems and processes in the body. Their biogenesis takes place in two compartments of a cell, commencing in the nucleus and concluding in the cytoplasm. The process begins in the nucleus with the transcription of the miRNA gene by RNA-polymerase II, producing a long primary miRNA (pri-miRNA). A processing complex comprising of an RNA binding protein and RNase III Drosha is then bound to the long pri-miRNA. The Drosha enzyme is responsible for the initial stages of maturation of the miRNA as it cleaves the pri-miRNA into a hairpin structured precursor miRNA (pre-miRNA). Through Exportin 5, the pre-miRNA is transported from the nucleus to the cytoplasm of the cell to initiate the miRNA's final maturation stages. In the cytoplasm, the Dicer enzyme recognizes the pre-miRNA via the two nucleotide long overhang on its 3' terminal and cuts off the terminal loop of the pre-miRNA's hairpin, generating a miRNA duplex in the process. Argonaute protein 2 (Ago2) in the cytoplasm accommodates and unwinds the miRNA duplex, leading to the degradation of one strand (the passenger strand) whilst the mature strand (guide strand) is used to form the miRNA-induced silencing complex (miRISC) important for gene expression regulation (Nemecz *et al*., 2016).

MiRNAs can be found in animals, plants, and viruses and are expressed in all cell types and involved in regulation of gene expression at the post-transcriptional level. This is achieved in either of two ways, inhibiting translation of messenger RNA (mRNA) into protein or amplifying the destruction of mRNA (Levy *et al*., 2017). The miRNAs, through complementary base pairing, carry out either of the aforementioned processes by binding to the 3' end of the untranslated (UTR)

region on target mRNA molecules. Circulating miRNAs play pivotal roles in the regulation of wide-ranging biological processes in the body (Felekkis & Papaneophytou, 2020). Whilst they are involved in normal cellular physiological processes, they have been implicated in disease and their aberrant expression is common in pathological states (Kontaraki *et al*., 2014; Liu *et al*., 2018), denoting the initial stages or progression of the disease (Cui *et al*., 2019). Many clinical studies have reported specific expression patterns in disease, highlighting their potential for use in disease diagnostics and prognostics (Navickas *et al*., 2016). The choice of sample used for miRNA profiling is important as variations in the observed miRNA expression profiles have been reported, depending on whether analysis was performed in plasma or serum samples (Mompeón *et al*., 2020). The systemic nature of cancer has seen whole blood being favoured for miRNA profiling in some studies with some comparable results observed between different sample types (whole blood versus serum), although variations in miRNA expression profiles still exist (Pascut *et al*., 2019). Nonetheless, whether serum, plasma or whole blood miRNAs are being investigated, the importance of miRNAs is clear and the crucial roles they play in disease cannot be discounted and makes them interesting targets for research. MiRNAs possess unique properties which allow them to: 1) regulate gene expression 2) target multiple genes and control the genome 3) act as tumour suppressors in cancers 4) show distinct expression profiles between normal states and disease (favouring biomarker profiling and targeting for therapy purposes) (Giza *et al*., 2014). These characteristics are important in deciphering physiological processes under the control of a particular miRNA or a group of miRNAs in disease (Chavali *et al*., 2013). All things considered, these molecules present opportunities for further study whose findings have potential of ushering in a new dawn in the field of disease diagnostics, therapy and prognostics.

2.8 Studies involving miRNAs in disease

The study of miRNAs has been undertaken in various scientific fields since their discovery 27 years ago (Lee *et al*., 1993), using various molecular methods like whole miRNA sequencing, microarrays and RT-PCRs. Whilst the vast majority of the work conducted on miRNAs thus far has been in the field of cancer, they have certainly been an emerging research target in CVDs. Over the past 10-15 years, research on miRNAs in various forms of HPT has been undertaken

globally, more so in Asia (Li *et al*., 2011; Kontaraki *et al*., 2014; Kontaraki *et al*., 2014; Yang *et al*., 2014; Cengiz, Yavuzer, *et al*., 2015; Li *et al*., 2016; Kaneto *et al*., 2017; Badawy *et al*., 2018; Liu *et al*., 2018; Li *et al*., 2020). MiRNA profiling and expression level determination in various body fluids and tissue in normal and diseased participants have paved a way for more research to be conducted on the pathophysiological role miRNAs play in BP regulation and the prognosis of HPT (Zhang *et al*., 2017). Certain miRNAs have shown consistent expression profiles (upregulated or downregulated) in diseased participants compared to healthy controls. As a result, such miRNAs have been proposed as having possible roles in the development of disease, with functional studies identifying the biological pathways and cellular processes that they interact with (Huang *et al*., 2016).

2.8.1 MiRNAs in cancer

The relative stability of miRNAs in various body tissues and their differential expression in normal and diseased individuals has facilitated the concept of targeting them as biomarkers of various diseases. Using liquid bead arrays and RT-qPCR techniques, Markou and colleagues demonstrated the differential expression of eight miRNAs in cancerous and non-cancerous tissue, reporting dysregulation of miR-10a and miR-30e-5p for the very first time and showing that expression of miR-30e-5p at higher levels was associated with a shorter overall survival (Markou *et al*., 2013). Gastric cancer is one of the leading causes of cancer related deaths worldwide claiming close to 1 million lives annually around the world. The symptoms present later on when the disease has progressed and this partially explains the high mortality rates related to the disease (Shin & Chu, 2014). Whilst a diagnostic measure like endoscopy exists, its invasive nature makes its use undesirable. In addition, endoscopy can only be used to diagnose the cancer once it has presented itself (which is usually late on in disease progression). As such, there is need for a non-invasive method that is able to detect gastric cancer early on to facilitate a reduction in gastric cancer mortality. Using next generation sequencing and various *in vivo* and *in vitro* techniques, Han and colleagues identified miR-29c as a tumour suppressing miRNA targeting the integrin β1 gene. On the basis of these findings, they were able to show that the development of gastric cancer was preceded by a loss in the expression of miR-29c, thus identifying it as a possible diagnostic or

prognostic biomarker for the carcinoma (Han *et al*., 2015). One study identified miR-1299 as an important role player in suppressing the growth of colon cancer cells via downregulation of the signal transducers and activators of transcription (STAT3), an important component in the heart's adaptation to elevated BP (Zouein *et al*., 2013; Wang *et al*., 2017). The prognosis of gastrointestinal cancer is poor when the Bmi1 protein is highly expressed and miR-30e was identified as a potential regulator of Bmi1 expression (Sugihara *et al*., 2013). With further investigations, this finding could present possible miRNA-based theraputic avenues for gastrointestinal cancer patients.

2.8.2 MiRNAs in diabetes mellitus

MiRNAs have also been extensively investigated in the field of diabetes over the past 5-10 years. They play various regulatory roles that are essential for maintaining physiologically viable glucose levels. This involves regulation of insulin signalling pathways and the fate of the pancreatic β-cells (apoptosis, survival, differentiation) (Feng *et al*., 2016). The maintenance of a normal mass of pancreatic β-cells is essential in the prevention of hyperglycaemia and in part, is achieved by the regulatory effects of miR-375 on genes that inhibit the growth of cells in the pancreas (Poy *et al*., 2009).

MiRNAs have also demonstrated stable differential expression in various glucose states, suggesting their potential as biomarkers of disease and/or therapeutic targets. Cui *et al*., 2016 compared the transcriptome in impaired fasting glucose (IFG) and T2DM and identified a panel of miRNAs (miR-192, miR-29a and miR-144) as potential targets for further investigation in T2DM and IFG. In a South African mixed-ancestry population where a high prevalence of diabetes has been previously described (Erasmus *et al*., 2012), Matsha and colleagues described the differential expression of a panel of miRNAs based on the participants' glycaemic status, with some of the differentially expressed miRNAs being involved in the regulation of genes relevant to the production and secretion of insulin, suggesting their possible roles in the development or progression of T2DM. A significantly different expression of miR-126-3p was seen between prediabetics and screen-detected diabetics (Matsha *et al*., 2018). Another study reported a significantly low expression of miR-126 in the plasma of T2DM and IFG patients in comparison

to the normal group and an inverse association between miR-126 expression and T2DM development (Zhang *et al*., 2013). In another study by Yan and colleagues, the plasma expression of miR-1249, -320b and -572 suggested their ability to differentiate prediabetics from type 2 diabetics (Yan *et al*., 2016). Weale *et al*, 2020 showed an association between dysglycaemia and the expression of miR-30a-5p and miR-182-5p, with the latter potentially predicting prediabetes. Put together, all these findings suggest the potential for a vast array of miRNAs to be used as biomarkers for identifying individuals at risk of T2DM development.

2.8.3 MiRNAs in hypertension and cardiovascular diseases

A key characteristic of miRNAs is their stability for long periods in stored serum or plasma samples, hence making them ideal biomarker targets for detection of disease, including screening for hypertension in especially resource limited settings (Shin & Chu, 2014). Amongst their diverse functional repertories, miRNAs, through the maintenance of vascular integrity, play a crucial role in the homeostatic regulation of blood pressure and ultimately HPT and CVDs. Alterations in the expression of various miRNAs have been linked to the development of HPT in various ways, including their effects on genes relevant to the RAAS and the vascular endothelium (Nemecz *et al*., 2016).

The importance of the endothelium towards BP regulation has been well described and disturbances to the angiogenic process have been linked to the development of HPT (Ferroni *et al*., 2012). They are also essential for maintaining physiologically optimal vascular resistance and through the release of vasodilators into the circulation, help reduce resistance in the vasculature. High BP exerts changes on the functionality and phenotype of ECs and an association between an inappropriately functioning vascular endothelium and HPT has been established. The role of miRNAs in the maintenance of EC functionality has also been previously described (Nemecz *et al*., 2016).

MiR-126 is an endothelial cell miRNA which plays an important role in angiogenesis, wound repair and maintenance of vascular integrity. The miRNA regulates the response of ECs to vascular endothelial growth factor (VEGF) by direct repression of the negative regulators of VEGF (Fish *et al*., 2008). VEGF is a signalling protein essential for the promotion of angiogenesis and

endothelial cell permeability. Its effects are mediated by receptors, of which VEGFR-2 is its primary receptor on endothelial cells. Binding of VEGF to VEGFR-2 initiates the recruitment of phosphatidylinositide-3-kinase (PI3) and then phosphorylation of endothelial nitric oxide synthase (eNOS), culminating in increased NO production. After diffusion of NO into the vascular smooth muscle cells (VSMCs) and activation of the guanylate cyclase within, there is an increase in cyclic guanosine monophosphate (cGMP) and eventually vasodilation. Besides EC dilation, activation of the VEGF pathway also influences migration and proliferation of ECs (Kružliak *et al*., 2014). Propositions have been made that HPT may develop as a result of inhibiting the VEGF-signalling pathway, thereby causing HPT either as a result of decreased NO production or the promotion of vasoconstriction through increased production of ET-1, an endogenous vasoconstrictor (Kappers *et al*., 2011).

Hijmans and colleagues showed significantly reduced expression of miR-126 in the plasma of hypertensive participants compared to their normotensive counterparts using RT-qPCR. They concluded that the dysregulation of miR-126 was associated with increased vascular risk for HPT and the development and progression of CVDs (Hijmans *et al*., 2018). In a cohort of 60 normotensive and hypertensive individuals, the expression of miR-126 in peripheral blood mononuclear cells (PBMCs) of normotensives was also significantly higher, relative to the hypertensives. In addition, miR-126 expression was positively associated with mean pulse pressure, a known predictor of target organ damage in HPT (Kontaraki *et al*., 2014). Interestingly, a study in China targeting miR-126 as a potential therapeutic target for essential HPT in 10 participants showed contrasting results. The expression of miR-126 was significantly higher in hypertensive participants when compared to the normotensives (Liu *et al*., 2018), whilst there was no difference in miR-126 expression based on HPT status in another study (Cengiz, Karatas, *et al*., 2015). Endothelial function is also regulated by let-7e and the miRNA's expression was increased in essential HPT (Badawy *et al*., 2018) and has also been associated with stroke when highly expressed in circulation (S. Huang *et al*., 2016).

Li *et al*., 2011 conducted a study to determine the miRNA expression profile in essential HPT and its links to human cytomegalovirus (HCMV) in a Chinese population. This was achieved by

performing miRNA expression profiling in plasma samples from hypertensive and normotensive participants using microarray technology. The findings were validated by two real-time PCR validation assays in two other different cohorts of hypertensive and healthy controls and their findings demonstrated differential expression of 27 miRNAs in hypertensive relative to the healthy controls.

An increase in the blood level of soluble ST2 (sST2), a receptor for interleukin-33 (IL-33), has been shown to be predictive of increased SBP (Ho *et al*., 2013) and HPT (Coglianese *et al*., 2012). In 2017, Wu and colleagues showed that single nucleotide polymorphisms in the interleukin 1 receptor-like (ST2) gene were associated with a risk of HPT, through their control of sST2 expression (Wu *et al*., 2017). The expression of miR-202-3p was shown to be induced by Ang II and associated with a protective role against essential HPT, through the antagonization of sST2 induction (Li *et al*., 2020).

Yang and colleagues provided a possible mechanism in which another miRNA may be involved in the pathogenesis of HPT and as such a possible diagnostic target. In three different male cohorts, the plasma expression of miR-505 in hypertensives was significantly higher in comparison to the normotensive group using microarray and RT-qPCR assays. Using cell culture, they showed that increased expression of miR-505 was associated with altered migration and formation of the endothelial cell and tube, respectively, through regulation of Fibroblast Growth Factor 18 (FGF18) expression, an angiogenic factor (Yang *et al*., 2014).

Based on the prior demonstration of the utility of miR-145, miR-24, miR-181a and miR-4516 in renin secretion, contraction of VSMCs, aldosterone synthesis and salt sensitivity and resistance, a study was conducted to assess their possible roles in HPT pathogenesis and it was demonstrated that downregulation of miR-145 and the upregulation of miR-4516 may be useful in the independent prediction of HPT (Özkan *et al*., 2019). In another study, miR-145 expression was significantly lower in hypertensive individuals compared to normotensives and the miRNA's involvement in the maintenance of the VSMC contractile phenotype suggests its differential expression has implications on BP and the development of CVD risk (Kontaraki *et al*., 2014). Santovito and colleagues reported increased expression of miR-145-5p in atherosclerotic plaques

from hypertensive patients (Santovito *et al*., 2013) and the miRNA's influence on the VSMC phenotype further highlights its role in BP regulation (Parmacek, 2009; Kontaraki *et al*., 2014). In human kidney cells, Marques and colleagues showed the potential involvement of miR-181a in the regulation of the *REN* gene required for making renin and the elevation in renin mRNA levels was attributed to reduced miR-181a expression (Marques *et al*., 2011). Furthermore, in an animal model, a negative correlation between renal miR-181a expression and BP was also reported and renin production, due to the effects of the sympathetic nervous system, was influenced by low expression of miR-181a, contributing to the development of HPT (Jackson *et al*., 2013). Two studies showed that miR-155-5p failure to regulate a single nucleotide polymorphism linked with HPT led to increased type 1 angiotensin II receptor expression and elevated BP (Sethupathy *et al*., 2007; Ceolotto *et al*., 2011). Another miRNA previously associated with HPT is miR-21. Li and colleagues reported overexpression of miR-21 in the cardiac mitochondria of spontaneously hypertensive rats (SHRs). Furthermore, they also demonstrated increased plasma miR-21 expression in HPT compared to normotension and a positive correlation between miR-21 expression and systolic and diastolic BP (Li *et al*., 2016), with similar findings reported in another study (Cengiz, Yavuzer, *et al*., 2015). Another miRNA of interest is miR-1299 and although it is yet to be reported in HPT, it has been implicated in Rheumatic Heart Disease (RHD), a common complication of which is pulmonary arterial hypertension (PAH) (Li *et al*. 2015). The close association between autophagy, programmed cell death and CVDs has been established and Su *et al*, 2020 showed that miR-30e-3p is involved in the inhibition of apoptosis and promotion of cardiomyocyte autophagy in an ischaemia/hypoxia environment.

As the understanding of miRNAs continues to grow, a multitude of studies continue to be conducted across the globe to profile and describe the roles played by novel and previously described miRNAs, at both molecular and cellular levels in the development and progression of HPT and CVDs. The identification of a consistently dysregulated panel of miRNAs in HPT offers possible therapeutic avenues. Anti-miRs are synthetic, antisense, oligonucleotide compounds that have a fully or partially complementary sequence to target miRNAs and are able to lower endogenous miRNA levels. When highly expressed in a disease state or condition, these

dysregulated miRNAs can be targeted using anti-miRs that dampen down or reverse their action, thus preventing further exacerbation of the disease (Van Rooij *et al*., 2012). However, because a single miRNA controls multiple genes and multiple miRNAs control a single gene, silencing miRNAs using anti-miRs may have unintended effects away from the target gene. It is therefore important that anti-miRs are used with the outmost care to ensure that only the intended, positive effects are realised with their use.

Findings from miRNA biomarker studies have not always been consistent and some level of variation and discordance has been observed (Wronska *et al*., 2015). This can, in part, be attributed to differences in experimental design. The kind of profiling assays used, the number of study participants, exclusion and inclusion criteria, the sample type used and participant socio-demographics might have a role to play in the discrepancies in miRNA expression results observed in studies of individuals suffering from the same disease or condition. It is essential that more studies on these miRNA expression profiles in HPT are conducted in different socio-economic settings with the aim of eventually standardising the profiling assays used, so that a universally accepted panel of biomarkers diagnostic of HPT can be identified.

Overall, it is evident from published literature that epigenetics and indeed miRNAs are major role players in various disease processes. As such, a clear understanding of their contributions to the development and progression of disease is essential if long lasting measures to curb the burden of diseases like HPT and CVDs are to be effectively implemented. Understanding the various mechanisms underlying the pathogenesis of HPT may help predict HPT and CVD-related complications, providing a window of opportunity for early intervention, saving lives and easing the financial strain associated with chronic medical conditions.

2.9 References

Babiker, F.A., Elkhalifa, L.A. & Moukhyer, M.E. 2013. Awareness of Hypertension and Factors Associated with Uncontrolled Hypertension in Sudanese Adults. *Cardiovasc J Afr*, 24(6): 208–212.

Badawy, H.K., Abo-Elmatty, D.M. & Mesbah, N.M. 2018. Differential Expression of microRNA let-7e and 296-5p in Plasma of Egyptian Patients with Essential Hypertension. *Heliyon*, 4(11): e00969.

Biino, G., Parati, G., Concas, M.P., *et al*. 2013. Environmental and Genetic Contribution to Hypertension Prevalence: Data from an Epidemiological Survey on Sardinian Genetic Isolates. *PLoS One*, 8(3): e59612.

Campbell, N.R.C., Gilbert, R.E., Leiter, L.A., *et al*. 2011. Hypertension in People with Type 2 Diabetes: Update on Pharmacologic Management. *Can Fam Physician*, 57(9): 997–1002, e347–e353.

Campbell, N.R.C., Padwal, R., Picone, D.S., Su, H. & Sharman, J.E. 2020. The Impact of Small to Moderate Inaccuracies in Assessing Blood Pressure on Hypertension Prevalence and Control Rates. *J Clin Hypertens (Greenwich)*, 22(6): 939–942.

Cengiz, M., Karatas, O.F., Koparir, E., *et al*. 2015. Differential Expression of Hypertension-Associated MicroRNAs in the Plasma of Patients With White Coat Hypertension. *Medicine (Baltimore)*, 94(13): e693.

Cengiz, M., Yavuzer, S., Klçkran Avc, B., *et al*. 2015. Circulating miR-21 and eNOS in Subclinical Atherosclerosis in Patients with Hypertension. *Clin Exp Hypertens*, 37(8): 643–649.

Ceolotto, G., Papparella, I., Bortoluzzi, A., *et al*. 2011. Interplay between miR-155, AT1R A1166C Polymorphism, and AT1R Expression in Young Untreated Hypertensives. *Am J Hypertens*, 24(2): 241–246.

Charles, L., Triscott, J. & Dobbs, B. 2017. Secondary Hypertension: Discovering the Underlying Cause. *Am Fam Physician*, 96(7): 453–461.

Chavali, S., Bruhn, S., Tiemann, K., *et al*. 2013. MicroRNAs Act Complementarily to Regulate Disease-related mRNA Modules in Human Diseases. *RNA*, 19(11): 1552–62.

Choi, H.M., Kim, H.C. & Kang, D.R. 2017. Sex Differences in Hypertension Prevalence and Control: Analysis of the 2010-2014 Korea National Health and Nutrition Examination Survey C. N. Spracklen, ed. *PLoS One*, 12(5): e0178334.

Coglianese, E.E., Larson, M.G., Vasan, R.S., *et al*. 2012. Distribution and Clinical Correlates of the Interleukin Receptor Family Member Soluble ST2 in the Framingham Heart Study. *Clin Chem*, 58(12): 1673–1681.

Cui, M., Wang, H., Yao, X., *et al*. 2019. Circulating MicroRNAs in Cancer: Potential and Challenge. *Front Genet*, 10: 626.

Cui, Y., Chen, W., Chi, J. & Wang, L. 2016. Comparison of Transcriptome Between Type 2 Diabetes Mellitus and Impaired Fasting Glucose. *Med Sci Monit*, 22: 4699–4706.

Danaei, G., Finucane, M.M., Lin, J.K., *et al*. 2011. National, Regional, and Global Trends in Systolic Blood Pressure Since 1980: Systematic Analysis of Health Examination Surveys and Epidemiological Studies with 786 Country-years and 5.4 Million Participants. *Lancet*, 377(9765): 568–577.

Davids, S.F.G., Matsha, T.E., Peer, N., Erasmus, R.T. & Kengne, A.P. 2019. Increase in Blood Pressure Over a 7-year Period in a Mixed-Ancestry South African Population. *S Afr Med J*, 109(7): 503–510.

Diaz, K.M. & Shimbo, D. 2013. Physical Activity and the Prevention of Hypertension. *Curr Hypertens Rep*, 15(6): 659–668.

Egan, B.M., Kjeldsen, S.E., Grassi, G., Esler, M. & Mancia, G. 2019. The Global Burden of Hypertension Exceeds 1.4 Billion People: Should a Systolic Blood Pressure Target Below 130 Become the Universal Standard? *J Hypertens*, 37(6): 1148–1153.

Erasmus, R.T., Soita, D.J., Hassan, M.S., *et al*. 2012. High Prevalence of Diabetes Mellitus and

Metabolic Syndrome in a South African Coloured Population: Baseline Data of a Study in Bellville, Cape Town. *S Afr Med J*, 102(11 Pt 1): 841–844.

Everett, B. & Zajacova, A. 2015. Gender Differences in Hypertension and Hypertension Awareness Among Young Adults. *Biodemography Soc Biol*, 61(1): 1–17.

Fan, R., Mao, S.-Q., Gu, T.-L., *et al*. 2017. Preliminary Analysis of the Association Between Methylation of the ACE2 Promoter and Essential Hypertension. *Mol Med Rep*, 15(6): 3905–3911.

Felekkis, K. & Papaneophytou, C. 2020. Challenges in Using Circulating Micro-RNAs as Biomarkers for Cardiovascular Diseases. *Int J Mol Sci*, 21(2): 561.

Feng, J., Xing, W. & Xie, L. 2016. Regulatory Roles of MicroRNAs in Diabetes. *Int J Mol Sci*, 17(10): 1–12.

Ferroni, P., Della-Morte, D., Palmirotta, R., *et al*. 2012. Angiogenesis and Hypertension: The Dual Role of Anti-Hypertensive and Anti-Angiogenic Therapies. *Curr Vasc Pharmacol*, 10(4): 479–493.

Fesler, A., Liu, H. & Ju, J. 2018. Modified miR-15a has Therapeutic Potential for Improving Treatment of Advanced Stage Colorectal Iancer through Inhibition of BCL2, BMI1, YAP1 and DCLK1. *Oncotarget*, 9(2): 2367–2383.

Fish, J.E., Santoro, M.M., Morton, S.U., *et al*. 2008. miR-126 Regulates Angiogenic Signaling and Vascular Integrity. *Dev Cell*, 15(2): 272–284.

Flack, J.M. & Adekola, B. 2020. Blood Pressure and the new ACC/AHA Hypertension Guidelines. *Trends Cardiovasc Med*, 30(3): 160–164.

Fuchs, F.D. & Whelton, P.K. 2020. High Blood Pressure and Cardiovascular Disease. *Hypertension*, 75(2): 285–292.

Fuster, V., Kelly, B.B. & Vedanthan, R. 2011. Global Cardiovascular Health: Urgent Need for an Intersectoral Approach. *J Am Coll Cardiol*, 58(12): 1208–1210.

Gamen, E., Seeger, W. & Pullamsetti, S.S. 2016. The Emerging Role of Epigenetics in Pulmonary Hypertension. *Eur Respir J*, 48(3): 903–917.

Gheorghe, A., Griffiths, U., Murphy, A., *et al*. 2018. The Economic Burden of Cardiovascular Disease and Hypertension in Low- and Middle-Income Countries: A Systematic Review. *BMC Public Health*, 18(1): 975.

Gilad, S., Meiri, E., Yogev, Y., *et al*. 2008. Serum MicroRNAs are Promising Novel Biomarkers. *PLoS One*, 3(9): e3148.

Gillis, E.E. & Sullivan, J.C. 2016. Sex Differences in Hypertension: Recent Advances. *Hypertension*, 68(6): 1322–1327.

Giza, D.E., Vasilescu, C. & Calin, G.A. 2014. Key Principles of miRNA Involvement in Human Diseases. *Discoveries (Craiova)*, 2(4): e34.

Gonzalez-Jaramillo, V., Portilla-Fernandez, E., Glisic, M., *et al*. 2019. The Role of DNA Methylation and Histone Modifications in Blood Pressure: A Systematic Review. *J Hum Hypertens*, 33(10): 703–715.

Ha, S.K. 2014. Dietary Salt Intake and Hypertension. *Electrolyte Blood Press*, 12(1): 7–18.

Han, T.-S., Hur, K., Xu, G., *et al*. 2015. MicroRNA-29c Mediates Initiation of Gastric Carcinogenesis by Directly Targeting ITGB1. *Gut*, 64(2): 203–214.

He, F.J., Li, J. & MacGregor, G.A. 2013. Effect of Longer Term Modest Salt Reduction on Blood Pressure: Cochrane Systematic Review and Meta-analysis of Randomised Trials. *BMJ*, 346: f1325.

Hijmans, J.G., DIehl, K.J., Bammert, T.D., *et al*. 2018. Association Between Hypertension and Circulating Vascular-related MicroRNAs. *J Hum Hypertens*, 32(6): 440–447.

Hinderliter, A.L., Voora, R.A. & Viera, A.J. 2018. Implementing ABPM into Clinical Practice. *Curr Hypertens Rep*, 20(1): 5.

Ho, J.E., Larson, M.G., Ghorbani, A., *et al*. 2013. Soluble ST2 Predicts Elevated SBP in the Community. *J Hypertens*, 31(7): 1431–1436.

Huang, S., Lv, Z., Guo, Y., *et al*. 2016. Identification of Blood Let-7e-5p as a Biomarker for Ischemic Stroke Y. Zheng, ed. *PLoS One*, 11(10): e0163951.

Huang, Y.Q., Huang, C., Chen, J.Y., Li, J. & Feng, Y.Q. 2016. The Association of Circulating

miR-30a, miR-29 and miR-133 with White-Coat Hypertension. *Biomark Med*, 10(12): 1231–1239.

Jackson, K.L., Marques, F.Z., Watson, A.M.D., *et al*. 2013. A Novel Interaction Between Sympathetic Overactivity and Aberrant Regulation of Renin by miR-181a in BPH/2J Genetically Hypertensive Mice. *Hypertension*, 62(4): 775–781.

Ji, A., Pan, C., Wang, H., *et al*. 2019. Prevalence and Associated Risk Factors of Chronic Kidney Disease in an Elderly Population from Eastern China. *Int J Environ Res Public Health*, 16(22): 4383.

Ji, L.D., Li, J.Y., Yao, B.B., *et al*. 2017. Are Genetic Polymorphisms in the Renin-Angiotensin-Aldosterone System Associated with Essential Hypertension? Evidence from Genome-Wide Association Studies. *J Hum Hypertens*, 31(11): 695–698.

Jiang, S.-Z., Lu, W., Zong, X.-F., Ruan, H.-Y. & Liu, Y. 2016. Obesity and Hypertension. *Exp Ther Med*, 12(4): 2395–2399.

Jones Buie, J.N., Goodwin, A.J., Cook, J.A., Halushka, P. V & Fan, H. 2016. The Role of miRNAs in Cardiovascular Disease Risk Factors. *Atherosclerosis*, 254: 271–281.

Kaneto, C.M., Nascimento, J.S., Moreira, M.C.R., *et al*. 2017. MicroRNA Profiling Identifies miR-7-5p and miR-26b-5p as Differentially Expressed in Hypertensive Patients with Left Ventricular Hypertrophy. *Braz J Med Biol Res*, 50(12): e6211.

Kappers, M.H.W., Smedts, F.M.M., Horn, T., *et al*. 2011. The Vascular Endothelial Growth Factor Receptor Inhibitor Sunitinib Causes a Preeclampsia-Like Syndrome With Activation of the Endothelin System. *Hypertension*, 58(2): 295–302.

Kazmi, N., Elliott, H.R., Burrows, K., *et al*. 2020. Associations Between High Blood Pressure and DNA Methylation. *PLoS ONE*, 15(1): e0227728.

Kearney, P.M., Whelton, M., Reynolds, K., *et al*. 2005. Global Burden of Hypertension: Analysis of Worldwide Data. *Lancet*, 365(9455): 217–223.

Kontaraki, J, Marketou, M., Zacharis, E., Parthenakis, F. & Vardas, P. 2014. Differential Expression of Vascular Smooth Muscle-modulating MicroRNAs in Human Peripheral Blood

Mononuclear Cells: Novel Targets in Essential Hypertension. *J Hum Hypertens*, 28(8): 510–516.

Kontaraki, JE, Marketou, M., Zacharis, E., Parthenakis, F. & Vardas, P. 2014. MicroRNA-9 and microRNA-126 Expression Levels in Patients with Essential Hypertension: Potential Markers of Target-Organ Damage. *J Am Soc Hypertens*, 8(6): 368–375.

Kresovich, J.K., Zhang, Z., Zheng, Y., *et al*. 2017. Histone 3 Modifications and Blood Pressure in the Beijing Truck Driver Air Pollution Study. *Biomarkers*, 22(6): 584–593.

Kružliak, P., Novák, J. & Novák, M. 2014. Vascular Endothelial Growth Factor inhibitor-induced Hypertension: From Pathophysiology to Prevention and Treatment Based on Long-acting Nitric Oxide Donors. *Am J Hypertens*, 27(1): 3–13.

Lee, R., Feinbaum, R.L. & Ambros, V. 1993. The C. elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. *Cell*, 75(5): 843–854.

Leggio, M., Lombardi, M., Caldarone, E., *et al*. 2017. The Relationship Between Obesity and Hypertension: An Updated Comprehensive Overview on Vicious Twins. *Hypertens Res*, 40(12): 947–963.

Levy, E., Spahis, S., Bigras, J.-L., Delvin, E. & Borys, J.-M. 2017. The Epigenetic Machinery in Vascular Dysfunction and Hypertension. *Curr Hypertens Rep*, 19(6): 52.

Li, H., Zhang, X., Wang, F., *et al*. 2016. MicroRNA-21 Lowers Blood Pressure in Spontaneous Hypertensive Rats by Upregulating Mitochondrial Translation. *Circulation*, 134(10): 734–751.

Li, L., Zhong, D., Xie, Y., *et al*. 2020. Blood microRNA 202-3p Associates with the Risk of Essential Hypertension by Targeting soluble ST2. *Biosci Rep*, 40(5): BSR20200378.

Li, N., Lian, J., Zhao, S., Zheng, D., Yang, X., Huang, X., *et al*. 2015. Detection of Differentially Expressed MicroRNAs in Rheumatic Heart Disease: miR-1183 and miR-1299 as Potential Diagnostic Markers. *Biomed Res Int*, 2015: 524519.

Li, S., Zhu, J., Zhang, W., *et al*. 2011. Signature microRNA Expression Profile of Essential Hypertension and its Novel Link to Human Cytomegalovirus Infection. *Circulation*, 124(2): 175–184.

Li, Y. & Kowdley, K. V. 2012. MicroRNAs in Common Human Diseases. *Genomics Proteomics Bioinformatics*, 10(5): 246–253.

Lim, S.S., Vos, T., Flaxman, A.D., *et al*. 2012. A Comparative Risk Assessment of Burden of Disease and Injury Attributable to 67 Risk Factors and Risk Factor Clusters in 21 Regions, 1990–2010: A Systematic Analysis for the Global Burden of Disease Study 2010. *Lancet*, 380(9859): 2224–2260.

Lindhorst, J., Alexander, N., Blignaut, J. & Rayner, B. 2007. Differences in Hypertension Between Blacks and Whites: An Overview. *Cardiovasc J Afr*, 18(4): 241–247.

Liu, Jia, Liu, Jiamei, Shi, L., *et al*. 2018. Preliminary Study of microRNA-126 as a Novel Therapeutic Target for Primary Hypertension. *Int J Mol Med*, 41(4): 1835–1844.

Lloyd-Sherlock, P., Beard, J., Minicuci, N., Ebrahim, S. & Chatterji, S. 2014. Hypertension Among Older Adults in Low-and Middle-Income Countries: Prevalence, Awareness and Control. *Int J Epidemiol*, 43(1): 116–128.

Mao, S., Fan, R., Gu, T., *et al*. 2016. *Hypermethylation of SCNN1A Gene-Body Increases the Risk of Essential Hypertension*.

Markou, A., Sourvinou, I., Vorkas, P.A., Yousef, G.M. & Lianidou, E. 2013. Clinical Evaluation of MicroRNA Expression Profiling in Non Small Cell Lung Cancer. *Lung Cancer*, 81(3): 388–396.

Marques, F.Z., Campain, A.E., Tomaszewski, M., *et al*. 2011. Gene Expression Profiling Reveals Renin mRNA Overexpression in Human Hypertensive Kidneys and a Role for microRNAs. *Hypertension*, 58(6): 1093–1098.

Matsha, T.E., Kengne, A.P., Hector, S., *et al*. 2018. MicroRNA Profiling and Their Pathways in South African Individuals with Prediabetes and Newly Diagnosed Type 2 Diabetes Mellitus. *Oncotarget*, 9(55): 30485–30498.

Matsha, T.E., Pheiffer, C., Humphries, S.E., *et al*. 2016. Genome-Wide DNA Methylation in Mixed Ancestry Individuals with Diabetes and Prediabetes from South Africa. *Int J Endocrinol*,

2016: 3172093.

Mayosi, B.M., Flisher, A.J., Lalloo, U.G., *et al*. 2009. The Burden of Non-Communicable Diseases in South Africa. *Lancet*, 374(9693): 934–947.

Mills, K.T., Bundy, J.D., Kelly, T.N., *et al*. 2016. Global Disparities of Hypertension Prevalence and Control: A Systematic Analysis of Population-Based Studies from 90 Countries. *Circulation*, 134(6): 441–450.

Mompeón, A., Ortega-Paz, L., Vidal-Gómez, X., Costa, T.J., Pérez-Cremades, D., Garcia-Blas, S., *et al*. 2020. Disparate miRNA Expression in Serum and Plasma of Patients with Acute Myocardial Infarction: A Systematic and Paired Comparative Analysis. *Sci Rep*, 10(1): 5373.

Mottl, A.K., Shoham, D.A. & North, K.E. 2008. Angiotensin II Type 1 Receptor Polymorphisms and Susceptibility to Hypertension: A HuGE review. *Genet Med*, 10(8): 560–574.

Muhamedhussein, M., Nagri, Z. & Manji, K. 2016. Prevalence, Risk Factors, Awareness, and Treatment and Control of Hypertension in Mafia Island, Tanzania. *Int J Hypertens*, 2016: 1281384.

Narkiewicz, K. 2006. Obesity and Hypertension - The Issue is More Complex Than We Thought. *Nephrol Dial Transplant*, 21(2): 264–267.

National Department of Health (NDoH), Statistics South Africa (Stats SA), South African Medical Research Council (SAMRC) & ICF. 2016. *South Africa Demographic and Health Survey 2016: Key Indicators*. Pretoria, South Africa and Rockville, Maryland, USA.

Navickas, R., Gal, D., Laucevičius, A., Taparauskaite, A., Zdanyte, M. & Holvoet, P. 2016. Identifying Circulating microRNAs as Biomarkers of Cardiovascular Disease: A Systematic Review. *Cardiovasc Res*, 111(4): 322–337.

Nemecz, M., Alexandru, N., Tanko, G. & Georgescu, A. 2016. Role of MicroRNA in Endothelial Dysfunction and Hypertension. *Curr Hypertens Rep*, 18(12): 87.

Ntuli, S.T., Maimela, E., Alberts, M., Choma, S. & Dikotope, S. 2015. Prevalence and Associated Risk Factors of Hypertension Amongst Adults in a Rural Community of Limpopo Province, South Africa. *Afr J Prim Health Care Fam Med*, 7(1): 847.

Özkan, G., Ulusoy, Ş., Geyik, E. & Erdem, Y. 2019. Down-regulation of miRNA 145 and Up-regulation of miRNA 4516 May Be Associated with Primary Hypertension. *J Clin Hypertens*, 21(11): 1724–1731.

Parchwani, D.N., Patel, D.D., Rawtani, J. & Yadav, D. 2018. Analysis of Association of Angiotensin II Type 1 Receptor Gene A1166C Gene Polymorphism with Essential Hypertension. *Ind J Clin Biochem*, 33(1): 53–60.

Parmacek, M.S. 2009. MicroRNA-modulated Targeting of Vascular Smooth Muscle Cells. *J Clin Invest*, 119(9): 2526–2528.

Pascut, D., Krmac, H., Gilardi, F., *et al*. 2019. A Comparative Characterization of the Circulating miRNome in Whole Blood and Serum of HCC Patients. *Sci Rep*, 9: 8265.

Peltzer, K. & Phaswana-Mafuya, N. 2013. Hypertension and Associated Factors in Older Adults in South Africa. *Cardiovasc J Afr*, 24(3): 66–72.

Pinto, E. 2007. Blood Pressure and Ageing. *Postgrad Med J*, 83(976): 109–114.

Poy, M.N., Hausser, J., Trajkovski, M., *et al*. 2009. MiR-375 Maintains Normal Pancreatic α- and β-cell Mass. *Proc Natl Acad Sci U S A*, 106(14): 5813–5818.

Puar, T.H.K., Mok, Y., Debajyoti, R., *et al*. 2016. Secondary Hypertension in Adults. *Singapore Med J*, 57(5): 228–232.

Rangel, M., Dos Santos, J.C., Ortiz, P.H.L., *et al*. 2014. Modification of Epigenetic Patterns in Low Birth Weight Children: Importance of Hypomethylation of the ACE Gene Promoter. *PLoS ONE*, 9(8): e106138.

Van Rooij, E., Purcell, A.L. & Levin, A.A. 2012. Developing MicroRNA therapeutics. *Circ Res Res*, 110(3): 496–507.

Rossier, B.C., Bochud, M. & Devuyst, O. 2017. The Hypertension Pandemic: An Evolutionary Perspective. *Physiology (Bethesda)*, 32(2): 112–125.

Sacks, F.M., Svetkey, L.P., Vollmer, W.M., *et al*. 2001. Effects on blood pressure of reduced

dietary sodium and the dietary approaches to stop hypertension (dash) diet. *N Engl J Med*, 344(1): 3–10.

Santovito, D., Mandolini, C., Marcantonio, P., *et al*. 2013. Overexpression of microRNA-145 in Atherosclerotic Plaques from Hypertensive Patients. *Expert Opin Ther Targets*, 17(3): 217–223.

Savoia, C., Sada, L., Zezza, L., *et al*. 2011. Vascular Inflammation and Endothelial Dysfunction in Experimental Hypertension. *Int J Hypertens*, 2011: 281240.

Sebo, P., Pechère-Bertschi, A., Herrmann, F.R., Haller, D.M., Bovier, P. 2014. Blood Pressure Measurements are Unreliable to Diagnose Hypertension in Primary Care. *J Hypertens*, 32(3): 509-17.

Sethupathy, P., Borel, C., Gagnebin, M., *et al*. 2007. Human microRNA-155 on Chromosome 21 Differentially Interacts with its Polymorphic Target in the AGTR1 3′ Untranslated Region: A Mechanism for Functional Single-Nucleotide Polymorphisms Related to Phenotypes. *Am J Hum Genet*, 81(2): 405–413.

Sharman, J.E., O'Brien, E., Alpert, B., *et al*. 2020. Lancet Commission on Hypertension Group Position Statement on the Global Improvement of Accuracy Standards for Devices that Measure Blood Pressure. *J Hypertens*, 38(1): 21-29.

Shin, V.Y. & Chu, K.-M. 2014. MiRNA as Potential Biomarkers and Therapeutic Targets for Gastric Cancer. *World J Gastroenterol*, 20(30): 10432–10439.

Silambarasan, M., Tan, J.R., Karolina, D.S., *et al*. 2016. MicroRNAs in Hyperglycemia Induced Endothelial Cell Dysfunction. *Int J Mol Sci*, 17(4): 518.

Spence, J.D. & Rayner, B.L. 2018. Hypertension in Blacks. Individualized Therapy Based on Renin/Aldosterone Phenotyping. *Hypertension*, 72(2): 263–269.

Spruill, T.M. 2010. Chronic Psychosocial Stress and Hypertension. *Curr Hypertens Rep*, 12(1): 10–16.

Stanaway, J.D., Afshin, A., Gakidou, E., *et al*. 2018. Global, Regional, and National Comparative Risk Assessment of 84 Behavioural, Environmental and Occupational, and Metabolic Risks or

Clusters of Risks for 195 Countries and Territories, 1990-2017: A Systematic Analysis for the Global Burden of Disease Stu. *Lancet*, 392(10159): 1923–1994.

Steyn, K., Gaziano, T.A., Bradshaw, D., Laubscher, R. & Fourie, J. 2001. Hypertension in South African Adults: Results from the Demographic and Health Survey, 1998. *J Hypertens*, 19(10): 1717–1725.

Stoll, S., Wang, C. & Qiu, H. 2018. DNA Methylation and Histone Modification in Hypertension. *Int J Mol Sci*, 19(4): 1174.

Su, B., Wang, X., Sun, Y., *et al*. 2020. miR-30e-3p Promotes Cardiomyocyte Autophagy and Inhibits Apoptosis via Regulating Egr-1 during Ischemia/Hypoxia. *Biomed Res Int*, 2020: 7231243.

Sugihara, H., Ishimoto, T., Watanabe, M., Sawayama, H., Iwatsuki, M., Baba, Y. 2013. Identification of miR-30e* Regulation of Bmi1 Expression Mediated by Tumor-Associated Macrophages in Gastrointestinal Cancer. *PLoS ONE*, 8(11): e81839.

Svetkey, L.P., Erlinger, T.P., Vollmer, W.M., *et al*. 2005. Effect of lifestyle modifications on blood pressure by race, sex, hypertension status, and age. *J Hum Hypertens*, 19(1): 21–31.

Unger, T., Borghi, C., Charchar, F., *et al*. 2020. 2020 International Society of Hypertension Global Hypertension Practice Guidelines. *Hypertension*, 75(6): 1334–1357.

Wagner, S., Buus, N.H., Jespersen, B., Ahrendt, P., *et al*. 2013. Measurement Adherence in the Blood Pressure Self-Measurement Room. *Telemed J E Health*, 19(11): 826-33.

Wang, Y., Lu, Z., Wang, N., Zhang, M., Zeng, X., Zhao, W. 2017. MicroRNA-1299 is a Negative Regulator of STAT3 in Colon Cancer. *Oncol Rep*, 37(6): 3227–34.

Weale, CJ., Matshazi, DM., Davids, SFG., *et al*. 2020. Circulating miR-30a-5p and miR-182-5p in Prediabetes and Screen-Detected Diabetes Mellitus. *Diabetes Metab Syndr Obes*, 13: 5037-47.

Wise, I.A. & Charchar, F.J. 2016. Epigenetic Modifications in Essential Hypertension. *Int J Mol Sci*, 17(4): 451.

Wronska, A., Kurkowska-Jastrzebska, I. & Santulli, G. 2015. Application of microRNAs in

Diagnosis and Treatment of Cardiovascular Disease. *Acta Physiol*, 213(1): 60–83.

Wu, F., Li, L., Wen, Q., *et al*. 2017. A Functional Variant in ST2 Gene is Associated with Risk of Hypertension via Interfering MiR-202-3p. *J Cell Mol Med*, 21(7): 1292–1299.

Xu, S.-J., Xu, W.-J., Zeng, Z., Zhang, M. & Zhang, D.-Y. 2020. MiR-424 Functions as Potential Diagnostic and Prognostic Biomarker in Melanoma. *Clinical Laboratory*, 66(7).

Yan, S., Wang, T., Huang, S., *et al*. 2016. Differential Expression of MicroRNAs in Plasma of Patients with Prediabetes and Newly Diagnosed Type 2 Diabetes. *Acta Diabetol*, 53(5): 693–702.

Yang, Q., Jia, C., Wang, P., *et al*. 2014. MicroRNA-505 Identified from Patients with Essential Hypertension Impairs Endothelial Cell Migration and Tube Formation. *Int J Cardiol*, 177(3): 925–934.

Yildirim, E., Ermis, E., Allahverdiyev, S., *et al*. 2019. Circulating miR-21 Levels in Hypertensive Patients with Asymptomatic Organ Damage. *Medicine*, 98(39): e17297.

Zhang, T., Lv, C., Li, L., *et al*. 2013. Plasma miR-126 is a Potential Biomarker for Early Prediction of Type 2 Diabetes Mellitus in Susceptible Individuals. *Biomed Res Int*, 2013: 761617.

Zhang, Z., Joyce, B.T., Kresovich, J.K., *et al*. 2017. Blood Pressure and Expression of MicroRNAs in Whole Blood. *PLoS One*, 12(3): e0173550.

Zhou, B., Bentham, J., Di Cesare, M., *et al*. 2017. Worldwide Trends in Blood Pressure from 1975 to 2015: A Pooled Analysis of 1479 Population-based Measurement Studies with 19.1 Million Participants. *Lancet*, 389(10064): 37–55.

Zouein, FA., Zgheib, C., Hamza, S., Fuseler, JW., Hall, JE., Soljancic, A., *et al*. 2013. Role of STAT3 in Angiotensin II-induced Hypertension and Cardiac Remodeling Revealed by Mice Lacking STAT3 Serine 727 Phosphorylation. *Hypertens Res*, 36(6): 496–503.

CHAPTER 3: MANUSCRIPT 1

Manuscript submitted to the Frontiers in Cardiovascular Medicine Journal.

MicroRNA profiles in normotensive and hypertensive South African individuals

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Abstract

Hypertension is a complex multifactorial condition whose symptoms appear in advanced disease. Dysregulation of gene expression regulatory factors like miRNAs has been reported in disease development. Identifying biomarkers which could help understand the pathogenesis and prognosis of hypertension is essential. The study's objective was to investigate miRNA expression profiles according to participant blood pressure status. Next generation sequencing was used to identify differentially expressed miRNAs in the whole blood of 48 body mass index-, smoking- and age-matched normotensive (n=12), screen-detected hypertensive (n=16) and known hypertensive (n=20) female participants. Quantitative reverse transcription polymerase chain reaction was used to validate the next generation sequencing findings in a larger, independent sample of 84 men and 179 women. Using next generation sequencing, 30 dysregulated miRNAs were identified and miR-1299 and miR-30a-5p were the most significantly differentially expressed. Both miRNAs were upregulated in known hypertensives or screen-detected hypertensives compared to the normotensives. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis indicated possible involvement of platelet activation, calcium signalling and aldosterone synthesis pathways. Further validation of miR-1299 and miR-30a-5p using quantitative reverse transcription polymerase chain reaction confirmed the sequencing results. These findings demonstrate miRNA dysregulation in hypertension and their expression may be related to genes and biological pathways essential for blood pressure homeostasis.

1. Introduction

The 8th report released by the Joint National Committee on Prevention, Detection and Evaluation of High Blood Pressure describes hypertension (HPT) as the persistent elevation of blood pressure (BP) above the 140/90 mmHg threshold (James *et al*., 2014; Abegaz *et al*., 2018). Despite efforts to understand the pathogenesis of the condition, HPT remains a leading public health concern affecting both developed and developing countries ((Wise & Charchar, 2016; Polsinelli *et al*., 2017). It has been identified as one of the most important modifiable risk factors for cardiovascular disease, renal disease, and stroke, and accounts for over 10 million deaths throughout the world annually (Peltzer & Phaswana-Mafuya, 2013; Babiker *et al*., 2013; Lim *et al*., 2013). In 90-95% of HPT patients, the cause is unknown, and is thus termed primary or essential HPT (Bátkai & Thum, 2012; Rossier *et al*., 2017). However, research has demonstrated the involvement of genetic and environmental factors in the development of HPT. The influence of epigenetic factors such as deoxyribonucleic (DNA) methylation and histone modification on the pathogenesis of HPT has been a subject of intense research, with Mu *et al*., 2011 showing that the induction of histone modulation led to the downstream development of salt-induced HPT. However, there is a paucity of research regarding miRNAs (miRNAs) in the context of HPT.

MiRNAs are a group of small, endogenous, non-coding ribonucleic (RNA) sequences that are 17-25 base pairs long (Shin & Chu, 2014). These molecules are involved in gene expression regulation at the post-transcriptional level. This is achieved by binding to the 3'untranslated region of complementary messenger RNA (mRNA) molecules and inhibiting translation into protein or inducing mRNA degradation (Levy *et al*., 2017). MiRNAs are present in almost every cell and disturbances in their regulation are usually associated with disease processes, including HPT (Chen *et al*., 2008; Nemecz *et al*., 2016). Herein, we investigated the miRNA profiles in South African individuals with normal BP, as well as those presenting with known or screen-detected HPT.

2. Materials and methods

2.1. Ethics statement

This investigation was based on the Cape Town Vascular and Metabolic Health (VMH) study, which was approved by the Research Ethics Committees of the Cape Peninsula University of Technology (CPUT) (NHREC: REC - 230 408 – 014) and Stellenbosch University (N14/01/003). Ethical approval was also obtained for this cross-sectional sub-study from the CPUT Health and Wellness Sciences Research Ethics Committee (CPUT/HW-REC 2019/H7). The study was conducted as per the provisions of the Declaration of Helsinki. All procedures were explained to the participants in their language of choice. Once the participants fully understood their participation, they signed informed consent forms to allow the collection of blood and anthropometric data.

2.2. Study design and procedures

Data collection and procedures have been described previously (Matsha *et al*., 2018). Briefly, participants underwent anthropometric and BP measurements, as well as oral glucose tolerance tests (OGTT). Anthropometric measurements for each participant were taken three times and the average reported. BP was measured according to the World Health Organisation (WHO) guidelines (Chalmers *et al*., 1999), using a semi-automatic digital BP monitor (Omron M6 comfort-preformed cuff BP Monitor, China) on the right arm in a sitting position and at rest for at least 10 minutes. Three BP readings were taken at three-minute intervals and the lowest systolic BP and corresponding diastolic BP values were used. Participants were grouped into three categories based on; the use of anti-hypertensive medication as known HPT, BP measurement of 140/90 mm Hg or greater as screen-detected HPT and normal BP measurement (less than 140/90 mm Hg) as normotensive. Body Mass Index (BMI) was calculated as weight per height squared (kg/m^2), where kg was the participant's weight in kilograms and m^2, the square of their height.

The following biochemical parameters were analyzed at an ISO 15189 accredited Pathology practice (PathCare Reference Laboratory, Cape Town, South Africa): glycated haemoglobin (HbA1c) by High Performance Liquid Chromatography (BioRad Variant Turbo, BioRad,

Hercules, CA, USA); serum insulin by a paramagnetic particle chemiluminescence assay (Beckman DXI, Beckman Coulter, South Africa); serum cotinine by Competitive Chemiluminescent Immunoassay (Immulite 2000, Siemens, Munich, Germany); plasma glucose by the enzymatic hexokinase method (Beckman AU, Beckman Coulter, Brea, CA, USA); total cholesterol (TC); high density lipoprotein cholesterol (HDL-c) by enzymatic immunoinhibition – end point (Beckman AU, Beckman Coulter, Brea, CA, USA); triglycerides (TG) by glycerol phosphate oxidase-peroxidase, end point (Beckman AU, Beckman Coulter, Brea, CA, USA); low density lipoprotein cholesterol (LDL) by enzymatic selective protection – end point (Beckman AU, Beckman Coulter, Brea, CA, USA); and ultrasensitive C-reactive protein (CRP) by Latex Particle Immunoturbidimetry (Beckman AU, Beckman Coulter, Brea, CA, USA). In addition, blood samples were collected in a Tempus RNA tube (ThermoFisher Scientific, Waltham, MA, USA) and stored at -80°C for total RNA extraction and analysis.

2.3. RNA isolation

Total RNA, including miRNAs, was isolated from whole blood using the MagMax for Stabilized Blood RNA isolation kit (ThermoFisher Scientific) according to manufacturer's instructions. The concentration and purity of each RNA extract was determined using a NanoDrop One spectrophotometer. Total RNA extracts with 260/280 values between 1.8 and 2.0, and concentrations greater than 20ng/µl were used for miRNA sequencing (miRNA-seq) using next generation sequencing (NGS) and quantitative reverse transcription PCR (RT-qPCR).

2.4. MiRNA sequencing

This was conducted on total RNA samples from 48 female participants representing three different HPT statuses. The inclusion of females only in this part of the study was to avoid introducing potential sources of variation due to gender effect in an already small cohort. Small RNA library construction, deep sequencing, and data processing were performed at Arraystar Inc., Rockville, USA as previously described by Matsha *et al*., 2018. Briefly, the total RNA of each sample was used to prepare the miRNA sequencing library as follows:1) 3'-adapter ligation with T4 RNA ligase 2 (truncated); 2) 5'-adapter ligation with T4 RNA ligase; 3) complementary DNA (cDNA) synthesis with RT primer; 4) PCR amplification; 5) extraction and purification of ~130-150 bp

PCR amplified fragments (correspond to ~15-35 nt small RNAs) from the polyacrylamide gel electrophoresis gel. The Agilent 2100 Bioanalyzer was used to quantify completed libraries, thereafter DNA fragments were denatured with 0.1M sodium hydroxide to generate single-stranded DNA molecules, then captured on Illumina flow cells, amplified in situ, and finally sequenced for 51 cycles on the Illumina HiSeq system according to the manufacturer's instructions. Raw sequences were generated as clean reads from the Illumina HiSeq using real-time base calling and quality filtering. The clean reads that passed the quality filter were processed to remove adaptor sequences as the trimmed reads. The trimmed reads (length ≥ 15 nt) were aligned to the human pre-miRNA in miRBase 21 (http://www.mirbase.org/), using NovoAlign software. The miRNA expression levels were measured and normalized as transcripts per million of total aligned miRNA reads. MiRNAs with fold changes ≥1.3, and p-values ≤ 0.1 were selected as the differentially expressed miRNAs. Novel miRNAs were predicted using miRDeep (Friedlander *et al*., 2008).

2.5. Gene Ontology and Functional Enrichment Analysis

The Gene Ontology (GO) analysis was performed to describe gene and gene product attributes (http://www.geneontology.org). The ontology covers three domains: Biological Process, Cellular Component and Molecular Function. Commonly predicted gene targets were subjected to functional analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG). A conservative Fisher's exact test and false discovery rate method were used to calculate the targeted pathways.

2.6. Validation of NGS miRNA expression results

To confirm the differential expression of miRNAs, the validation of NGS results was performed on total RNA from an independent sample of 263 male and female participants randomly selected from an existing database and 48 females on which NGS had been conducted. MiRNAs were converted to complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). The miRNA expression levels were assessed using TaqMan miRNA Assay primers on the QuantStudio 7 Flex real-time PCR instrument (Life Technologies, Carlsbad, CA, USA) analyser. In order to determine miRNA expression in each sample and between two groups, the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ (Livak

& Schmittgen, 2001), respectively were used and normalized using miR-16-5p as the endogenous control. The suitability of miR-16-5p as an endogenous control in RT-qPCR was assessed and confirmed, as there was minimal variation in its expression in normotensive and hypertensive participants.

2.7. Statistical analysis

Data were analysed using R statistical software version 3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria) and TIBCO Statistica version 13.5.0.17 (TIBCO Software Inc, California, USA). The Shapiro-Wilk W test was employed to determine whether the data were normally distributed, based on probability thresholds of $p>0.1$. Continuous variables were summarized as mean and standard deviation (SD) when normally distributed, while median, and 25^{th} and 75^{th} percentiles were used for skewed variables, whilst categorical variables were reported as counts and percentages. When comparing groups, for continuous variables, the analysis of variance (ANOVA) was used for normally distributed data; Kruskal Wallis-H test with Dunn post-hoc test was used for skewed data, whilst the chi-square test was used for categorical variables. A p-value <0.05 was used to characterize statistically significant results.

3. Results

3.1. General participant characteristics

Of the 1988 VMH survey participants, 311 (227, 73.0% female) were selected for inclusion into this sub-study. Of these, 48 (all female) took part in the NGS part of the study while an additional 263 randomly selected male and female participants were included in the RT-qPCR validation study. The distribution of the NGS and RT-qPCR participants by BP status is shown in Table 1. The 48 women in the NGS sample included 20 with known HPT, 16 with screen-detected HPT and 12 normotensives, whilst the validation sample included 106 known hypertensives, 52 screen-detected hypertensives and 105 normotensives. The expected differences by status for HPT in the cardiovascular risk profile were apparent across the two sub-samples (Table 1).

3.2. NGS miRNA expression profiling

All 48 samples met the quality control standards. We generated Heat Map and Unsupervised Hierarchical Clustering on all miRNAs that were expressed in at least one sample, to produce miRNA or condition trees that would allow us to pick out groups of similar miRNAs. The result of hierarchical clustering on conditions showed a distinguishable miRNA expression profile amongst the groups (Figures 1A-C). For the identification of differentially expressed miRNAs, we computed "fold changes" (i.e., the ratio of the group averages) and *p*-values between each group. MiRNAs with fold changes ≥ 1.3 and *p*-values ≤ 0.1 were selected as the differentially expressed miRNAs. Based on pre-specified criteria, we then used volcano plots to visualize the significantly differentially expressed miRNAs between the study groups as shown in Figures 2A-C. A total of 30 significantly differentially expressed mature miRNAs were identified at varying expression levels and are summarized in Table 2. Of the thirty differentially expressed miRNAs, two (6.7%) were novel, and whilst one of these novel miRNAs was upregulated in known HPT versus normotensive, the other was upregulated in known HPT versus screen-detected HPT. Whilst miR-1299 exhibited the highest fold change of all significantly upregulated miRNAs as seen in screen-detected HPT versus normotensive (fold change = 3.38, p=0.081), miR-30a-5p upregulation was greatest in known HPT versus normotensive (fold change = 2.44, p=0.063) and known HPT versus screen detected HPT (fold change = 2.02, p=0.072; Table 2).

KEGG pathway analysis revealed 84 pathways, five of which are essential for platelet activation, calcium signalling, vascular smooth muscle contraction, vasopressin-mediated water reabsorption and aldosterone synthesis and secretion. Based on GO analyses, we retrieved the biological processes, cellular components and molecular functions of dysregulated miRNAs. In Figure 3, we present the top enrichment scores for biological processes of dysregulated miRNAs in hypertensive versus normotensive participants.

3.3. Next generation sequencing results validation

The RT-qPCR data were normalized using miR-16-5p and the raw Ct values, showing its suitability as an endogenous control in our cohort, are shown in Supplementary Figure 1. The two miRNAs with the highest fold change between study groups using NGS were selected for validation with RT-qPCR, namely miR-30a-5p and miR-1299. The relative expressions ($2^{-\Delta Ct}$) of each target miRNA in the three participant groups are shown in Figure 4. Both miR-30a-5p and miR-1299 were upregulated in known HPT compared to normotensive or screen-detected HPT, $p \leq 0.015$, whilst miR-30a-5p was also significantly upregulated in screen-detected HPT vs normotensive, $p=0.023$. Using the $2^{-\Delta\Delta Ct}$ formula to compute fold changes between two groups, miR-30a-5p expression was 2.58-fold higher in known HPT versus normotensive and 1.69-fold higher versus screen-detected HPT. In screen-detected HPT, miR-30a-5p expression was 1.52-fold higher when compared to the normotensives. As for miR-1299, there was a 3.93-fold and 2.78-fold higher expression in known HPT versus normotensives and screen-detected HPT respectively. However, there was not a great difference in the expression of miR-1299 between screen-detected HPT vs normotensive as shown by the 1.41-fold difference in expression.

4. Discussion

To our knowledge, no study has been conducted on miRNA expression in relation to HPT in populations from Africa. Using NGS, we identified 30 (including two novel) mature miRNAs that were differentially expressed in 48 South African women with either screen-detected or treated HPT. These miRNAs were associated with pathways such as platelet activation, calcium signalling and vascular smooth muscle contraction pathways which are particularly important in cardiovascular pathogenesis (Gkaliagkousi *et al*., 2010; Landstrom *et al*., 2017; Touyz *et al*., 2018). Two miRNAs, namely miR-1299 and miR-30a-5p were the most significantly dysregulated in hypertensive individuals and this was validated using RT-qPCR in 311 study participants, confirming the miRNA sequencing results while yielding new findings.

Several studies have reported on a number of dysregulated miRNAs in HPT using different tissues, but results remain inconsistent (Li *et al*., 2011; Jairajpuri *et al*., 2017; Chen *et al*., 2018b; Özkan *et al*., 2019; Yildirim *et al*., 2019). A study similar to ours reported 27 dysregulated miRNAs in plasma collected from 18 normotensive and hypertensive males and female participants (Li *et al*., 2011), although the miRNAs were not similar to ours. A recurring theme within these miRNA profiling studies in HPT is the inter-study inconsistency of findings. For example, expression of various miRNAs such as miR-21, miR-145-5p, miR-155-5p, miR-181a (Santovito *et al*., 2013; Cengiz M *et al*., 2015; Sethupathy *et al*., 2007; Li *et al*., 2016; Ceolotto *et al*., 2011; Jackson *et al*., 2013; Marques *et al*., 2011; Parmacek, 2009; Kontaraki *et al*., 2014) that had been previously associated with BP and HPT were not found in this study. We suspect this may partially be attributed to the diverse genetic makeup of Africans and in particular, our study participants whose heterogeneous genetic makeup comprises 32-43% Khoisan, 20–36% Bantu-speaking Africans, 21–28% European and 9–11% Asian ancestry (De Wit *et al*., 2010). Furthermore, differences in the methods used could account for the discordance in inter-study findings as the tissue specific nature of some miRNAs has been previously described (Ludwig *et al*., 2016). In our study, discordant results with regards to miR-1299 were evident between NGS and RT-qPCR. Other studies have employed the candidate miRNA approach and reported on miRNAs that have not necessarily been identified using microarrays or sequencing, highlighting the need for more studies

employing the same methodologies and experimental designs and standardised sample preparation before these miRNAs can be utilised as new biomarkers.

In a previous study, miR-30 was down-regulated in the plasma of patients with essential HPT (Huang, 2016). In contrast, our findings using both sequencing and RT-qPCR showed an upregulation of miR-30a-5p in both screen-detected or known hypertensive (on antihypertensive treatment) individuals. This difference in expression may be partially explained by the differences in the sample type used for analysis. Whilst our study utilised whole blood (composed of plasma, red blood cells, platelets and white blood cells) for total miRNA expression, the other study made use of plasma (cell-deficient). Pre-analytical sample manipulation using centrifugation, which is required for obtaining plasma from whole blood, affects miRNA expression profiles, as it removes from the plasma, cell-specific miRNAs that would otherwise have been detected in whole blood (Felekkis & Papaneophytou, 2020). Findings similar to ours were also reported by Huang and colleagues, who demonstrated increased plasma expression of miR-30a in essential and white coat HPT, relative to normotensive participants (Huang *et al*., 2016). Overexpression of miR-30a has been reported to interfere with the removal of damaged or dead endothelial cells, promoting atherosclerosis and predisposing individuals to cardiovascular complications, like heart attacks (Zhang *et al*., 2015). Similarly, other miRNAs in the miR-30 family have been associated with cardiovascular diseases and suggestions made that they act as predictors for acute myocardial infarction and heart failure (Zhang *et al*., 2015; Maciejak *et al*., 2018). For instance, the overexpression of miR-30b-5p was shown to have a downregulatory effect on a muscleblind-like splicing regulator 1 (MBLN1) transcript in atherosclerosis, possibly playing a role in the regulation of vascular smooth muscle cells VSMCs (Woo *et al*., 2020). Another miRNA with interesting results was miR-1299, which was significantly upregulated in screen-detected HPT when compared to the normotensive group, fold change = 3.38. This was also confirmed with RT-qPCR, which indicated a 1.41-fold increase in expression of the miRNA in screen-detected HPT compared to the normotensive group. Although this finding is yet to be reported in HPT by other groups, the miRNA has been implicated in Rheumatic Heart Disease (RHD), a common complication of which is pulmonary arterial hypertension (PAH) (Li *et al*. 2015). One study

identified miR-1299 as an important role player in suppressing the growth of colon cancer cells via downregulation of the signal transducers and activators of transcription (STAT3). STAT3 is as an important component in the heart's adaptation to elevated BP (Zouein *et al*., 2013; Wang *et al*., 2017). It is possible that elevated expression of miR-1299, as seen in the hypertensive participants, may be a contributing factor in protecting against cardiovascular events associated with elevated BP levels.

As seen in KEGG analysis, the significantly differentially expressed miRNAs had possible involvement in various pathways relevant to HPT, including vascular smooth muscle contraction, vasopressin-mediated water reabsorption, platelet activation, calcium signalling and aldosterone synthesis and secretion. Alterations to the vascular smooth muscle cells (VSMCs) phenotype has implications in vascular resistance, BP and HPT and Kontaraki and colleagues demonstrated differential expression of five miRNAs (miR-1, -21, -133, 143 and -145) previously implicated in the alteration of the VSMC phenotype (Kontaraki *et al*., 2014). Water retention is also essential in BP regulation and fluid volume maintenance and various miRNAs have been implicated in these processes. Through repression of the methyl CpG binding protein 2 (*Mecp2*) gene and MeCP2 protein, miR-132 regulates vasopressin synthesis and as such, fluid retention (Bijkerk *et al*., 2018), whilst miR-32 and -137 regulate water retention by targeting kidney water channels controlled by vasopressin (Gomes *et al*., 2018; Kim *et al*., 2015). Dysregulations in aldosterone production or secretion pathways may be a risk for the development of HPT and aldosterone production is reduced due to miR-24 targeting of mRNA from the *CYP11B2* gene (Robertson *et al*, 2013), whilst angiotensin II-mediated overexpression of miR-21 leads to increased aldosterone secretion (Romero *et al*., 2008). Dysregulation of calcium signalling leads to altered responses by the vasculature, a common characteristic in HPT (Wilson *et al*., 2019). In a murine model, Wu and colleagues demonstrated the regulation of calcium signalling in the kidney by the miR-30 family (Wu *et al*., 2015), whilst another study reported miR-214 as a regulator of the calcium pathways through repression of mRNA encoding the sodium-calcium exchanger protein, Ncx1 (Aurora *et al*., 2012).

Our study had some limitations. Firstly, we did not investigate the effect of antihypertensive drugs, which is likely to have influenced the differential miRNA expression between treated (known HPT) and untreated (screen-detected HPT) hypertensive individuals. For instance, in a murine model of salt-sensitive HPT, a high salt diet was accompanied by reduced expression of miR-27a, miR-29a and miR-133a. However, Nebivolol prevented the high salt-mediated lower expression of miR-27a, whilst there was complete and partial reversal of high salt-induced miR-29a decrease by Nebivolol and Atenolol respectively. Both medications were able to prevent a decrease in miR-133a expression (Ye *et al*., 2013). Second, in our NGS analysis, miRNA expression screening was done in only 48 female participants. However, the RT-qPCR validation was performed in a larger sample that also included male participants. Lastly, only two of the 30 significantly dysregulated miRNAs as shown by NGS were chosen for validation by RT-qPCR. As such, the relationship between these 28 miRNAs and HPT in our cohort was not explored.

In conclusion, our study demonstrated miRNA dysregulation in hypertensive individuals and to our knowledge, is the first study to do so in a sub-Saharan African population. Based on our findings, we have shown a number of miRNAs, particularly, miR-30a-5p and miR-1299 that could be explored further for a potential prognostic role, or as therapeutic targets.

5. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Author contributions

Conceptualization, T.E.M., R.T.E. and A.P.K.; methodology, D.M.M. S.R. and C.J.W.; formal analysis, D.M.M. and S.F.G.D.; investigation, D.M.M and C.J.W.; resources, T.E.M.; data curation, D.M.M. S.R. and S.F.G.D.; writing—original draft preparation, D.M.M.; writing—review and editing, T.E.M., R.T.E., A.P.K., G.M.D., S.R. and S.H.; validation, D.D.M., C.J.W., S.R.; visualization, D.M.M., S.F.G.D. and T.E.M.; supervision, T.E.M.; G.M.D. and S.H.; project administration, T.E.M., S.F.G.D., funding acquisition, T.E.M, R.T.E and A.P.K. All authors have read and agreed to the published version of the manuscript.

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9. References

Abegaz TM, Abdela OA, Bhagavathula AS, Teni FS. Magnitude and Determinants of Uncontrolled Blood Pressure Among Hypertensive Patients in Ethiopia: Hospital-based Observational Study. Pharm Pract (Granada). (2018) 16(2):1173.

Aurora AB, Mahmoud AI, Luo X, Johnson BA, Van Rooij E, Matsuzaki S, *et al*. MicroRNA-214 Protects the Mouse Heart from Ischemic Injury by Controlling Ca2+ Overload and Cell Death. J Clin Invest. (2012) 122(4):1222–32.

Babiker FA, Elkhalifa LA, Moukhyer ME. Awareness of Hypertension and Factors Associated with Uncontrolled Hypertension in Sudanese Adults. Cardiovasc J Afr. (2013) 24(6):208–12.

Bátkai S, Thum T. MicroRNAs in Hypertension: Mechanisms and Therapeutic Targets. Curr Hypertens Rep. (2012) 14(1):79–87.

Bijkerk R, Trimpert C, van Solingen C, de Bruin RG, Florijn BW, Kooijman S, *et al*. MicroRNA-132 Controls Water Homeostasis through Regulating MECP2-mediated Vasopressin Synthesis. Am J Physiol Ren Physiol (2018) 315(4):F1129–38.

Cengiz M, Yavuzer S, Klçkran Avc B, Yürüyen M, Yavuzer H, Dikici SA, *et al*. Circulating miR-21 and eNOS in Subclinical Atherosclerosis in Patients with Hypertension. Clin Exp Hypertens. (2015) 37(8):643–9.

Ceolotto G, Papparella I, Bortoluzzi A, Strapazzon G, Ragazzo F, Bratti P, *et al*. Interplay between miR-155, AT1R A1166C Polymorphism, and AT1R Expression in Young Untreated Hypertensives. Am J Hypertens. (2011) 24(2):241–6.

Chalmers J, MacMahon S, Mancia G, Whitworth J, Beilin L, Hansson L, *et al*. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Clin Exp Hypertens. (1999) 21(5–6):1009–60.

Chen S, Chen R, Zhang T, Lin S, Chen Z, Zhao B, *et al*. Relationship of Cardiovascular Disease Risk Factors and Noncoding RNAs with Hypertension: A Case-Control Study. BMC Cardiovasc Disord. (2018) 18(1):58.

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, *et al*. Characterization of microRNAs in Serum: A Novel Class of Biomarkers for Diagnosis of Cancer and Other Diseases. Cell Res. (2008) 18(10):997–1006.

De Wit E, Delport W, Rugamika CE, Meintjes A, Möller M, Van Helden PD, *et al*. Genome-wide Analysis of the Structure of the South African Coloured Population in the Western Cape. Hum Genet. (2010) 128(2):145–53.

Felekkis K, Papaneophytou C. Challenges in Using Circulating Micro-RNAs as Biomarkers for Cardiovascular Diseases. Int J Mol Sci. (2020) 21(2):561.

Friedländer MR, Chen W, Adamidi C, Maaskola J, *et al*. Discovering microRNAs from Deep Sequencing Data using miRDeep. *Nat Biotechnolol.* (2008) 26(4): 407-15.

Gkaliagkousi E, Passacquale G, Douma S, Zamboulis C, Ferro A. Platelet Activation in Essential Hypertension: Implications for Antiplatelet Treatment. Am J Hypertens. (2010) 23(3):229–36.

Gomes A, da Silva I V., Rodrigues CMP, Castro RE, Soveral G. The Emerging Role of microRNAs in Aquaporin Regulation. Front Chem. (2018) 6:238.

Huang Y, Chen J, Zhou Y, Yu X, Huang C, Li J, *et al*. Circulating miR-30 Is Related to Carotid Artery Atherosclerosis. Clin Exp Hypertens. (2016) 38(5):489–94.

Huang YQ, Huang C, Chen JY, Li J, Feng YQ. The Association of Circulating miR-30a, miR-29

and miR-133 with White-Coat Hypertension. Biomark Med. (2016) 10(12):1231–9.

Jackson KL, Marques FZ, Watson AMD, Palma-Rigo K, Nguyen-Huu TP, Morris BJ, *et al*. A Novel Interaction Between Sympathetic Overactivity and Aberrant Regulation of Renin by miR-181a in BPH/2J Genetically Hypertensive Mice. Hypertension. (2013) 62(4):775–81.

Jairajpuri DS, Malalla ZH, Mahmood N, Almawi WY. Circulating microRNA Expression as Predictor of Preeclampsia and its Severity. Gene. (2017) 627:543–8.

James PA, Oparil S, Carter BL, Cushman WC, Dennison-Himmelfarb C, Handler J, *et al*. 2014 Evidence-based Guideline for the Management of High Blood Pressure in Adults: Report from the Panel Members Appointed to the Eighth Joint National Committee (JNC 8). JAMA. (2014) 311(5):507–20.

Kim J-E, Jung HJ, Lee Y-J, Kwon T-H. Vasopressin-regulated miRNAs and AQP2-targeting miRNAs in Kidney Collecting Duct Cells. Am J Physiol Ren Physiol. (2015) 308(7):F749–64.

Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. Differential Expression of Vascular Smooth Muscle-modulating MicroRNAs in Human Peripheral Blood Mononuclear Cells: Novel Targets in Essential Hypertension. J Hum Hypertens. (2014) 28(8):510–6.

Landstrom AP, Dobrev D, Wehrens XHT. Calcium Signaling and Cardiac Arrhythmias. Circ Res. (2017) 120(12):1969–93.

Levy E, Spahis S, Bigras J-L, Delvin E, Borys J-M. The Epigenetic Machinery in Vascular Dysfunction and Hypertension. Curr Hypertens Rep. (2017) 19(6):1–12.

Li H, Zhang X, Wang F, Zhou L, Yin Z, Fan J, *et al*. MicroRNA-21 Lowers Blood Pressure in Spontaneous Hypertensive Rats by Upregulating Mitochondrial Translation. Circulation. (2016) 134(10):734–51.

Li N, Lian J, Zhao S, Zheng D, Yang X, Huang X, *et al*. Detection of Differentially Expressed MicroRNAs in Rheumatic Heart Disease: miR-1183 and miR-1299 as Potential Diagnostic Markers. Biomed Res Int. (2015) 2015:524519.

Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM, *et al*. Signature microRNA Expression

Profile of Essential Hypertension and its Novel Link to Human Cytomegalovirus Infection. Circulation. (2011) 124(2):175–84.

Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, *et al*. A Comparative Risk Assessment of Burden of Disease and Injury Attributable to 67 Risk Factors and Risk Factor Clusters in 21 Regions, 1990–2010: A Systematic Analysis for the Global Burden of Disease Study 2010. Lancet. (2012) 380(9859):2224–60.

Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods. (2001) 25(4):402–8.

Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, *et al*. Distribution of miRNA Expression Across Human Tissues. Nucleic Acids Res. (2016) 44(8):3865–77.

Maciejak A, Kostarska-Srokosz E, Gierlak W, Dluzniewski M, Kuch M, Marchel M, *et al*. Circulating miR-30a-5p as a Prognostic Biomarker of Left Ventricular Dysfunction After Acute Myocardial Infarction. Sci Rep. (2018) 8(1):9883.

Marques FZ, Campain AE, Tomaszewski M, Zukowska-Szczechowska E, Yang YHJ, Charchar FJ, *et al*. Gene Expression Profiling Reveals Renin mRNA Overexpression in Human Hypertensive Kidneys and a Role for microRNAs. Hypertension. (2011) 58(6):1093–8.

Matsha TE, Kengne AP, Hector S, Mbu DL, Yako YY, Erasmus RT. MicroRNA Profiling and Their Pathways in South African Individuals with Prediabetes and Newly Diagnosed Type 2 Diabetes Mellitus. Oncotarget. (2018) 9(55):30485–98.

Mu S, Shimosawa T, Ogura S, Wang H, Uetake Y, Kawakami-Mori F, *et al*. Epigenetic Modulation of the Renal β-adrenergic–WNK4 Pathway in Salt-Sensitive Hypertension. Nat Med. (2011) 17(5):573–80.

Nemecz M, Alexandru N, Tanko G, Georgescu A. Role of MicroRNA in Endothelial Dysfunction and Hypertension. Curr Hypertens Rep. (2016) 18(12):87.

Özkan G, Ulusoy Ş, Geyik E, Erdem Y. Down-regulation of miRNA 145 and Up-regulation of miRNA 4516 May Be Associated with Primary Hypertension. J Clin Hypertens. (2019)

21(11):1724–31.

Parmacek MS. MicroRNA-modulated Targeting of Vascular Smooth Muscle Cells. J Clin Invest. (2009) 119(9):2526–8.

Peltzer K, Phaswana-Mafuya N. Hypertension and Associated Factors in Older Adults in South Africa. Cardiovasc J Afr. (2013) 24(3):66–72.

Polsinelli VB, Satchidanand N, Singh R, Holmes D, Izzo JL. Hypertension and Aging in Rural Haiti: Results from a Preliminary Survey. J Hum Hypertens. (2017) 31(2):138–44.

Robertson S, Mackenzie SM, Alvarez-Madrazo S, Diver LA, Lin J, Stewart PM, *et al*. MicroRNA-24 is a Novel Regulator of Aldosterone and Cortisol Production in the Human Adrenal Cortex. Hypertension. (2013) 62(3):572–8.

Romero DG, Plonczynski MW, Carvajal CA, Gomez-Sanchez EP, Gomez-Sanchez CE. Microribonucleic Acid-21 Increases Aldosterone Secretion and Proliferation in H295R Human Adrenocortical Cells. Endocrinology. (2008) 149(5):2477–83.

Rossier BC, Bochud M, Devuyst O. The Hypertension Pandemic: An Evolutionary Perspective. Physiol. (2017) 32(2):112–25.

Santovito D, Mandolini C, Marcantonio P, De Nardis V, Bucci M, Paganelli C, *et al*. Overexpression of microRNA-145 in Atherosclerotic Plaques from Hypertensive Patients. Expert Opin Ther Targets. (2013) 17(3):217–23.

Sethupathy P, Borel C, Gagnebin M, Grant GR, Deutsch S, Elton TS, *et al*. Human microRNA-155 on Chromosome 21 Differentially Interacts with its Polymorphic Target in the AGTR1 3′ Untranslated Region: A Mechanism for Functional Single-Nucleotide Polymorphisms Related to Phenotypes. Am J Hum Genet. (2007) 81(2):405–13.

Shin VY, Chu K-M. MiRNA as Potential Biomarkers and Therapeutic Targets for Gastric Cancer. World J Gastroenterol. (2014) 20(30):10432–9.

Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, *et al*. Vascular Smooth Muscle Contraction in Hypertension. Cardiovasc Res. (2018) 114(4):529–39.

Wang Y, Lu Z, Wang N, Zhang M, Zeng X, Zhao W. MicroRNA-1299 is a Negative Regulator of STAT3 in Colon Cancer. Oncol Rep. (2017) 37(6):3227–34.

Wilson C, Zhang X, Buckley C, Heathcote HR, Lee MD, McCarron JG. Increased Vascular Contractility in Hypertension Results from Impaired Endothelial Calcium Signaling. Hypertension. (2019) 74(5):1200–14.

Wise IA, Charchar FJ. Epigenetic Modifications in Essential Hypertension. Int J Mol Sci. (2016) 17(4):451.

Woo CC, Liu W, Lin XY, Dorajoo R, Lee KW, Richards AM, *et al*. The Interaction Between 30b-5p miRNA and MBNL1 mRNA is Involved in Vascular Smooth Muscle Cell Differentiation in Patients with Coronary Atherosclerosis. Int J Mol Sci. (2020) 21(1):11.

Wu J, Zheng C, Wang X, Yun S, Zhao Y, Liu L, *et al*. MicroRNA-30 Family Members Regulate Calcium/Calcineurin Signaling in Podocytes. J Clin Invest. (2015) 125(11):4091–106.

Xanthakis V, Vasan RS. Aldosterone and the Risk of Hypertension. Curr Hypertens Rep. (2013) 15(2):102–7.

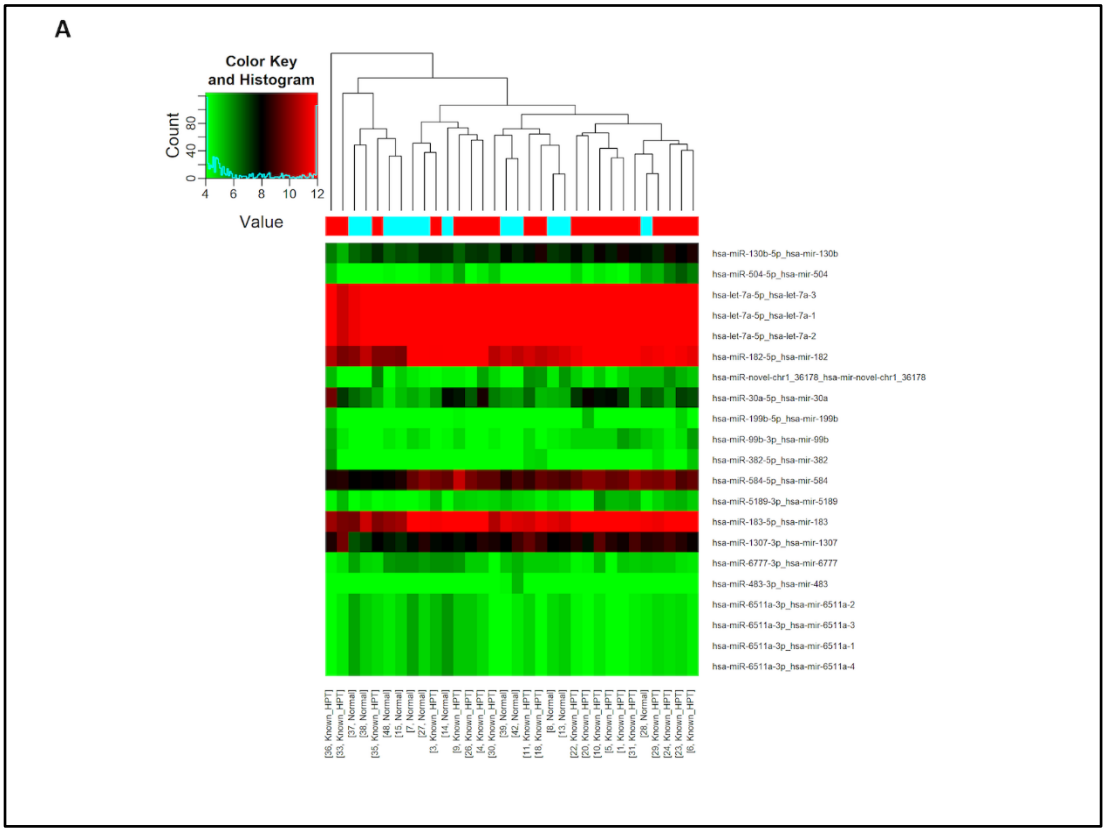
Ye H, Ling S, Castillo AC, Thomas B, Long B, Qian J, *et al*. Nebivolol Induces Distinct Changes in Profibrosis microRNA Expression Compared with Atenolol, in Salt-sensitive Hypertensive Rats. Hypertension. (2013) 61(5):1008–13.

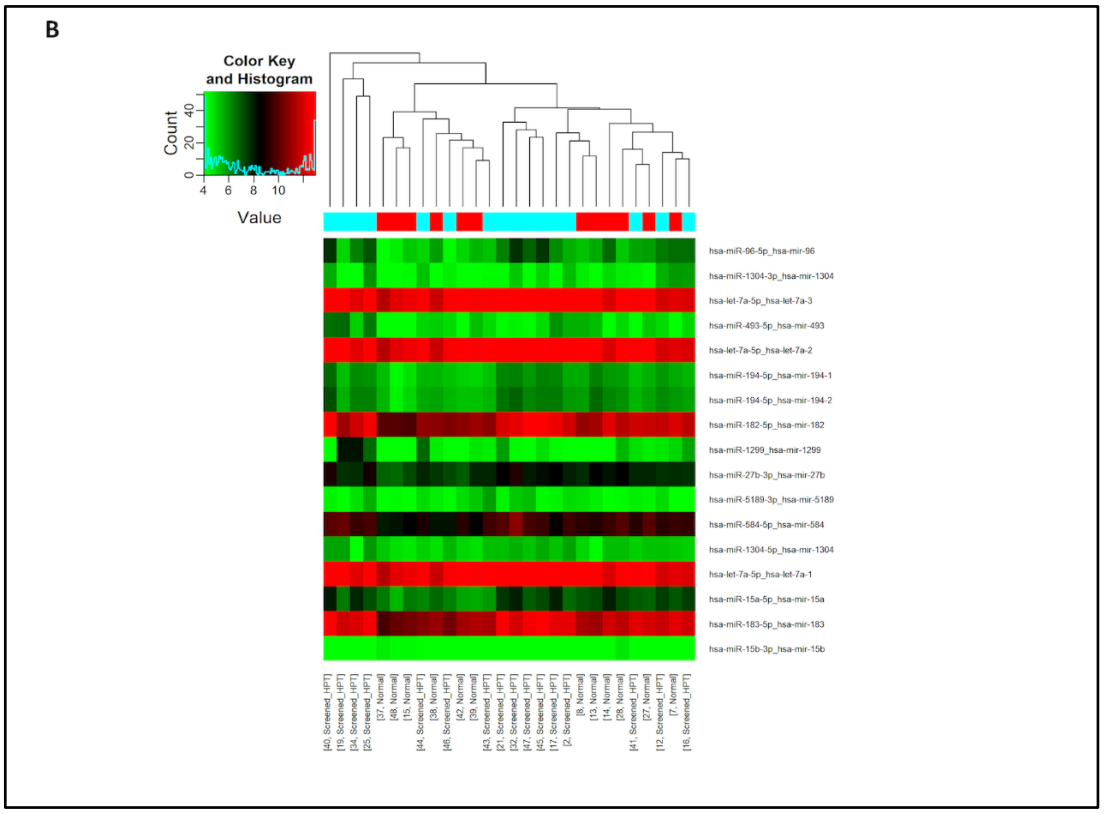
Yildirim E, Ermis E, Allahverdiyev S, Ucar H, Yavuzer S, Cengiz M. Circulating miR-21 Levels in Hypertensive Patients with Asymptomatic Organ Damage. Medicine (Baltimore). (2019) 98(39):e17297.

Zhang T, Tian F, Wang J, Jing J, Zhou SS, Chen YD. Endothelial Cell Autophagy in Atherosclerosis is Regulated by miR-30-mediated Translational Control of ATG6. Cell Physiol Biochem. (2015) 37(4):1369–78.

Zouein FA, Zgheib C, Hamza S, Fuseler JW, Hall JE, Soljancic A, *et al*. Role of STAT3 in Angiotensin II-induced Hypertension and Cardiac Remodeling Revealed by Mice Lacking STAT3 Serine 727 Phosphorylation. Hypertens Res. (2013) 36(6):496–503.

10. Figure legends





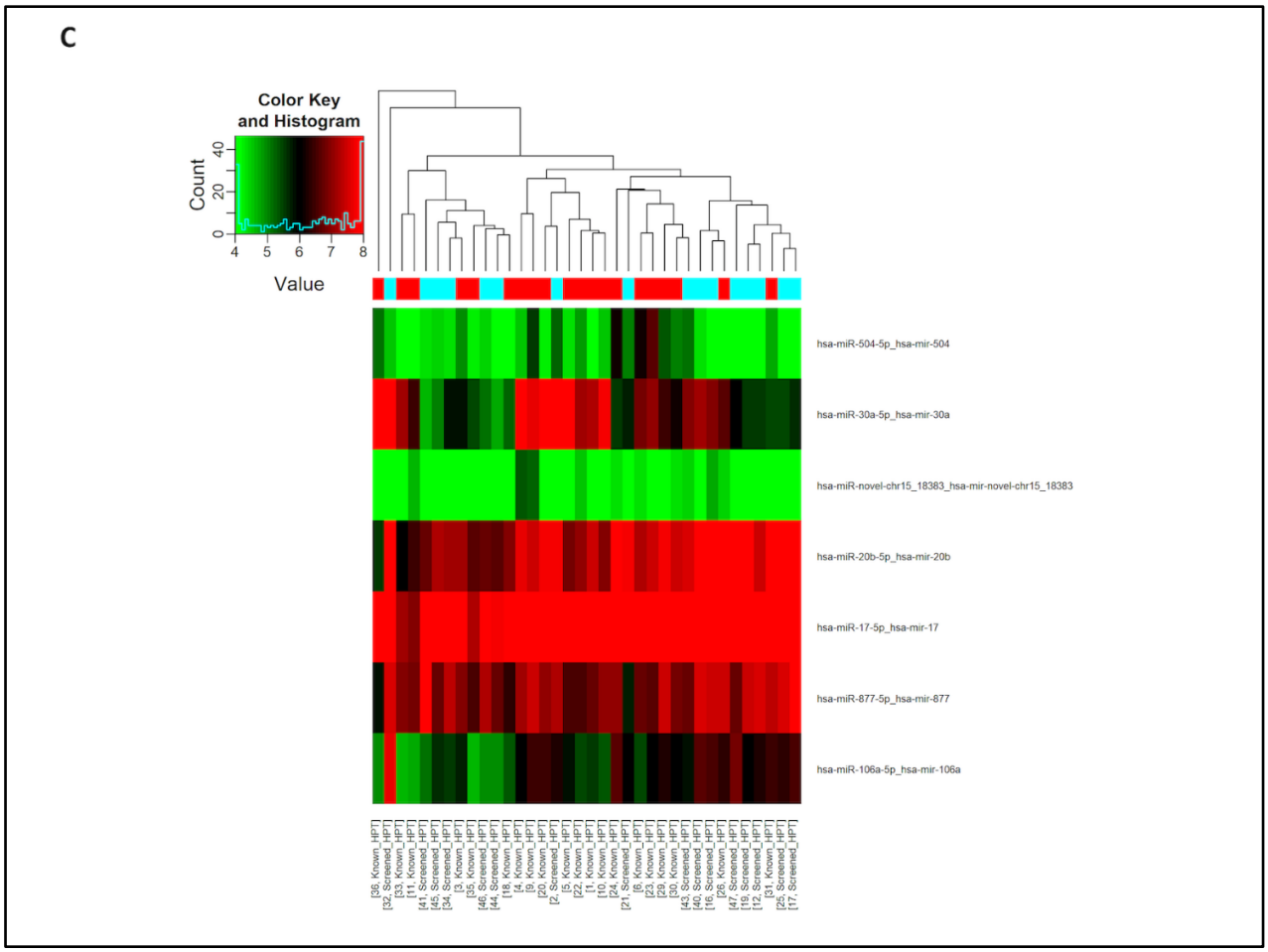
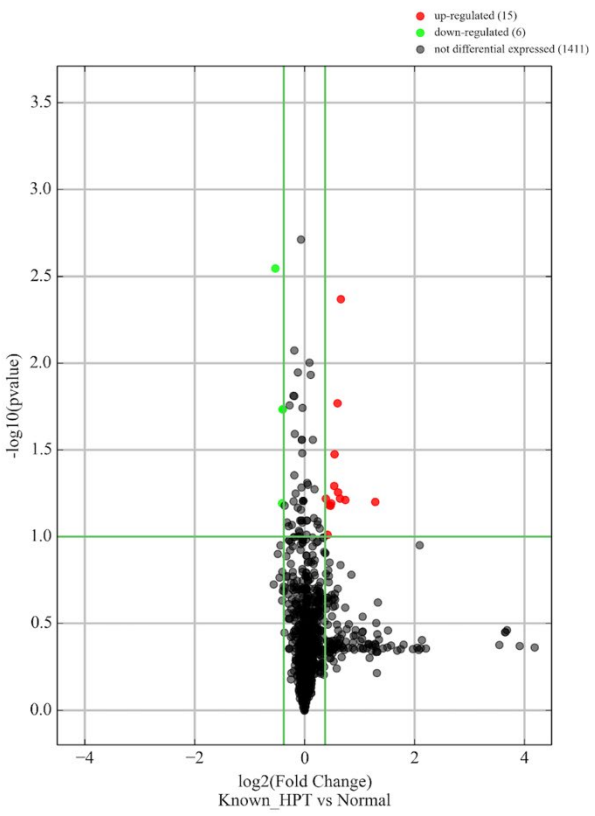


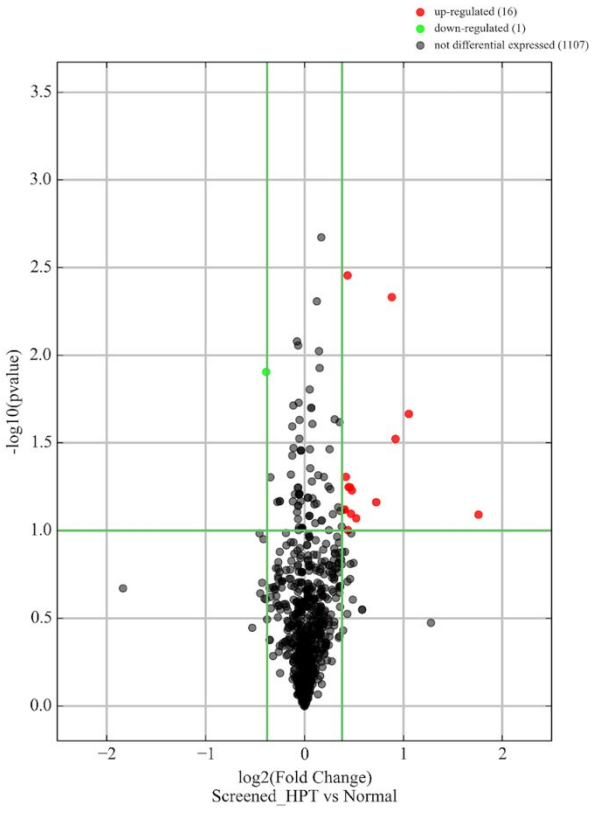
Figure 1. Differential miRNA expression according to HPT status.

The heatmap shows all differentially expressed miRNAs at adjusted *p*-value < 0.05. (**A**) normotensive versus known HPT; (**B**) normotensive versus screen-detected HPT; (**C**) known HPT versus screen-detected HPT.

A


up-regulated (15)
down-regulated (6)
not differential expressed (1411)
-log10(pvalue)
log2(Fold Change)
Known_HPT vs Normal

B


up-regulated (16)
down-regulated (1)
not differential expressed (1107)
-log10(pvalue)
log2(Fold Change)
Screened_HPT vs Normal

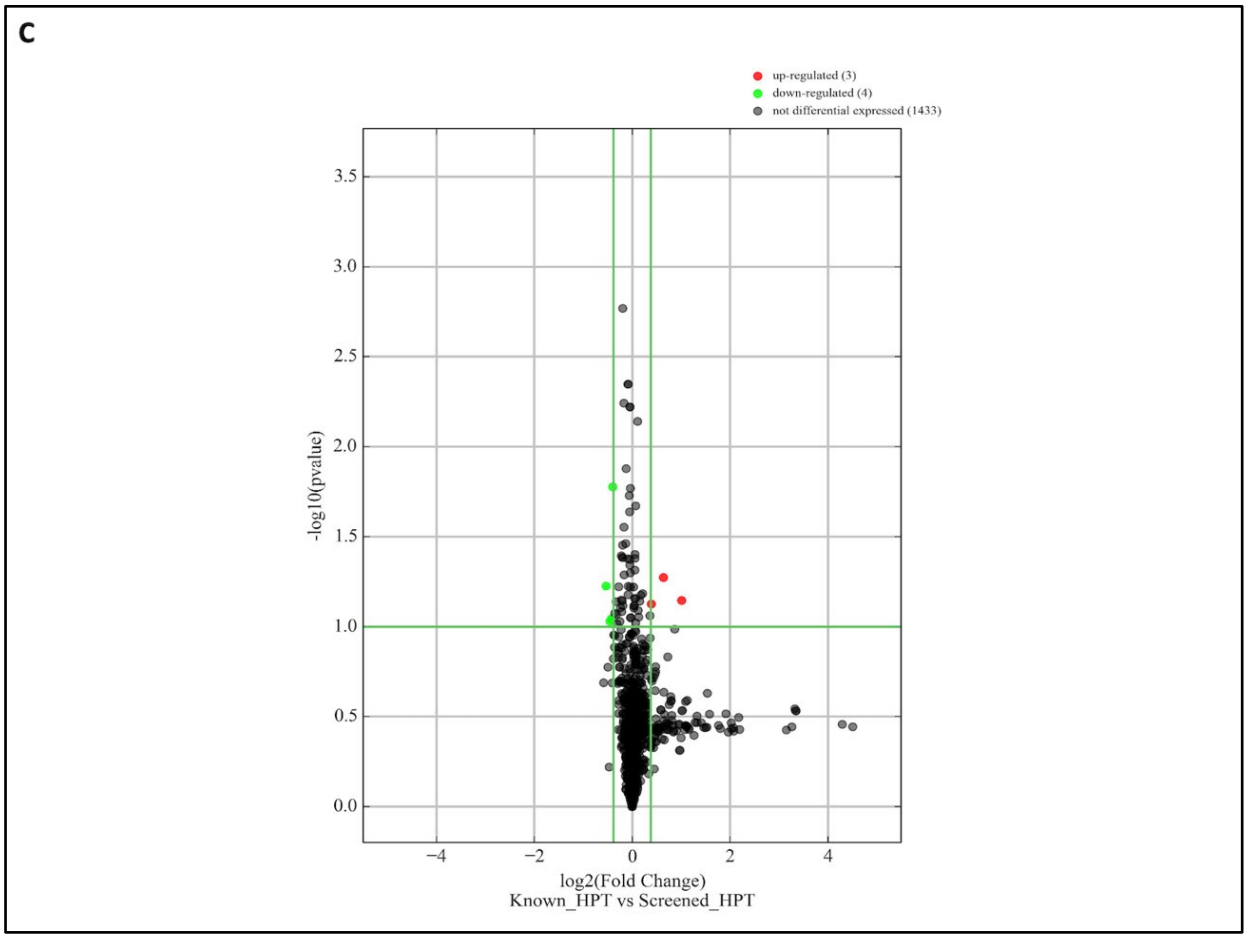
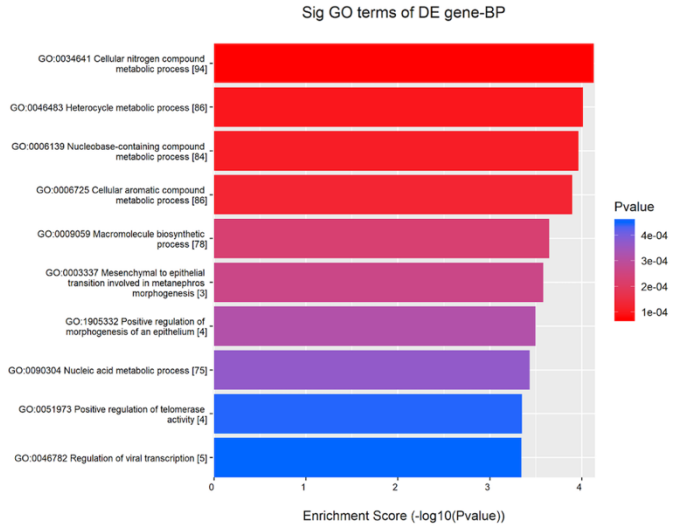


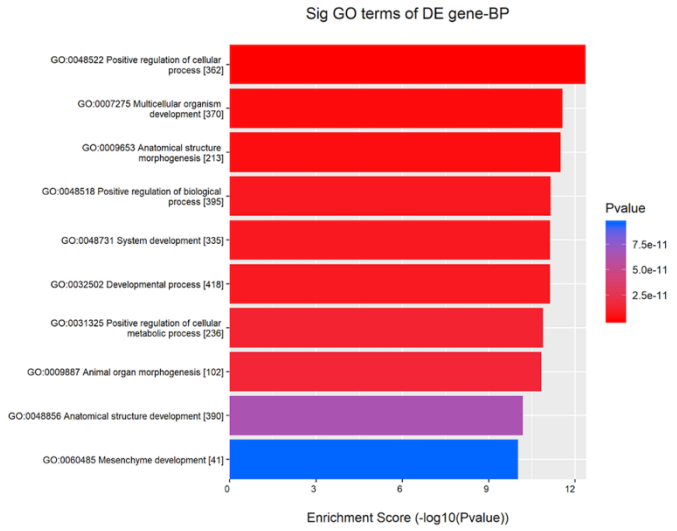
Figure 2. Differentially expressed miRNAs in comparison between blood pressure groups.

(**A**) is a comparison between known hypertensives and normotensives. (**B**) is a comparison between screen-detected HPT and normotensives. (**C**) is a comparison between known HPT and screen-detected HPT. Significantly different expression of a miRNA was those in which there was a ≥1.3-fold change difference in expression between comparison groups and $p \leq 0.1$. Red and green dots signify upregulated and downregulated miRNAs respectively. No differential expression was signified with a black dot.

A


Sig GO terms of DE gene-BP
GO:0034641 Cellular nitrogen compound metabolic process [94]
GO:0046483 Heterocycle metabolic process [86]
GO:0006139 Nucleobase-containing compound metabolic process [84]
GO:0006725 Cellular aromatic compound metabolic process [86]
GO:0009059 Macromolecule biosynthetic process [78]
GO:0003337 Mesenchymal to epithelial transition involved in metanephros morphogenesis [3]
GO:1905332 Positive regulation of morphogenesis of an epithelium [4]
GO:0090304 Nucleic acid metabolic process [75]
GO:0051973 Positive regulation of telomerase activity [4]
GO:0046782 Regulation of viral transcription [5]
0
1
2
3
4
Enrichment Score (-log10(Pvalue))
Pvalue
4e-04
3e-04
2e-04
1e-04

B


Sig GO terms of DE gene-BP
GO:0048522 Positive regulation of cellular process [362]
GO:0007275 Multicellular organism development [370]
GO:0009653 Anatomical structure morphogenesis [213]
GO:0048518 Positive regulation of biological process [395]
GO:0048731 System development [335]
GO:0032502 Developmental process [418]
GO:0031325 Positive regulation of cellular metabolic process [236]
GO:0009887 Animal organ morphogenesis [102]
GO:0048856 Anatomical structure development [390]
GO:0060485 Mesenchyme development [41]
0
3
6
9
12
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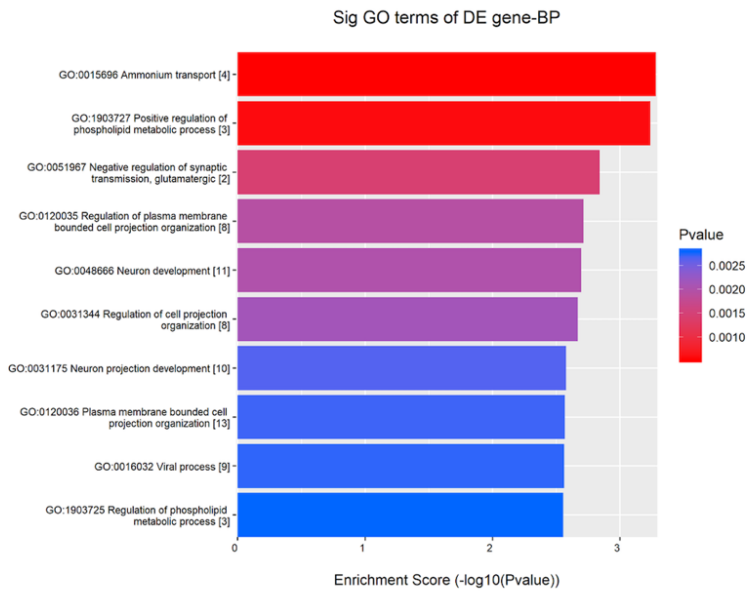
Pvalue
7.5e-11
5.0e-11
2.5e-11

C


Sig GO terms of DE gene-BP
GO:0015696 Ammonium transport [4]
GO:1903727 Positive regulation of phospholipid metabolic process [3]
GO:0051967 Negative regulation of synaptic transmission, glutamatergic [2]
GO:0120035 Regulation of plasma membrane bounded cell projection organization [8]
GO:0048666 Neuron development [11]
GO:0031344 Regulation of cell projection organization [8]
GO:0031175 Neuron projection development [10]
GO:0120036 Plasma membrane bounded cell projection organization [13]
GO:0016032 Viral process [9]
GO:1903725 Regulation of phospholipid metabolic process [3]
0
1
2
3
Enrichment Score (-log10(Pvalue))
Pvalue
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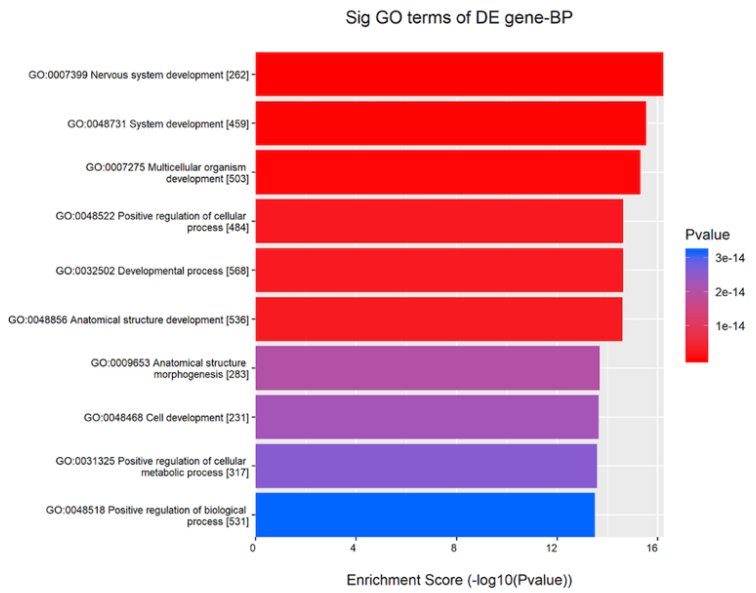
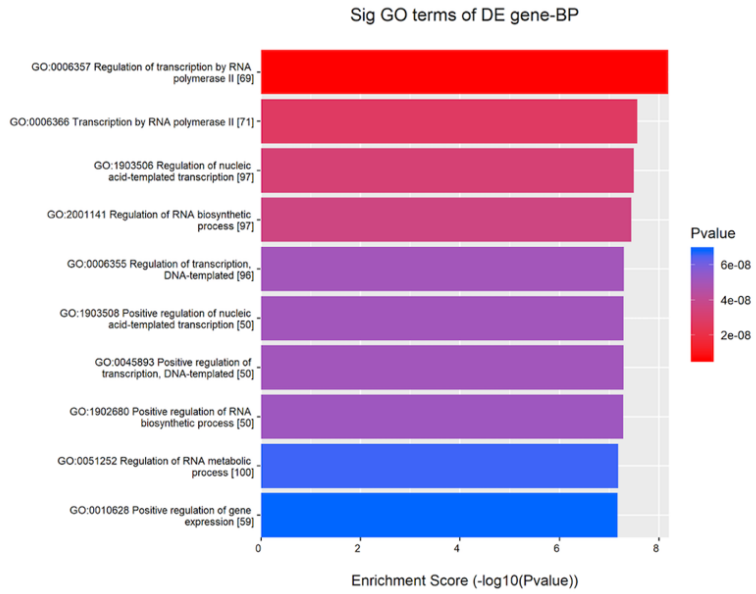
0.0020
0.0015
0.0010

D


Sig GO terms of DE gene-BP
GO:0007399 Nervous system development [262]
GO:0048731 System development [459]
GO:0007275 Multicellular organism development [503]
GO:0048522 Positive regulation of cellular process [484]
GO:0032502 Developmental process [568]
GO:0048856 Anatomical structure development [536]
GO:0009653 Anatomical structure morphogenesis [283]
GO:0048468 Cell development [231]
GO:0031325 Positive regulation of cellular metabolic process [317]
GO:0048518 Positive regulation of biological process [531]
0
4
8
12
16
Enrichment Score (-log10(Pvalue))
Pvalue
3e-14
2e-14
1e-14

E


Sig GO terms of DE gene-BP
GO:0006357 Regulation of transcription by RNA polymerase II [69]
GO:0006366 Transcription by RNA polymerase II [71]
GO:1903506 Regulation of nucleic acid-templated transcription [97]
GO:2001141 Regulation of RNA biosynthetic process [97]
GO:0006355 Regulation of transcription, DNA-templated [96]
GO:1903508 Positive regulation of nucleic acid-templated transcription [50]
GO:0045893 Positive regulation of transcription, DNA-templated [50]
GO:1902680 Positive regulation of RNA biosynthetic process [50]
GO:0051252 Regulation of RNA metabolic process [100]
GO:0010628 Positive regulation of gene expression [59]
0
2
4
6
8
Enrichment Score (-log10(Pvalue))
Pvalue
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4e-08
2e-08

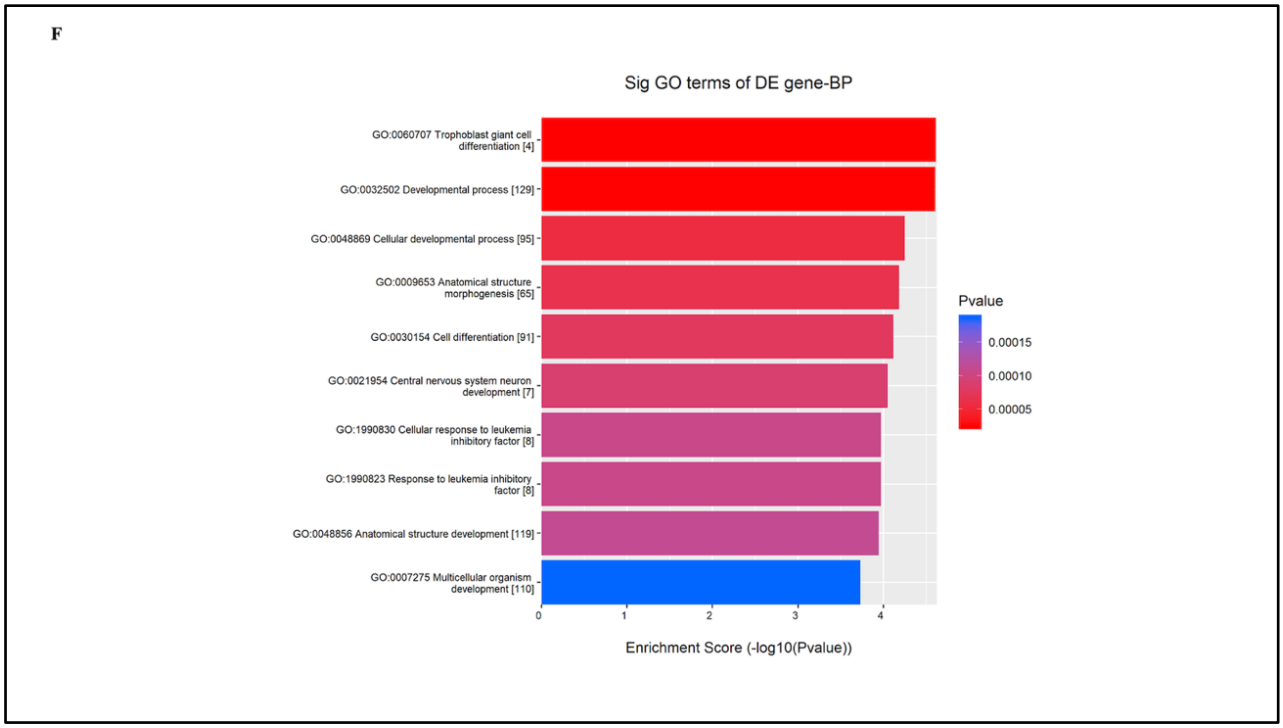

Figure 3. GO analysis - Biological Processes. The bar plots show the top ten enrichment score values of the significant enrichment terms.

X-axis: GOID's enrichment score value; it equals −log10(*p*-value); Y-axis: GO category. (**A**) upregulated in known HPT vs. normotensive. (**B**) downregulated in known HPT vs. normotensive. (**C**) upregulated in screen-detected HPT vs. normotensive. (**D)** downregulated in screen-detected HPT vs. normotensive. (**E**) upregulated in known HPT vs. screen-detected HPT. (**F**) downregulated in known HPT vs. screen-detected HPT.

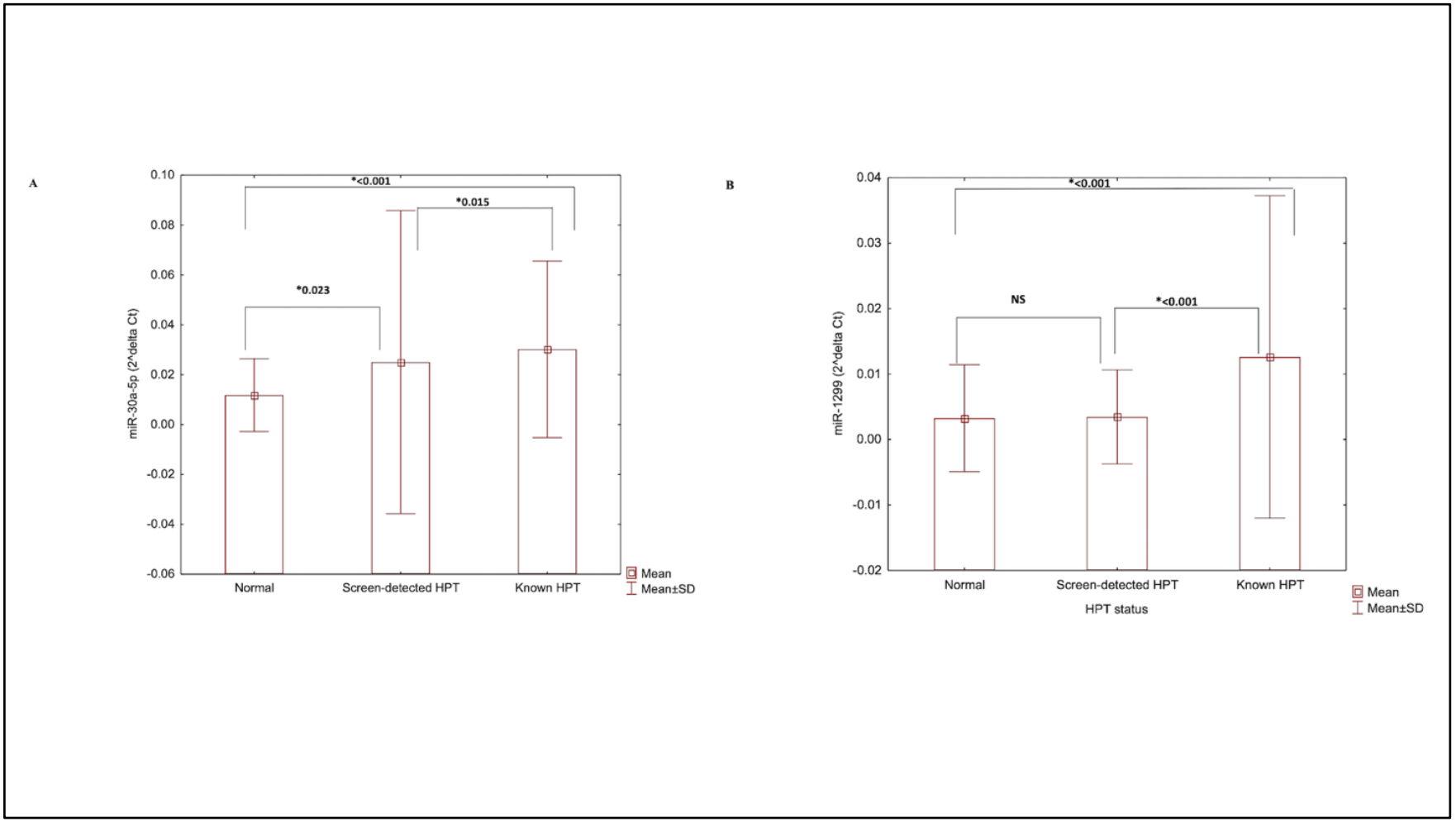

Figure 4. Relative expression of miR-30a-5p and miR-1299.

(A). miR-30a-5p in normotensive (n=116), screen-detected HPT (n=66) and known HPT (n=124). (B). miR-1299p in normotensive, screen-detected HPT and known HPT. Data are represented graphically as the mean ± SD. Not significant (NS) if p>0.05 and significant p-value denoted by an asterisk*.

Table 1. Characteristics of the participants, based on hypertension status.

	Next Generation Sequencing sample				Validation sample (RT-qPCR)			
	Normotensive, n=12	**Screen-detected HPT, n=16**	**Known HPT, n=20**	**p-value**	**Normotensive, n=105**	**Screen-detected HPT, n=52**	**Known HPT, n=106**	**p-value**
Female, n(%)	12 (100%)	16 (100%)	20 (100%)	-	57 (54.29)	36 (69.23)	86 (81.13)	<0.001
Male, n(%)	-	-	-	-	48 (45.71)	16 (30.77)	20 (18.87)	
Age (years)	49.6 ± 9.3	52.8 ± 7.1	56.1 ± 7.7	0.086	40 ± 15.32	51.12 ± 13.43	61.1 ± 10.6	<0.001
Body mass index (kg/m^2)	29.1 ± 8.1	30.6 ± 9.0	32.3 ± 6.4	0.509	25.08 ± 6.45	28.71 ± 7.96	30.85 ± 7.06	<0.001
Waist circumference (cm)	87.5 ± 16.2	92.0 ± 22.1	97.9 ± 11.4	0.226	81.99 ± 13.71	91.32 ± 16.71	95.86 ± 14.75	<0.001
Hip circumference (cm)	101.7 ± 17.7	106.1 ± 18.4	108.8 ± 14.2	0.504	97.55 ± 12.69	103.76 ± 15.3	106.05 ± 13.85	<0.001
Waist to hip Ratio	0.86 ± 0.06	0.86 ± 0.09	0.90 ± 0.06	0.139	0.84 ± 0.07	0.88 ± 0.08	0.90 ± 0.08	<0.001
Systolic blood pressure (mmHg)	113.4 ± 14.5	147.7 ± 22.2	144.7 ± 27.7	<0.001	118.77 ± 12.95	149.12 ± 19.96	148.50 ± 23.81	<0.001
Diastolic blood pressure (mmHg)	74.8 ± 11.9	90.6 ± 14.8	89.7 ± 17.7	0.018	74.95 ± 10.47	97.19 ± 12.32	89.11 ± 13.56	<0.001
Fasting blood glucose (mmol/L)	6.18 ± 3.87	7.50 ± 4.58	8.28 ± 4.27	0.413	4.87 ± 1.43	5.55 ± 2.72	6.70 ± 3.49	<0.001
2-hour fasting glucose	8.63 ± 4.39	9.84 ± 6.53	12.83 ± 4.54	0.172	5.69 ± 2.8	6.80 ± 4.37	7.74 ± 4.47	0.002
HbA1c (%)	6.28 ± 1.60	7.10 ± 2.82	7.77 ± 2.67	0.276	5.79 ± 1.14	6.19 ± 1.49	6.70 ± 1.74	<0.001
Fasting Insulin (mIU/L)	5.88 ± 3.49	7.89 ± 3.89	15.44 ± 8.66	<0.001	6.81 ± 6.65	8.03 ± 6.24	11.14 ± 14.33	0.011
Diabetes Mellitus, n(%)	5 (41.7)	6 (37.5)	13 (65.0)	0.093	7 (6.7)	7 (13.7)	35 (33.3)	<0.001
Triglycerides-S (mmol/L) *	1.12 (0.86-1.64)	1.26 (1.00-1.50)	1.74 (1.43-3.31)	0.008	1.05 (0.72; 1.42)	1.28 (0.9; 1.67)	1.40 (1.05; 1.83)	<0.001
Total Cholesterol (mmol/L)	5.93 ± 1.14	5.66 ± 1.12	5.93 ± 1.23	0.757	4.75 ± 1.18	5.13 ± 0.97	5.42 ± 1.04	<0.001
LDL-cholesterol (mmol/L)	3.76 ± 1.09	3.48 ± 0.97	3.96 ± 1.06	0.402	2.86 ± 1.00	3.13 ± 0.96	3.37 ± 0.91	0.001
HDL-cholesterol (mmol/L)	1.57 ± 0.50	1.48 ± 0.59	1.17 ± 0.21	0.032	1.36 ± 0.41	1.35 ± 0.38	1.37 ± 0.34	0.984
usCRP (mg/L)	6.32 ± 8.79	9.73 ± 13.20	11.00 ± 6.89	0.44	7.32 ± 13.51	6.24 ± 7.09	7.24 ± 14.03	0.871
Serum Cotinine (ng/mL) *	10.0 (10.0-22.5)	209.5 (10.0-261.0)	99.4 (10.0-195.5)	0.146	137 (10; 265.5)	10 (10; 287)	10 (10; 135.75)	0.002
JIS MetS criteria	4 (33.33)	9 (56.25)	18 (90.00)	0.014	22 (21.15)	21 (41.18)	61 (58.65)	<0.001

Values presented as mean±SD unless marked with an asterisk*, in which case the median and (25th-75th percentiles) are reported. The Kruskal-Wallis test and analysis of variance (ANOVA) were used to compare the median and mean baseline characteristics respectively across blood pressure groups. SD = standard deviation, usCRP = ultrasensitive CRP, MetS = Metabolic Syndrome

Table 2. Dysregulated mature miRNAs in screen-detected & known HPT compared to normotensive participants. A comparison of dysregulated miRNAs between screen-detected HPT and known HPT participants is also shown

Mature miRNA	miRNA accession number	Known HPT vs Normotensive fold change	p-value	BH FDR	Mature miRNA	miRNA accession number	Screen-detected HPT vs Normotensive fold change	p-value	BH FDR	Mature miRNA	miRNA accession number	Known HPT vs Screen-detected HPT	p-value	BH FDR
miR-30a-5p	MIMAT0000087	2.44	0.063	0.7403	miR-1299	MIMAT0005887	3.38	0.081	0.8106	miR-30a-5p	MIMAT0000087	2.02	0.072	0.7031
miR-504-5p	MIMAT0002875	1.67	0.062	0.7403	miR-182-5p	MIMAT0000259	2.08	0.022	0.8106	miR-504-5p	MIMAT0002875	1.56	0.053	0.7031
miR-5189-3p	MIMAT0027088	1.58	0.004	0.7403	miR-96-5p	MIMAT0000095	1.89	0.030	0.8106	miR-novel-chr15_18383	miR-novel-chr15_18383	1.31	0.075	0.7031
miR-182-5p	MIMAT0000259	1.57	0.060	0.7403	miR-183-5p	MIMAT0000261	1.84	0.005	0.8106	miR-877-5p	MIMAT0004949	0.76	0.017	0.7031
miR-183-5p	MIMAT0000261	1.53	0.056	0.7403	miR-493-5p	MIMAT0002813	1.65	0.069	0.8106	miR-106a-5p	MIMAT0000103	0.75	0.091	0.7031
miR-1307-3p	MIMAT0005951	1.52	0.017	0.7403	miR-1304-3p	MIMAT0022720	1.44	0.085	0.8106	miR-17-5p	MIMAT0000070	0.73	0.093	0.7031
miR-novel-chr1_36178	miR-novel-chr1_36178	1.46	0.034	0.7403	miR-5189-3p	MIMAT0027088	1.39	0.059	0.8106	miR-20b-5p	MIMAT0001413	0.69	0.060	0.7031
miR-382-5p	MIMAT0000737	1.45	0.051	0.7403	miR-584-5p	MIMAT0003249	1.38	0.080	0.8106					
miR-584-5p	MIMAT0003249	1.4	0.064	0.7403	miR-27b-3p	MIMAT0000419	1.38	0.057	0.8106					
miR-130b-5p	MIMAT0004680	1.39	0.066	0.7403	miR-194-5p	MIMAT0000460	1.36	0.057	0.8106					
let-7a-5p	MIMAT0000062	1.37	0.066	0.7403	miR-15a-5p	MIMAT0000068	1.36	0.099	0.8106					
miR-199b-5p	MIMAT0000263	1.34	0.098	0.7403	miR-1304-5p	MIMAT0005892	1.35	0.004	0.8106					
miR-99b-3p	MIMAT0004678	1.31	0.060	0.7403	let-7a-5p	MIMAT0000062	1.32	0.076	0.8106					
miR-6511a-3p	MIMAT0025479	0.76	0.019	0.7403	miR-15b-3p	MIMAT0004586	0.76	0.013	0.8106					
miR-483-3p	MIMAT0002173	0.75	0.064	0.7403										
miR-6777-3p	MIMAT0027455	0.69	0.003	0.7403										

BH FDR, Benjamini-Hochberg False Discovery Rate corrected *p*-value

CHAPTER 4: MANUSCRIPT 2

Manuscript submitted to the Molecular Diagnostic and Therapy Journal.

Circulating levels of microRNAs associated with hypertension

Short title: MicroRNA profiling in hypertension

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Abstract

MicroRNAs are non-coding, post-transcriptional regulators of gene expression and their dysregulation has been associated with the development of various diseases. Understanding their role in disease development and progression is essential. Prior research focusing on microRNAs in disease has provided a basis for understanding disease prognosis and offers possible channels for therapeutic interventions. This study aimed to investigate possible differences in microRNA expression profiles of participants based on their blood pressure status in order to elucidate possible roles played by select microRNAs in hypertension development. Using quantitative reverse transcription polymerase chain reaction, we evaluated the expression levels of miR-126-3p, 30a-5p, 182-5p, 30e-3p and 1299 in the whole blood of 1456 participants, normotensive (n=573), screen-detected hypertensive (n=304) and known hypertensive (n=579). The expression of miR-126-3p and 182-5p was significantly higher in known hypertensives when compared to both screen-detected hypertensives and normotensives and also in screen-detected hypertensives vs normotensives. There was also a significant association between the expression of miR-126-3p, 182-5p and 30a-5p and known hypertension. The study was able to demonstrate the dysregulation of three microRNAs in hypertension, whilst further highlighting its association with miR-126, 182-5p and 30a-5p expression.

1. Introduction

Hypertension is a complex and multifactorial disease responsible for considerable loss of life globally (Bromfield & Muntner, 2013; Mills *et al*., 2020). It is an important, modifiable risk factor for cardiovascular disease (Touyz *et al*., 2018; Mills *et al*., 2020), whose prevalence varies globally and is on an upward trajectory in sub-Saharan Africa (Campbell *et al*., 2015; Mills *et al*., 2016). Hypertension prevalence rates in some sub-Saharan African countries currently rank among the highest globally, in stark contrast to the picture from a few decades ago when the region had the lowest blood pressure levels (Danaei *et al*., 2011). Inroads into understanding the pathophysiology of HPT and advancing treatment options have been made over the years. The pathogenesis has been linked to various biological processes, including endothelial dysfunction, impaired angiogenesis, dysregulation of the renin-angiotensin-aldosterone axis and platelet activation (Taddei *et al*., 2001; Gkaliagkousi *et al*., 2010; Touyz *et al*., 2018). Sizeable financial investments have been made to study the genetic and environmental determinants of hypertension and some of these studies have linked the Liddle syndrome. This is a hereditary disorder characterised by overactivity of the renal tubular epithelial sodium channel (ENaC) as a result of mutations in the *SCNN1B* and *SCNN1G* genes), resulting in the development of HPT through sodium retention accompanied by potassium excretion, and low plasma renin and aldosterone levels. The syndrome continues to play its part towards the high HPT prevalence levels currently observed in sub-Saharan Africa (Spence & Rayner, 2018). However, in up to 95% of HPT cases, the aetiology remains unknown and primary hypertension continues to be a leading cause of morbidity and premature mortality globally (Carretero & Oparil, 2000; He & Macgregor, 2007). Described as a “silent killer”, affected individuals may be asymptomatic or symptoms only manifest in the later stages of the condition when possible target organ damage has taken place (Moore, 2005).

Processes involved in blood pressure homeostasis are tightly regulated by various systems in the body. Amongst others, cellular processes like differentiation, growth and metabolism, are known to be under the control of microRNAs (miRNAs) (Vidigal & Ventura, 2015). These miRNAs are 18-25 base pair long, non-coding, post-transcriptional regulators of gene expression and their dysregulation has been linked to the development of cancer, essential hypertension, viral disease and endothelial dysfunction (Chen *et al*., 2008; Li & Kowdley, 2012; Silambarasan *et al*., 2016; Nemecz *et al*., 2016). It is plausible that dysregulation of miRNAs may lead to disturbances in the body’s blood pressure regulatory mechanisms and

play an important role in the development of HPT. Herein, we investigated circulating levels of miR-1299, miR-30a-5p, miR-30e-3p, miR-126-3p and miR-182-5p in participants with normal blood pressure, as well as screen-detected and known hypertensives on anti-hypertensive treatment. These miRNAs were chosen as possible targets in our study as next generation sequencing data had previously shown their association with diabetes and hypertension (Matsha *et al*., 2018; unpublished data).

2. Materials and methods

2.1. Ethical considerations

This investigation was based on the ongoing Cape Town Vascular and Metabolic Health (VMH) study, which was approved by the Research Ethics Committees of the Cape Peninsula University of Technology (CPUT) and Stellenbosch University (respectively, NHREC: REC - 230 408 – 014 and N14/01/003). Ethical approval was also obtained for this sub-study from the CPUT Health and Wellness Sciences Research Ethics Committee (CPUT/HW-REC 2019/H7). The study was conducted as per the provisions of the Declaration of Helsinki and institutional guidelines. All procedures were explained to the participants in their language of choice and once participants fully understood their participation, they signed informed consent forms to allow the collection of blood and anthropometric data.

2.2. Study design and procedures

This cross-sectional study involved male and female participants from the VMH study, an extension of the previously described Cape Town Bellville South Study (Matsha *et al*., 2012). Data collection and procedures have been reported previously (Matsha *et al*., 2018). Briefly, each participant underwent anthropometric measurements and these were reported as the average of three separate readings. Body Mass Index (BMI) was calculated as weight per height squared (kg/m^2) where kg is a participant's weight in kilograms and m^2 is the square of their height in metres. Blood pressure was measured according to the World Health Organisation (WHO) guidelines (Chalmers *et al*., 1999), using a semi-automatic digital blood pressure monitor (Omron M6 comfort-preformed cuff blood pressure monitor, China) on the right arm in a sitting position and at rest for at least 10 minutes. Three blood pressure readings were taken at three-minute intervals and the lowest systolic blood pressure and corresponding diastolic blood pressure values were used. Participants were grouped into three categories based on; the use of anti-hypertensive medication as known hypertension, blood pressure measurement of 140/90 mm Hg as screen-detected hypertension and normal blood pressure measurement as normotensive.

Various biochemical parameters were measured in an ISO 15189 accredited pathology practice (PathCare Reference Laboratory, Cape Town, South Africa) using different analytical methods as follows: glycated haemoglobin (HbA1c) by High Performance Liquid Chromatography (BioRad Variant Turbo, BioRad, Hercules, CA, USA); serum insulin by a paramagnetic

particle chemiluminescence assay (Beckman DXI, Beckman Coulter, South Africa); serum cotinine by Competitive Chemiluminescent **(**Immulite 2000, Siemens, Munich, Germany); plasma glucose by enzymatic hexokinase method (Beckman AU, Beckman Coulter, Brea, CA, USA); total cholesterol (TC); high density lipoprotein cholesterol (HDL-c) by enzymatic immunoinhibition – end point (Beckman AU, Beckman Coulter, Brea, CA, USA); triglycerides (TG) by glycerol phosphate oxidase-peroxidase, end point (Beckman AU, Beckman Coulter, Brea, CA, USA); low density lipoprotein cholesterol (LDL) by enzymatic selective protection – end point (Beckman AU, Beckman Coulter, Brea, CA, USA); and ultrasensitive C-reactive protein (CRP) by Latex Particle Immunoturbidimetry (Beckman AU, Beckman Coulter, Brea, CA, USA). These analyses were conducted at an ISO 15189 accredited pathology practice (PathCare Reference Laboratory, Cape Town, South Africa). Blood samples for miRNA expression analysis were collected in Tempus Blood RNA tubes (ThermoFisher Scientific, Waltham, MA, USA) and stored at -80°C for total RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis.

2.3. RNA isolation

Total RNA, including miRNA, was isolated from 3 mL of whole blood using the MagMax Total RNA isolation kit (ThermoFisher Scientific) according to manufacturer's instructions, with the nucleic acid washing and elution steps conducted on the Kingfisher Flex system. The concentration and purity of each total RNA extract was determined using a NanoDrop One spectrophotometer and total RNA extracts with 260/280 values between 1.8 and 2.0, and concentrations greater than 20 ng/µl were used for RT-qPCR.

2.4. Quantitative reverse transcription PCR

RT-qPCR was performed to determine the miRNA expression levels. Briefly, using the TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA), 2µl of total RNA was converted into cDNA through sequential conduction of poly-A tailing, adaptor ligation, reverse transcription and miR-Amp steps as per the manufacturer's recommendation. The miRNA expression levels were then determined using TaqMan miRNA Assay primers (ThermoFisher Scientific, Waltham, MA, USA) on the QuantStudio 7 Flex real-time PCR instrument (Life Technologies, Carlsbad, CA, USA). In order to determine relative miRNA expression in each sample, the $2^{-\Delta Ct}$ method was used whilst the $2^{-\Delta\Delta Ct}$ method was used to compute fold change differences in miRNA

expression between the study groups using miR-16-5p (ThermoFisher Scientific, Waltham, MA, USA) as the endogenous control (Livak & Schmittgen, 2001).

2.5. Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) v.26 software (IBM Corp, USA). Results are reported as count (and percentages), mean (and standard deviation) for normally distributed variables or median (25^{th}-75^{th} percentiles) for asymmetrically distributed variables. The Kruskal-Wallis test and analysis of variance (ANOVA) were used to compare the median and mean baseline characteristics respectively across blood pressure groups. Spearman's partial correlations, adjusted for age, gender and BMI, were used to assess the relationship between miRNAs and other cardiovascular risk profile variables whilst multivariable logistic regression models were used to assess the association of miRNAs with screen-detected hypertension and known hypertension with crude and adjusted odds ratio (OR). A *p*-value less than 0.05 signified statistically significant findings.

3. Results

3.1. Participant characteristics

A total of 1456 participants were included in this study. A summary of the participants characteristics is shown in Table 1. There were 386 (67.4%) females and 187 (32.6%) males in the normotensive group with an average age of 39.3±13.7 years, 209 (68.8%) females and 95 (31.2%) males in the screen-detected hypertension group with an average age of 48.9±13.4 years whilst the known hypertension group comprised of 478 (82.6%) females and 101 (17.4%) males whose average age was 58.9±11.0 years. As expected for hypertension, there was a significant difference in the average age of participants across the three groups (p<0.001). Serum gamma-Glutamyltransferase (Gamma GT-S) levels differed significantly between the three groups and the other expected differences (BMI, age, systolic blood pressure, waist circumference, HDL-c and LDL cholesterol) by hypertension status in the cardiovascular risk profile were apparent between the participant groups as shown in Table 1.

3.2. Relative miRNA expression

Normalization of relative miRNA expression was done with reference to the expression of miR-16-5p. Overall, the expression of miR-126-3p and 182-5p differed significantly across all blood pressure groups. Figure 1A shows that in participants with known hypertension, the relative expression ($2^{-\triangle Ct}$) of miR-126-3p was significantly higher than that of the normotensives and screen-detected hypertensives, (p<0.001). For miR-182-5p, the relative expression in known hypertension was significantly higher (4.3604) compared to both normotensive (1.7105) and screen detected hypertension (2.3124), both p<0.001 as shown in Figure 1B. However, for miR-30a-5p, 1299 and 30e-3p, whilst there were significant differences in their relative expression ($2^{-\triangle Ct}$) in known hypertension (0.0677; 0.0105 and 0.0141) vs normotensive (0.033; 0.0044 and 0.0059) respectively (all p<0.001), that significance was not seen when screen-detected hypertension was compared to the normotensives, all p≥0.114 as shown in Figures 2A-C.

3.3. Fold change computation

When compared to the normotensives, there was at least a 2.2-fold increase in expression of miR-126-3p, 182-5p, 30a-5p, 1299 and 30e-3p in the known hypertension group. MiR-126-3p was the most expressed of the five miRNAs, particularly in known hypertension versus normotensive (fold change=2.64) and known hypertension versus screen-detected

hypertension (fold change=1.99). However, the fold difference in expression of all five miRNAs in the screen-detected hypertension participants compared to the normotensives was ≤1.65-fold, with the lowest fold difference in expression between these two blood pressure groups observed for miR-30e-3p, whose expression was 1.17-fold higher in screen-detected hypertension compared to the normotensive.

3.4. MiRNA expression correlation with anthropometric measurements

There was a significant positive correlation between the expression of the five miRNAs across the blood pressure groups (r≥0.71, p<0.001), with the highest correlation coefficient in expression seen between miR-126-3p and miR-182-5p (r=0.983, p<0.001) as shown in Table 2. Detailed correlations between the expression of each miRNA and biochemical parameters are shown in Supplementary Tables S1-5. The expression of all miRNAs correlated negatively with waist circumference, with the highest correlation coefficients seen in the screen-detected hypertension group for both miR-126-3p (r = -0.748, p<0.001) and miR-30e-3p (r = -0.729, p<0.001). There was a significant, though weak correlation between the expression of both miR-182-5p and miR-30a-5p with systolic blood pressure in the known hypertension group. However, there was no significant association between the expression of miR-30e-3p, miR-126-3p and 1299 with systolic blood pressure regardless of blood pressure status. Whilst there was no correlation between the expression of any of the five miRNAs and TC, there was a significant positive correlation with HDL-c, with the highest correlation coefficients seen in the known hypertension group with respect to miR-182-5p (r=0.629, p=0.001), miR-30a-5p (r=0.615, p=0.002), miR-30e-3p (r=0.608, p=0.002) miR-126-3p (r=0.595, p=0.003) and finally miR-1299 (r=0.508, p=0.013). Gamma GT-S also showed a significantly positive correlation with the expression of all but miR-1299 in the screen-detected hypertension group.

3.5. Multivariable regression analysis

The results of multivariable regression analysis are shown in Table 3. With regards to miR-126-3p, the crude odds (age and gender adjusted only) ratio was 1.16 (95% confidence interval (CI): 1.05-1.27, p=0.003) for screen-detected hypertension, whilst for known hypertension, the OR was 1.58 (95% CI: 1.46-1.71, p<0.001). For miR-30a-5p, the OR was 1.24 (95% CI: 1.00-1.55, p=0.053) for screen-detected hypertension whilst for known hypertension, the OR was 1.63 (95% CI: 1.36-1.95, p<0.001). The crude odds ratio for miR-182-5p was 1.11 (95% CI: 1.05-1.17, p<0.001) for screen-detected hypertension whilst for known hypertension, the OR was 1.24 (95% CI: 1.18-1.30, p<0.001). The associations between the expression of these miR-

126-3p, 182-5p and 30a-5p and hypertension (both screen-detected and known) remained significant even when the model was adjusted for BMI, TC, TG and HbA1c. When further adjusted for duration of known hypertension diagnosis, the associations also remained significant for these three miRNAs. As for miR-30e-3p, the only significant association was with known hypertension which had an OR of 1.71 (95% CI: 1.46-2.01, $p<0.001$) whilst for screen-detected hypertension, the OR was 1.07 (95% CI: 0.91-1.26, $p=0.393$). There was no significant association between the expression of miR-1299 and screen-detected or known hypertension.

4. Discussion

This study demonstrated a significantly higher expression of miR-126-3p and miR-182-5p in hypertensives (both screen-detected and known hypertension) when compared to the normotensives. However, when the expression levels of miR-30a-5p, 30e-3p and 1299 were compared between normotensives and screen-detected hypertension, no significant difference was observed. Although multivariable logistic regressions showed no association between miR-30e-3p and miR-1299, we observed an association between the expression of miR-126-3p, 182-5p and 30a-5p with screen-detected and known hypertension, particularly in the latter. Even after adjustment of the crude model for age, sex, BMI, HbA1c, TG and TC, the associations remained significant.

Angiogenesis and maintenance of vascular integrity are vital blood pressure regulatory processes in which miR-126 has an essential role (Wang *et al*., 2008). During hypertensive states, there is loss of endothelial cell function and blood perfusion to capillaries becomes limited, leading to capillary disappearance. This is described as microvascular rarefaction and is a distinctive characteristic of hypertension (Humar *et al*., 2009). Its effects contribute to hypertension related complications such as organ damage and stroke. It has been reported that miR-126, through the stimulation of proangiogenic activity of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), counteracts microvascular rarefaction by encouraging the formation of blood vessels. This is achieved through the quelling of Spred-1 expression, a known inhibitor of angiogenic signalling (Wang *et al*., 2008). Our study demonstrated a higher expression of miR-126-3p in hypertensive participants when compared to the normotensives, and this finding has also been reported in another study (Liu *et al*., 2018). It is plausible that the high expression of miR-126 in hypertensives may represent a response to lower the blood pressure through promotion of blood vessel formation, whilst repressing the effects of anti-angiogenic Spred-1. Whilst our study demonstrated a higher expression of miR-126-3p in the whole blood of hypertensives compared to the normotensives, another study demonstrated lower expression of miR-126 in hypertensives when compared to healthy controls, albeit in peripheral blood mononuclear cells (PBMCs) (Kontaraki *et al*., 2014). There was no significant difference in miR-126 expression between hypertensive and normotensive participants in the study by Chen and co-workers (Chen *et al*., 2018). These discrepancies could in part be explained by the type of sample used and participants recruitment criteria. It has also been demonstrated that anti-hypertensive medications like nebivolol and atenolol affect

miRNA expression (Ye *et al*., 2013). Kontaraki and colleagues excluded anyone on anti-hypertensive medication from their study, while Chen and colleagues' hypertensive group included any participants who had been on anti-hypertensive therapy for longer than three months (Chen *et al*., 2018).

The efficacy of miR-182 against glioblastoma multiforme, a therapy-resistant cancer of the brain has been previously reported (Kouri *et al*., 2015). It has been established as an oncogenic miRNA and its interactions in several types of cancers have been reviewed (Wei *et al*., 2015). The expression of miR-182-5p was significantly higher in both hypertension groups when compared to the normotensives in our study. However, there is a paucity of studies indicating a role for miR-182-5p in essential hypertension. Nonetheless, in a study evaluating the miRNA expression profiles in the placenta of pregnant participants, the expression of miR-182 was found to be elevated in participants with preeclampsia, a mid-term complication of pregnancy characterised by proteinuria and hypertension, when compared to normal, pregnant controls (Pineles *et al*., 2007). Hypertension is a known risk factor for coronary artery disease (CAD) (Weber *et al*., 2016) and in participants with unprotected left main CAD, the plasma expression of miR-182-5p was significantly higher compared to the non-CAD control group and further analyses indicated its high diagnostic power for uncontrolled left main CAD (Zhu *et al*., 2019). Whether miR-182-5p plays a role in haemodynamic regulation or indeed the pathogenesis of hypertension remains to be elucidated.

In our study, the expression of miR-30a-5p was significantly higher in known hypertensives compared to normotensives. The upregulation of miR-30a in hypertensive participants compared to normal controls was also reported in another study and identified as a possible biomarker target for differentiating white coat hypertension from essential hypertension and normotensives (Huang *et al*., 2016). Delta-like ligand 4 (dll4) is mainly expressed in the vascular endothelium and exerts its effects through Notch signalling by attachment to its receptor Notch1. The ligand is a key inhibitor of angiogenesis and the expression of miR-30a works against the expression of dll4 and in doing so, promotes angiogenesis (Jiang *et al*., 2013). The promotion of angiogenesis through the expression of miR-30a-5p can lead to lowering of the blood pressure and could have been the case in our cohort of known hypertensives.

The large number of participants involved in the project provided sufficient power to the study as each blood pressure group was adequately represented. Whilst the cross-sectional nature of the study precludes inference about causal relationships between miRNA expression and

development of hypertension, it provided a basis for setting up longitudinal cohorts in which functional studies can be conducted to further clarify the roles that these non-coding RNAs play in the pathogenesis of hypertension.

In conclusion, our study demonstrated the differential expression of miRNAs in the whole blood of participants on the basis of their blood pressure status. Furthermore, prior findings on miR-126 and the miR-30 family were further validated and their roles in the pathogenesis of hypertension warrant further investigation as they could offer potential prognostic and therapeutic avenues for cardiovascular diseases.

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6. Conflict of Interest

The authors declare that they have no competing interests.

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8. Authors' contributions

DMM: wrote the first draft, experimental procedures, data analysis and interpretation. **CJW:** experimental procedures, data analysis and interpretation. **RTE:** conception, interpretation of the data, revising it for intellectual content. **APK:** conception, interpretation of the data, revising it for intellectual content. **SFGD:** recruitment and screening of cohort, statistical analysis and interpretation of data. **SR:** interpretation of data, editing and revising it for intellectual content. **GMD:** editing and revising it for intellectual content. **TEM:** conception

and design of the study, analysis and interpretation of the data, revising it for intellectual content. All authors read and approved the final manuscript.

9. References

Bromfield S, Muntner P. High Blood Pressure: The Leading Global Burden of Disease Risk Factor and the Need for Worldwide Prevention Programs. Curr Hypertens Rep. 2013;15(3):134–6.

Campbell NRC, Bovet P, Schutte AE, Lemogoum D, Nkwescheu AS. High Blood Pressure in Sub-Saharan Africa: Why Prevention, Detection, and Control are Urgent and Important. J Clin Hypertens. 2015;17(9):663–7.

Carretero OA, Oparil S. Essential Hypertension. Part I: Definition and Etiology. Circulation. 2000;101(3):329–35

Chalmers J, MacMahon S, Mancia G, Whitworth J, Beilin L, Hansson L, *et al*. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Clin Exp. 1999;21(5–6):1009–60.

Chen S, Chen R, Zhang T, Lin S, Chen Z, Zhao B, *et al*. Relationship of Cardiovascular Disease Risk Factors and Noncoding RNAs with Hypertension: A Case-Control Study. BMC Cardiovasc Disord. 2018;18(1).

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, *et al*. Characterization of microRNAs in Serum: A Novel Class of Biomarkers for Diagnosis of Cancer and Other Diseases. Cell Res. 2008 Oct;18(10):997–1006.

Danaei G, Finucane MM, Lin JK, Singh GM, Paciorek CJ, Cowan MJ, *et al*. National, Regional, and Global Trends in Systolic Blood Pressure Since 1980: Systematic Analysis of Health Examination Surveys and Epidemiological Studies with 786 Country-years and 5.4 Million Participants. Lancet. 2011;377(9765):568–77.

Gkaliagkousi E, Passacquale G, Douma S, Zamboulis C, Ferro A. Platelet Activation in Essential Hypertension: Implications for Antiplatelet Treatment. Am J Hypertens. 2010;23(3):229–36

He FJ, Macgregor GA. Blood Pressure is the Most Important Cause of Death and Disability in the World. Eur Hear J;9(Suppl. B):B23–8.

Huang YQ, Huang C, Chen JY, Li J, Feng YQ. The Association of Circulating miR-30a, miR-29 and miR-133 with White-Coat Hypertension. Biomark Med. 2016;10(12):1231–9.

Humar R, Zimmerli L, Battegay E. Angiogenesis and Hypertension: An Update. J Hum Hypertens. 2009;23(12):773–82.

Jiang Q, Lagos-Quintana M, Liu D, Shi Y, Helker C, Herzog W, *et al*. MiR-30a Regulates Endothelial Tip Cell Formation and Arteriolar Branching. Hypertension. 2013;62(3):592–8.

Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. MicroRNA-9 and microRNA-126 Expression Levels in Patients with Essential Hypertension: Potential Markers of Target-Organ Damage. J Am Soc Hypertens. 2014;8(6):368–75.

Kouri FM, Hurley LA, Daniel WL, Day ES, Hua Y, Hao L, *et al*. MiR-182 Integrates Apoptosis, Growth, and Differentiation Programs in Glioblastoma. Genes Dev. 2015;29(7):732–45.

Li Y, Kowdley K V. MicroRNAs in Common Human Diseases. Genomics Proteomics Bioinformatics. 2012;10(5):246–53.

Liu J, Liu J, Shi L, Zhang F, Yu L, Yang X, *et al*. Preliminary Study of microRNA-126 as a Novel Therapeutic Target for Primary Hypertension. Int J Mol Med. 2018;41(4):1835–44.

Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods. 2001;25(4):402–8.

Matsha TE, Hassan MS, Kidd M, Erasmus RT. The 30-Year Cardiovascular Risk Profile of South Africans with Diagnosed Diabetes, Undiagnosed Diabetes, Pre-diabetes or Normoglycaemia: The Bellville, South Africa Pilot Study. Cardiovasc J Afr. 2012;23(1):5–11.

Matsha TE, Kengne AP, Hector S, Mbu DL, Yako YY, Erasmus RT. MicroRNA Profiling and Their Pathways in South African Individuals with Prediabetes and Newly Diagnosed Type 2 Diabetes Mellitus. Oncotarget. 2018;9(55):30485–98.

Mills KT, Bundy JD, Kelly TN, Reed JE, Kearney PM, Reynolds K, *et al*. Global Disparities of Hypertension Prevalence and Control: A Systematic Analysis of Population-Based Studies from 90 Countries. Circulation. 2016 Aug 9;134(6):441–50.

Mills KT, Stefanescu A, He J. The Global Epidemiology of Hypertension. Nat Rev Nephrol. 2020;16(4):223–37.

Moore J. Hypertension: Catching the Silent Killer. Nurse Pr. 2005;30(10):16–8, 23–4, 26–7, 36–7.

Nemecz M, Alexandru N, Tanko G, Georgescu A. Role of MicroRNA in Endothelial Dysfunction and Hypertension. Curr Hypertens Rep. 2016;18(12):87.

Pineles BL, Romero R, Montenegro D, Tarca AL, Han YM, Kim YM, *et al*. Distinct Subsets of microRNAs are Expressed Differentially in the Human Placentas of Patients with Preeclampsia. Am J Obs Gynecol. 2007;196(3):261.e1-261.e6.

Silambarasan M, Tan JR, Karolina DS, Armugam A, Kaur C, Jeyaseelan K. MicroRNAs in Hyperglycemia Induced Endothelial Cell Dysfunction. Int J Mol Sci. 2016;17(4):518.

Spence JD, Rayner BL. Hypertension in Blacks. Individualized Therapy Based on Renin/Aldosterone Phenotyping. Hypertension. 2018;72(2):263–9.

Taddei S, Virdis A, Ghiadoni L, Sudano I, Salvetti A. Endothelial Dysfunction in Hypertension. J Cardiovasc Pharmacol. 2001;38(Suppl 2):S11–4.

Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, *et al*. Vascular Smooth Muscle Contraction in Hypertension. Cardiovasc Res. 2018;114(4):529–39.

Vidigal JA, Ventura A. The Biological Functions of miRNAs: Lessons from in vivo Studies. Trends Cell Biol. 2015;25(3):137–47.

Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, *et al*. The Endothelial-Specific MicroRNA miR-126 Governs Vascular Integrity and Angiogenesis. Dev Cell. 2008;15(2):261–71.

Weber T, Lang I, Zweiker R, Horn S, Wenzel RR, Watschinger B, *et al*. Hypertension and Coronary Artery Disease: Epidemiology, Physiology, Effects of Treatment, and Recommendations: A Joint Scientific Statement from the Austrian Society of Cardiology and the Austrian Society of Hypertension. Wien Klin Wochenschr. 2016;128(13–14):467–79.

Wei Q, Lei R, Hu G. Roles of miR-182 in Sensory Organ Development and Cancer. Thorac Cancer. 2015;6(1):2–9.

Ye H, Ling S, Castillo AC, Thomas B, Long B, Qian J, *et al*. Nebivolol Induces Distinct Changes in Profibrosis MicroRNA Expression Compared with Atenolol, in Salt-sensitive Hypertensive Rats. Hypertens. 2013 61(5):1008–13.

Zhu L, Chen T, Ye W, Wang J-Y, Zhou J-P, Li Z-Y, *et al*. Circulating miR-182-5p and miR-5187-5p as Biomarkers for the Diagnosis of Unprotected Left Main Coronary Artery Disease. J Thorac Dis. 2019;11(5):1799–808.

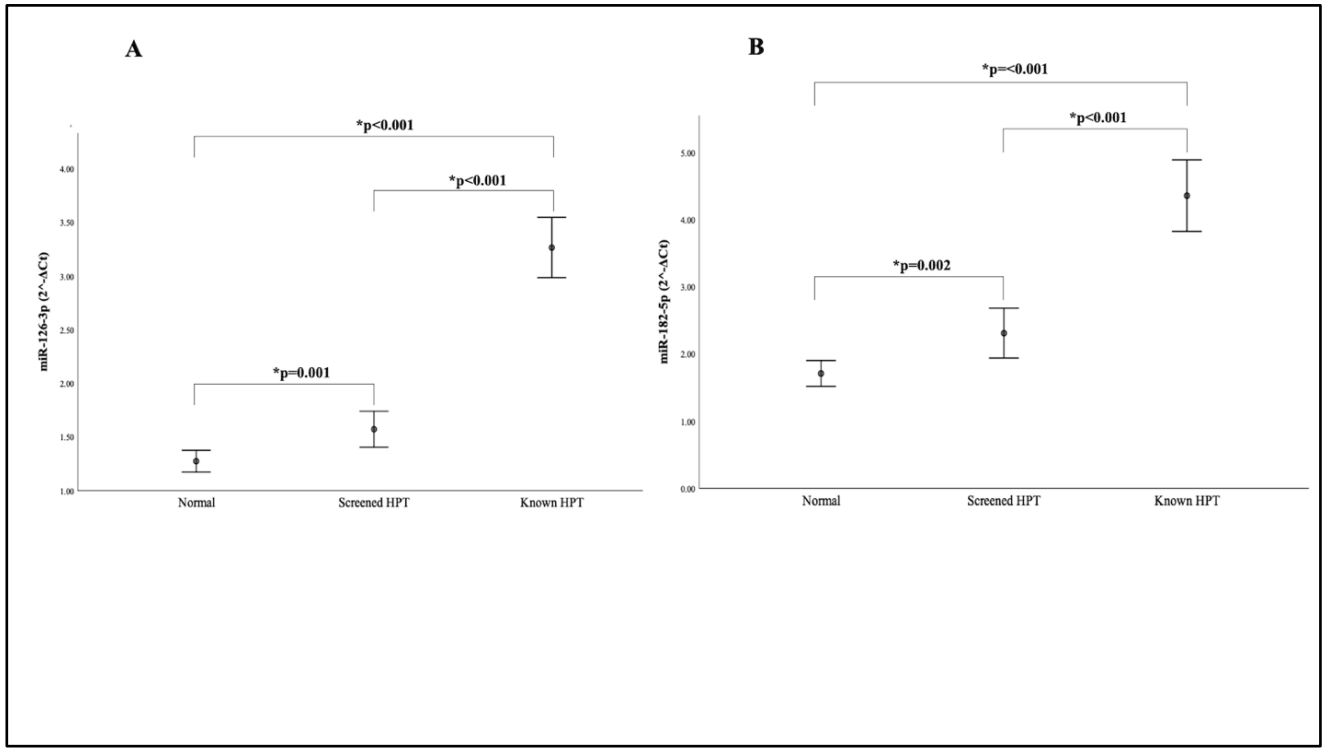

Figure 1. Comparison of the relative expression of miR-126-3p and 182-5p in the three blood pressure groups.

A. The expression of miR-126-3p was significantly higher in known hypertension when compared to screen-detected hypertension (p<0.001) and the normotensives (p<0.001). There was also a significant difference in expression when screen-detected hypertensives were compared to the normotensives (p=0.001). **B.** There was a significantly higher expression of miR-182-5p in known hypertension when compared to screen-detected hypertension (p<0.001) and the normotensives (p<0.001). When screen-detected hypertensives were compared to the normotensives, that significant difference in expression remained (p=0.002). The relative miRNA expressions were calculated using the $2^{-\Delta Ct}$ method and a one-way ANOVA used to compare differences in miRNA expression between groups.

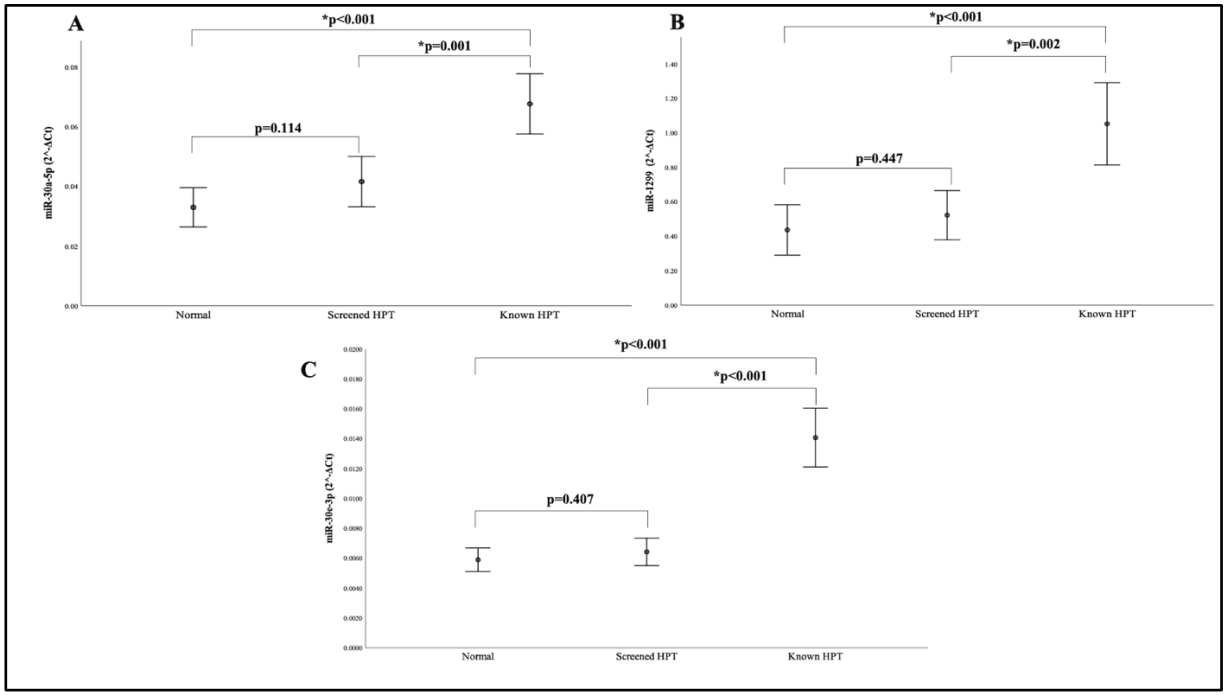

Figure 2. Comparison of the relative expression of miR-30a-5p, miR-1299 and miR-30e-3p.

A. The expression of the miR-30a-5p was significantly higher in known hypertension compared to screen-detected hypertension (p=0.001) and the normotensives (p<0.001). However, there was no significant difference in expression between screen-detected hypertensives and normotensives (p=0.114). **B.** There was a significantly higher expression of miR-1299 in known hypertension when compared to screen-detected hypertension (p=0.002) and the normotensives (p<0.001). There was no significant difference in expression when screen-detected hypertensives were compared to the normotensives (p=0.447). **C.** The expression of miR-30e-3p was significantly higher in known hypertension when compared to screen-detected hypertension (p<0.001) and the normotensives (p<0.001). There was no significant difference in expression when screen-detected hypertensives were compared to the normotensives (p=0.407). The relative miRNA expressions were calculated using the $2^{-\Delta Ct}$ method and a one-way ANOVA used to compare differences in miRNA expression between groups.

Table 1. Study participant characteristics based on blood pressure status

	Normotensive (n=573) mean±SD	Screen-detected HPT (n=304) mean±SD	Known HPT (n=579) mean±SD	All *p*-value	Normotensive vs Screen-detected HPT *p*-value	Normotensive vs Known HPT *p*-value	Screen-detected HPT vs Known HPT *p*-value
Gender							
Female, n(%)	386 (67.4)	209 (68.8)	478 (82.6)	<0.001	0.676	<0.001	<0.001
Male, n(%)	187 (32.6)	95 (31.2)	101 (17.4)				
Age (years)	39.3 ± 13.7	48.9 ± 13.4	58.9 ± 11.0	<0.001	<0.001	<0.001	<0.001
Body mass index (kg/m^2)	25.9 ± 7.0	28.4 ± 8.5	31.4 ± 7.7	0.426	<0.001	<0.001	<0.001
Waist circumference (cm)	84.5 ± 15.3	91.2 ± 16.3	98.5 ± 16.4	0.684	<0.001	<0.001	<0.001
Hip circumference (cm)	98.2 ± 15.0	102.1 ± 16.4	108.5 ± 16.2	0.807	<0.001	<0.001	<0.001
Waist to Hip ratio	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.728	<0.001	<0.001	0.006
Systolic blood pressure (mmHg)	115.5 ± 12.7	151.7 ± 19.5	148.2 ± 26.4	0.025	<0.001	<0.001	0.041
Diastolic blood pressure (mmHg)	74.7 ± 9.1	95.8 ± 11.6	90.7 ± 15.4	0.875	<0.001	<0.001	<0.001
Fasting Blood glucose(mmol/L)*	4.6 (4.3; 5.0)	4.9 (4.6; 5.5)	5.3 (4.9; 6.8)	<0.001	<0.001	<0.001	<0.001
2-hour glucose (mmol/L)*	5.3 (4.3; 6.6)	6.1 (5.0; 7.7)	6.9 (5.5; 8.6)	<0.001	<0.001	<0.001	<0.001
HbA1c (%)	5.7 ± 1.0	5.9 ± 1.3	6.8 ± 1.9	0.032	0.006	<0.001	<0.001
HbA1c (mmol/mol)	38.6 ± 11.0	40.9 ± 13.8	50.7 ± 21.1	0.032	0.006	<0.001	<0.001
Fasting Insulin (mIU/L)*	5.6 (3.4; 8.8)	6.1 (3.9; 9.6)	8.0 (5.0; 13.1)	<0.001	0.065	<0.001	<0.001
2-hour Insulin (mIU/L)*	28.8 (15.0; 53.4)	34.7 (19.1; 64.0)	48.7 (26.5; 88.2)	<0.001	0.014	<0.001	<0.001
Triglycerides (mmol/L)*	1.0 (0.7; 1.4)	1.2 (0.9; 1.7)	1.4 (1.0; 1.9)	<0.001	<0.001	<0.001	<0.001
Total Cholesterol (mmol/L)	4.8 ± 1.1	5.2 ± 1.1	5.4 ± 1.2	0.296	<0.001	<0.001	<0.001
HDL-cholesterol (mmol/L)	1.3 ± 0.4	1.4 ± 0.5	1.3 ± 0.3	<0.001	<0.001	0.359	<0.001
LDL-cholesterol (mmol/L)	3.0 ± 1.0	3.2 ± 1.0	3.3 ± 1.0	0.230	0.025	<0.001	0.021
C-Reactive Protein (mg/L)*	2.7 (1.1; 6.9)	3.7 (1.5; 9.3)	5.2 (2.5; 10.1)	<0.001	0.012	<0.001	<0.001
Gamma GT (IU/L)*	25 (18; 39)	30 (21; 50)	32 (22; 53)	<0.001	<0.001	<0.001	<0.001
Serum Creatinine (µmol/L)	59.5 ± 12.0	62.7 ± 28.9	71.8 ± 61.0	0.558	0.021	<0.001	0.014

Values presented as mean±SD unless marked with an asterisk*, in which case the median and (25^{th}-75^{th} percentiles) are reported. The Kruskal-Wallis test and analysis of variance (ANOVA) were used to compare the median and mean baseline characteristics respectively across blood pressure groups. HPT = hypertension, SD = standard deviation.

Table 2. Partial correlations between miRNA relative expression and anthropometric and biochemical parameters

	miR-30a-5p $2^{-\Delta Ct}$		**miR-126-3p $2^{-\Delta Ct}$**		**miR-182-5p $2^{-\Delta Ct}$**		**miR-30e-3p $2^{-\Delta Ct}$**		**miR-1299 $2^{-\Delta Ct}$**	
	r	*p*-value	r	*p*-value	r	*p*-value	r	*p*-value	r	*p*-value
miR-30a-5p $2^{-\Delta Ct}$	1.000		0.901	<0.001	0.937	<0.001	0.901	<0.001	0.710	<0.001
miR-1299 $2^{-\Delta Ct}$	0.710	<0.001	0.731	<0.001	0.738	<0.001	0.721	<0.001	1.000	
miR-182-5p $2^{-\Delta Ct}$	0.937	<0.001	0.983	<0.001	1.000		0.968	<0.001	0.738	<0.001
miR-30e-3p $2^{-\Delta Ct}$	0.901	<0.001	0.973	<0.001	0.968	<0.001	1.000		0.721	<0.001
miR-126-3p $2^{-\Delta Ct}$	0.901	<0.001	1.000		0.983	<0.001	0.973	<0.001	0.731	<0.001
Waist circumference (cm)	-0.485	0.019	-0.535	0.009	-0.518	0.011	-0.538	0.008	-0.521	0.011
Hip circumference (cm)	-0.154	0.482	-0.053	0.809	-0.100	0.651	-0.084	0.703	0.048	0.828
Waist to Hip ratio	0.001	0.997	-0.113	0.608	-0.060	0.787	-0.083	0.705	-0.131	0.553
Systolic blood pressure (mmHg)	0.313	0.146	0.293	0.174	0.304	0.158	0.275	0.204	0.268	0.216
Diastolic blood pressure (mmHg)	0.224	0.304	0.217	0.319	0.228	0.295	0.202	0.354	0.233	0.284
Fasting Blood glucose (mmol/L)	0.133	0.546	0.077	0.728	0.109	0.621	0.119	0.590	0.040	0.856
2-hour glucose (mmol/L)	0.046	0.834	0.019	0.932	0.044	0.841	0.058	0.792	0.102	0.644
HbA1c (%)	-0.201	0.357	-0.170	0.439	-0.190	0.385	-0.153	0.485	-0.130	0.555
Fasting insulin (mIU/L)	0.272	0.209	0.241	0.267	0.267	0.218	0.250	0.250	0.216	0.322
2-hour insulin (mIU/L)	0.049	0.826	0.024	0.913	0.051	0.815	0.045	0.838	0.078	0.723
Triglycerides-S (mmol/L)	0.037	0.868	0.018	0.935	0.008	0.971	-0.024	0.915	-0.079	0.719
Total cholesterol (mmol/L)	0.108	0.624	0.019	0.930	0.039	0.858	0.015	0.945	0.041	0.854
HDL-cholesterol (mmol/L)	0.445	0.033	0.401	0.058	0.431	0.040	0.443	0.034	0.421	0.045
LDL-cholesterol (mmol/L)	0.065	0.769	-0.017	0.939	-0.003	0.991	-0.025	0.908	0.021	0.925
C-Reactive Protein (mg/L)	0.145	0.509	0.139	0.528	0.144	0.511	0.173	0.429	0.152	0.487
Gamma GT (IU/L)	0.094	0.669	0.047	0.830	0.078	0.722	0.063	0.775	0.043	0.845
Serum creatinine (µmol/L)	-0.349	0.443	-0.390	0.387	-0.377	0.404	-0.385	0.394	-0.337	0.460

Age, gender and BMI adjusted Spearman's partial correlations were used to assess the association between miRNA relative expression and anthropometric and biochemical parameters across the three blood pressure groups.

Table 3. Multivariable regression analysis of miRNAs for the presence of screen-detected and known hypertension

	Screen-detected HPT			**Known HPT**		
	OR	**95% CI**	***p-value***	**OR**	**95% CI**	***p-value***
miR 30a-5p*						
Model 1	1.24	(1.00;1.55)	0.053	1.63	(1.36;1.95)	<0.001
Model 2	1.35	(1.07;1.72)	0.013	1.98	(1.58;2.48)	<0.001
Model 3	1.35	(1.06;1.71)	0.015	1.97	(1.57;2.48)	<0.001
Model 4	1.37	(1.07;1.74)	0.011	1.99	(1.57;2.51)	<0.001
Model 5	1.35	(1.06;1.72)	0.014	1.97	(1.56;2.48)	<0.001
Model 6	-	-	-	1.81	(1.4;2.34)	<0.001
*miR 30e-3p****						
Model 1	1.07	(0.91;1.26)	0.393	1.58	(1.4;1.78)	<0.001
Model 2	1.14	(0.96;1.36)	0.130	1.71	(1.46;1.99)	<0.001
Model 3	1.15	(0.96;1.37)	0.121	1.71	(1.46;2.01)	<0.001
Model 4	1.16	(0.97;1.39)	0.094	1.74	(1.48;2.05)	<0.001
Model 5	1.17	(0.98;1.39)	0.089	1.74	(1.48;2.05)	<0.001
Model 6	-	-	-	1.81	(1.42;2.30)	<0.001
*miR 126-3p**						
Model 1	1.16	(1.05;1.27)	0.003	1.58	(1.46;1.71)	<0.001
Model 2	1.23	(1.11;1.37)	<0.001	1.72	(1.55;1.9)	<0.001
Model 3	1.21	(1.09;1.35)	<0.001	1.69	(1.52;1.87)	<0.001
Model 4	1.23	(1.11;1.38)	<0.001	1.71	(1.54;1.91)	<0.001
Model 5	1.23	(1.1;1.37)	<0.001	1.71	(1.53;1.91)	<0.001
Model 6	-	-	-	1.93	(1.65;2.26)	<0.001
*miR 1299***						
Model 1	1.07	(0.94;1.21)	0.329	1.22	(1.11;1.35)	<0.001
Model 2	1.59	(0.42;5.96)	0.495	1.18	(1.05;1.33)	0.005
Model 3	1.05	(0.92;1.2)	0.466	1.19	(1.05;1.34)	0.006
Model 4	1.08	(0.94;1.24)	0.308	1.21	(1.06;1.37)	0.005
Model 5	1.09	(0.94;1.25)	0.254	1.22	(1.07;1.39)	0.004
Model 6	-	-	-	1.23	(1.05;1.44)	0.009
*miR 182-5p**						
Model 1	1.11	(1.05;1.17)	<0.001	1.24	(1.18;1.3)	<0.001
Model 2	1.14	(1.07;1.21)	<0.001	1.31	(1.24;1.39)	<0.001
Model 3	1.14	(1.07;1.21)	<0.001	1.31	(1.23;1.39)	<0.001
Model 4	1.14	(1.08;1.22)	<0.001	1.31	(1.23;1.4)	<0.001
Model 5	1.14	(1.07;1.22)	<0.001	1.31	(1.23;1.39)	<0.001
Model 6	-	-	-	1.31	(1.21;1.41)	<0.001

Model 1: Crude; Model 2: included age and sex; Model 3: included age, sex and BMI; Model 4: included age, sex, BMI, HbA1c; Model 5: included age, sex, BMI, HbA1c,triglycerides, total cholesterol; Model 6: included age, sex, BMI, HbA1c, triglycerides, total cholesterol; duration of disease *calculated for 0.1 unit increase; ** calculated for 0.01 unit increase; ***calculated for 0.001 unit increase.

CHAPTER 5: MANUSCRIPT 3

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Two novel microRNAs and their association with absolute blood pressure parameters in an urban South African community

Running title: Novel microRNAs in hypertension

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Abstract

Introduction: MicroRNAs play an important role in gene expression regulation and thus have a significant role in disease development and progression. The description of novel microRNAs in various populations around the world continues to add to the pool of existing microRNAs that can be targeted for diagnostic and therapeutic purposes in disease.

Objective: This study aimed to describe novel microRNAs in a normotensive and hypertensive African population and relate their expression to blood pressure parameters and hypertension status.

Methods: Next-generation sequencing was conducted on blood from 48 normotensive and hypertensive participants and findings confirmed by quantitative reverse transcription polymerase chain reaction in a cohort of 881 participants. The relationship between the novel microRNAs and systolic and diastolic blood pressure as well as mean arterial pressure was also investigated in the same cohort.

Results: Two novel microRNAs (miR-novel-chr1_36178 and miR-novel-chr15_18383) were significantly dysregulated by hypertension status. The expression of miR-novel-chr1_36178 differed according to sex, correlated with mean arterial pressure and systolic and diastolic blood pressure at higher levels of expression and was associated with screen-detected hypertension.

Discussion:

The association of miR-novel-chr1_36178 expression with mean arterial pressure and systolic and diastolic blood pressure, as well as its dysregulation according to hypertension status suggests its possible utility as a biomarker target for hypertension diagnosis and/or therapeutics. Furthermore, its association with screen detected hypertension and dose-response relationship with blood pressure suggests the microRNA may be used to identify and monitor individuals at risk of hypertension. Further characterisation of the novel miRNA's interactions with various genes and cellular processes are important in understanding its role in the development and progression of hypertension.

Keywords: hypertension, novel microRNA, blood pressure, Africa, RT-qPCR

1. Introduction

Ever since the discovery of the first non-coding ribonucleic acid (RNA) close to 30 years ago (Lee *et al*., 1993), more than 2000 mature human microRNAs (miRNAs) have since been described (Alles *et al*., 2019). Using small RNA sequencing techniques, epigenome studies continue to describe novel miRNAs in various body tissues and disease conditions (Wake *et al*., 2016; Matsha *et al*., 2018). These miRNAs are 18-22 base pairs long (Yildirim *et al*., 2019) and are involved in the regulation of gene expression at the post transcription level, either through targeted messenger RNA (mRNA) degradation or inhibition of translation (Krol *et al*., 2010; Wake *et al*., 2016). As such, they have a crucial role in maintaining the homeostatic balance of the human body's processes and dysregulations in their expression may be related to disease processes (Li & Kowdley, 2012; Shin & Chu, 2014).

An individual's phenotype is strongly influenced by the complex interplay between their genetic makeup and the environment they inhabit (Baye *et al*., 2011). Identifying variations in the genetic (particularly the epigenome) makeup of different populations may help us understand and explain why different populations respond to the same disease and/or medications in a different manner. As the impact of miRNAs in the world of science and disease continues to grow steadily, their study in various diseases, including hypertension (HPT), has gained considerable impetus in high income Asian (Li *et al*., 2011; Huang *et al*., 2016; Liu *et al*., 2018) and European (Kontaraki *et al*., 2014) countries with potentially interesting therapeutic implications (Baumann & Winkler, 2014). However, there is a paucity of studies focusing on miRNAs and HPT in the African population, despite its well-described genetic diversity (Campbell & Tishkoff, 2008) and steadily increasing HPT prevalence (Mills *et al*., 2016).

Herein, we present two previously undescribed miRNAs exhibiting distinct expression patterns in a hypertensive African population when compared to normotensives from the same community. We further interrogated the relationship between the expression of these novel miRNAs and blood pressure parameters (systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP)). Establishment of specific epigenomic signatures with clinical parameters of disease may offer alternative diagnostic and prognostic avenues, in addition to the potential development of effective, population-targeted therapeutic measures, instead of blanket approaches for genetically diverse populations.

2. Materials and methods

2.1 Ethical approval

Ethical approval for this study was obtained from the CPUT Health and Wellness Sciences Research Ethics Committee (CPUT/HW-REC 2019/H7). It is a sub-study of the larger and ongoing Cape Town Vascular and Metabolic Health (VMH) study, which was approved by the Research Ethics Committees of the Cape Peninsula University of Technology (CPUT) and Stellenbosch University (respectively, NHREC: REC - 230 408 – 014 and N14/01/003). The study was conducted according to the recommendations and guidelines of the Declaration of Helsinki. All procedures were explained to the participants in their language of choice and once they fully understood their participation, they signed informed consent forms to allow the collection of blood and anthropometric data.

2.2 Study design and procedures

This was a cross-sectional study composed of male and female participants from the VMH study, an extension of the Cape Town Bellville South study, previously described (Matsha *et al*., 2012). Data collection and procedures have been reported previously (Matsha *et al*., 2018). Briefly, each participant underwent anthropometric measurements and these were reported as the average of three separate readings. Body Mass Index (BMI) was calculated as weight per height squared (kg/m^2) where kg is a participant's weight in kilograms and m^2 is the square of their height in metres. Blood pressure (BP) was measured according to the World Health Organisation (WHO) guidelines (Chalmers *et al*., 1999), using a semi-automatic digital BP monitor (Omron M6 comfort-preformed cuff blood pressure monitor, China) on the right arm in a sitting position and at rest for at least 10 minutes. Three BP readings were taken at three-minute intervals and the lowest SBP and corresponding DBP values were used. Participants were grouped into two categories based on BP measurement of 140/90 mm Hg as screen-detected HPT and normal BP measurement as normotensive.

Various biochemical parameters were measured in an ISO 15189 accredited pathology practice (PathCare Reference Laboratory, Cape Town, South Africa) using different analytical methods as follows: glycated haemoglobin (HbA1c) by High Performance Liquid Chromatography (BioRad Variant Turbo, BioRad, Hercules, CA, USA); serum insulin by a paramagnetic particle chemiluminescence assay (Beckman DXI, Beckman Coulter, South Africa); serum cotinine by Competitive Chemiluminescent **(**Immulite 2000, Siemens, Munich, Germany);

plasma glucose by enzymatic hexokinase method (Beckman AU, Beckman Coulter, Brea, CA, USA); total cholesterol (TC); high density lipoprotein cholesterol (HDL-c) by enzymatic immunoinhibition – end point (Beckman AU, Beckman Coulter, Brea, CA, USA); triglycerides (TG) by glycerol phosphate oxidase-peroxidase, end point (Beckman AU, Beckman Coulter, Brea, CA, USA); low density lipoprotein cholesterol (LDL) by enzymatic selective protection – end point (Beckman AU, Beckman Coulter, Brea, CA, USA); and ultrasensitive C-reactive protein (CRP) by Latex Particle Immunoturbidimetry (Beckman AU, Beckman Coulter, Brea, CA, USA). These analyses were conducted at an ISO 15189 accredited pathology practice (PathCare Reference Laboratory, Cape Town, South Africa). Blood samples for miRNA expression analysis were collected in Tempus Blood RNA tubes (ThermoFisher Scientific, Waltham, MA, USA) and stored at -80°C for total RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis.

2.3 RNA isolation

Total RNA, including miRNA, was isolated from 3mL of whole blood using the MagMax Total RNA isolation kit (ThermoFisher Scientific) according to manufacturer's instructions, with the nucleic acid washing and elution steps conducted on the Kingfisher Flex system. The concentration and purity of each total RNA extract was determined using a NanoDrop One spectrophotometer and total RNA extracts with 260/280 values between 1.8 and 2.0, and concentrations greater than 20ng/µl were used for RT-qPCR.

2.4 MicroRNA sequencing using next generation sequencing

This was conducted on samples from a cohort of 48 age-matched, normotensive, screen-detected hypertensive and known hypertensive female participants. Small RNA library construction, deep sequencing, and data processing were performed at Arraystar Inc., Rockville, USA as previously described (Matsha *et al*., 2018). Briefly, the total RNA of each sample was used to prepare the miRNA sequencing library as follows:1) 3'-adapter ligation with T4 RNA ligase 2 (truncated); 2) 5'-adapter ligation with T4 RNA ligase; 3) cDNA synthesis with RT primer; 4) PCR amplification; 5) extraction and purification of ~130-150 bp PCR amplified fragments (correspond to ~15-35 nt small RNAs) from the PAGE gel. Completed libraries were quantified using the Agilent 2100 Bioanalyzer, followed by denaturation of DNA fragments using 0.1M sodium hydroxide to generate single-stranded DNA molecules, which were then captured on Illumina flow cells, amplified *in situ*, and sequenced on the Illumina HiSeq system for 51 cycles according to the manufacturer's

instructions. Raw sequences were generated as clean reads from the Illumina HiSeq using real-time base calling and quality filtering. Adaptor sequences were removed from clean reads that passed the quality filter to produce trimmed reads of length ≥ 15 nucleotides. Using NovoAlign software, the trimmed reads were aligned to the human pre-miRNA in miRBase 21. The miRNA expression levels were measured and normalized as transcripts per million (TPM) of total aligned miRNA reads. MicroRNAs with fold changes ≥1.3, and *p*-values ≤ 0.1 were selected as the differentially expressed miRNAs. Novel miRNAs were predicted using miRDeep.

2.5 Quantitative reverse transcription PCR

RT-qPCR was performed to determine the novel miRNA expression levels in an independent sample of 881 normotensive, screen-detected hypertensive and known hypertensive male and female participants, as determined by next generation sequencing. Briefly, using the TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA), 2µl of total RNA was converted into cDNA through sequential conduction of poly-A tailing, adaptor ligation, reverse transcription and miR-Amp steps as per the manufacturer's recommendation. The miRNA expression levels were then determined using TaqMan miRNA Assay primers (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) on the QuantStudio 7 Flex real-time PCR instrument (Life Technologies, Carlsbad, CA, USA). In order to determine relative miRNA expression in each sample, the $2^{-\Delta Ct}$ method was used whilst the $2^{-\Delta\Delta Ct}$ method was used to compute fold change differences in miRNA expression between the study groups using miR-16-5p (ThermoFisher Scientific, Waltham, MA, USA) as the endogenous control (Livak & Schmittgen, 2001).

2.6 Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) v.26 software (IBM Corp, USA). Normally distributed variables are reported as count (and percentages), mean (and standard deviation) whilst asymmetrically distributed variables are reported as median (25^{th}-75^{th} percentiles). Comparisons of median and mean baseline characteristics across BP groups were done using the Kruskal-Wallis test and analysis of variance (ANOVA) respectively. Spearman's correlations, adjusted for age and sex, were used to assess the relationship between miRNAs and other cardiovascular risk profile variables whilst multivariable logistic regression models were used to assess the association of miRNAs

with screen-detected HPT and known HPT with crude and adjusted odds ratio (OR). A *p*-value less than 0.05 signified statistically significant findings.

3. Results

3.1 Cohort description

The study was made up of 598 (67.9%) female and 283 (32.1%) male participants with a mean age of 42.6 years. BP parameters (SBP, DBP and MAP) did not show any significant difference by sex ($p \geq 0.085$). The expression of miR-novel-chr1_36178 significantly differed according to sex ($p=0.002$), with greater expression in males compared to females. Other significant variations based on sex are shown in Table 1. Briefly, anthropometric, glycaemic and lipid indices were significantly raised in women, $p \leq 0.043$ whilst GGT was reduced, $p=0.002$.

3.2 Whole genome miRNA sequencing

Two novel miRNAs (miR-novel-chr15_18383 and miR-novel-chr1_36178) were discovered using next generation sequencing techniques and their characteristics are described in Table 2. miR-novel-chr15_18383 is transcribed from a gene located on chromosome 15, whilst miR-novel-chr1_36178 is from a gene located on chromosome 1. Their expression differed significantly according to HPT status and whilst the expression of miR-novel-chr1_36178 was significantly higher in known hypertensives on therapy compared to the normotensives (fold difference = 1.5), the expression of miR-novel-chr15_18383 was significantly higher in known hypertensives on therapy compared to screen-detected hypertensives (fold difference = 1.3).

3.3 Linear correlations

As shown in Table 3, there was a weak, positive correlation between the expression of miR-novel-chr15_18383 and miR-novel-chr1_36178 ($r=0.48$, $p<0.001$). There was no correlation between the expression of miR-novel-chr15_18383 and BP parameters. Interestingly, although very weak, there was a positive correlation between miR-novel-chr1_36178 expression and both DBP and MAP ($r \leq 0.08$, $p \leq 0.024$).

3.4 Linear regression models

Table 4 shows models used to predict absolute BP values based on the expression levels of the two novel miRNAs. For models 1 and 2, the variables were measured at the continuous level. MiRNA expression data values were log-transformed before analysis to correct for skewness. Whilst expression levels of miR-novel-chr15_18383 could not significantly predict the SBP, DBP and MAP as shown in Model 2, there was statistical significance in the expression of miR-novel-chr1_36178 and prediction of SBP, DBP and MAP. For every 1-unit increase in the

expression of miR-novel-chr1_36178, there is a 0.03 increase in the SBP (p=0.003), a 0,01 increase in the DBP (p= 0.035) and 0.02 increase in the MAP (p=0.008).

A dose response assessment was done to determine if the effect of the novel miRNAs on BP variables was dependant on the expression levels of the miRNAs. The miRNA expression data was divided into five quantiles (Q1-5), with Q1 being used as the reference. Although a significant relationship between the expression of miR-novel-chr1_36178 and BP variables was shown in Model 1, the significance of that relationship remained only when the miRNA was expressed at levels falling in quantile 5 (Q5) (p≤0.004). Findings in Model 4 concurred with Model 2 findings, indicating that even when expressed at high levels, miR-novel-chr15_18383 had no significant relationship with SBP, DBP and MAP.

3.5 Regression analysis

Age and sex adjusted odds ratios (OR) were calculated to quantify the strength of the relationship between miRNA expression and screen-detected HPT, using the normotensives as a reference. As shown in Table 5, the expression of miR-novel-chr1_36178 and miR-novel-chr15_18383 was significantly associated with screen-detected HPT, OR = 1.36 (95% CI: 1.08-1.70), p=0.008 and OR = 1.31 (95% CI: 1.05-1.63), p=0.016. As for the dose response analysis, a significant association between the expression of miR-novel-chr1_36178 and the presence of screen-detected HPT was seen only when the miRNA was expressed at the highest level (Q5), OR = 2.13 (95% CI: 1.32-3.45), p=0.002. In contrast, there was no dose dependant association between the expression of miR-novel-chr15_18383 and the presence of screen-detected HPT, even at Q5 levels.

Table 1. Characteristics of participants that took part in the study.

	All	**Female**	**Male**	*p*-value
	n=881 mean ± SD	n=598 mean ± SD	n=283 mean ± SD	
miR-novel-chr1_36178 ($2^{-\Delta Ct}$)*	0.659 (0.2471;1.5976)	0.569 (0.2205;1.4008)	0.858 (0.2986;1.9741)	0.002
miR-novel-chr15_18383 ($2^{-\Delta Ct}$)*	0.001 (0.0004;0.0037)	0.001 (0.0004;0.0037)	0.001 (0.0005;0.0038)	0.848
Age (years)	42.6 ± 14.3	42.9 ± 14.4	42.0 ± 14.2	0.415
Body mass index	26.8 ± 7.6	28.6 ± 8.0	23.0 ± 5.3	<0.001
Waist circumference (cm)	86.8 ± 16.0	89.3 ± 16.3	81.5 ± 13.8	<0.001
Hip circumference (cm)	99.6 ± 15.6	103.3 ± 15.8	91.6 ± 11.6	<0.001
Waist to Hip ratio*	0.87 (0.82;0.92)	0.86 (0.81;0.92)	0.88 (0.84;0.94)	<0.001
Systolic Blood Pressure (mmHg)	128.1 ± 23.1	127.8 ± 23.2	128.6 ± 22.8	0.663
Diastolic Blood Pressure (mmHg)	82.1 ± 14.2	82.6 ± 13.5	80.9 ± 15.3	0.085
Mean Arterial Pressure	97.4 ± 16.1	97.7 ± 15.7	96.8 ± 17.0	0.422
Fasting blood glucose (mmol/L)*	4.7 (4.4;5.2)	4.7 (4.5;5.2)	4.7 (4.3;5.2)	0.043
2-hour glucose (mmol/L)*	5.5 (4.5;6.9)	5.9 (4.9;7.2)	4.7 (3.9;6)	<0.001
HbA1c (%)*	5.6 (5.3;5.9)	5.6 (5.3 5.9)	5.5 (5.3;5.8)	0.046
HbA1c (mmol/mol)*	37.7 (34.4;41.0)	37.7 (34.4;41.0)	36.6 (34.4;39.9)	0.046
Fasting insulin (mIU/L)*	5.8 (3.6;9.1)	6.6 (4.2;9.6)	4.3 (2.7;7.3)	<0.001
2-hour insulin (mIU/L)*	30.8 (15.7;56.8)	38.4 (22.0;70.9)	18.4 (8.4;36.0)	<0.001
Triglycerides (mmol/L)*	1.07 (0.78;1.52)	1.05 (0.76;1.48)	1.11 (0.81;1.63)	0.093
Total cholesterol (mmol/L)	4.97 ± 1.14	5.1 ± 1.15	4.69 ± 1.07	<0.001
HDL-cholesterol (mmol/L)	1.35 ± 0.41	1.38 ± 0.41	1.29 ± 0.39	0.002
LDL-cholesterol (mmol/L)	3.0 ± 0.1	3.2 ± 0.1	2.8 ± 0.9	<0.001
C-Reactive Protein (mg/L)*	3.0 (1.2;7.8)	3.5 (1.4;8.5)	2.3 (1.0;6.0)	0.008
Gamma GT-S* (IU/L)*	27 (19;42)	26 (18;40)	29 (21;47)	0.002

Values presented as mean±SD unless marked with an asterisk*, in which case the median and (25^{th}-75^{th} percentiles) are reported. The Kruskal-Wallis test and analysis of variance (ANOVA) were used to compare the median and mean baseline characteristics respectively between sex. SD = standard deviation.

Table 2. Description of significantly differentially expressed novel microRNAs discovered by next generation sequencing

Mature ID	Pre-miRNA accession ID	Mature seed sequence	Mature length	Mature sequence
hsa-miR-novel-chr1_36178	MYNO2414	UCCAGC	17	CUCCAGCCUGGGCAACA
hsa-miR-novel-chr15_18383	MYNO1379	GCUCCC	22	UGCUCCCCCUCCCUUCCUGGGA

Table 3. Spearman correlations for blood pressure parameters and novel miRNA expression

	miR-novel-chr1_36178 ($2^{-\Delta Ct}$)		miR-novel-chr15_18383 ($2^{-\Delta Ct}$)	
	r	***p*-value**	**r**	***p*-value**
miR-novel-chr1_36178 ($2^{-\Delta Ct}$)	1.000		0.477	<0.001
miR-novel-chr15_18383 ($2^{-\Delta Ct}$)	0.477	<0.001	1.000	
Systolic blood pressure (mmHg)	0.064	0.060	0.026	0.453
Diastolic blood pressure (mmHg)	0.082	0.016	0.048	0.162
Mean arterial pressure (mmHg)	0.076	0.024	0.045	0.195

Table 4. Age and sex adjusted linear regression models for determinants of absolute BP levels and Mean Arterial Pressure#

	SBP		DBP		MAP	
	B (95% CI)	***p*-value**	**B (95% CI)**	***p*-value**	**B (95% CI)**	***p*-value**
Model 1						
miR-novel-chr1_36178	0.03 (0.01; 0.05)	0.003	0.01 (0; 0.03)	0.035	0.02 (0; 0.03)	0.008
Model 2						
miR-novel-chr15_18383	0.02 (0; 0.04)	0.123	0.01 (0; 0.02)	0.073	0.01 (0; 0.03)	0.071
Model 3						
miR-novel-chr1_36178 Q2	1.91 (-2.39; 6.22)	0.382	-0.37 (-3.21; 2.47)	0.800	0.39 (-2.71; 3.5)	0.803
miR-novel-chr1_36178 Q3	3.09 (-1.2; 7.39)	0.158	0.29 (-2.54; 3.13)	0.839	1.23 (-1.88; 4.33)	0.438
miR-novel-chr1_36178 Q4	2.24 (-2.08; 6.55)	0.309	0.33 (-2.52; 3.17)	0.821	0.96 (-2.15; 4.08)	0.544
miR-novel-chr1_36178 Q5	6.99 (2.66; 11.32)	0.002	4.2 (1.35; 7.06)	0.004	5.13 (2.01; 8.26)	0.001
Model 4						
miR-novel-chr15_18383 Q2	-3.91 (-8.39; 0.56)	0.086	-0.45 (-3.39; 2.49)	0.766	-1.6 (-4.83; 1.62)	0.330
miR-novel-chr15_18383 Q3	0.97 (-3.53; 5.46)	0.673	0.89 (-2.06; 3.84)	0.554	0.92 (-2.32; 4.16)	0.579
miR-novel-chr15_18383 Q4	0.15 (-4.32; 4.62)	0.947	1.84 (-1.1; 4.78)	0.219	1.28 (-1.94; 4.5)	0.437
miR-novel-chr15_18383 Q5	0.94 (-3.54; 5.42)	0.680	1.27 (-1.67; 4.21)	0.397	1.16 (-2.07; 4.39)	0.481

#Linear regression models showing the relationship between SBP, DBP and MAP. In Model 1 and 2, log-transformed variables were used to cater for the skewness in miRNA expression data. In Model 3 and 4, the dose response for each miRNA was determined by dividing the relative miRNA expression into five quintiles, with Quintile 1 (Q1) as the reference.

Table 5. Age and sex adjusted odds ratios (with 95% confidence intervals) for determinants of screen-detected hypertension

	Normotensive vs Screen-detected hypertension		
	Odds ratio	**95% CI**	**p-value**
Model 1			
miR-novel-chr1_36178	1.36	(1.08; 1.70)	0.008
Model 2			
miR-novel-chr15_18383	1.31	(1.05; 1.63)	0.016
Model 3			
miR-novel-chr1_36178 Q1	1		
miR-novel-chr1_36178 Q2	1.32	(0.82; 2.14)	0.255
miR-novel-chr1_36178 Q3	1.52	(0.94; 2.46)	0.086
miR-novel-chr1_36178 Q4	1.5	(0.93; 2.43)	0.098
miR-novel-chr1_36178 Q5	2.13	(1.32; 3.45)	0.002
Model 4			
miR-novel-chr15_18383 Q1	1		
miR-novel-chr15_18383 Q2	1.04	(0.64; 1.70)	0.871
miR-novel-chr15_18383 Q3	1.38	(0.85; 2.24)	0.188
miR-novel-chr15_18383 Q4	1.55	(0.96; 2.51)	0.072
miR-novel-chr15_18383 Q5	1.37	(0.84; 2.22)	0.208

For Model 1 and 2, log-transformed variables were used to cater for skewed miRNA expression data. In Model 3 and 4, the dose response for each of the novel miRNAs was determined by dividing the miRNA expression into five quintiles, with Quantile 1 (Q1) as the reference.

4. Discussion

Whole genome sequencing showed the differential expression of two novel microRNAs (miR-novel-chr1_36178 and miR-novel-chr15_18383) of length 17 and 22 nucleotides respectively, in peripheral blood from normotensive and hypertensive individuals. These findings were confirmed in a larger, independent sample by RT-qPCR. The expression of miR-novel-chr15_18383 had no association with sex and did not correlate with any of the three BP parameters at lower or higher levels of expression. In contrast, the expression of miR-novel-chr1_36178 significantly differed according to sex. At lower levels of expression, the miRNA had no association with screen-detected HPT or SBP, DBP and MAP. Interestingly, when expressed at greater levels, the miRNA was associated with all three BP parameters and the odds of screen-detected HPT in the presence of miR-novel-chr1_36178 doubled.

Whilst the influence of miRNAs on gene regulation has been previously described (Filipowicz *et al*., 2008; Catalanotto *et al*., 2016), the effects of the extent to which a miRNA is expressed relative to its function remains an understudied aspect. However, Shu and colleagues demonstrated the dose-dependent function of a panel of miRNAs (let-7a-7f and the miR-17-92 cluster) and postulated that target mRNA selection may not only be dependent on sequence homology as commonly described, but also on the endogenous expression levels of the particular miRNA (Shu *et al*., 2012). It is plausible that the miR-novel-chr1_36178 described in our study, also exhibits this dose-dependent characteristic in BP regulation.

Whilst the relationship between expression of these two novel miRNAs and BP variables or HPT status cannot be directly compared to similar studies (as our findings are novel), differential expression of miRNAs in HPT has been previously reported (Kontaraki *et al*., 2014; Yildirim *et al*., 2019) and expression of miRNAs has also been related to BP parameters. For instance, one study showed a significant correlation between SBP and the expression of miR-21, miR-126 and miR-34a has been reported, whilst miR-146a expression was correlated with SBP and DBP (Hijmans *et al*., 2018). An elevated expression of miR-21 in hypertensives compared to normotensives has previously been reported and there was a positive correlation between miR-21 expression and clinical and ambulatory SBP (Cengiz *et al*., 2015), suggestive of a potential relationship between the miRNA and the regulation of BP. The maintenance of vascular integrity is one of the important processes carried out by endothelial cells (Wang *et al*., 2008) and miR-126, one of the miRNAs showing a differential expression pattern in hypertensives and correlated to BP parameters in a study by Hijmans and colleagues, plays an

integral role in this. As such, changes in the concentrations of miRNAs in hypertensives may represent an increased risk of disease in the vasculature of older people, increasing the possibility of a cardiovascular event.

In conclusion, miR-novel-chr1_36178 showed significant dysregulation in hypertensives and its expression at higher levels, was related to SBP, DBP and MAP. As such, it warrants further study to understand its dose dependant relationship with BP parameters and the possible role it plays in the pathogenesis of HPT.

Our study had some limitations. As HPT is deemed a "silent killer", its diagnosis is often made when subclinical hypertension-mediated organ damage (HMOD) has taken place (Piskorz, 2020). It is plausible that HMOD was present in our screen-detected and known hypertensive participants and influenced the observed novel miRNA expression profiles. As such, it may be argued that the novel miRNA expression profiles may be biomarkers of HMOD and this was not further investigated in our study. Another limitation to our study was that the networks and pathways targeted by the miRNAs as well as the nature of their interactions are currently not well established as these are novel miRNAs. Further studies into this may shed more light on how its expression impacts BP regulation. In addition, not much is known about these novel miRNAs and as such, findings could not be directly compared to findings from other studies at this stage. However, the discovery of these novel miRNAs adds to the pool of miRNAs that can be targeted for study in HPT and CVDs.

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6. Conflicts of interest

The authors declare no conflicts of interest.

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8. Author contributions

DMM: wrote the first draft, experimental procedures, data analysis and interpretation. **CJW:** experimental procedures, data analysis and interpretation. **RTE:** conception, interpretation of the data, revising it for intellectual content and final approval of the version to be published. **APK:** conception, interpretation of the data, revising it for intellectual content and final approval of the version to be published. **SFGD:** recruitment and screening of cohort, statistical analysis and interpretation of data. **SR:** interpretation of data, editing and revising it for intellectual content. **GMD:** editing and revising it for intellectual content. **TEM:** conception and design of the study, analysis and interpretation of the data, revising it for intellectual content and final approval of the version to be published.

9. References

Alles, J., Fehlmann, T., Fischer, U., *et al*. 2019. An Estimate of the Total Number of True Human miRNAs. *Nucleic Acids Res*, 47(7): 3353–3364.

Baumann, V. & Winkler, J. 2014. MiRNA-based Therapies: Strategies and Delivery Platforms for Oligonucleotide and Non-Oligonucleotide Agents. *Future Med Chem*, 6(17): 1967–1984.

Baye, T.M., Abebe, T. & Wilke, R.A. 2011. Genotype-Environment Interactions and their Translational Implications. *Per Med*, 8(1): 59–70.

Campbell, M.C. & Tishkoff, S.A. 2008. African Genetic Diversity: Implications for Human Demographic History, Modern Human Origins, and Complex Disease Mapping. *Annu Rev Genomics Hum Genet*, 9: 403–433.

Catalanotto, C., Cogoni, C. & Zardo, G. 2016. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int J Mol Sci*, 17(10): 1712.

Cengiz, M., Karatas, O.F., Koparir, E., *et al*. 2015. Differential Expression of Hypertension-Associated MicroRNAs in the Plasma of Patients With White Coat Hypertension. *Medicine (Baltimore)*, 94(13): e693.

Chalmers, J., MacMahon, S., Mancia, G., *et al*. 1999. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. *Clin Exp Hypertens*, 21(5–6): 1009–1060.

Filipowicz, W., Bhattacharyya, S.N. & Sonenberg, N. 2008. Mechanisms of Post-transcriptional Regulation by microRNAs: Are the Answers in Sight? *Nat Rev Genet*, 9(2): 102–114.

Hijmans, J.G., DIehl, K.J., Bammert, T.D., *et al*. 2018. Association Between Hypertension and Circulating Vascular-related MicroRNAs. *J Hum Hypertens*, 32(6): 440–447.

Huang, Y.Q., Huang, C., Chen, J.Y., Li, J. & Feng, Y.Q. 2016. The Association of Circulating miR-30a, miR-29 and miR-133 with White-Coat Hypertension. *Biomark Med*, 10(12): 1231–1239.

Kontaraki, J.E., Marketou, M.E., Zacharis, E.A., Parthenakis, F.I. & Vardas, P.E. 2014. MicroRNA-9 and microRNA-126 Expression Levels in Patients with Essential Hypertension: Potential Markers of Target-Organ Damage. *J Am Soc Hypertens*, 8(6): 368–375.

Krol, J., Loedige, I. & Filipowicz, W. 2010. The Widespread Regulation of microRNA

Biogenesis, Function and Decay. *Nat Rev Genet*, 11(9): 597–610.

Lee, R., Feinbaum, R.L. & Ambros, V. 1993. The C. elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. *Cell*, 75(5): 843–854.

Li, S., Zhu, J., Zhang, W., *et al*. 2011. Signature microRNA Expression Profile of Essential Hypertension and its Novel Link to Human Cytomegalovirus Infection. *Circulation*, 124(2): 175–184.

Li, Y. & Kowdley, K. V. 2012. MicroRNAs in Common Human Diseases. *Genomics Proteomics Bioinformatics*, 10(5): 246–253.

Liu, Jia, Liu, Jiamei, Shi, L., *et al*. 2018. Preliminary Study of microRNA-126 as a Novel Therapeutic Target for Primary Hypertension. *Int J Mol Med*, 41(4): 1835–1844.

Livak, K.J. & Schmittgen, T.D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. *Methods*, 25(4): 402–408.

Matsha, T.E., Hassan, M.S., Kidd, M. & Erasmus, R.T. 2012. The 30-Year Cardiovascular Risk Profile of South Africans with Diagnosed Diabetes, Undiagnosed Diabetes, Pre-diabetes or Normoglycaemia : The Bellville, South Africa Pilot Study. *Cardiovasc J Afr*, 23(1): 5–11.

Matsha, T.E., Kengne, A.P., Hector, S., *et al*. 2018. MicroRNA Profiling and Their Pathways in South African Individuals with Prediabetes and Newly Diagnosed Type 2 Diabetes Mellitus. *Oncotarget*, 9(55): 30485–30498.

Mills, K.T., Bundy, J.D., Kelly, T.N., *et al*. 2016. Global Disparities of Hypertension Prevalence and Control: A Systematic Analysis of Population-Based Studies from 90 Countries. *Circulation*, 134(6): 441–450.

Piskorz D. 2020. Hypertensive Mediated Organ Damage and Hypertension Management. How to Assess Beneficial Effects of Antihypertensive Treatments? *High Blood Press Cardiovasc Prev,* 27(1): 9-17.

Shin, V.Y. & Chu, K.-M. 2014. MiRNA as Potential Biomarkers and Therapeutic Targets for Gastric Cancer. *World J Gastroenterol*, 20(30): 10432–10439.

Shu, J., Xia, Z., Li, L., *et al*. 2012. Dose-dependent Differential mRNA Target Selection and Regulation by let-7a-7f and miR-17-92 Cluster microRNAs. *RNA Biol*, 9(10): 1275–1287.

Wake, C., Labadorf, A., Dumitriu, A., *et al*. 2016. Novel microRNA Discovery Using Small RNA Sequencing in Post-Mortem Human Brain. *BMC Genomics*, 17(1): 776.

Wang, S., Aurora, A.B., Johnson, B.A., *et al*. 2008. The Endothelial-Specific MicroRNA miR-126 Governs Vascular Integrity and Angiogenesis. *Dev Cell*, 15(2): 261–271.

Yildirim, E., Ermis, E., Allahverdiyev, S., *et al*. 2019. Circulating miR-21 Levels in Hypertensive Patients with Asymptomatic Organ Damage. *Medicine*, 98(39): e17297.

CHAPTER 6: DISCUSSION

In this section, results reflecting work conducted to achieve the study objectives are discussed. This includes novel findings and their possible clinical relevance. In addition, recommendations for future projects are made to circumvent limitations cited in this study.

6.1 Summary of novel findings

Manuscript 1:

This was the first study in Africa to conduct whole genome miRNA sequencing in HPT and identified a panel of 30 miRNAs that were dysregulated in hypertensives. Whilst some of the dysregulated miRNAs in our study had been previously reported in other studies, we reported a unique miRNA profile for this population, including the dysregulation of two novel miRNAs. The dysregulation of these miRNAs was associated with various biological pathways including platelet activation, calcium signalling and vascular smooth muscle contraction pathways. The findings from this part of the study informed the choice of miRNAs investigated in a later part of the study.

Manuscript 2:

Herein, the top five dysregulated miRNAs in whole genome miRNA profiling were chosen and investigated in a larger, independent sample using RT-qPCR. The expression of three of these miRNAs (miR-182-5p, 30e-3p and 1299) had not been previously investigated in HPT. They had however been implicated in various forms of CVDs or conditions characterised by high BP, including pre-eclampsia, coronary artery disease, heart failure and rheumatic heart disease. The other two miRNAs (miR-126-3p and miR-30a-5p) had been previously investigated in HPT, albeit with inconsistent findings. Whilst some studies reported either no difference or low expression of miR-126 in hypertensives, our study reported higher expression of miR-126-3p and miR-30a-5p in hypertensives. In addition, we reported for the first time, miR-182-5p dysregulation in HPT.

Manuscript 3:

This part of the study investigated the relationship between clinical BP measures and the expression of two novel miRNAs from miRNA sequencing that have never been previously described in any population. Whilst the findings were not conclusive for one of the miRNAs, when highly expressed, the other novel miRNA was associated with SBP and DBP and screen-

detected HPT. As such, further characterisation of the miRNA in the population and further investigation of its specific roles in BP regulation is warranted.

6.2 Clinical relevance

6.2.1 MicroRNAs as possible therapeutic targets

Despite the success of anti-hypertensive medications in symptom management and control, resistant forms of HPT which represent, in part, failure of therapeutic interventions, continue to persist and pose increased cardiovascular risk, particularly to elderly patients (Calhoun *et al*., 2008). In our study, we reported significant differences in miRNA expression between hypertensives on therapy and newly diagnosed hypertensives that were not on therapy and this difference in expression could, in part, be attributed to the effect of anti-hypertensive medication on miRNA expression. The expression of miR-19, miR-101 and let-7e has been associated with a response to the beta-blockers metoprolol and atenolol, suggesting a regulatory role for these miRNAs in genes involved in pharmacodynamics (Solayman *et al*., 2019). Taking the angiotensin receptor blocker Olmesartan for six months has been shown to significantly decrease the expression of both miR-221 and miR-2226 in hypertensives (Mandraffino *et al*., 2017). The evidence suggests a bi-directional relationship, where the expression of some miRNAs is influenced by the anti-hypertensive medication in use, whilst in other cases, the miRNAs that are expressed have an effect the efficacy of the medication. A full appreciation of the influence of antihypertensive medication on miRNA expression and the specific functional contributions of various miRNAs at different stages of HPT development opens up the possibility of targeting these miRNAs for therapy development and monitoring and prognostic purposes in HPT. Given that antihypertensive medication does influence the expression of miRNAs, which have gene expression regulatory functions, it is important that development of new BP lowering strategies or medications is done with miRNAs as an integral component of the process.

The gene expression regulation capabilities of miRNAs means they have vast control on millions of biological processes, including those critical for BP regulation. Our study reported dysregulated vascular smooth muscle contraction, vasopressin-mediated water reabsorption, platelet activation, calcium signalling and aldosterone synthesis and secretion biological pathways. There was evidence of dysregulation of different biological pathways between known and newly diagnosed hypertensives in our study and some of these pathways have been linked to HPT. Whilst platelet activation and renin secretion pathways were dysregulated in

known hypertensives, in screen-detected hypertensives, the dysregulated pathways were those involved in vascular smooth muscle contraction and vasopressin-regulated water reabsorption. All these pathways are important in maintaining the BP within physiologically accepted limits. Studies have shown that miR-132 regulates vasopressin synthesis and thus water retention (Bijkerk *et al*., 2018). The *CYP11B2* gene is important in fluid volume regulation as it regulates sodium retention and potassium excretion through production of aldosterone synthase (Xanthakis & Vasan, 2013). Altered aldosterone production and thus higher susceptibility to HPT has been linked to the effects of miR-24 on the *CYP11B2* gene products (Robertson *et al*., 2013). By inhibiting vascular contraction and enhancing vasodilation, the vascular endothelium maintains vascular tone through the production and release of vasoactive elements and this is dependent on functional calcium signalling pathways. Dysregulation of calcium signalling leads to altered responses by the vasculature, a common characteristic in HPT (Wilson *et al*., 2019). The miR-30 family regulates calcium signalling in the kidney (Wu *et al*., 2015), whilst miR-214, through repression of mRNA encoding the sodium-calcium exchanger protein, Ncx1 regulates calcium pathways (Aurora *et al*., 2012). The evidence points towards miRNA involvement in various biological processes essential for BP regulation and further highlights their role in the pathogenesis of HPT. However, due to the complex nature in which miRNAs function, further investigations into the network of miRNA-pathway interactions using biological functionality assays need to be conducted if an understanding of how they result in disease is to be reached. At that point, they may form a subset of therapeutic targets that can manipulated to either reverse or enhance their action, depending on their pathophysiological role in disease.

Expression profiles in disease may show an upregulation or downregulation of the miRNAs in question. In situations where increased expression of a particular miRNA favours development of a disease, dampening down its expression may be a viable corrective option. This can be achieved through delivery of inhibitory oligonucleotides targeting miRNAs *in vivo* and diminishing their function (Krützfeldt *et al*., 2005; Ma *et al*., 2010). Where the development or progression of disease is due to reduced expression of a particular miRNA, reconstitution of the miRNA pool by synthetic miRNA mimics is a possible therapeutic avenue. For example, in pulmonary arterial HPT where there is reduced miR-193 expression, using murine models of pulmonary HPT, Sharma and colleagues showed that administration of a synthetic miR-193 mimic to the lungs lowered right ventricular systolic pressure (Sharma *et al*., 2014). A similar

study also reported a significant drop in right ventricular systolic pressure after reconstitution of miR-424 and miR-503 in PAH murine models (Kim *et al*., 2013).

It is clear that, be it supplementary reconstitution of an under expressed, albeit desirable miRNA or the inhibition of an overexpressed, problematic miRNA, these non-coding RNAs are an interesting therapeutic target in HPT and related diseases. The use of synthetic oligonucleotides targeting or mimicking miRNAs implicated in disease is a promising therapeutic approach that warrants further investigation.

6.2.2 MiRNAs as biomarkers of hypertension and cardiovascular disease

Given that HPT affects more than 1 billion people (Mills *et al*., 2016) and annually, accounts for over 10 million deaths globally (Stanaway *et al*., 2018), and that more than 50% of hypertensives are unaware of their condition (Babiker *et al*., 2013; Peltzer & Phaswana-Mafuya, 2013), it is safe to classify it as a greatly concerning public health problem requiring urgent intervention. The intervention strategies may take various forms, including development of alternative diagnostic or therapeutic strategies to supplement currently adopted ones. Presently, HPT diagnostic recommendations advise that a diagnosis be made after at least two separate office/clinical visits unless the BP readings are substantially greater than the recommended SBP and DBP limits. However, in some resource limited settings, HPT diagnosis after a single clinical visit or without strict adherence to recommendations remains a possibility. In addition, whilst ambulatory blood pressure measurement is the preferred method for monitoring BP and making a HPT diagnosis, the relatively higher financial need for that limits its routine availability. As such, investigations into the development of an alternative or supplementary HPT diagnostic procedure is warranted. As a result, microRNAs are now being investigated not only as potential biomarkers for HPT diagnosis, but with the added advantage of potentially being used for the development of therapy.

By definition, biomarkers are reproducible and accurately measurable objective indicators of the medical state of the patient, as observed from the outside (Strimbu & Tavel, 2010). Some miRNAs exhibit different expression patterns in disease when compared to the normal state and as such have been candidates for biomarker research. In addition, to their differential expression in disease, their stability in circulation due to their resistance to RNase activity, pH and temperature fluctuations (Chen *et al*., 2008; Mitchell *et al*., 2008) further accentuates their appeal as biomarkers of disease. Various studies have reported the potential utility of miRNAs as biomarker targets in disease and their clinical relevance has been reported in T2DM (Matsha

et al., 2018; Pordzik *et al*., 2019), prostate cancer (Dybos *et al*., 2018), large B-cell lymphoma (Lawrie *et al*., 2008), endothelial dysfunction (Nemecz *et al*., 2016) and other human diseases (Li & Kowdley, 2012).

The significant differences in the level of expression of miR-126-3p, 182-5p, 30a-5p and miR-1299 in the blood of normotensive and hypertensive individuals in our study makes them potential biomarker targets for prognosis or diagnosis of HPT. Additionally, the association of BP parameters like SBP and DBP with the expression of miR-novel-chr1_36178 points towards its possible utility in prediction and monitoring fluctuations in BP levels. Dysregulation of miRNAs investigated in our study has been previously reported in HPT and CVDs. For instance, just as reported by Liu and colleagues, our study also demonstrated increased miR-126 expression in hypertensives in comparison to the normotensives (Liu *et al*., 2018). However, this finding was in contrast to two other studies which either reported no difference in miR-126 expression between hypertensives and normotensives (Chen *et al*., 2018) or increased miR-126 expression in normotensives relative to hypertensives (Kontaraki *et al*., 2014). In PAH patients, plasma miR-30a-5p expression was higher in comparison to normal controls (Tan *et al*., 2019), whilst Huang and colleagues reported miR-30a dysregulation in essential and white coat HPT, echoing our findings in which we also reported miR-30a-5p upregulation in hypertensives relative to normotensives (Huang *et al*., 2016). One study reported miR-182-5p as a suitable biomarker for unprotected left main coronary artery disease whilst (Zhu *et al*., 2019) whilst significantly low plasma miR-182-3p expression was reported in pulmonary arterial hypertension patients, compared to healthy controls in another study (Sun *et al*., 2020). Sometimes similar studies do not report the same findings, even in reference to the same miRNA within the same disease. However, discordant findings in miRNA profiling are not uncommon and intra- and inter-population genetic diversity, participant inclusion and exclusion criteria, human sample type used, the miRNA profiling methods and the choice of normalizers for the generated qPCR data could explain some of the observed discrepancies. Owing to the lack a standardised procedure for conducting miRNA profiling, inter-study comparison of findings is very challenging and is an aspect of the field that warrants further improvement (Glinge *et al*., 2017).

Besides the miRNAs investigated in our study, differential expression of other miRNAs relevant to HPT and CVDs has been previously reported and this includes increased expression of miR-21 and lower miR-143 and -145 expression in hypertensives (Yildirim *et al*., 2019; Li

et al., 2016; Kontaraki *et al*., 2014). Just like (Huang *et al*., 2016), Kontaraki and colleagues also reported downregulation of miR-133 in hypertensives compared to the controls (Kontaraki *et al*., 2014). Upregulation of miR-155 in hypertensives and a positive correlation with SBP, DBP and other blood pressure measurements has also been reported (Huang *et al*., 2020). In another study, a combination of miR-122-5p, -199a-3p, -208a-3p and -223-3p was reported to be of good diagnostic utility for HPT in an Asian population (Zhang *et al*., 2018).

Overall, it is encouraging that there seems to be a distinction in miRNA expression patterns in diseased individuals, relative to healthy controls. This presents an opportunity to further study those miRNAs as they may have key roles in either preserving the healthy state or promoting the development of disease. This is particularly important as differential miRNA expression in disease and indeed HPT, may be a representation of either of two scenarios: 1) a counteractive response to reverse the disease state or progression or 2) a consequence of dysregulation of a biological or physiological process linked to the development of disease. It is essential to further analyse direction of the relationship between miRNA expression and HPT, so as to understand the pathophysiologic role of these non-coding RNAs in HPT and CVDs in general.

The aforementioned findings from our study are important for several reasons. Firstly, they confirmed findings from similar studies, whilst further highlighting miRNA dysregulation in HPT. Furthermore, given the paucity in miRNA expression data for hypertensives in Africa, the two novel miRNAs identified in this study added to the pool of non-coding RNAs that can be targeted for further evaluation in hypertensive African populations. The establishment of a panel of miRNAs indicative of the presence of HPT risks or disease may be a valuable addition to the diagnostic process. With the conduction of intense, standardised miRNA-based research in HPT, it is a distinct possibility that miRNAs may eventually form surrogate endpoints in HPT, with important implications for the diagnostic, monitoring and treatment processes.

6.3 Strengths and limitations

The miRNAs selected for investigation in this study were based on whole genome miRNA profiling findings from participants within the same cohort. Whole genome miRNA profiling made use of NGS technology, which is considered a robust screening platform for miRNA expression (Tam *et al*., 2014). Furthermore, the cross-platform validation of whole genome miRNA profiling results with RT-qPCR added significant strength to the study. In addition, these study investigations were conducted in the largest, well-characterised cohort for miRNA investigations in people with HPT to date. A limitation of the study was its cross-sectional

nature, which meant that causal inferences could not be made between miRNA expression and HPT. Additionally, financial constraints meant that NGS could only be conducted on 48 female mixed ancestry individuals. Although the use of admixture populations in genetic studies has its advantages, considering the diversity of African populations, future studies including other populations from the region are advised. Investigations in this study were conducted on whole blood, whilst other studies have made use of serum, plasma and tissue samples, thus complicating inter-study comparisons. At present, there is no standardised method for conducting these investigations and as such, it is important that dysregulated miRNAs are investigated in a smaller cohort, using different tissue types to facilitate standardisation of miRNA expression measurement methods. Nonetheless, our findings offered a platform for the development of longitudinal studies in which the relationship between expression of certain miRNAs and development of HPT and associated complications could be scrutinised further.

Based on findings from this study, we recommend conducting the following work in future to advance the knowledge gained thus far:

- MicroRNA profiling of participants whose HPT diagnosis has changed (for the better or worse) or have had a cardiovascular event (since their miRNA expression was last profiled) and comparing with their previous miRNA expression profiles. This would enable a longitudinal analysis, potentially shedding light on the causal-effect relationship between expression of certain miRNAs and occurrence of HPT or related cardiovascular complications.
- Comparison of miRNA expression profiles in incident hypertensives and known hypertensives to assess the effect of different antihypertensive drug class on the expression of miRNAs that are important in HPT.
- Profiling of other significantly dysregulated miRNAs in HPT that were not investigated in this study due to financial constraints.
- The use of alternative sample types like tissue, PBMCs, platelets, plasma or serum to conduct miRNA expression profiling.
- Increasing and strengthening African collaboration efforts on genetic studies to mitigate the financial constraints faced by many institutions in the region.

6.4 References

Aurora, A.B., Mahmoud, A.I., Luo, X., *et al*. 2012. MicroRNA-214 Protects the Mouse Heart from Ischemic Injury by Controlling Ca2+ Overload and Cell Death. *J Clin Invest*, 122(4): 1222–1232.

Babiker, F.A., Elkhalifa, L.A. & Moukhyer, M.E. 2013. Awareness of Hypertension and Factors Associated with Uncontrolled Hypertension in Sudanese Adults. *Cardiovasc J Afr*, 24(6): 208–212.

Bijkerk, R., Trimpert, C., van Solingen, C., *et al*. 2018. MicroRNA-132 Controls Water Homeostasis through Regulating MECP2-mediated Vasopressin Synthesis. *Am J Physiol Renal Physiol*, 315(4): F1129–F1138.

Calhoun, D.A., Jones, D., Textor, S., *et al*. 2008. Resistant Hypertension: Diagnosis, Evaluation, and Treatment. *Circulation*, 117(25): e510–e526.

Chen, S., Chen, R., Zhang, T., *et al*. 2018. Relationship of Cardiovascular Disease Risk Factors and Noncoding RNAs with Hypertension: A Case-Control Study. *BMC Cardiovasc Disord*, 18(1): 58.

Chen, X., Ba, Y., Ma, L., *et al*. 2008. Characterization of microRNAs in Serum: A Novel Class of Biomarkers for Diagnosis of Cancer and Other Diseases. *Cell Res*, 18(10): 997–1006.

Dybos, S.A., Flatberg, A., Halgunset, J., *et al*. 2018. Increased Levels of Serum miR-148a-3p are Associated with Prostate Cancer. *APMIS*, 126(9): 722–731.

Glinge, C., Clauss, S., Boddum, K., *et al*. 2017. Stability of Circulating Blood-based microRNAs - Pre-Analytic Methodological Considerations. *PLoS One*, 12(2): e0167969.

Huang, Y. qing, Huang, C., Zhang, B. & Feng, Y. qing. 2020. Association of Circulating miR-155 Expression Level and Inflammatory Markers with White Coat Hypertension. *J Hum Hypertens*, 34(5): 397–403.

Huang, Y.Q., Huang, C., Chen, J.Y., Li, J. & Feng, Y.Q. 2016. The Association of Circulating miR-30a, miR-29 and miR-133 with White-Coat Hypertension. *Biomark Med*, 10(12): 1231–1239.

Kim, J., Kang, Y., Kojima, Y., *et al*. 2013. An Endothelial Apelin-FGF Link Mediated by miR-424 and miR-503 is Disrupted in Pulmonary Arterial Hypertension. *Nat Med*, 19(1): 74–82.

Kontaraki, J, Marketou, M., Zacharis, E., Parthenakis, F. & Vardas, P. 2014. Differential

Expression of Vascular Smooth Muscle-modulating MicroRNAs in Human Peripheral Blood Mononuclear Cells: Novel Targets in Essential Hypertension. *J Hum Hypertens*, 28(8): 510–516.

Kontaraki, JE, Marketou, M., Zacharis, E., Parthenakis, F. & Vardas, P. 2014. MicroRNA-9 and microRNA-126 Expression Levels in Patients with Essential Hypertension: Potential Markers of Target-Organ Damage. *J Am Soc Hypertens*, 8(6): 368–375.

Krützfeldt, J., Rajewsky, N., Braich, R., *et al*. 2005. Silencing of microRNAs in vivo with 'antagomirs'. *Nature*, 438(7068): 685–689.

Lawrie, C.H., Gal, S., Dunlop, H.M., *et al*. 2008. Detection of Elevated Levels of Tumour-associated microRNAs in Serum of Patients with Diffuse Large B-cell Lymphoma. *Br J Haematol*, 141(5): 672–675.

Li, H., Zhang, X., Wang, F., *et al*. 2016. MicroRNA-21 Lowers Blood Pressure in Spontaneous Hypertensive Rats by Upregulating Mitochondrial Translation. *Circulation*, 134(10): 734–751.

Li, Y. & Kowdley, K. V. 2012. MicroRNAs in Common Human Diseases. *Genomics Proteomics Bioinformatics*, 10(5): 246–253.

Liu, Jia, Liu, Jiamei, Shi, L., *et al*. 2018. Preliminary Study of microRNA-126 as a Novel Therapeutic Target for Primary Hypertension. *Int J Mol Med*, 41(4): 1835–1844.

Ma, L., Reinhardt, F., Pan, E., *et al*. 2010. Therapeutic Silencing of miR-10b Inhibits Metastasis in a Mouse Mammary Tumor Model. *Nat Biotechnol*, 28(4): 341–347.

Mandraffino, G., Aragona, C.O., Cairo, V., *et al*. 2017. Circulating Progenitor Cells in Hypertensive Subjects: Effectiveness of a Treatment with Olmesartan in Improving Cell Number and miR Profile in Addition to Expected Pharmacological Effects. *PLoS One*, 12(3): e0173030.

Matsha, T.E., Kengne, A.P., Hector, S., *et al*. 2018. MicroRNA Profiling and Their Pathways in South African Individuals with Prediabetes and Newly Diagnosed Type 2 Diabetes Mellitus. *Oncotarget*, 9(55): 30485–30498.

Mills, K.T., Bundy, J.D., Kelly, T.N., *et al*. 2016. Global Disparities of Hypertension Prevalence and Control: A Systematic Analysis of Population-Based Studies from 90 Countries. *Circulation*, 134(6): 441–450.

Mitchell, P.S., Parkin, R.K., Kroh, E.M., *et al*. 2008. Circulating microRNAs as Stable Blood-

based Markers for Cancer Detection. *Proc Natl Acad Sci USA*, 105(30): 10513–10518.

Nemecz, M., Alexandru, N., Tanko, G. & Georgescu, A. 2016. Role of MicroRNA in Endothelial Dysfunction and Hypertension. *Curr Hypertens Rep*, 18(12): 87.

Peltzer, K. & Phaswana-Mafuya, N. 2013. Hypertension and Associated Factors in Older Adults in South Africa. *Cardiovasc J Afr*, 24(3): 66–72.

Pordzik, J., Jakubik, D., Jarosz-Popek, J., *et al*. 2019. Significance of Circulating microRNAs in Diabetes Mellitus Type 2 and Platelet Reactivity: Bioinformatic Analysis and Review. *Cardiovasc Diabetol*, 18(1): 113.

Robertson, S., Mackenzie, S.M., Alvarez-Madrazo, S., *et al*. 2013. MicroRNA-24 is a Novel Regulator of Aldosterone and Cortisol Production in the Human Adrenal Cortex. *Hypertension*, 62(3): 572–578.

Sharma, S., Umar, S., Potus, F., *et al*. 2014. Apolipoprotein A-I Mimetic Peptide 4F Rescues Pulmonary Hypertension by Inducing microRNA-193-3p. *Circulation*, 130(9): 776–785.

Solayman, M.H., Langaee, T.Y., Gong, Y., *et al*. 2019. Effect of Plasma MicroRNA on Antihypertensive Response to Beta Blockers in the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) Studies. *Eur J Pharm Sci*, 131: 93–98.

Stanaway, J.D., Afshin, A., Gakidou, E., *et al*. 2018. Global, Regional, and National Comparative Risk Assessment of 84 Behavioural, Environmental and Occupational, and Metabolic Risks or Clusters of Risks for 195 Countries and Territories, 1990-2017: A Systematic Analysis for the Global Burden of Disease Stu. *Lancet*, 392(10159): 1923–1994.

Strimbu, K. & Tavel, J.A. 2010. What are Biomarkers? *Curr Opin HIV AIDS*, 5(6): 463–466.

Sun, L., Lin, P., Chen, Y., *et al*. 2020. miR-182-3p/Myadm Contribute to Pulmonary Artery Hypertension Vascular Remodeling via a KLF4/p21-dependent Mechanism. *Theranostics*, 10(12): 5581–5599.

Tam, S., De Borja, R., Tsao, M.S. & Mcpherson, J.D. 2014. Robust Global microRNA Expression Profiling using Next-Generation Sequencing Technologies. *Lab Invest*, 94(3): 350–358.

Tan, H., Yao, H., Lie, Z., *et al*. 2019. MicroRNA-30a-5p Promotes Proliferation and Inhibits Apoptosis of Human Pulmonary Artery Endothelial Cells Under Hypoxia by Targeting YKL-40. *Mol Med Rep*, 20(1): 236–244.

Wilson, C., Zhang, X., Buckley, C., *et al*. 2019. Increased Vascular Contractility in Hypertension Results from Impaired Endothelial Calcium Signaling. *Hypertension*, 74(5): 1200–1214.

Wu, J., Zheng, C., Wang, X., *et al*. 2015. MicroRNA-30 Family Members Regulate Calcium/Calcineurin Signaling in Podocytes. *J Clin Invest*, 125(11): 4091–4106.

Xanthakis, V. & Vasan, R.S. 2013. Aldosterone and the Risk of Hypertension. *Curr Hypertens Rep*, 15(2): 102–107.

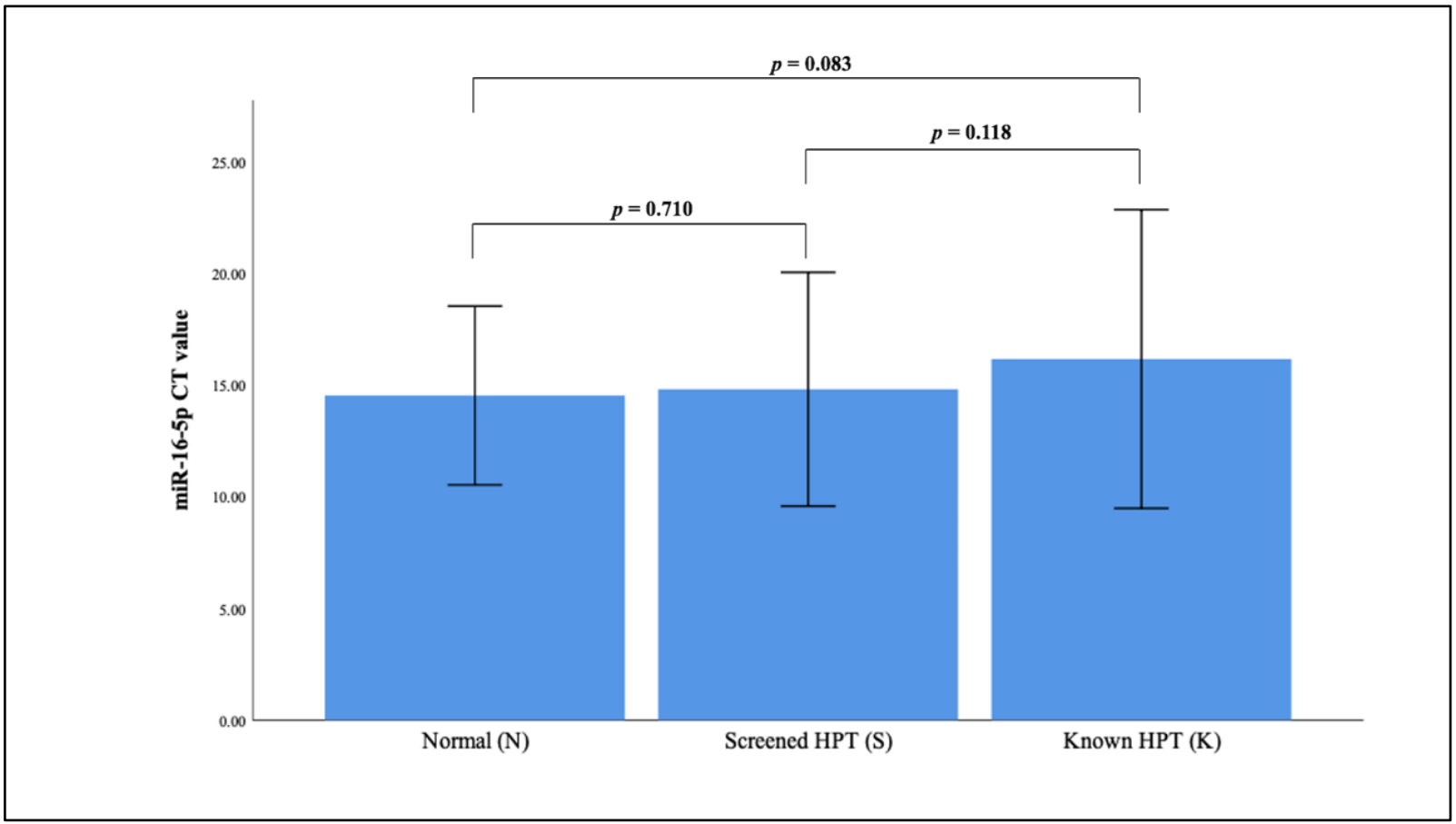
Yildirim, E., Ermis, E., Allahverdiyev, S., *et al*. 2019. Circulating miR-21 Levels in Hypertensive Patients with Asymptomatic Organ Damage. *Medicine*, 98(39): e17297.

Zhang, X., Wang, X., Wu, J., *et al*. 2018. The Diagnostic Values of Circulating MiRNAs for Hypertension and Bioinformatics Analysis. *Biosci Rep*, 38(4): 1–11.

Zhu, L., Chen, T., Ye, W., *et al*. 2019. Circulating miR-182-5p and miR-5187-5p as Biomarkers for the Diagnosis of Unprotected Left Main Coronary Artery Disease. *J Thorac Dis*, 11(5): 1799–1808.

APPENDIX

Appendix A: Supplementary material for Manuscript 1



Supplementary Figure 1. A comparison of the raw Ct values of the RT-qPCR normalizer, miR-16-5p in the three blood pressure groups.

No significant difference in expression was observed between groups. Known HPT v normotensives, p=0.083; known HPT v screen-detected HPT, p=0.118 and normotensive v screen-detected HPT, p=0.710.

Appendix B: Supplementary material for Manuscript 2

Supplementary Table S1. Age, gender and BMI adjusted partial correlation coefficients for the association between miR-30a-5p relative expression and anthropometric and biochemical parameters according to blood pressure status.

	All		**Normotensive**		**Screen-detected HPT**		**Known HPT**	
	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
miR-30a-5p $2^{-\Delta Ct}$	1.000		1.000		1.000		1.000	
miR-1299 $2^{-\Delta Ct}$	0.710	<0.001	0.643	0.001	0.734	<0.001	0.774	<0.001
miR-182-5p $2^{-\Delta Ct}$	0.937	<0.001	0.934	<0.001	0.923	<0.001	0.945	<0.001
miR-30e-3p $2^{-\Delta Ct}$	0.901	<0.001	0.880	<0.001	0.867	<0.001	0.934	<0.001
miR-126-3p $2^{-\Delta Ct}$	0.901	<0.001	0.891	<0.001	0.878	<0.001	0.915	<0.001
Waist circumference(cm)	-0.485	0.019	-0.518	0.011	-0.602	0.002	-0.416	0.048
Hip circumference (cm)	-0.154	0.482	-0.048	0.827	-0.278	0.199	-0.022	0.921
Waist to Hip ratio	0.001	0.997	0.024	0.913	0.127	0.564	-0.063	0.774
Systolic blood pressure (mmHg)	0.313	0.146	-0.019	0.931	0.376	0.077	0.448	0.032
Diastolic blood pressure (mmHg)	0.224	0.304	0.051	0.817	0.239	0.272	0.309	0.152
Fasting Blood glucose (mmol/L)	0.133	0.546	0.253	0.244	0.238	0.275	0.012	0.957
2-hour glucose (mmol/L)	0.046	0.834	0.202	0.355	0.244	0.262	-0.123	0.576
HbA1c (%)	-0.201	0.357	-0.151	0.492	-0.066	0.763	-0.197	0.368
Fasting insulin (mIU/L)	0.272	0.209	0.418	0.047	0.185	0.397	0.075	0.734
2-hour insulin (mIU/L)	0.049	0.826	0.222	0.308	0.001	0.997	-0.053	0.811
Triglycerides-S (mmol/L)	0.037	0.868	0.079	0.721	0.028	0.900	0.089	0.686
Total cholesterol (mmol/L)	0.108	0.624	0.140	0.524	0.342	0.110	0.255	0.240
HDL-cholesterol (mmol/L)	0.445	0.033	0.417	0.048	0.565	0.005	0.615	0.002
LDL-cholesterol (mmol/L)	0.065	0.769	0.128	0.561	0.284	0.189	0.177	0.419
C-Reactive Protein (mg/L)	0.145	0.509	0.109	0.619	0.184	0.402	0.255	0.240
Gamma GT (IU/L)	0.094	0.669	-0.068	0.758	0.471	0.023	0.182	0.407
S-Creatinine (µmol/L)	-0.349	0.443	-0.283	0.539	-0.472	0.285	-0.242	0.601
Duration of disease (years)	-	-	-	-	-	-	0.358	0.430

Supplementary Table S2. Age, gender and BMI adjusted partial correlation coefficients for the association between miR-1299 relative expression and anthropometric and biochemical parameters according to blood pressure status.

	All		**Normotensive**		**Screen-detected HPT**		**Known HPT**	
	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
miR-30a-5p $2^{-\Delta Ct}$	0.710	<0.001	0.643	0.001	0.734	<0.001	0.774	<0.001
miR-1299 $2^{-\Delta Ct}$	1.000		1.000		1.000		1.000	
miR-182-5p $2^{-\Delta Ct}$	0.738	<0.001	0.708	<0.001	0.692	<0.001	0.795	<0.001
miR-30e-3p $2^{-\Delta Ct}$	0.721	<0.001	0.678	<0.001	0.690	<0.001	0.796	<0.001
miR-126-3p $2^{-\Delta Ct}$	0.731	<0.001	0.678	<0.001	0.697	<0.001	0.807	<0.001
Waist circumference (cm)	-0.521	0.011	-0.513	0.012	-0.493	0.017	-0.526	0.010
Hip circumference (cm)	0.048	0.828	0.213	0.330	-0.015	0.945	0.048	0.828
Waist to Hip ratio	-0.131	0.553	-0.154	0.482	0.073	0.742	-0.222	0.309
Systolic blood pressure (mmHg)	0.268	0.216	0.014	0.948	0.362	0.089	0.367	0.085
Diastolic blood pressure (mmHg)	0.233	0.284	0.143	0.516	0.347	0.105	0.259	0.233
Fasting Blood glucose (mmol/L)	0.040	0.856	0.144	0.512	0.123	0.575	-0.056	0.799
2-hour glucose (mmol/L)	0.102	0.644	0.207	0.342	0.362	0.090	-0.078	0.724
HbA1c (%)	-0.130	0.555	0.031	0.887	-0.146	0.506	-0.158	0.473
Fasting insulin (mIU/L)	0.216	0.322	0.367	0.085	0.088	0.689	0.039	0.860
2-hour insulin (mIU/L)	0.078	0.723	0.215	0.324	0.104	0.637	-0.039	0.860
Triglycerides-S (mmol/L)	-0.079	0.719	-0.047	0.830	-0.116	0.598	0.017	0.937
Total cholesterol (mmol/L)	0.041	0.854	0.058	0.794	0.250	0.250	0.220	0.313
HDL-cholesterol (mmol/L)	0.421	0.045	0.502	0.015	0.527	0.010	0.508	0.013
LDL-cholesterol (mmol/L)	0.021	0.925	0.035	0.874	0.210	0.335	0.188	0.392
C-Reactive Protein (mg/L)	0.152	0.487	0.165	0.453	0.233	0.284	0.201	0.358
Gamma GT (IU/L)	0.043	0.845	0.058	0.792	0.299	0.166	0.063	0.776
S-Creatinine (µmol/L)	-0.337	0.460	-0.298	0.517	-0.441	0.322	-0.247	0.593
Duration of disease (years)	-	-	-	-	-	-	0.202	0.664

Supplementary Table S3. Age, gender and BMI adjusted partial correlation coefficients for the association between miR-182-5p relative expression and anthropometric and biochemical parameters according to blood pressure status.

	All		**Normotensive**		**Screen-detected HPT**		**Known HPT**	
	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
miR-30a-5p $2^{-\Delta Ct}$	0.937	<0.001	0.934	<0.001	0.923	<0.001	0.945	<0.001
miR-1299 $2^{-\Delta Ct}$	0.738	<0.001	0.708	<0.001	0.692	<0.001	0.795	<0.001
miR-182-5p $2^{-\Delta Ct}$	1.000		1.000		1.000		1.000	
miR-30e-3p $2^{-\Delta Ct}$	0.968	<0.001	0.961	<0.001	0.949	<0.001	0.980	<0.001
miR-126-3p $2^{-\Delta Ct}$	0.983	<0.001	0.974	<0.001	0.968	<0.001	0.982	<0.001
Waist circumference(cm)	-0.518	0.011	-0.547	0.007	-0.685	<0.001	-0.453	0.030
Hip circumference (cm)	-0.100	0.651	0.051	0.816	-0.218	0.318	0.011	0.959
Waist to Hip ratio	-0.060	0.787	-0.048	0.826	0.036	0.870	-0.133	0.544
Systolic blood pressure (mmHg)	0.304	0.158	-0.021	0.923	0.327	0.128	0.415	0.049
Diastolic blood pressure (mmHg)	0.228	0.295	0.095	0.667	0.233	0.285	0.284	0.189
Fasting Blood glucose (mmol/L)	0.109	0.621	0.191	0.384	0.239	0.273	0.006	0.980
2-hour glucose (mmol/L)	0.044	0.841	0.184	0.402	0.248	0.253	-0.131	0.550
HbA1c (%)	-0.190	0.385	-0.175	0.426	0.021	0.924	-0.215	0.325
Fasting insulin (mIU/L)	0.267	0.218	0.422	0.045	0.220	0.314	0.041	0.853
2-hour insulin (mIU/L)	0.051	0.815	0.230	0.291	0.039	0.860	-0.081	0.714
Triglycerides-S (mmol/L)	0.008	0.971	0.018	0.935	0.134	0.541	0.013	0.952
Total cholesterol (mmol/L)	0.039	0.858	0.097	0.661	0.235	0.281	0.220	0.312
HDL-cholesterol (mmol/L)	0.431	0.040	0.459	0.028	0.486	0.019	0.629	0.001
LDL-cholesterol (mmol/L)	-0.003	0.991	0.077	0.728	0.185	0.398	0.146	0.508
C-Reactive Protein (mg/L)	0.144	0.511	0.169	0.442	0.154	0.484	0.157	0.474
Gamma GT (IU/L)	0.078	0.722	-0.052	0.814	0.472	0.023	0.143	0.514
S-Creatinine (µmol/L)	-0.377	0.404	-0.335	0.463	-0.503	0.249	-0.268	0.561
Duration of disease (years)	-	-	-	-	-	-	0.318	0.487

Supplementary Table S4. Age, gender and BMI adjusted partial correlation coefficients for the association between miR-30e-3p relative expression and anthropometric and biochemical parameters according to blood pressure status.

	All		**Normotensive**		**Screen-detected HPT**		**Known HPT**	
	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
miR-30a-5p $2^{-\Delta Ct}$	0.901	<0.001	0.880	<0.001	0.867	<0.001	0.934	<0.001
miR-1299 $2^{-\Delta Ct}$	0.721	<0.001	0.678	<0.001	0.690	<0.001	0.796	<0.001
miR-182-5p $2^{-\Delta Ct}$	0.968	<0.001	0.961	<0.001	0.949	<0.001	0.980	<0.001
miR-30e-3p $2^{-\Delta Ct}$	1.000		1.000		1.000		1.000	
miR-126-3p $2^{-\Delta Ct}$	0.973	<0.001	0.970	<0.001	0.958	<0.001	0.981	<0.001
Waist circumference (cm)	-0.538	0.008	-0.534	0.009	-0.729	<0.001	-0.465	0.025
Hip circumference (cm)	-0.084	0.703	0.049	0.826	-0.174	0.426	-0.001	0.996
Waist to Hip ratio	-0.083	0.705	-0.035	0.874	-0.018	0.934	-0.144	0.513
Systolic blood pressure (mmHg)	0.275	0.204	-0.017	0.940	0.342	0.110	0.408	0.053
Diastolic blood pressure (mmHg)	0.202	0.354	0.105	0.633	0.260	0.231	0.268	0.216
Fasting Blood glucose (mmol/L)	0.119	0.590	0.205	0.347	0.257	0.236	0.006	0.978
2-hour glucose (mmol/L)	0.058	0.792	0.211	0.335	0.263	0.226	-0.124	0.572
HbA1c (%)	-0.153	0.485	-0.110	0.616	0.022	0.919	-0.192	0.379
Fasting insulin (mIU/L)	0.250	0.250	0.398	0.060	0.182	0.407	0.043	0.844
2-hour insulin (mIU/L)	0.045	0.838	0.231	0.290	0.012	0.957	-0.080	0.717
Triglycerides-S (mmol/L)	-0.024	0.915	-0.052	0.813	0.099	0.654	0.035	0.875
Total cholesterol (mmol/L)	0.015	0.945	0.036	0.871	0.165	0.451	0.233	0.284
HDL-cholesterol (mmol/L)	0.443	0.034	0.483	0.019	0.485	0.019	0.608	0.002
LDL-cholesterol (mmol/L)	-0.025	0.908	0.012	0.958	0.112	0.609	0.165	0.451
C-Reactive Protein (mg/L)	0.173	0.429	0.248	0.254	0.163	0.458	0.151	0.492
Gamma GT (IU/L)	0.063	0.775	-0.066	0.763	0.445	0.033	0.136	0.536
S-Creatinine (µmol/L)	-0.385	0.394	-0.329	0.471	-0.539	0.212	-0.277	0.547
Duration of disease (years)	-	-	-	-	-	-	0.317	0.489

Supplementary Table S5. Age, gender and BMI adjusted partial correlation coefficients for the association between miR-126-3p relative expression and anthropometric and biochemical parameters according to blood pressure status.

	All		**Normotensive**		**Screen-detected HPT**		**Known HPT**	
	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
miR-30a-5p $2^{-\Delta Ct}$	0.901	<0.001	0.891	<0.001	0.878	<0.001	0.915	<0.001
miR-1299 $2^{-\Delta Ct}$	0.731	<0.001	0.678	<0.001	0.697	<0.001	0.807	<0.001
miR-182-5p $2^{-\Delta Ct}$	0.983	<0.001	0.974	<0.001	0.968	<0.001	0.982	<0.001
miR-30e-3p $2^{-\Delta Ct}$	0.973	<0.001	0.970	<0.001	0.958	<0.001	0.981	<0.001
miR-126-3p $2^{-\Delta Ct}$	1.000		1.000		1.000		1.000	
Waist circumference (cm)	-0.535	0.009	-0.539	0.008	-0.748	<0.001	-0.456	0.029
Hip circumference (cm)	-0.053	0.809	0.104	0.636	-0.134	0.542	0.041	0.852
Waist to Hip ratio	-0.113	0.608	-0.088	0.689	-0.073	0.741	-0.163	0.456
Systolic blood pressure (mmHg)	0.293	0.174	-0.038	0.863	0.360	0.092	0.384	0.070
Diastolic blood pressure (mmHg)	0.217	0.319	0.068	0.759	0.279	0.197	0.256	0.239
Fasting Blood glucose (mmol/L)	0.077	0.728	0.173	0.430	0.192	0.380	-0.040	0.855
2-hour glucose (mmol/L)	0.019	0.932	0.177	0.420	0.206	0.346	-0.159	0.468
HbA1c (%)	-0.170	0.439	-0.094	0.670	-0.006	0.977	-0.230	0.292
Fasting insulin (mIU/L)	0.241	0.267	0.382	0.072	0.204	0.351	0.045	0.837
2-hour insulin (mIU/L)	0.024	0.913	0.206	0.345	0.013	0.955	-0.082	0.711
Triglycerides-S (mmol/L)	0.018	0.935	0.017	0.940	0.196	0.371	0.048	0.827
Total cholesterol (mmol/L)	0.019	0.930	0.048	0.829	0.185	0.398	0.270	0.212
HDL-cholesterol (mmol/L)	0.401	0.058	0.431	0.040	0.449	0.031	0.595	0.003
LDL-cholesterol (mmol/L)	-0.017	0.939	0.031	0.889	0.137	0.534	0.209	0.339
C-Reactive Protein (mg/L)	0.139	0.528	0.184	0.401	0.174	0.426	0.122	0.580
Gamma GT (IU/L)	0.047	0.830	-0.080	0.717	0.440	0.035	0.116	0.598
S-Creatinine (µmol/L)	-0.390	0.387	-0.346	0.447	-0.503	0.250	-0.281	0.542
Duration of disease (years)	-	-	-	-	-	-	0.254	0.583

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