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Predictive value of microRNAs and cytokines in the development of type 2 diabetes mellitus and hypertension in a South African mixed ancestry population.

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DECLARATION

I, Daniël Johannes Botes, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed  **Date 28 March 2025**

ABSTRACT

The increasing burden of cardiometabolic diseases (CMDs) like Type 2 diabetes mellitus (T2DM) and hypertension in South Africa is a matter of significant concern, particularly among individuals of mixed ancestry, and the diagnosis of these conditions with currently adopted methodologies remains sub-optimal. The pathophysiology of these two CMDs is strongly influenced by inflammatory pathways mediated by cytokines, which regulate chronic inflammation in CMDs. These cytokines are in turn regulated by short, non-coding RNAs such as microRNAs (miRNAs). Altered expression of miRNAs and cytokines has been associated with CMD development, and as such may be leveraged as early diagnostic markers for CMDs to supplement existing methods. As such, using RNA isolation and quantitative reverse transcription polymerase chain reaction methodologies, this study explored the relationship between circulatory extracellular vesicle (EV)-derived miR-92a and miR-29a and cytokine profiles in a mixed ancestry population living with T2DM and hypertension, to illuminate our understanding of the mechanisms underlying these CMDs. The investigations did not show a significant difference in the expression of miR-92a-3p and miR-29a in various glycaemic and hypertension groups ($p \geq 0.234$), although the expression of miR-92a-3p increased with increasing blood pressure and thus was notably higher in hypertensive than in normotensives, whilst the highest expression of miR-29a-3p was seen in normotensives, and lowest in the pre-hypertensive group. TNF-α did not indicate any significant difference in expression levels between different glycaemic or hypertensive states ($p \geq 0.344$). However, TNF-α levels significantly correlated with monocyte levels in the pre-hypertensive group and AST levels in normoglycaemic individuals. Overall, the expression patterns of both miRNAs in this study did not support their use as biomarkers for the diagnosis of either dysglycaemia or hypertension. The expression of both miRNAs correlated with monocytes and AST ($r \geq 0.649$, $p \leq 0.044$). Overall, the expression patterns of both miRNAs and TNF-α in this study did not support their use as biomarkers for the diagnosis of either dysglycaemia or hypertension, despite uncovering relations between these miRNAs and cytokines with other clinical parameters that can be further investigated.

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ABBREVIATIONS

Abbreviation	Definition
CMD	Cardio metabolic disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DBS	Diastolic blood pressure
DHS	Demographic and Health Survey
DNAs	Deoxyribonucleic acids
EV	Extracellular vesicles
GDM	Gestational diabetes mellitus
HbA1c	Haemoglobin A1C
HDL	High density lipoprotein
HLA	Human leukocyte antigen
hs-CRP	High sensitivity C- reactive protein
HUVECs	Human umbilical vein endothelial cells
IDF	International diabetes federation
IL	Interleukin
LMICs	Low- and middle-income countries
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
miRNAs	MicroRNAs
NAFLD	Non-alcoholic fatty liver disease
NCDs	Non-communicable disease
OGTT	Oral glucose tolerance test
Ox-LDL	Oxidized low-density lipoprotein
PAH	Pulmonary arterial hypertension
PRRs	Pattern recognition receptors
RAAS	Renin angiotensin aldosterone system
RNAs	Ribonucleic acids
SADHS	South African Demographic and Health Survey
SAGE	Global Aging and Adult Health survey
SBP	Systolic blood pressure
SGK3	Serum and glucocorticoid inducible kinase 3
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-β1	Transforming growth factor beta 1
THBS2	Thrombospondin-2
TNF-α	Tumour necrosis factor alpha
TRBP	Dicer/TAR RNA binding protein

CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Non-communicable diseases (NCDs) are the leading cause of death globally, with the majority of mortalities attributed to cardiovascular diseases (CVD) in comparison to other NCDs (Roth et al., 2017). Two of the major contributors to CVD are type 2 diabetes mellitus (T2DM) and hypertension. The prevalences of these two cardiometabolic diseases (CMDs) are on the rise in low-to-middle income countries (LMICs), which make up the majority of the African continent (Jamison, 2006; Kappa et al., 2020). South Africa has the 4th highest age-adjusted diabetes prevalence globally at 10.8%, and the highest number of people living with diabetes on the African continent (International Diabetes Federation, 2021). Worryingly, 52.4% of people living with diabetes remain undiagnosed (International Diabetes Federation, 2021). The CMD burden is further compounded by hypertension, which is prevalent in 10.2% of South Africans aged between 15 and 25 years (Day et al., 2015), and in 40% of people aged 25 years and above. Although the pathophysiology of T2DM and hypertension is somewhat different, both diseases share similar risk factors, and the onset of one greatly increases the chance of developing the other (Mitchell et al., 1990; Akalu & Belsti, 2020). Additionally, both T2DM and hypertension have complex aetiologies in which inflammation, oxidative stress and epigenetic mechanisms play significant roles (Reddy & Natarajan, 2011).

In CMDs like diabetes, timeous interventions are crucial if downstream complications are to be avoided, and these timeous interventions are facilitated by early diagnosis. However, currently adopted diagnostic methods are unable to diagnose these CMDs in the early disease process. Additionally, these methods are not applicable to all populations. For example, in people with haemoglobinopathies, the use of glycated haemoglobin (HbA1c) to diagnose or monitor diabetes is suboptimal. As such, there is a need to explore alternative diagnostic methods to counter these shortcomings. Genes are involved in blood glucose metabolism and elevated blood pressure which are respectively associated with T2DM and hypertension (Franceschini & Le, 2014; Li et al., 2020; Wang et al., 2023). MicroRNAs (miRNAs) play a key role in gene regulation in different proteins that are critical for blood glucose, insulin secretion and endothelial dysfunction in the development of T2DM and hypertension (Filipowicz et al., 2008; Nemecz et al., 2016; Zhu & Leung, 2023; Shrivastav & Singh, 2024). Considering this, an epigenetic and personalised diagnostic approach seems to be the next big step in population-based therapy (García-Calzón et al., 2020; Kowluru & Mohammed, 2022; Yang et al., 2022; Shrivastav & Singh, 2024).

1.2 Diabetes mellitus

1.2.1 Classification of diabetes mellitus

Diabetes mellitus (DM) is a group of chronic disorders characterised by dysregulated glycaemic levels (Forouhi & Wareham, 2010), which present as persistently elevated blood glucose or abnormal spikes in glucose levels after carbohydrate rich meals (Bogardus et al., 1984; Reaven 2005; Her et al., 2020). This may be due to defective insulin action and/or secretion (Kobayashi & Olefsky, 1979; Bizzotto et al., 2021). A combination of factors such as family history of diabetes, obesity, ethnicity, age, environmental exposures like pathogens and stress as well as sedentary lifestyles and a lack of physical activity may favour a shift from euglycemia to hyperglyceamia (Bereda, 2022; Ho et al., 2023; Khalil et al., 2023).

DM can be classified into type 1 diabetes mellitus (T1DM) which accounts for close to 10% of diabetes cases andT2DM, which accounts for 90% of diabetes-related cases. Other types including gestational diabetes mellitus (GDM), maturity onset diabetes of the young (MODY) and latent autoimmune diabetes in adults (LADA) account for 1% of the cases (International Diabetes Federation, 2021). The two main forms, which constitute the majority of diabetes cases are: T1DM – which occurs idiopathically or as a result of autoimmune destruction of pancreatic β-cells, and T2DM – which occurs as a result of insulin resistance attributed to physio-pathological changes such as reduction in available insulin receptor binding spots, mutation of receptors and insulin receptors (IR) blocked by antibodies (American Diabetes Association, 2011; Li et al., 2022).

1.2.1.1 Type 1 diabetes mellitus

Genetics play a significant role in the development of T1DM. In 1987, Risch showed that there was a 44% risk of developing T1DM through genetic inheritance of the human leukocyte antigen (HLA), whilst in 1996, Noble et al. reported a 53% risk of developing insulin dependent DM through HLA class II allele inheritance (Risch, 1987; Noble et al, 1996). Another risk factor for T1DM stems from destruction of pancreatic β cells by autoantibodies produced by the immune system. Pancreatic β cells are even more susceptible to the immune system because the islet cells of the pancreas are highly vascularised, which favours interaction with immune cells. Furthermore, the insulin and granules secreted by the pancreatic β cells are target antigens of islet autoimmunity (Mallone & Eizirik, 2020). In stage one of T1DM development, glycaemic control is still normal, whilst in stage 2, glycaemic control becomes compromised, but without notable symptoms. From stage 3, a clinical diagnosis can be made, whilst stage 4 represents overt T1DM (Greenbaum et al., 2018).

1.2.1.2 Type 2 diabetes mellitus

T2DM results from the human body's inability to regulate blood glucose levels and the failure to adapt to extreme and constant hyperglycaemia. As the transportation of glucose is interrupted once it arrives in the bloodstream by mediators such as insulin which promotes the transport of glucose out of the blood. The reduced interaction of insulin is due to various factors such as reduction of IR, over worked β cells that produce more insulin growth like factors that bind poorly to IR (Mallone & Eizirik, 2020; Li et al., 2022). Risk factors for T2DM include being overweight, physical inactivity, family history, age, race, smoking and high blood pressure (Harris et al., 1999; Garber, 2012; Maddatu et al., 2017; Kral et al., 2019; Nwankwo et al., 2019; Sattar et al., 2019; Lee et al., 2020; Yang et al., 2024). Poor glucose control leads to long term complications such as neuropathy, nephropathy, retinopathy, CVDs, foot complications and impaired wound healing (Dangwal et al., 2015; Wang et al., 2017; Maiya et al., 2018; Feldman et al., 2019).

1.2.1.3 Gestational diabetes mellitus

The onset of GDM presents itself during the third trimester of pregnancy, as insulin insensitivity develops rapidly as the pregnancy progresses (Catalano et al., 1991; Buchanan & Xiang, 2005; Sonagra et al., 2014). However, the condition resolves itself after pregnancy. However, individuals who have developed GDM are at an increased risk of developing T2DM and so are their offspring.

1.2.1.4 Other types of diabetes mellitus

DM can be categorised as polygenic or monogenic, with T1DM and T2DM being the polygenic forms, whilst syndromic and non-syndromic forms of diabetes such as MODY and neonatal diabetes are described as monogenic forms of diabetes (Tosur & Philipson, 2022). Other specific types of diabetes include Wolfram Syndrome, Alström Syndrome, LADA, Type 3c diabetes, steroid-induced diabetes and cystic fibrosis diabetes (Alstrom et al., 1959; Inoue et al., 1998; Strom et al., 1998; Marshall et al., 2005; Hwang & Weiss, 2014; Liu et al., 2020; Wu et al., 2020).

1.2.2 Epidemiology of type 2 diabetes mellitus

The prevalence of diabetes is rising at an alarming rate both regionally and globally, and as per the International Diabetes Federation (IDF) Diabetes Atlas, in 2021, a staggering 536.6 million people between the ages of 20 to 79 years were reported to be affected by diabetes (International Diabetes Federation, 2021). Furthermore, this worldwide estimate is expected to increase by 46% (783 million) by the year 2045.

Moreover, although previously only considered an epidemic in developed countries, the growing incidence is extending to developing countries, with concerns being raised in LMICs such as those in Africa (Godman et al., 2020).

Worryingly, the African region can expect a disproportionate 129% rise in the number of individuals living with diabetes by the year 2045, the highest projected increase globally (International Diabetes Federation, 2021). In addition, the region has the highest rates of undiagnosed diabetes, amongst all IDF regions, at 54% (International Diabetes Federation, 2021). One of many factors could be the rapid urbanisation, as a study from West Africa found the prevalence of T2DM and impaired fasting glucose (6.2%) in urbanised areas was more than double that of rural areas (2.5%) (Issaka et al., 2023). Figure 1.1 below shows globally how far more severe Africa will be affected by current estimations compared to other IDF regions.

According to the IDF, the prevalence of diabetes in South Africa is 11.3%, and 4.2 million adults are currently living with diabetes (International Diabetes Federation, 2021). The first round of the South African National Health and Nutrition Examination Survey (SANHANES-1) reported the following ethnic diabetes prevalence estimates amongst individuals aged 15 years and above from the South African general population: African ancestry (8.9%), mixed ancestry (9.9%), Caucasian (16%), and Indian (32.2%) (Sifunda et al., 2023).

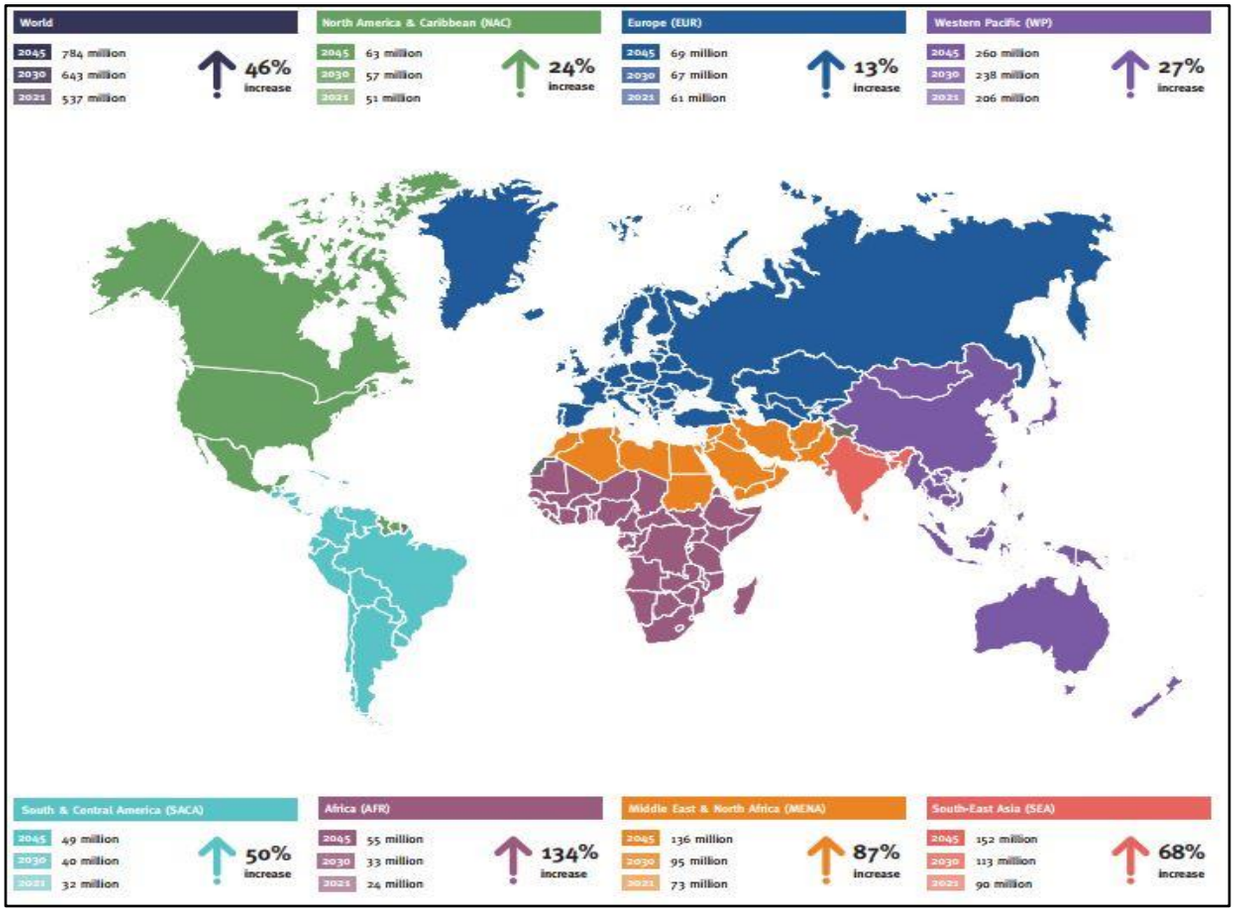

Figure 1.1: Adults living with diabetes around the globe (International Diabetes Federation, 2021).

1.2.3 Diagnosis of type 2 diabetes mellitus

Dysregulation in glycaemia can be measured by various tests, but the predominant ones are measuring fasting glucose levels, administering an oral glucose tolerance test, and HbA1c test.

1.2.3.1 Fasting blood glucose

Participants are required to fast a minimum of eight hours before a blood sample is collected from them to determine blood glucose values. Normal fasting blood glucose values range between 3.9 mmol/l and 5.6 mmol/l, whilst fasting blood glucose values for prediabetes lie between 5.6mmol/l to 6.9mmol/l, and for those living with diabetes 7 mmol/l and above (Zhou et al., 2016). A few drawbacks to this test include a lengthy conduction time, and the result accuracy depends on participants' adherence to the fasting requirement before testing. Furthermore, the test results require additional verification by other confirmatory tests, hence on its own, the fasting blood glucose test functions as a screening method.

1.2.3.2 Oral glucose tolerance test (OGTT)

The OGTT is the current gold standard method for T2DM diagnosis. The participant is administered 75g of glucose and the glycaemic changes are observed (WHO Expert Committee, 1980). A plasma glucose reading of 7.8-11mmol/l indicates impaired glucose tolerance (prediabetes), whilst a plasma glucose reading above 11mmol/l indicates diabetes. However, the OGTT comprises of both a measurement of fasting blood glucose value and then glucose administration test to be conducted for diagnosis to be made. The OGTT method is used to rule out possible falsely elevated readings obtained in the fasting glucose measurement or in HbA1c readings (Alzahrani et al., 2023). Some pitfalls with the current use of OGTT and fasting blood glucose test is how much time it consumes to perform. Additionally, to administer a patient with a load of glucose while their glycaemic control is already poorly regulated adds unnecessary strain to validate the outcomes. As such, research to identify a less time intensive and stress inducing alternative is warranted.

1.2.3.3 Glycated haemoglobin (HbA1c)

Glycated haemoglobins form about 6% of total haemoglobins, and HbA1c is a ketoamine which takes over a span of 120 days to form and is the product of condensation of a haemoglobin and glucose (Bunn et al., 1975; Bunn et al., 1976). Due to the gradual process leading to the formation of HbA1c, it gives a relative indication of blood glucose trends over two to three months. If the HbA1c reading is between 5.7% and 6.5%, an OGTT needs to be conducted to rule out any false indications, and anything above 6.5% is also considered to be a diabetic state (Petersmann et al., 2019). Some concerns relating to HbA1c are that there are cases of genetic predisposition to increased HbA1c levels or underestimating through HIV treatment inhibiting nucleoside reverse transcriptase (Bergman et al., 2020). A meta-analysis also found HbA1c to have a low sensitivity towards prediabetes diagnosis and a high number of false negative results (Barry et al., 2017).

1.2.4 Aetiology of type 2 diabetes mellitus

The pathogenesis of T2DM is multifactorial, and genetic predisposition, environmental factors such as sedentary lifestyle, dietary intake, excessive alcohol consumption and cigarette smoking increase the risk for the progression of hyperglycaemia (DeFronzo, 2004). Furthermore, the risk of development of hyperglycaemia is increased in individuals who are overweight/obese and/or have a family history of T2DM. The risk is further exacerbated by the presence of hypertension (Mouri & Badireddy, 2022). T2DM is a

multisystem disease and increases the risk of developing CVDs like peripheral vascular disease, accelerated atherosclerosis, premature coronary artery disease and cerebrovascular disease through the immense strain put on the micro- and macrovascular systems (Haffner et al., 1998; Beckman et al., 2002; Nesto, 2004). Gallicia-Garcia and colleagues highlighted the complexity of T2DM as it affects several molecular systems leading to β cell dysfunction, gut dysbiosis, metabolic memory, dysregulated miRNA expression, oxidative stress, inflammation and endothelial dysfunction (Galicia-Garcia et al., 2020). Metabolic memory refers to the adverse effects of short-term abnormalities in glucose metabolism that translate into long term health concerns (Dong et al., 2024).

The development of T2DM is due to the nature of the physiological changes that take place when glycaemic control changes over time. This is demonstrated in the desensitization of the IR undergoing endocytosis in HepG2 cells by the Src homology phosphatase and mitogen activated-protein kinase pathways (Hall et al, 2020). Most of these IR are found in the liver, muscle tissue and adipose tissue (Escribano et al., 2017). The continuous endocytosis process of the IR is otherwise known as insulin resistance (Escribano et al, 2017; Hall et al, 2020). Normal blood glucose levels start off slightly elevated and the hormonal control of insulin becomes less sensitive. This, in essence, is when prediabetes starts, as the normal mean value of blood glucose is above the normal healthy ranges but below the diabetic ranges (Rooney et al., 2023). Different mechanisms and modulators play a direct and indirect role on insulin activity and regulation. It should be noted that a vast number of studies indicate that with the expansion of adipose tissue, there is an increase of immune cells which drive chronic inflammation and dysregulated metabolic pathways such as insulin resistance (Schipper et al., 2012; Xu et al., 2013; Russo & Lumeng, 2018; Bäckdahl et al., 2021; Kolb, 2022).

1.3 Hypertension

Prolonged increase of circulating blood pressure, where normal resting blood pressure values are elevated is termed hypertension (Whelton, 1994; Mills et al., 2020). It is a leading modifiable risk factor for CVDs and contributes to premature death (Mills et al., 2020). Hypertension is categorised into essential/primary hypertension when there is no identifiable cause of the prolonged blood pressure increase, and secondary hypertension when there are known causes for the above normal blood pressure levels (Sánchez-Lozada et al., 2023; Hall et al., 2024). The former accounts for 90-95% cases of hypertension, whilst the latter accounts for the remaining 5-10% of cases.

1.3.1 Epidemiology of hypertension

Worldwide, hypertension is the leading cause of cardiovascular related disease (Stanaway et al., 2018). Estimations on a global scale suggest that 31.1% of adults in 2010 had hypertension (Roth et al., 2018). Even with the widespread use of antihypertensive medication, the prevalence of hypertension has and continues to increase, especially in LMICs (Mills et al., 2016). In 2019, the estimated global hypertension prevalence was 32%, with over 65% of people living with hypertension residing in LMICs (Lu et al., 2019). If measures are not taken to address this trend, the burden of NCDs will continue to wreak havoc on healthcare delivery systems, particularly in these LMICs.

Hypertension is more prevalent in LMICs in comparison to high income countries (Ibrahim & Damasceno, 2012), and its prevalence on the African continent ranges between 37% to 75%, depending on the country (Akpa et al., 2020). It was reported that between 1998 and 2003, the prevalence of hypertension in South Africa rose dramatically from 54% in 1998, to 78% in 2003 in people 55 years and older (Ibrahim & Damasceno, 2012).

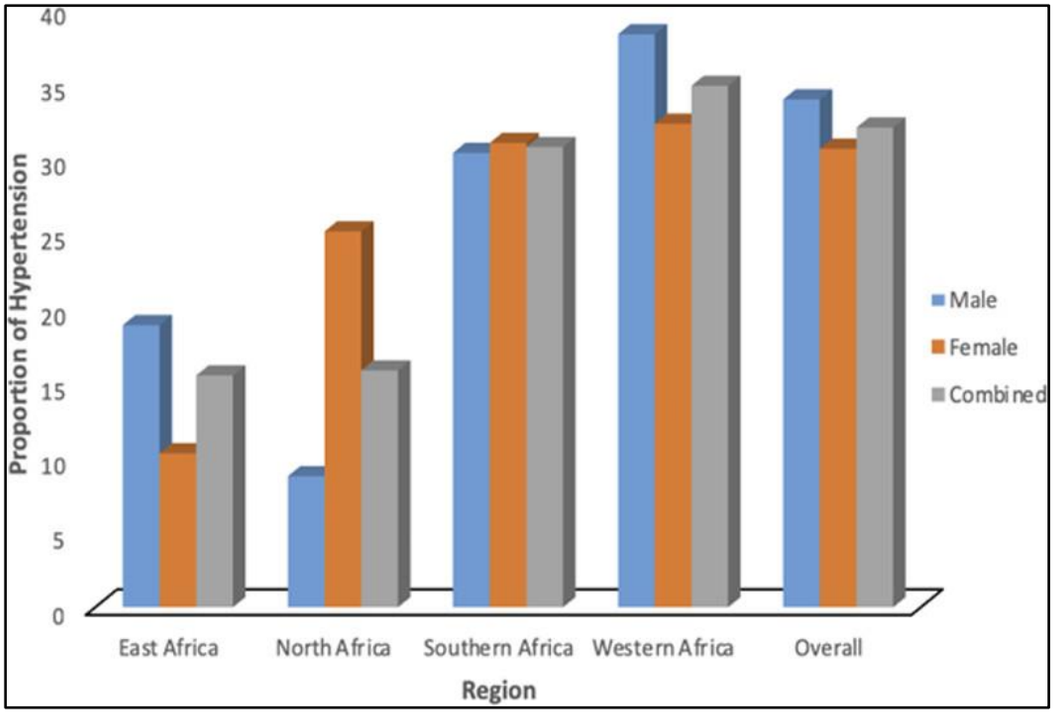

Figure 1.2: Age-adjusted regional hypertension prevalence (Kappa et al., 2020).

1.3.2 Diagnosis of hypertension

The international guidelines for hypertension in an office setting are shown in Figure 1.3 below.

Category	Systolic (mm Hg)		Diastolic (mm Hg)
Normal BP	<130	and	<85
High-normal BP	130–139	and/or	85–89
Grade 1 hypertension	140–159	and/or	90–99
Grade 2 hypertension	≥160	and/or	≥100

Figure 1.3: International guidelines for diagnosing hypertension within office settings (Unger et al., 2020).

The guidelines for defining hypertension for study purposes were defined by Mancia et al., (2013) and adjusted in 2014 by Seedat et al., for applicability in a South African setting (Mancia et al., 2013; Seedat et al., 2014). The office blood pressure has set groupings as follows: Normal <120 mmHg systolic blood pressure (SBP) and <80 mmHg diastolic blood pressure (DBP); Optimal: SBP of 120-129 mmHg and DBP of 80-84 mmHg, High normal: 130-139 SBP mmHg and 85-89 DBP mmHg, Grade 1 hypertension: 140-159 SBP mmHg and 90-99 DBP mmHg, Grade 2 hypertension: 160-179 SBP mmHg and 100-109 DBP mmHg, Grade 3 hypertension: SBP >180 mmHg and DBP >110 mmHg. Hypertension in the South African setting ranges within the three grades outlined above. However, it should be noted that those who are in the high normal ranges are already at risk for CVD (Seedat et al., 2014). Current guidelines used to diagnose hypertension on the African continent are adapted from European data, although numerous studies have reported the vast differences between these populations. As such, there is a need to explore more population-specific guidelines for hypertension diagnosis.

1.3.3 Aetiology of hypertension

The pathogenesis of hypertension and the progression towards disease states is influenced by non-modifiable factors such as genetics, gender and advanced age (Poznyak et al., 2022). Whilst age is an established risk factor for hypertension, one study reported that earlier onset of hypertension resulted in a 24.5% increase in target end-organ damage affecting two or more organs (Suvila et al., 2019). Additionally, as hypertension falls under geriatric disease when only age is at work, the disease can be triggered much earlier through modifiable factors such as smoking, alcohol consumption, physical inactivity and body weight (Ng et al.,2020; Rausch et al., 2022).

It has been previously reported that being hypertensive is associated with a 2.5-fold risk of developing T2DM, making diabetes a risk factor of hypertension (Gress et al., 2000). Hypertension contributes to macro-vascular dysfunction, and this in turn, contributes to the progression towards T2DM development, indicating the vascular relationship these pathological conditions share and making hypertension a risk factor of T2DM (Climie et al., 2019). The anomalous vasoconstriction action within the circulatory system which is constantly engaged while vasodilators actions are inhibited and is sustained by inflammation further influence the disruption of circulatory homeostasis (Valentin et al., 2007). Additionally, a study conducted on Sub-Saharan Africans, found the biggest comorbidity for T2DM to be hypertension (Ekoru et al., 2019). Both T2DM and hypertension have pathophysiological homeostatic shifts such as endothelial dysfunction driven by low grade chronic inflammation (Sinha & Haque, 2022). This just briefly goes to show the complex relationship between both diseases and how chronic inflammation plays a vital role in both.

1.4 Chronic Inflammation in the pathogenesis of type 2 diabetes and hypertension

Inflammation is a mechanism the body uses to remove foreign and harmful stimuli while restoring the functionality of involved structures (Takeuchi & Akira, 2010). Recognition of these detrimental stimuli is done through pattern recognition receptors (PRRs) such as mannose receptors, glucan receptors, scavenger receptors and Toll-like receptors (Barret et al., 2016). Innate immune response is normally triggered by PRRs and includes the release of cytokines by various cells including natural killer cells, neutrophils, dendritic cells, mast cells, macrophages and eosinophils, depending on the physiological need (Turvey & Broide, 2010). Cytokines are protein molecules that are produced by many cells for communication and immune response regulation (Kany et al., 2019). The immune system responds to invading organisms or foreign substances through the short-lived release of cytokines and other protective molecules. However, when there is an imbalance in this protective response, it persists beyond its initial requirement, potentially causing more harm than good, through chronic inflammation.

Pro-inflammatory cytokines are produced by activated immune cells and mediate cell communication and inflammation. Chronic inflammation is normally at the base of disrupting homeostasis, leading to pathological conditions such as T2DM and hypertension (Zhang & An., 2007). In the pathological progression of T2DM, which is tightly linked to the altered proliferation, functions and components of the adaptive and innate immunity, it is important to investigate cytokines and how they influence metabolic disturbances (Zhou et al., 2018).

Inflammation is regulated through the action of pro-inflammatory and anti-inflammatory cytokines (Dinarello, 1997; Opal & DePalo, 2000). In chronic inflammation, the imbalance is clear, where pro-inflammatory

cytokines are overexpressed, as is characteristic of CMDs such as T2DM and hypertension (Sinah & Haque, 2022). Dysregulation of the innate immune cells, predominantly neutrophils, drives sterile metabolic inflammation, which is also known as chronic inflammation (van de Vyfer, 2023). However, the key drivers behind chronic metabolic inflammation are adipose tissue expansion and insulin resistance (van de Vyfer, 2023). A chronic inflammatory state increases susceptibility for infections and cancers as the immune system becomes exhausted (Shruthi et al., 2022). As such, the body has measures in place to ensure tight regulation of the inflammatory process. For example, the pleiotropic cytokine interleukin 10 (IL-10) has a fundamental role in cell homeostasis. It acts as an anti-inflammatory cytokine to prevent uncontrolled immune response through the Janus kinases/ non-receptor tyrosine-protein kinase (Jak1/Tyk2) and STAT3 signalling pathway (Carlini et al., 2023).

1.4.1 Inflammation in type 2 diabetes mellitus

Clinically, a correlation has been observed between insulin resistance and chronic inflammation (Wieser et al., 2013). Skeletal muscle and other tissues develop insulin resistance from pro-inflammatory mediators released by adipose tissue, the liver and associated immune cells (Shoelson et al., 2006). However, studies that identified key cytokines in the development of T2DM found little therapeutic ability in blocking these cytokines, suggesting a more complex aetiology beyond cytokine action only (Dominguez et al., 2005; Gonzalez-Gay et al., 2006). In obesity-driven inflammation, it was observed that it leads to systemic insulin resistance and decrease of β cell mass and is indicative that inflammation on that scale contributes to β cell death (Bays, 2011).

Certain cytokines seem to be more significantly differentially expressed in the prediabetes state, as is the case with IL-8 (Lopez et al., 2017). IL-8 is a chemoattractant cytokine produced by monocytes, macrophages, fibroblasts but distinctly targets neutrophils (Matsushima & Oppenheim, 1989; Bickel, 1993). IL-8 possibly contributes towards pathogenesis and complications in T2DM by infiltrating and activating adipose tissue (Elgazar-Carmon et al., 2008; Makki et al., 2013). In T2DM and obesity, the upregulation of IL-8 is heavily influenced by oxidative stress (Tomita et al., 2003; Marseglia et al., 2014). Other studies also found increased expression in IL-8 in T2DM patients compared to healthy controls (Cimini et al., 2017; Mohammed et al., 2018). IL-6 is also associated with the pathogenesis of T2DM, and it promotes insulin sensitivity and is associated with glucose metabolism (Akbari & Hassan-Zadeh, 2018). While dysregulation of the aforementioned cytokines predominantly contributes to the pathogenesis of dysglycaemia, there are other ones such as IL-10, an anti-inflammatory cytokine, which are important for tissue homeostasis (Iyer & Cheng, 2012).

In one study, the expression of IL-10 was reported to be lower in a T2DM population compared to those living without T2DM (van Excel et al., 2002), whilst another study echoed those results by reporting a decreased expression of IL-10 and elevated HbA1c reading in their T2DM population (Acharya et al., 2015). However, a study from South Africa scoping hypercoagulation and abnormal clot formation found upregulation of IL-10 in T2DM patients (Randeria et al., 2019). These discordant findings on the expression of IL-10 in T2DM population warrants further investigation. While a biomarker on its own can indicate one thing, a ratio between them can further indicate if the change was a homeostatic response or potentially pathogenic. It was observed that the ratio of IL-1β / IL-10 was greater in an African descendant population compared to Caucasian population and lower expression of IL-10 in Caucasian population than in African descendent population in a study done within South Africa (Crouch et al., 2020). As such, difference in ancestral genetics might explain differences in IL-10 expression.

Tumour necrosis factor alpha (TNF-α) is a regulatory cytokine renowned for its multiple roles and is involved in inflammation, cell apoptosis, cytotoxicity, production of cytokines and onset of insulin resistance (Duprez et al., 2011; Günther et al., 2011). As mentioned earlier, obesity is associated with T2DM and TNF-α is a key driver of insulin resistance in adipose tissue (Hotamisligil et al., 1996; Yuan et al., 2001; Malone & Hansen, 2019). However, it should be noted that in some cases, there are indications of obese states in which systemic insulin sensitivity is preserved (Ruderman et al., 1981; Unger & Scherer, 2010; Vishvanath & Gupta, 2019).

1.4.2 Inflammation in hypertension

Some of the characteristics in hypertensive inflammatory driven states are conditions such as arterial remodelling, vascular fibrosis, endothelial dysfunction, and atherosclerosis (Sinah & Haque, 2022). Increased C-Reactive Protein (CRP) activity promotes the activity of pro-inflammatory cytokines such as IL-6, IL-1β and tumour necrosis factor- α (TNF-α). CRP can mediate phagocytosis, expression of adhesion molecules and immune responses which promote further inflammation and is considered a prototypic marker of inflammation (Devaraj et al., 2011; Akhmedov & Sharipov, 2023). While elevated concentration of CRP is observed with vasodilator dysfunction and hypertension, it does not directly contribute to the elevation of blood pressure (Fichtlscherer et al., 2000; Smith et al., 2005). Cytokines produced in hypertension by T cells like IL-17A and interferon gamma (IFN-γ) are related to end-organ damage and vascular dysfunction (Caillon et al., 2019). IL-17A activity is driven by hepatic stellate cells activation and it is the resultant positive feedback to those hepatic stellate cells and gamma delta T (γδT) cells that propagates inflammation and

fibrosis. These physiological changes promoted by IL-17A causes vascular dysfunction and hypertension through thickening of vascular circulatory lining, atherosclerosis and cardiac muscle alterations (Caillon et al., 2019). In addition to the above mentioned pro-inflammatory cytokines, monocytes and macrophages also play a major role in the inflammatory cascade in hypertension (Loperena et al., 2018).

Some studies report that the activity of cytokines could be influenced or mediated by small, non-coding RNA transcripts known as miRNAs, by regulating the expression of genetic information used to direct the assembly of immune cells (Asirvatham et al., 2009). As miRNAs are key components in interacting with cytokines, they may be potentially pivotal regulators of the chronic inflammation underlying T2DM and hypertension development and progression. For example, miR-92a is regulated by IL-6 in human pulmonary artery endothelial cells (HPAEC) by signalling through STAT3, which leads to a reduction of bone morphogenetic protein receptor type 2 BMPR2, a pathogenic hallmark of pulmonary hypertension (Brock et al., 2009). Another case is where profibrogenic cytokine production is mediated by miRNAs in hepatic stellate cells (HSC) (Eguchi et al., 2020). It should be noted that miRNAs also adjust immune responses, as exosomal miRNAs have auto-and-paracrine regulatory abilities, thus posing more a back-and-forth interaction between miRNAs and cytokines (Iftikhar & Carney, 2016; Artimovič et al., 2024). By leveraging the influence of circulatory miRNAs on immune responses, we can get a better understanding of the inflammatory processes related to T2DM and hypertension, as well as the epigenetic mechanisms involved in their development and progression (Corrêa & Rogero, 2018; Qu et al., 2018).

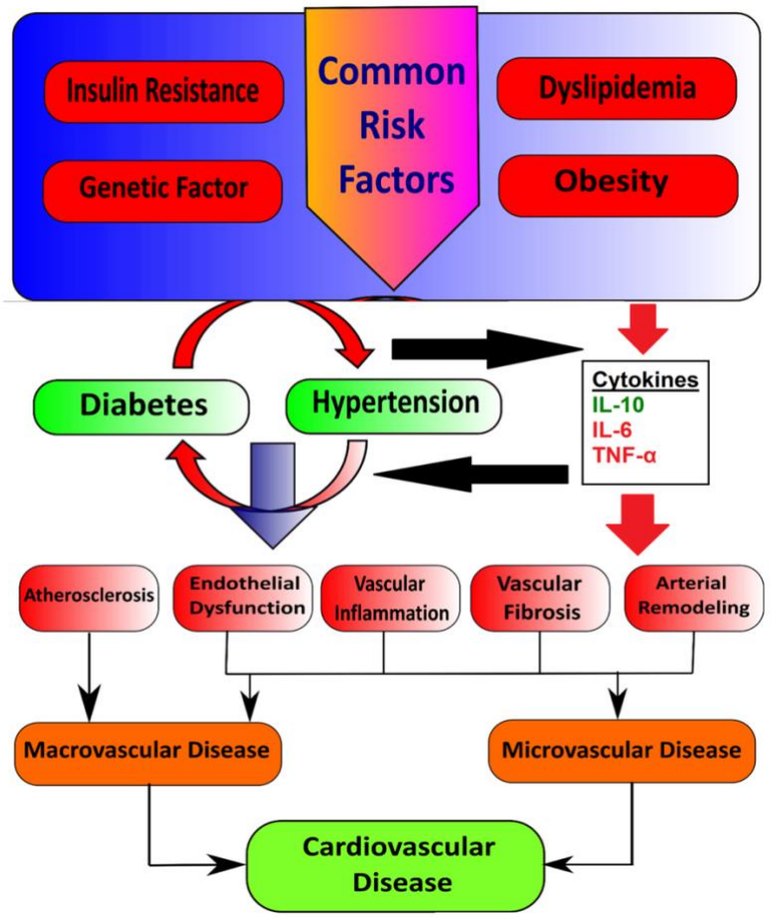

Figure 1.4: Summary and interplay of risk factors, cytokines, disease states that lead to CVD (Adapted from Sinha & Haque, 2022).

1.5 MicroRNAs

MiRNAs form part of a group of endogenous small non-coding RNAs whose length ranges from 16 to 27 nucleotides (nt) in length, although the majority of these mature miRNAs are 22nt in length (Fang et al., 2013). Most miRNAs are located in intracellular spaces but can also be found in extracellular environments within plasma (Lee et al., 1993; Nelson et al., 2003; O'Brien et al., 2018; Zhang et al., 2020) and are involved in diverse cellular and physiological processes including differentiation, proliferation, regulation, angiogenesis and apoptosis (Bartel, 2009; Li et al., 2009; Shu et al., 2017 Bartel, 2018).

1.5.1 Brief history on MicroRNAs

The joint discovery of the first miRNA, *lin-4*, was in nematodes *Caenorhabditis elegans* (*C. elegans*) by two laboratories in 1993, and this finding set down the pioneering groundwork for miRNA studies (Lee et al.,

1993; Wightman & Ruvkun, 1993). It was not until seven years later that a second miRNA, a hetero chronic gene of *C. elegans* named *let-7*, was identified (Reinhart et al., 2000), and over the last two decades, approximately 2300 mature human miRNAs have been described (Alles et al., 2019). A new profound focus grew on miRNAs as a possible novel angle for cancer research after miRNA discovery. MiRNAs were predominantly targeted in cancer research due to their dysregulated expression in various cancers, as well as their tumour suppressor and oncogene activity (Garzon et al., 2009). The diversity in applied research branched out extensively from cancer to cardiovascular, autoimmune, neurodegenerative diseases and molecular biomarkers of disease (Kwon et al., 2005; Sonkoly et al., 2007; Fiore et al., 2008). Currently, miRNA expression patterns are being investigated as biomarkers for cellular pathways in various human samples, including tissue (cancer biopsies), plasma, urine, saliva, serum and other human fluids. This further opens the possibility to explore diverse methods for obtaining accurate sources for biomarkers (Cortez & Calin, 2009).

With studies focusing on CMDs such as T2DM and hypertension, it became evident that miRNAs play a profound role at different stages of these diseases. For example, it has been reported that miRNAs are involved in insulin production, secretion and interaction, hence may have a role to play in the development of diabetes (Shantikumar et al., 2012). It has also been shown that miRNAs regulate endothelial functioning, nitric oxide production/release and the Renin-Angiotensin-Aldosterone-System (RAAS), thus suggesting their possible role in blood pressure regulation and the development of hypertension (Nemecz et al., 2016). From the extensive range of different miRNAs and their broad influence on different biological processes, their role in disease is quite complex, though an understanding of their biogenesis may make the picture clearer.

A growing interest in using miRNAs as a more accurate and less invasive way of identifying individuals with prediabetes and other CMDs could be the future for better diagnostic options (Matsha et al., 2018; La Sala et al., 2019). The expression of a wide range of miRNAs can be measured in bodily fluids such as plasma, tears, seminal fluid, urine, breast milk, saliva, pleural fluid, amniotic fluid, bronchial lavage, colostrum, cerebrospinal fluid and peritoneal fluid (Weber et al., 2010). MiRNAs may be carried by exosomes, which are extracellular membranous vesicles released by cells and thus mediate cell-to-cell communication and regulate various physiological pathways involved in paracrine functioning (Mathivanan et al., 2010). Over the years, extracellular vesicle-derived miRNAs have been shown to yield great diagnostic value for diseases of cardiovascular and metabolic origin (La Sala et al., 2019; Ojha et al., 2019; Zhang & Huang, 2021). Due to the non-specificity observed when investigating whole blood miRNA expression in disease, the need has arisen for thorough investigations relating to the value of more specific extracellular derived miRNAs, and

the diagnostic value they have for onset of a disease and treatment (Xu et al, 2022). As such, using selected miRNAs that cater for cellular signalling with a specific cytokine profile may improve our understanding of the immune-mediated and epigenetic mechanisms in the pathogenesis of T2DM and hypertension, thus bridging the gap on how these diseases can be diagnosed or treated.

1.5.2 Biogenesis of microRNAs

The biogenesis of miRNAs and the different ways in which they are secreted into circulation is illustrated in figure 1.5 below. Primary miRNAs (pri-miRNAs) are transcribed from miRNA genes via RNA polymerase II activity. Following that, the resultant pri-miRNA transcripts are cleaved by Drosha/DGCR8 to form preliminary miRNAs (pre-miRNAs). These pre-miRNAs are transported across the nuclear membrane into the cytoplasm via Exportin-5 and are cleaved further by dicer/TAR RNA binding protein (TRBP) producing miRNA duplexes, however only one duplex of miRNA matures (Nelson et al., 2003; Zhang et al., 2020). Following biogenesis, mature miRNAs are then transported into circulation via three main mechanisms, namely: shuttling via lipoprotein complexes such as high-density lipoprotein (HDL), RNA binding proteins such as argonaut-2 (Ago2), as well as shuttling via extracellular vesicles (EVs), such as exosomes, micro vesicles and apoptotic bodies.

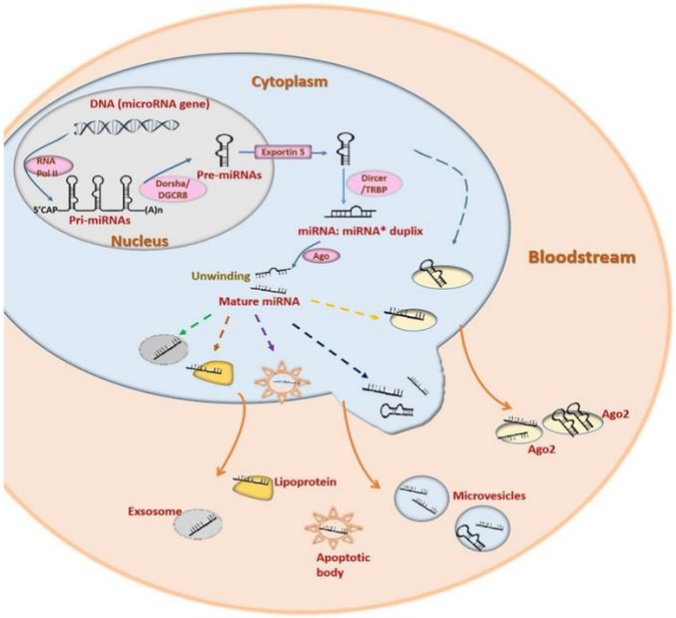

Figure 1.5: MicroRNA biogenesis from pri-miRNAs to pre-miRNAs to mature miRNAs (Zhang et al., 2020).

The two main sources of extracellular vesicles include exosomes and micro-vesicles as depicted in figure 1.6. Exosomes form from the inward budding of the endosomal membrane and progress towards multi

vesicular endosomes during the maturing phase. Micro-vesicles, on the other hand, form from outward budding and fission of the plasma membrane (Van Niel et al., 2018; Raposo & Stahl, 2019). Exosome derived miRNAs' main aim is towards post-transcriptional regulation and gene expression (Zheng et al., 2021).

Various studies have promoted miRNAs as candidate biomarkers for cancers and CMDs (Zernecke et al., 2009; Kosaka et al., 2013; Zhou et al., 2014), particularly, EV-derived miRNAs, which serve as potentially significant biomarkers as they mediate cell-to-cell communication (Fabbri et al., 2012). Moreover, these EV-derived miRNAs have been further reported as demonstrating regulatory functionalities within inflammation as well as dysregulation in disease states (Fritz et al., 2016). Thus, in combination with their functions in inter-tissue crosstalk, EV-derived miRNAs have been touted as potential biomarkers for CMDs (Prattichizzo et al., 2021).

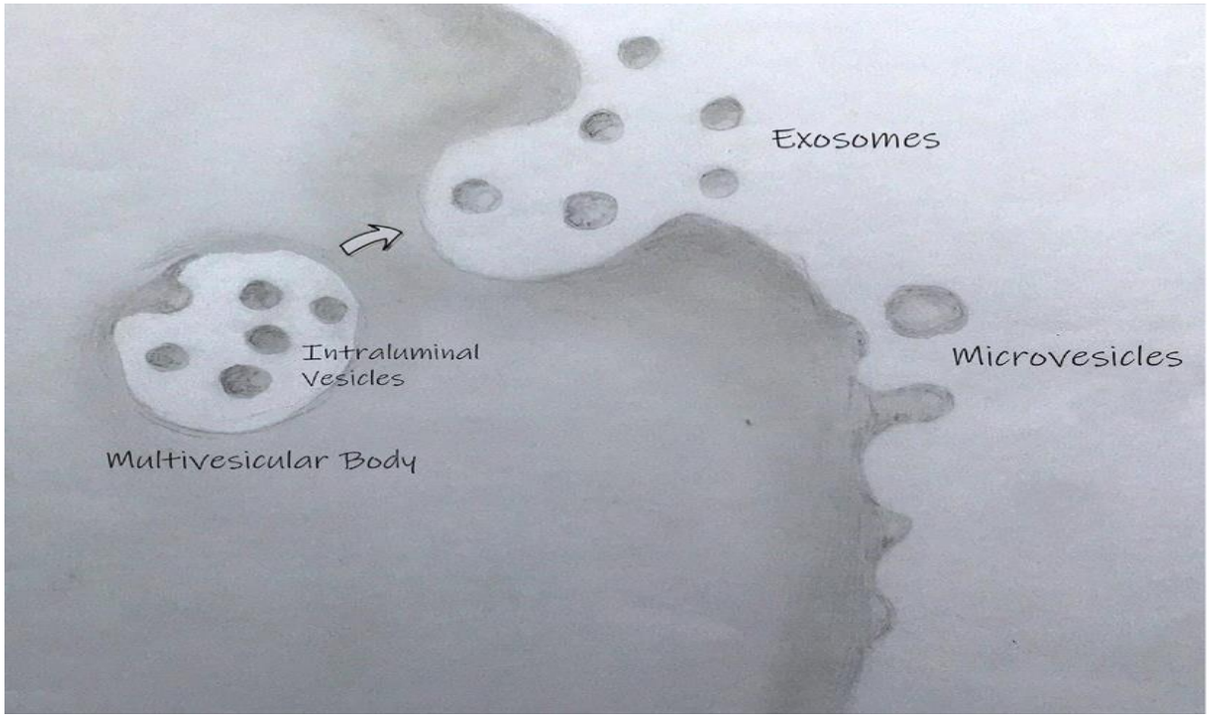

Figure 1.6: Extracellular vesicle biogenesis (Couch et al., 2021).

1.5.3 MicroRNAs in cardiometabolic diseases

Globally, it is suggested that miRNAs play a role in most biological processes, and studies have indicated their involvement in CMDs (Najafi-Shoushtari, 2011). Some of these miRNAs have been reported to contribute to vascular health by maintaining a functional endothelium (Araldi & Suárez, 2016), whilst others

regulate the vascular smooth muscles, further indicating the diversity and complexity of miRNAs functionality (Quintavalle et al., 2011). Their complexity is further highlighted in cardiac development and functioning, as the dysregulation of a single miRNA like miR-1 may contribute to multiple abnormalities such as arrhythmias, defective ventricular septation, cardiac hypertrophy and myocyte hyperplasia (Callis & Wang, 2008). Impaired endothelial functioning and stiffening of vascular smooth muscle cells is significantly related to hypertensive pathology (Akhmedov & Sharipov, 2023; Wang et al., 2019).

Other CMDs like chronic kidney disease (CKD) have been investigated via making use of miRNAs such as miR-125b, -145 and -155(Chen et al., 2013). Because of their involvement with vascular smooth muscle cells and resulting in a more proliferative phenotyping, they partake in regulating these cells and altered remodelling (Chen et al., 2013). Another study showed that in CKD, the expression levels of miR-130a-3b and miR-126-3p were reduced in coronary artery disease patients, which led to reduced proliferation in endothelial extracellular vesicle-recipient cells (Zietzer, 2022). Non-alcoholic fatty liver disease (NAFLD) falls under CMDs and is the most common form of liver disease (Younossi et al., 2018). A study of miR-21, 34a & -122 expression in NAFLD reported a correlation between miR-122 expression with fibrosis stage and inflammation activity in NAFLD patients (Cheung et al., 2008; Cermelli et al., 2011; Yamada et al., 2013). Extensive research has also been done on stroke and showed how up regulation of miR-15a, -16, -17-5p and downregulation of miR-23a were diagnostic markers for the condition (Jia et al., 2015; Wu et al., 2015; Tian et al., 2016). Thus, miRNAs have been used to do some considerable work in understanding CMDs.

MiRNAs have the capacity to be considered as biomarkers, as they are measurable characteristics of the human body that can indicate towards health, disease or response to intervention. A whole range of miRNAs are differently expressed in T2DM and hypertension development as they are involved in insulin signalling, glucose metabolism and vascular regulation (Chakraborty et al., 2014; Agbu & Carthew, 2021).

1.5.3.1 MicroRNAs in type 2 diabetes mellitus

The glycaemic response of the body is vital for ensuring a homeostatic environment for all other organs and tissues to function properly. Key modulators in this include the liver, which utilizes gluconeogenesis and glycogenolysis, and the pancreas through the secretion of insulin, proinsulin and amylin and glucagon (Adeva-Andany et al., 2016; Karpińska & Czauderna, 2022). MiRNAs are being recognised as regulators of glucose metabolism and homeostasis and are being pursued as potential biomarkers for metabolic status and disease (Mirra et al., 2018; Agbu & Carthew, 2021). For instance, circulating miR-126-3p has been proposed as a promising biomarker for T2DM, and has been reported to have cardioprotective properties in

states of hyperglycaemia (He et al., 2023). Circulating miR-126 has been reported to act as a preponderantly intercellular messenger stemming from endothelial cells and invoking internalization within vascular smooth muscle cells (VSMC) and monocytes (Wang et al., 2009). Another key miRNA linked to glucose homeostasis is miR-375. Pancreatic β cells are critical in terms of glycaemic homeostasis as they are the main source of insulin, and miR-375 which is expressed in pancreatic islets, has been identified as a regulator of β cell proliferation and insulin secretion via insulin exocytosis (Poy et al., 2004; Marchetti et al., 2018).

In the quest to find miRNAs that act as early indicators in the progressive development of T2DM, both miR-375 and miR-9 have been identified as possible indicators of the prediabetic stage (Al-Muhtaresh & Al-Kafaji, 2018). Using the luciferase assay, miR-9 was observed to be a regulator of syntaxin-binding protein 1 (*Stxbp1*) gene expression by targeting stxbp 1 messenger RNA 3′UTR and further plays a significant role in insulin secretion (Wang et al., 2018). In-depth appraisal for therapeutics in T2DM via EV-shuttled miRNAs were explored in a study where their preclinical findings suggest that altered secretion and loading of miRNAs were affected by diet and exercise with a need for further investigation (Prattichizzo et al., 2021). Furthermore, they suggest miR-155 enriched EVs promote insulin resistance, and circulating set of miRNAs (miR-122, -192, -27a & -27b) targets peroxisome proliferator-activated receptor α (PPARα) to induce hepatic insulin resistance (Prattichizzo et al., 2021). A study done by Monfared et al., (2022) showed that individuals with diabetes had a 6-fold increase of miR-182-5p in their saliva and serum, and serum miR-182-5p demonstrated a positive correlation with HbA1c (Monfared et al., 2022). There might be a pattern of pre-emptive rise of miR-182-5p in early stages or transitioning to pathological conditions, as the beneficial functioning of miR-182-5p either gets downregulated or stays the same, while other factors change. Although there are exosome studies done on miR-182-5p, mostly bone marrow derived, information regarding its effect in diabetes and the progression of the diseases is quite limited, especially regarding plasma exosomes. Furthermore, the majority of inflammatory exosome miR-182-5p studies focus on primarily cardiac or neurological function, with a paucity of studies focused on hypertension or T2DM. However, a study done on the expression profile of circulating miRNAs in whole blood found significant correlation between known diabetes and normal tolerant using miR-182-5p in a T2DM population (Weale et al., 2021). Other researchers further illuminated how miR-182-5p was significantly upregulated in prediabetes compared to those newly diagnosed with T2DM (Karolina et al., 2011). Additionally, miR-182-5p demonstrated greater diagnostic capabilities for prediabetes compared to HbA1c (Weale et al., 2021). This trend seems consistent as miR-182-5p is upregulated in the early stages of T2DM, and the expression decreases with time (Mir et al., 2022). A study that speculated that when miR-182-5p is downregulated it

could serve as a protective response found the opposite when investigating its role in myocardial infarction (Niu et al., 2023).

With improvements in technology, research into EV-derived miRNAs has made some significant strides. Pokharel and colleagues evaluated circulating plasma levels of miR-29a and miR-192 and found that the former could strongly indicate T2DM with an area under the curve (AUC) value of 0.95, whilst the latter was more indicative of prediabetes, with an AUC of 0.747 (Pokharel et al., 2024). Whilst a number of miRNAs have been investigated in the context of T2DM, not much has been done with regards to miR-182-5p, much less so in EV-derived sources relative to whole blood samples. However, with the few studies done on miR-182-5p, it has been shown to improve glucose control, diabetic wound healing, early detection of T2DM development, and the paramount role it plays in the inner workings of T2DM is clear (Sedgeman et al., 2018; Weale et al, 2020; Li et al., 2023).

1.5.3.2 MicroRNAs in hypertension

Investigating the role of miRNAs play in hypertension narrows down the scope of focus to organs such as heart, kidneys, lungs and circulatory-and-endocrine system. Within them, we find physiological structures such as vascular smooth muscle cells, endothelial cells and regulatory hormones being affected by how miRNAs are regulated. Then there is a debate on whether atherosclerosis is a cause or consequence of hypertension. However, what is clear is that the build-up of plaque and elevated blood pressure, both contribute to the deterioration of the circulatory system (Lonardo et al., 2018). It is speculated that arterial intimal thickening is one of the earliest contributors of physiological change to atherosclerosis (Milutinović et al., 2020). MiR-143 and miR-145 have been implicated in controlling intimal thickening after vascular injury (Cheng et al., 2009; Cordes et al., 2009), by targeting the KLF4, KLF5, ELK1, SP1 and myocardin pathways (Cheng et al., 2009; Khachigian, 2019). Vascular endothelium is pivotal for the regulation of the vessels within the circulatory system and miR-126 plays a pivotal role in modulating vascular development and homeostasis (Fish, et al 2009; Wang et al., 2009).

In the search for less invasive diagnostic information, studies zoomed in on miR-182-5p. One such study has shown how this miRNA inhibits the proliferation of vascular smooth muscle cells and activating the NF-kB, PI3K/AKT and extracellular signal-regulated kinase (ERK) signalling pathways relating to atherosclerosis (Jin et al., 2020). Making use of the weighted gene co-expression network analysis (WGCNA), researchers found an association between miR-182 and hypertension and described it as a critical role player in the angiogenesis-induced hypertrophic response of the heart (Li et al., 2016). Previous miRNAs that were

identified to be linked to hypertension in different populations were comprehensively examined in one study and showed the combination of miR-199a-3p, 208a-3p, 122-5p, and 223-3p had strong diagnostic performance for hypertension (AUC: 0.80) (Zhang et al., 2018).

1.5.4 MicroRNAs in cardiometabolic disease in Africa

Across the African continent, research progress is slowly taking foot on miRNAs pertaining to CMD. In Egypt, there were studies done on the impact of increased expression of miR-122 on insulin resistance. Their findings concluded that miR-122 could be a possible role player in the pathogenesis of obesity in children, and an early predictor for metabolic dysfunction (Rashad et al., 2019; Abdou et al., 2024; Abdou et al., 2024). In Burkina Faso, a study was done investigating the role of miR-33a, -33b in cholesterol metabolism in T2DM patients and found that those with a high prevalence for miR-33a was in the diabetic population (Koumaré et al., 2019).

Using RNA-sequencing, Matsha and co-workers identified already established and novel population-specific miRNAs associated with diabetes in a Bellville South community in Cape Town, South Africa, (Matsha et al., 2018). The validation study that followed investigated miR-182-5p as a potential screening and diagnostic option for prediabetes, and findings demonstrated the miRNA's superior discriminatory powers over HbA1c (Weale et al, 2020; Weale et al, 2021). In addition, it was reported that miR-126-3p, 182-5p and 30a-5p were associated with hypertension and highlighted its possible contribution in CMD development (Matshazi et al, 2021). Another miRNA which has been extensively investigated in cells and circulation is miR-29a, due to its influence on insulin sensitivity, as well as other metabolic pathways (Bagge et al., 2012; Lin et al., 2020). However, it is yet to be investigated in a mixed ancestry population, particularly in the South African context as the risk of T2DM and other CMDs is quite pronounced in that population. Furthermore, another miRNA that has demonstrated relevance to T2DM and hypertension pathogenesis, is miR-92a, as it has been investigated as a potential inflammatory biomarker (Wang et al., 2019).

1.5.4.1 MiR-29a

Huang and co-workers conducted a study and confirmed that exposing human umbilical vein endothelial cells (HUVECs) to high glucose concentrations led to reduced proliferation and increased apoptosis. The study found that an overexpression of miR-29a inhibits the effect of high glucose to increase Bax expression in HUVECs. However, overexpression of Bax reversed the effects of overexpressed miR-29a (Huang et al, 2019). Overexpressed miR-29a seems to be a general indicator for pathology in T2DM, as it appears to cause upregulated glucose levels as well as inhibition of glucose-stimulated insulin secretion in the insulinoma cell

line, INS-1E and β cells, which are considered well performing insulin responsive cells. Even though the inhibition of miR-29a increased the glucose-stimulated insulin secretion, there was no significant change in the effect that high glucose levels had on insulin (Bagge et al., 2012). Another study looking into miR-29a-3p found that when it was inhibited, ROS production and apoptosis were increased via the targeting of Bax (Zhang et al., 2019). This was further substantiated in a study using serum and aortic vascular tissue from atherosclerotic mice that had apparent lower expression of miR-29a-3p. They went further and proposed that overexpression of miR-29a-3p supresses cell proliferation, migration and foray of ox-LDL-induced vascular smooth muscle cells (You, et al., 2020). Another study has reported an association between upregulated levels of miR-29a-3p and gestational hypertension and GDM (Hromadnikova et al., 2020).

Investigations into miRNAs and cytokines could provide more context to the complex metabolic processes and chronic inflammation seen in CMDs. A study done by Chen and colleagues found that the upregulation of miR-29a in dendritic cells inhibits the ox-LDL pro-inflammatory cytokines, whereas another study by Qiu et al., in 2007 indicated that the downregulation of miR-29a increased pro-inflammatory cytokine secretion. Proteinuria is a common development during disease states such as T2DM and hypertension. In one study, miR-29a strongly correlated with CRP, transforming growth factor beta 1 (TGF-β1) and urine albumin-to-creatinine ratio in patients living with macroalbuminuria (Huang et al, 2018). The increased expression of miR-29a in obese children (~12 years) may indicate its involvement in the inflammatory pathway, as it positively correlates with high sensitivity C-reactive protein (hs-CRP), IL-6 and TNF-α (Mohany et al., 2021). From this, miR-29a seems to be a good base indicator and would require other biomarkers to delineate towards more specific disease types.

Studies using exosomes derived from bone marrow mesenchymal stem cells for miR-29a found that it played key roles in angiogenesis (Lu et al., 2020). Both exosome isolated miR-29a and miR-92a are hypoxia-inducible and indicative towards immune suppressive myeloid derived suppressor cells and play a role within glioma exosomal miRNAs studies (Guo et al., 2019). MiRNA exosomes, specifically miR-29a that is derived from macrophages within adipose tissue has been linked to insulin sensitivity (Liu et al., 2019).

1.5.4.2 MiR-92a

The progressive stiffening of arterial vessels and forming of micro and macro cardiac disease states seems to be linked with the change in miR-92a expression. A study done on a population of T2DM patients observed that those who developed CVD had a significantly higher serum expression of miR-92a (Wang et al, 2019). Although a previous study indicated a pathological link to the increase of miR-92a, within CD34+ cells, miR-92a was shown to have protective functioning against the development of diabetes retinopathy (Bhatwadekar

et al., 2015).

A study focussing on atherosclerosis induced by factors like hypertension and hyperlipidaemia found that increased expression of miR-92a-3p promoted ox-LDL induced apoptosis of HUVECs by regulating sirtuin6 and mitogen activated protein kinase (MAPK) pathway (Xu et al., 2021). Furthermore, higher circulating levels of miR-92a were shown to correlate with atherosclerosis and hypertension and played an impactful role in the regulation of inflammatory pathways (Huang et al, 2017). MiR-92a effects the endothelial inflammatory pathways by interacting with kruppel-like-factor 2 (KLF2) and kruppel-like-factor 4 (KLF4) (Huang et al, 2017). Endothelial cell homeostasis is regulated by KLF2 through various mechanisms such as endothelial anticoagulant gene expression, thrombotic function, regulation of endothelial nitric oxide synthase (eNOS) and pro-inflammatory activation of the endothelium (SenBanerjee et al., 2004; Lin et al., 2005). There is indication that miR-92a will most likely increase as inflammatory pathways cause strain and would be more prevalent within the hypertension group. Another study that narrowed down on EV-derived miR-92-3p from the colon cells examined the effect it had on endothelial cells. They observed acceleration of cell cycle, mitosis which led to endothelial cell proliferation, then with adhesion cells loosening which promotes cell migration (Yamada et al., 2019).

1.6 Rationale

Whilst various studies have reported the involvement of cytokines and miRNAs in the pathophysiology of T2DM and hypertension, the interactions between these molecules and their potential contributions to disease development and progression have yet to be fully investigated, especially in high-risk populations like the mixed ancestry community, where a higher-than-average prevalence of T2DM and hypertension has been previously reported. Furthermore, investigations of miRNA expression in human biological samples like extracellular exosomes, which are known to facilitate cell-to-cell communication, may potentially add vital context to studies conducted in cell-free samples like serum, saliva, urine and cell-rich samples like whole blood. An improved understanding of the pathophysiology of T2DM and hypertension may not only facilitate early patient diagnosis but also provide alternative therapeutic options to mitigate current shortcomings. It is for the aforementioned reasons that we proposed to conduct this study in a mixed ancestry Bellville South community where the burden of T2DM and hypertension remains very high, posing an even greater risk for CVDs.

1.7 Aim of the study

The aim of the study was to investigate the use of serum extracellular derived miR-29a and miR-92a and cytokine expression levels as potential biomarkers for dysglycaemia and hypertension.

1.8 Objectives of the study

The objectives of this study were:

- To extract and quantify serum extracellular vesicle-derived miR-29a and miR-92a expression levels, using reverse transcription quantitative PCR (RT-qPCR), and comparing the expression levels between different glycaemic and blood pressure states.
    - Normoglycaemia, Prediabetes and screen detected T2DM.
    - Normotensive, pre-hypertension as well as screen detected hypertension.
- To measure and compare serum cytokine profiles, using a Bio-Plex Pro Human Cytokine Screening Panel 27-plex, from individuals with different glycaemic and blood pressure states.
- To determine the associations between the expression of extracellular vesicle-derived miR-29a and miR-92a and cytokine expression between varied states of glycaemia and blood pressure.
- To assess the predictive and diagnostic value of extracellular vesicle-derived miR-29a and miR-92a expression, in combination with determined cytokine profiles for T2DM within a mixed ancestry population.

CHAPTER TWO: RESEARCH METHODOLOGY

2.1 Ethical clearance

This project was a sub-study, falling under the larger parent study for which ethical clearance was obtained from the Cape Peninsula University of Technology (CPUT) Research Ethics Committee (NHREC: REC- 230 408 – 014) and the Stellenbosch University Research Ethics Committee (N14/01/003). This study sought and obtained ethical clearance from CPUT Research Ethics Committee (No: CPUT/HWS-REC 2024/H3). The serum samples for this study were chosen from participants who were recruited between 2014 and 2016, and written, informed consent was obtained from these participants for genetic testing. The research followed the conducted in alignment with the code of ethics of the World Medical Association (Declaration of Helsinki).

2.2 Study design

This was a cross-sectional study, involving a subset of participants (a total of 150 males and females) recruited from Bellville South, Cape Town as part of the ongoing Vascular and Metabolic Health (VMH) study (Matsha et al., 2012).

2.2.1 Inclusion and exclusion criteria

Only participants residing in the Bellville South community, Cape Town were eligible for the recruitment into this study. The participants had to be between the age 18 and 70 years. Furthermore, participants included could not harbour acute or chronic illness other than T2DM or hypertension This was confirmed by a qualified medical professional who was part of the recruitment team. Lastly females that were pregnant were not recruited for this study.

2.2.2 Study population

Participants for this sub-study were conveniently selected (to populate the different groups whilst minimising variability in age, gender and BMI) from a cohort of 1989 mixed ancestry participants who were recruited between 2014 and 2016 from Bellville South and Belhar, Cape Town, South Africa. Upon recruitment, the parent study made use of a well put together protocol around questionnaires and physical examination for the collection of relevant data.

Anthropometric parameters assessed included variables such as height, weight, hip circumference and waist circumference, all measured in alignment with standardized practises (De Onis & Habicht, 1996). Height was measured in centimetres (cm) using a stadiometer. Measurements conducted for weight were recorded in kilograms (kg) assessed using a calibrated Sunbeam EB710 digital bathroom scale. Body mass index (BMI) was subsequently calculated using weight in kilograms per square meter (kg/m^2). Furthermore, to determine waist circumference, a non-elastic tape was used to measure the narrowest circumference between the ribs and the iliac crest in cm. Hip circumference was also determined by measuring the maximal circumference over the Gluteus Maximus in cm.

Participant blood pressure measurements were also taken, in accordance with the World Health Organization (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 of a participant who had been 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 categorized according to their blood pressure measurements as follows: normotensive, ≤120/80 mmHg; screen-detected and known hypertension, ≥140/90 mmHg, in alignment with WHO specifications (Chalmers et al., 1999). For the purposes of our sub-study, participants were re-categorized to include the prehypertension group, which was defined as systolic blood pressure (SBP) between 120-129 mmHg and/or diastolic blood pressure (DBP) between 80-89 mmHg.

In addition, the glycaemic statuses were defined in accordance with the World Health Organization criteria: normoglycaemia characterised by a fasting plasma glucose ≥ 7.0 mmol/L or 2-hour plasma glucose ≥ 11.1mmol/L; prediabetes defined as a fasting plasma glucose 6.1 – 6.9 mmol/L or 2-hour plasma glucose 7.8 – 11.0 mmol/L; T2DM diagnosed by a fasting plasma glucose ≥ 7 mmol/L or 2-hour plasma glucose ≥ 11.1 mmol/L, (Alberti, 1998; WHO, 2020). HbA1c was determined by high performance liquid chromatography (Biorad Variant Turbo, BioRad, South Africa). To determine participants' insulin levels a paramagnetic particle chemiluminescence assay (Beckman DXI, Beckman Coulter, South Africa) was done. Blood sampling collection was conducted by drawing six blood tubes per participant of which three fasting and three postprandial. The daily acquired blood was processed at PathCare an ISO 15189 accredited, Reference Laboratory (Cape Town, South Africa). An enzymatic hexokinase method was used to measure blood glucose levels (Beckman AU, Beckman Coulter, South Africa), whilst HbA1c levels were determined through high-performance liquid chromatography (HPLC) (BioRad Variant Turbo, BioRad, South Africa). Serum insulin was assessed via paramagnetic particle chemiluminescence (Beckman DXI, Beckman Coulter, South Africa). Serum triglycerides were estimated through glycerol phosphate oxidase-peroxidase

endpoint (Beckman AU, Beckman Coulter, South Africa). Moreover, serum high-density lipoprotein cholesterol (Cholesterol HDL) was measured using an enzymatic immune-inhibition endpoint assay (Beckman AU, Beckman Coulter, South Africa), whereas serum low-density lipoprotein cholesterol (Cholesterol LDL) was determined by enzymatic selective protection endpoint analysis (Beckman AU, Beckman Coulter, South Africa). Hs-CRP was quantified using Latex Particle immunoturbidimetry, whilst serum cotinine (Cotinine Serum) was measured by competitive chemiluminescence immunoassay (Immulite 2000, Siemens, South Africa). Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured using the IFCC-Rate method (Beckman AU, Beckman Coulter, South Africa) and serum gamma-glutamyl transferase (GGT) was assessed using the International Federation of Clinical Chemistry and Laboratory Medicine standardized reagents on a Beckman AU (Beckman Coulter, South Africa). In addition, serum creatinine was determined using the modified Jaffe kinetic method (Beckman AU, Beckman Coulter, South Africa).

2.3 Methods

2.3.1 Exosome isolation

Serum extracellular vesicles were isolated using Qiagen miRCURY® Exosome Serum/Plasma Kit (Qiagen, Hilden, Germany cat. #76603) according to manufacturer specifications (Figure 2.1). In brief, frozen serum samples were thawed from -20°C to 4°C, after which they were centrifuged for 10min and 27seconds at 3000g to pellet unwanted cells and other cellular debris. 500µl of serum sample was used as starting volume for exosome isolation. Incubation with a buffer solution for at least an hour was then followed by 30 min and 12 seconds centrifugation at 1500g to form pellets. Supernatant was removed and pelleted exosomes were resuspended in 200µl buffer solution. After exosome isolation, RNA extraction was immediately done.

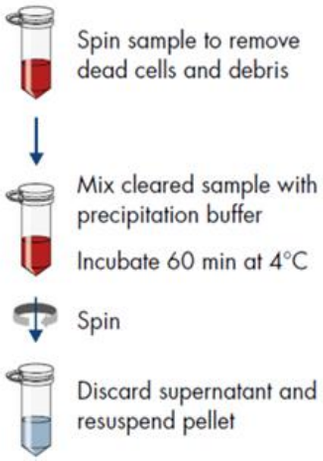

Figure 2.1: Qiagen miRCURY® Exosome Serum/Plasma kit overview (Qiagen, 2017).

2.3.2 RNA extraction

Total RNA including miRNAs was extracted from extracellular vesicles using the miRNeasy Serum/Plasma Advanced Kit (Cat. # 217204) (Qiagen, Hilden, Germany) (Figure 2.2). The phenol-free protocol allows for all of the steps to be performed on the bench and removes the need for phase separation, allowing for a more autonomic process.

The purification of total RNA was achieved using the miRNeasy Serum/Plasma Advance Kit via the implementation of a combination of steps including guanidine-based lysis of samples, inhibitor removal and silica-membrane base capture of nucleic acids. The RNA extraction began with transferring 200µl exosome isolated from serum after a quick 5 second vortex, to 2ml tubes using a 20-200µl pipet. 60µl of buffer RPL were added to the exosome aliquot, vortexed for 30 seconds and then incubated at room temperature for 3 minutes. Samples were lysed in the buffer RPL containing guanidine thiocyanate and detergents that facilitate lysis and denaturation of protein complexes and RNases. 20µl Buffer RPP was added, vortexed for 40 seconds and incubated for 3 minutes at room temperature to precipitate inhibitors which are mostly proteins by means of centrifugation of 12000g for 3 minutes and 27 seconds. After the centrifugation, a clear separation was observed from whitish pellets and clear fluid. The supernatant which was roughly 230µl containing the RNA was transferred to a new 2ml tube with the addition of 230µl isopropanol and mixed by vortexing it for 30 seconds. This allowed for optimal binding conditions for the RNA molecules from an

average of 18 nucleotides and upwards to the spin column membrane. The entire mixture was transferred to a spin column and centrifuged at 8500g for 27 seconds and flow through was discarded after centrifugation. 700µl of buffer RWT was added to the spin column and centrifuged at 8500g for 27 seconds and the flow through discarded afterwards. Following this, 500µl of buffer RPE was added to the spin column, centrifuged at 8500g for 27 seconds and the flow through discarded. Addition of 500µl alcohol (80% ethanol) to the spin column was then followed by centrifugation at 8500g for 2 minutes and 27 seconds. The spin columns were placed in new 2ml collection tubes, centrifuged at 16162g for 5 minutes and 27 seconds with open lids in order to dry the membrane. Resultant RNA was obtained by adding 20µl of RNase free water to the spin column with an incubation period of 60 seconds, followed by centrifugation at 16162g for a minute to get roughly 18µl of extracted RNA. Samples were vortexed for 5 seconds and spun down at 400g for 20 seconds. The purity and concentration of the resultant RNA samples was then assessed using a NanoDrop™ One (Nanodrop Technologies, Wilmington, United States), and only samples with a concentration > 2.4ng/ml, and an OD (optical density) ratio $^{A260}/_{A280}$ > 1.8, were deemed adequate for further processing (Kumar et al., 2020). For all centrifugation steps of 8500g and above, 27 seconds were added as an optimization step for the kit to cater for the extra time needed by the centrifuge to reach the prescribed centrifugation speed.

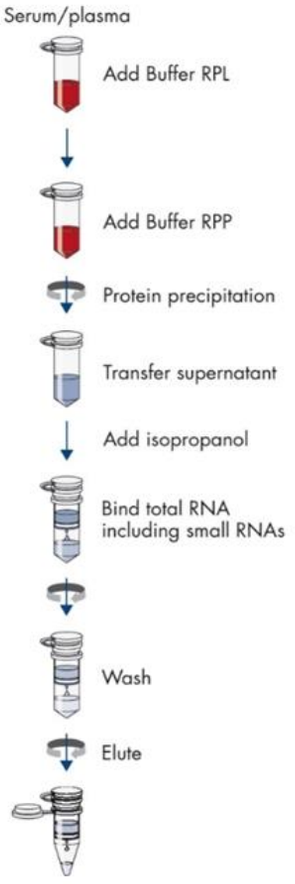

Figure 2.2: Qiagen miRNeasy Serum/Plasma Advanced Kit overview (Qiagen, 2021)

2.3.3 Reverse transcription and PCR

The miRCURY LNA Reverse Transcription kit (cat. # 339340) (Qiagen, Hilden, Germany) was used to convert the extracted RNA samples to complementary DNA (cDNA) in a single reaction step. All RNA sample concentrations were normalized to 5ng/µl before reverse transcription commenced. The reverse transcription master mix was made by combining all the components highlighted in blue in Table 1 below for approximately 20% overage. 8µl of the master mix was added to each well of the 96 well PCR plate, followed by 2µl of the appropriate RNA sample.

Table 2.1: Components for reverse transcription reaction

Components:	PCR Assay
5x miRCURY RT Reaction Buffer	2µl
RNase-free water	4.5µl
10x miRCURY RT Enzyme Mix	1µl
Synthetic RNA spike-ins	0.5µl
Template RNA	2µl
Total reaction volume:	**10µl**

Afterwards, the 96 well PCR plate was sealed off, placed on shaker at 700 rpm for one minute, then centrifuged for 5 minutes at 1200 RPM at 4ºC. The 96 well PCR plate was placed in the QuantStudio 7 flex thermocycler under the following conditions: **Initial incubation** at 42ºC for 60 minutes, followed by another **incubation** at 95ºC for 5 minutes to activate the reverse transcriptase. This was followed by **cooling** to 4ºC and the resultant cDNA (10µl) was then transferred from the plate to each of the labelled 1.5ml microcentrifuge tubes and stored at -20ºC.

After reverse-transcription, miRNA expression levels were determined using the Qiagen miRCURY LNA miRNA PCR Assays and primers (Cat. # 339306), as per manufacturer instructions (Qiagen, Hilden, Germany). All undiluted cDNA was thawed and diluted to a 1:60 ratio by using 2µl undiluted cDNA and 118µl RNase free water in preparation for the PCR. The remaining undiluted cDNA was stored at -20ºC. A PCR master mix was made by adding together the components highlighted in purple, shown in Table 2 below.

Table 2.2: PCR Reaction mix setup

Components:	PCR Assay
2x miRCURY SYBR Green Master Mix	5µl
ROX Reference Dye	0.05µl
PCR Primer Mix	1µl
RNase-free water	0.95µl
cDNA template (Diluted)	3µl
Total reaction volume:	**10µl**

PCR Primer mix consisted of: miR-29a-3p (YP00204698), miR-92a-3p (YP00204258) and miR-16-5p (YP00205702), which was made up from adding 200µl RNase free water to the tube containing the primer. The plates were sealed and shaken for a minute and centrifuged at 1200RPM for 5 minutes before placing in the QuantStudio 7 Flex thermocycler. The PCR was run under the following cycling conditions: **Initial heating** of PCR plate contents to 95ºC for 2 minutes, **Denaturation** at 95ºC for 10 seconds, followed by **annealing and extension** at 56ºC for 60 seconds for 40 cycles. The generated qPCR data was normalized using miR-16-5p as the endogenous control and verified a minimal variation between subgroups.

2.3.4 Cytokine profiling

Serum cytokine levels were determined (in duplicate) on a subgroup of 36 participants from the total 150. The samples were conveniently selected to populate the different study groups whilst minimising variability in age, gender and BMI. The profiling was done using the Bio-Plex Pro Human Cytokine Screening Panel (27-plex No. M500KCAF0Y), as per manufacturer's guidelines. The Bio-Plex kit allowed for the simultaneous detection of 27 different pro-and anti-inflammatory cytokines. However, for the purposes of this study, only TNF-α findings were reported as this was the target cytokine.

Prior to starting the 27-plex assay, all assay components were taken out of cold storage and brought to room temperature. The Bio-Rad machine was switched on and allowed to warm up for 30 minutes, following which calibration (Bio Rad catalogue #171203060) and validation (Bio Rad catalogue #171203001) were done to make ensure fluid and optics were functioning properly. For the wash station, wash buffer was prepared by diluting 1 part of the 10x stock solution with 9 parts deionized water.

Samples were then thawed and kept on ice in preparation for sample dilutions. Sample dilutions were done using a 1:5 ratio using 25µl serum sample and 125µl sample diluent HB. Following this, the lyophilized standards and control were reconstituted in 250µl standard diluent HB. The reconstituted standard and control were vortex for 5 seconds and incubated on ice for 30 minutes. After the incubation, a series of dilutions of the standard were done by initially filling seven (S2-S8) of eight (S1-S8) tubes with 150µl standard diluent HB. S1 received the total 250µl of reconstituted standard, of which 50µl was then transferred to S2. After mixing thoroughly, 50µl of the contents of tube S2 was transferred to S3. This process was repeated until tube S8 as indicated below in Figure 2.3.

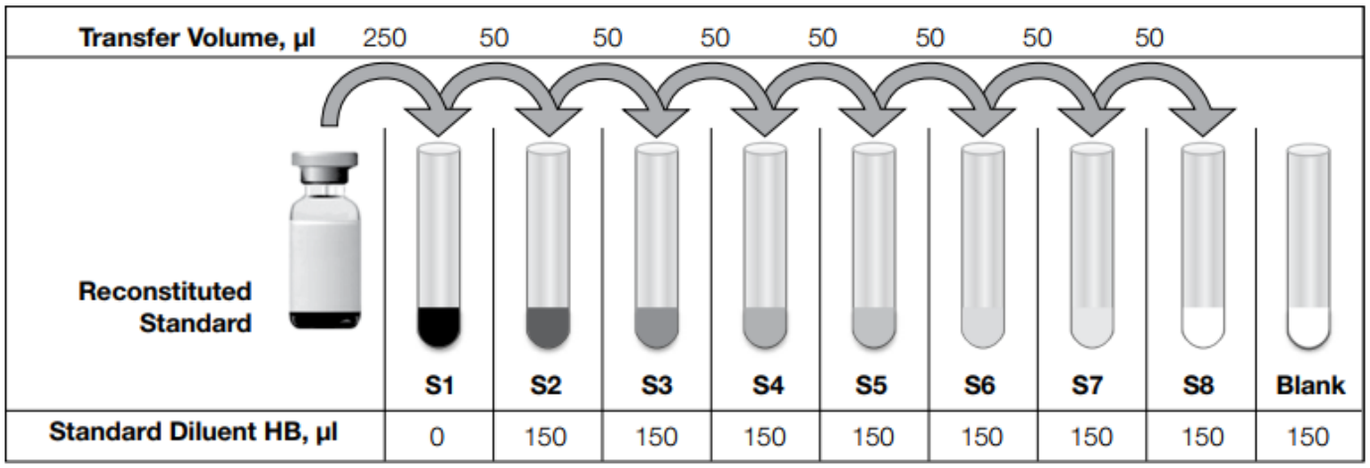

Figure 2.3: Procedure of dilution series for the multiplex (Adapted from Bio-Rad Laboratories Inc., 2023).

Coupled beads were prepared by diluting the 10x stock (570µl) with assay buffer (5130µl) in order to have diluted 1x stock bead solution (5700µl). The 1x diluted bead stock solution was vortexed for 30 seconds and then 50µl added to each well. The plate was then transferred to wash station where each well was washed twice with 100µl Bio Plex Wash Buffer. All of the samples, standards, control and blanks were vortexed for 5 seconds each before 50µl was transferred to their respective wells.

The plate was then sealed and placed on the shaker to incubate at 850rpm for 30 minutes. During the incubation period, the detection antibodies were prepared by diluting the 10x Detection Antibodies (300µl) with Detection Antibody Diluent HB (2700µl) to get to 1x diluted Detection Antibodies (3000µl). When incubation was done, the plate seal was removed and the plate washed three times at the magnetic wash station programmed for 100µl wash buffer per well. Then 25µl of the 1x diluted Detection Antibodies were added to all of the in wells. The plate was sealed again with new tape and incubated on the shaker for 30 minutes at 850rpm. While the incubation was in progress, the streptavidin—phycoerythrin (SA-PE) was prepared by diluting the 100x SA-PE (60µl) with assay buffer (5940µl) to yield a 1x SA-PE (6000µl).

As the incubation period reached the end, the sealing tape was removed, and the plate was put into the magnetic washer where it went through three wash cycles of 100µl wash buffer per well. Then 1x SA-PE buffer of 50µl was pipetted into each well. The plate was then sealed and put on the shaker for 10 minutes at 850rpm, followed by the 100µl of wash buffer three cycles in the magnetic wash is performed. The beads were resuspended by pipetting 125µl assay buffer into each well, the plate sealed and put on the shaker for 30 seconds at 850rpm and then seal removed and put into the BioPlex reader. The software specifications were 50 beads per region with the doublet discriminator set to 5,000 for low and 25,000 for high.

This multiplex approach enabled for quantification of the analytes in smaller starting volumes of serum sample, and with a quicker turnaround time in comparison to conventional immunoassay techniques. Fluorescently dyed magnetic bead sets were used as a substrate to facilitate antibody sandwiching of the analytes in solution, with subsequent detection observed through fluorescent techniques (Bio-Rad Laboratories Inc., 2023). Captured antibodies directed against the desired biomarkers was covalently coupled to the magnetic beads. Coupled beads reacted with the serum samples containing the cytokines, and after multiple wash steps to remove unbound protein, a biotinylated detection antibody was added to facilitate a sandwich complex. Final detection of the subsequent complex occurred with the addition of a SA-PE conjugate, of which the phycoerythrin serves as a fluorescent reporter. A flow diagram below in Figure 2.4 shows the workflow.

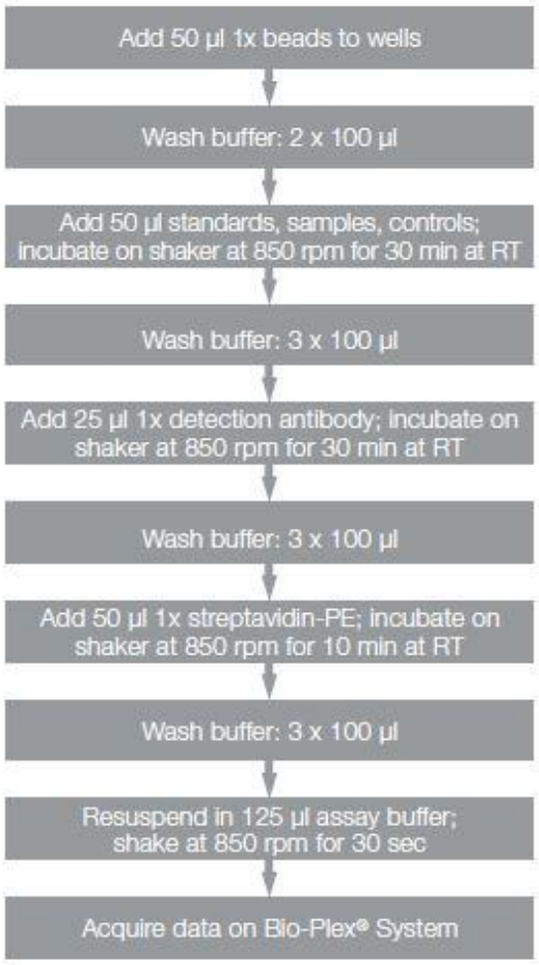

Figure 2.4: Bio-Plex Pro Human Cytokine Screening Panel overview (Bio-Rad Laboratories Inc., 2023).

2.4 Sample size calculation

G Power software (version 3.1) was used to calculate the minimum sample size required for this study. The alpha error probability value was set at 0.05 and the power of the study set at 0.8, to detect a medium effect size (f) of 0.3. As a result, the study required at least 111 participants to be appropriately powered.

2.5 Statistical analysis

The Statistical Package for the Social Sciences (SPSS) V29.0.2.0 software (IBM Corp, Armonk, NY, USA) was used for statistical analysis, and the Shapiro-Wilk test was employed to determine whether the data were normally distributed or skewed. Continuous variables were summarized as mean and standard deviation (SD) when normally distributed, while median, and 25th and 75th percentiles were used for skewed variables. Counts and percentages were reported for categorical variables. The analysis of variance (ANOVA) and Kruskal-Wallis tests were used to group differences in variables including miRNA and cytokine expression. Spearman partial correlations were used to determine the relationship between miRNA and cytokine expression levels, and other clinical parameters. Multivariable logistic regression analysis was performed to investigate the relationship between the miRNA and cytokine expression with both disease groups. A threshold p-value < 0.05 was set for statistical significance.

CHAPTER THREE: RESULTS

3.1 Cohort description and clinical parameters according to hypertension status

Table 3.1 illustrates the general characteristics of the 150 participants included in the study, grouped according to their hypertension status (Normal, n = 69; Pre-hypertension, n = 54; Hypertension, n = 27). Of these participants, 121 (80.7%) were female. As expected, median SBP and DBP increased significantly across hypertension categories normotensive, prehypertensive, and hypertensive indicating a clear trend with increasing hypertension severity ($p < 0.001$) Similarly, the median age, weight, body mass index (BMI), average waist circumference (Ave Waist), Triglycerides, low density lipoprotein cholesterol (Cholesterol LDL) and cholesterol significantly differed according to the hypertension status ($p \leq 0.047$) and showed an upward trend as the blood pressure increased. Whilst 2-hour glucose (Glucose 2 HRs), Fasting Blood Glucose, alanine transaminase (ALT) and gamma-glutamyl transferase (GGT) also significantly differed according to the hypertension status ($p \leq 0.030$), their levels dipped in participants with pre-hypertension before rising in those with hypertension, to levels relatively higher than in the normotensive participants. Lastly, HbA1c levels were significantly higher in the hypertensive group compared to the other two groups ($p < 0.001$).

3.2 Normalisation of qPCR data

As shown in Figure 3.1, there were no significant differences in the cycle threshold (Ct) values of the endogenous control (miR-16-5p) used for normalizing our qPCR results across all blood pressure groups (all $p > 0.05$), confirming the appropriateness of the normaliser for further analysis.

Table 3.1: Study participant characteristics according to hypertension status

	Overall (n=150) Median (25th; 75th percentile)	Normal (N) (n=69) Median (25th; 75th percentile)	Pre-hypertension (PreHPT) (n=54) Median (25th; 75th percentile)	Hypertension (HPT) (n=27) Median (25th; 75th percentile)	p-value			
					Overall	N vs PreHPT	N vs HPT	P vs HPT
Age(years)	51.5 (40; 61)	47 (31; 56.5)	52 (42.8; 61)	60 (53; 65)	**0.001**	0.069	**<0.001**	**0.011**
Sex								
Female, %(n)	80.7 (121)	81.2 (56)	83.3 (45)	74.1 (20)	0.604	0.755	0.442	0.324
Male, %(n)	19.3 (29)	18.8 (13)	16.7 (9)	25.9 (7)				
Weight (kg)	72.9 (56.8; 85.9)	68.3 (53.9; 80.9)	74.4 (61.5; 91.1)	82.6 (57.2; 93.5)	**0.047**	**0.036**	0.052	0.695
Height (cm)	157.5 (153.5; 163)	157.5 (154.1; 163)	156.5 (152.3; 161.3)	158.0 (151.0; 163.0)	0.543	0.316	0.443	0.835
BMI	29.2 (22.4; 35.1)	27.2 (21.3; 31.3)	30.2 (22.8; 37.9)	31.5 (23.4; 40.4)	**0.015**	**0.013**	**0.024**	0.745
BMI status								
Normal, %(n)	33.8 (50)	41.2 (28)	28.3 (15)	25.9 (7)	**0.016**	**0.009**	**0.025**	0.971
Overweight, %(n)	21.6 (32)	29.4 (20)	15.1 (8)	14.8 (4)				
Obese, %(n)	44.6 (66)	29.4(20)	56.6 (30)	59.3 (16)				
Ave Waist (cm)	93.7 (80.2; 106.8)	91.8 (77.3; 99.9)	99.5 (81.1; 109.5)	101.5 (87.3; 116.5)	**0.014**	**0.032**	**0.008**	0.483
Ave Hip (cm)	103.5 (93.6; 115.2)	100.8 (93.5; 107.8)	106.5 (94.5; 120.9)	109.5 (95.5; 124.5)	0.058	**0.038**	0.069	0.855
SBP (mmHg)	120.5 (108; 136)	108 (101; 113)	128 (122.8; 136)	152 (140; 169)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
DBP (mmHg)	79.0 (72; 87.0)	72.0 (66; 76)	85 (82; 87.3)	96 (90; 102)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Glucose 2 HRs (mmol/L)	8.2 (6.0; 10.9)	8.0 (5.4; 9.2)	7.8 (5.8; 9.1)	13.6 (11.9; 17.3)	**<0.001**	0.425	**<0.001**	**<0.001**
Fasting Blood Glucose (mmol/L)	5.1 (4.6; 5.9)	5.0 (4.5; 5.5)	4.9 (4.6; 5.4)	7.4 (5.6; 9.9)	**<0.001**	0.591	**<0.001**	**<0.001**
HbA1c (%)	5.8 (5.4; 6.4)	5.8 (5.3; 6.2)	5.8 (5.3; 6.3)	7.4 (6.1; 8.2)	**<0.001**	0.689	**<0.001**	**<0.001**
Diabetes status								
Normal (N), %(n)	36 (54)	39.1 (27)	50 (27)	0 (0)	**<0.001**	**0.001**	**<0.001**	**<0.001**
Prediabetes (P), %(n)	36 (54)	39.1 (27)	50 (27)	0 (0)				
Screened-Diabetes (D), %(n)	28 (42)	21.7 (15)	0 (0)	100 (27)				
Insulin 120 Minutes (mIU/L)	56.1 (28.6; 96.2)	51.6 (25.1; 92.6)	63.3 (32.8; 112.2)	56.8 (29.9; 81.8)	0.487	0.226	0.739	0.580
Insulin Fasting (mIU/L)	8.0 (4.3; 11.8)	7.6 (3.7; 12)	8.4 (5.2; 10.9)	8.8 (4.3; 17.4)	0.621	0.557	0.368	0.595
Triglycerides (mmol/L)	1.2 (0.8; 1.7)	1.0 (0.7; 1.4)	1.2 (0.9; 1.7)	1.7 (1.2; 2.7)	**0.001**	0.067	**<0.001**	**0.004**
Cholesterol LDL (mmol/L)	3.2 (2.5; 3.9)	3.0 (2.5; 3.7)	3.1 (2.4; 3.7)	3.7 (3.1; 4.4)	**0.034**	0.965	**0.013**	**0.023**
Cholesterol HDL (mmol/L)	1.2 (1.1; 1.5)	1.2 (1.1; 1.5)	1.2 (1.1; 1.5)	1.2 (1.0; 1.4)	0.791	0.886	0.545	0.525
Cholesterol (mmol/L)	5.1 (4.3; 6.0)	4.9 (4.2; 5.7)	5.1 (4.2; 5.7)	6.0 (4.8; 6.7)	**0.006**	0.605	**0.003**	**0.004**
CRP Ultrasensitive (mg/L)	4.9 (2.0; 12.6)	4.8 (1.2; 11.1)	4.9 (2.0; 11.8)	5.3 (3.5; 13.6)	0.542	0.834	0.275	0.379
Cotinine Serum (ng/mL)	10.0 (10.0; 244.5)	43.0 (10.0; 294.0)	10.0 (10.0; 191.5)	10.0 (10.0; 238.5)	0.243	0.095	0.498	0.510
ALT (IU/L)	18.5 (14.0; 27.3)	18.0 (13.5; 30.5)	17.0 (13; 23)	24.0 (17.0; 36.0)	**0.030**	0.178	0.143	**0.006**
AST (IU/L)	23.0 (19.0; 29.0)	23.0 (19.0; 29.0)	21.5 (19.0; 26.0)	23.0 (19.0; 38.0)	0.439	0.491	0.460	0.197
GGT (IU/L)	30.5 (20.8; 52.3)	29.0 (20.0; 49.5)	26.5 (19.8; 41.3)	53.0 (34.0; 77.0)	**0.001**	0.37	**0.002**	**<0.001**
Creatinine (umol/L)	56.0 (50.0; 67.0)	59.0 (52.0; 71.5)	55 (47.8; 65.0)	55.0 (50.0; 69.0)	0.090	**0.027**	0.639	0.264
Tobacco use								
Non-smoker, %(n)	47.7 (71)	42.6 (29)	53.7 (29)	48.1 (13)	0.800	0.458	0.84	0.89
Past smoker, %(n)	4.7 (7)	5.9 (4)	3.7 (2)	3.7 (1)				
Current smoker, %(n)	47.7 (71)	51.5 (35)	42.6 (23)	48.1 (13)				
Alcohol use								
No, %(n)	71.6 (106)	72.1 (49)	67.9 (36)	77.8 (21)	0.648	0.622	0.568	0.357
Yes, %(n)	28.4 (42)	27.9 (19)	32.1 (17)	22.2 (6)				
miR-16-5p CT*	28.5 ± 2.4	28.6 ± 2.5	28.3 ± 2.5	28.6 ± 1.7	0.706	0.712	1.000	0.824
miR-92a-3p $2^{-\Delta CT}$	0.612 (0.283; 2.664)	0.590 (0.233; 2.487)	0.638 (0.317; 4.727)	0.681 (0.327; 1.288)	0.944	0.757	0.828	0.895
miR-29a-3p $2^{-\Delta CT}$	0.077 (0.035; 0.171)	0.095 (0.04; 0.1755)	0.058 (0.028; 0.2733)	0.077 (0.033; 0.118)	0.439	0.234	0.463	0.565

*mean ± standard deviation, SBP: systolic blood pressure, DBP: diastolic blood pressure, Cholesterol LDL: low density lipoprotein cholesterol, Cholesterol HDL: high density lipoprotein cholesterol, BMI: Body mass index, ALT: Alanine transaminase, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase.

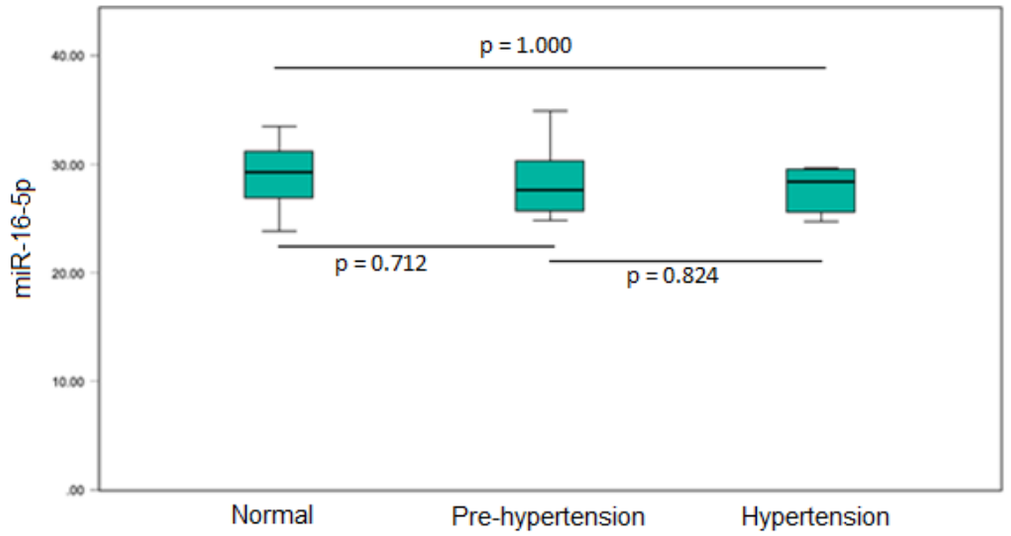

Figure 3.1: miR-16-5p Ct values per hypertension status with an overall p = 0.706 which indicates no significant difference in expression between the subgroups.

3.3 Primary analysis: microRNA expression analysis according to participant hypertension and diabetes statuses

3.3.1 MicroRNA expression analysis according to hypertension status

As illustrated in Figures 3.2 and 3.3, there were no statistically significant differences in the expression levels of miR-92a-3p and miR-29a-3p between the blood pressure groups ($p \geq 0.234$). However, miR-92a-3p expression increased with increasing blood pressure and thus was notably higher in hypertensives than in normotensives. Contrary to miR-92a-3p, the highest expression of miR-29a-3p was seen in normotensives, whilst the lowest expression was in the pre-hypertensive group.

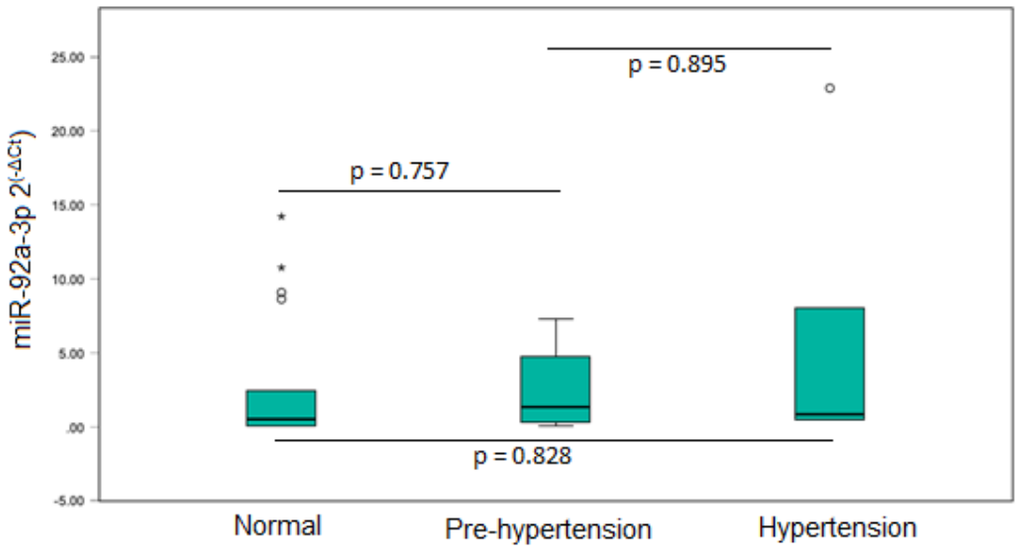

Figure 2: miR-92a-3p 2(-ΔCt) according to hypertension status.

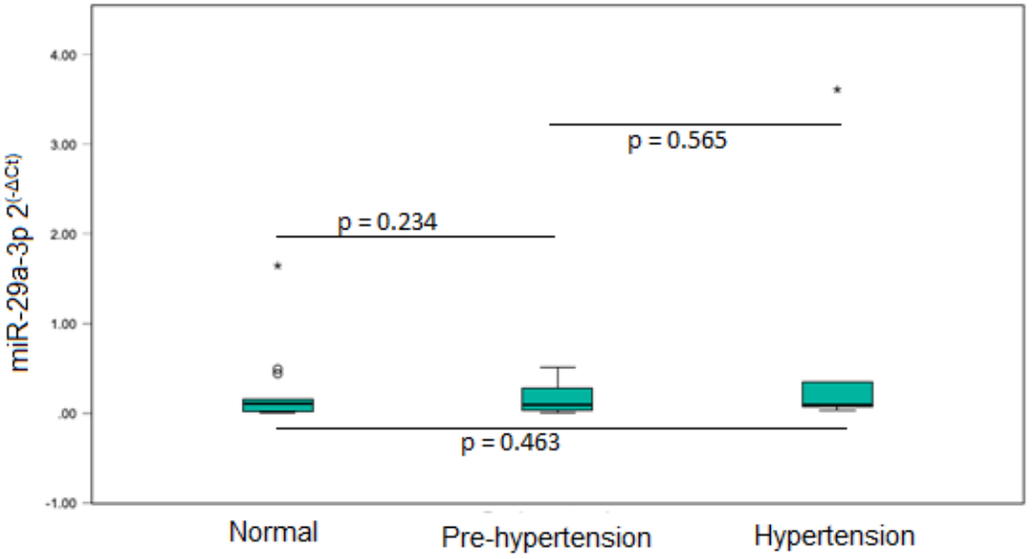

Figure 3.3: miR-29a-3p $2^{(-\Delta Ct)}$ according to hypertension status.

3.3.2 Spearman correlations between microRNA expression and clinical and anthropometric parameters according to hypertension status

Spearman correlations adjusted for age and waist circumference were conducted in order to assess the relationships between the expression of the target miRNAs and various anthropometric and biochemical parameters as shown in Table 3.2. There was a strong positive correlation between the expression of miR-92a-3p and miR-29a-3p ($r = 0.849$, $p < 0.001$) across all comparison groups, although the correlation was slightly weaker in the hypertensive group. Furthermore, there was an overall weak positive correlation between miR-92a-3p and DBP ($r = 0.243$) and 2-hour glucose ($r = 0.269$) (all $p \leq 0.040$). After post hoc analysis, the correlation between miR-92a-3p expression was borderline significant ($p = 0.051$) and significant ($p = 0.033$) with DBP and Glucose 2 HRs respectively. Additionally, there was a weak positive correlation between AST and miR-92a-3p expression in the pre-hypertension group ($r = 0.311$, $p = 0.028$). There was an overall weak negative correlation between creatinine and miR-92a-3p expression ($r = -0.178$, $p = 0.033$), and this correlation remained in the normotensive group ($p = 0.045$) after post hoc analysis.

Table 3.2: Correlation of miR-92a-3p adjusted for age and waist circumference according to hypertension status

	Overall		Normal		Pre-hypertension		Hypertension	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{-\Delta CT}$	1.000		1.000		1.000		1.000	
miR-29a-3p $2^{-\Delta CT}$	**0.849**	**<0.001****	**0.890**	**<0.001****	**0.894**	**<0.001****	**0.561**	**0.005***
Weight (kg)	-0.137	0.102	-0.153	0.223	-0.222	0.121	0.082	0.695
Height (cm)	-0.052	0.539	0.012	0.924	-0.163	0.258	0.096	0.647
BMI	-0.130	0.121	-0.178	0.160	-0.165	0.251	-0.096	0.646
Ave Hip (cm)	-0.067	0.426	-0.158	0.207	-0.041	0.776	-0.114	0.586
SBP (mmHg)	0.058	0.492	0.077	0.541	-0.121	0.403	-0.257	0.215
DBP (mmHg)	**0.171**	**0.040**	**0.243**	**0.051**	0.241	0.092	-0.036	0.866
Glucose 2 HRs (mmol/L)	**0.182**	**0.029**	**0.269**	**0.033**	0.201	0.161	-0.283	0.171
Fasting Blood Glucose (mmol/L)	0.067	0.423	0.177	0.161	0.038	0.794	-0.135	0.520
HbA1c (%)	0.034	0.689	-0.074	0.563	0.112	0.438	-0.162	0.448
Insulin 120 Minutes (mIU/L)	0.023	0.792	-0.092	0.485	0.135	0.348	0.243	0.253
Insulin Fasting (mIU/L)	0.078	0.352	0.117	0.355	0.075	0.605	0.177	0.396
Triglycerides (mmol/L)	0.076	0.368	0.083	0.513	0.086	0.552	0.104	0.628
Cholesterol LDL (mmol/L)	-0.057	0.499	0.032	0.799	-0.073	0.614	-0.230	0.280
Cholesterol HDL (mmol/L)	-0.006	0.947	-0.036	0.777	0.203	0.158	-0.384	0.064
Cholesterol (mmol/L)	-0.033	0.692	0.033	0.793	0.050	0.728	-0.347	0.089
CRP Ultrasensitive (mg/L)	-0.108	0.197	-0.030	0.814	-0.236	0.100	-0.253	0.222
Cotinine Serum (ng/mL)	-0.051	0.547	0.005	0.972	-0.044	0.763	-0.264	0.213
ALT (IU/L)	0.084	0.317	-0.012	0.925	0.215	0.134	0.124	0.554
AST (IU/L)	0.093	0.268	-0.059	0.641	**0.311**	**0.028**	0.144	0.492
GGT (IU/L)	0.079	0.346	-0.070	0.579	0.201	0.162	0.041	0.847
Creatinine (umol/L)	**-0.178**	**0.033**	**-0.250**	**0.045**	-0.203	0.157	0.249	0.230

The expression of miR-29a-3p had an overall weak positive correlation with Glucose 2 HRs (r = 0.209) and Fasting Blood Glucose (r = 0.228), all $p \leq 0.018$, across all comparison groups**.** Furthermore, a weak positive correlation between miR-29a-3p expression and AST (r = 0.302, p = 0.044), and a borderline significant, albeit weak correlation with ALT (r = 0.291, p = 0.053) was observed, but only in the pre-hypertension group, as shown in Table 3.3.

Table 3.3: Correlation of miR-29a-3p adjusted for age and waist circumference according to hypertension status

	Overall		**Normal**		**Pre-hypertension**		**Hypertension**	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{-\Delta CT}$	**0.849**	**<0.001****	**0.890**	**<0.001****	**0.894**	**<0.001****	**0.561**	**0.005***
miR-29a-3p $2^{-\Delta CT}$	1.000		1.000		1.000		1.000	
Weight (kg)	-0.111	0.212	-0.071	0.602	-0.157	0.302	-0.089	0.686
Height (cm)	0.004	0.968	-0.045	0.746	-0.063	0.683	0.112	0.611
BMI	-0.135	0.129	-0.044	0.751	-0.094	0.540	-0.106	0.632
Ave Hip (cm)	-0.028	0.756	-0.020	0.884	-0.004	0.980	-0.148	0.501
SBP (mmHg)	-0.094	0.290	-0.025	0.855	-0.130	0.395	-0.300	0.165
DBP (mmHg)	0.056	0.527	0.185	0.172	0.237	0.117	0.253	0.245
Glucose 2 HRs (mmol/L)	**0.209**	**0.018**	0.245	0.074	0.216	0.154	0.009	0.967
Fasting Blood Glucose (mmol/L)	**0.228**	**0.010**	0.217	0.112	0.173	0.256	0.240	0.270
HbA1c (%)	0.054	0.549	-0.158	0.250	0.094	0.539	0.035	0.878
Insulin 120 Minutes (mIU/L)	-0.167	0.067	-0.182	0.201	0.004	0.979	-0.316	0.152
Insulin Fasting (mIU/L)	-0.020	0.821	0.041	0.768	-0.005	0.972	-0.250	0.251
Triglycerides (mmol/L)	0.092	0.305	0.090	0.514	0.135	0.378	0.146	0.517
Cholesterol LDL (mmol/L)	-0.025	0.780	-0.051	0.710	-0.086	0.576	0.040	0.857
Cholesterol HDL (mmol/L)	0.041	0.649	0.008	0.955	0.243	0.108	-0.222	0.308
Cholesterol (mmol/L)	0.010	0.912	-0.056	0.683	0.041	0.791	-0.020	0.928
CRP Ultrasensitive (mg/L)	-0.086	0.337	-0.140	0.304	-0.108	0.482	0.055	0.802
Cotinine Serum (ng/mL)	-0.016	0.860	-0.110	0.434	0.062	0.684	0.009	0.969
ALT (IU/L)	0.095	0.284	-0.166	0.222	**0.291**	**0.053**	0.035	0.875
AST (IU/L)	0.092	0.303	-0.123	0.370	**0.302**	**0.044**	0.119	0.588
GGT (IU/L)	0.094	0.294	-0.130	0.340	0.257	0.088	-0.184	0.400
Creatinine (umol/L)	-0.083	0.350	-0.241	0.073	-0.073	0.632	0.136	0.535

3.3.3 Logistic regression

To investigate the associations between the expression levels of miR-92a-3p and miR-29a-3p and hypertension, logistic regression analysis was performed, using the normotensive group as the reference group. In the crude, as well as adjusted models, neither miRNA was significantly associated with the likelihood of hypertension ($p \geq 0.514$) in Table 3.5 & 3.5.

Table 3.4: Logistic regression of miR-92a-3p for Normal vs Hypertension

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.966	0.872	1.071	0.514
Model 2	0.979	0.874	1.096	0.714
Model 3	0.969	0.860	1.091	0.602
Model 4	0.983	0.876	1.102	0.768
Model 5	0.972	0.862	1.097	0.644

Model 1: Crude, Model 2: Crude + Age + Ave waist, Model 3: Crude + Age + Ave waist + Creatinine, Model 4: Crude + Age + Ave waist + Cholesterol, Model 5: Crude + Age + Ave waist + Creatinine + Cholesterol

Table 3.5: Logistic regression of miR-29a-3p for Normal vs Hypertension

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	1.220	0.470	3.163	0.683
Model 2	1.392	0.445	4.353	0.570
Model 3	1.039	0.309	3.490	0.950

Model 1: Crude, Model 2: Crude + Age + Ave waist, Model 3: Crude + Age + Ave waist + Glucose 2hr + Fasting Blood Glucose

3.3.4 Receiver-operating characteristic (ROC) curve analysis by hypertension status

In order to investigate the diagnostic capabilities of the target miRNAs for hypertension, receiver operating characteristic (ROC) curve analysis was performed (Figure 3.4). Subsequent results demonstrated that the established clinical parameters of blood pressure (SBP and DBP) performed as expected when discriminating pre-hypertension and hypertension (AUC ≥ 0.825, $p < 0.001$), whilst the miR-92a-3p and miR-29a-3p did not indicate any diagnostic superiority (AUC ≤ 0.567, $p = 0.243$). Further information of ROC curve results can be viewed in Appendix A: Supplementary results.

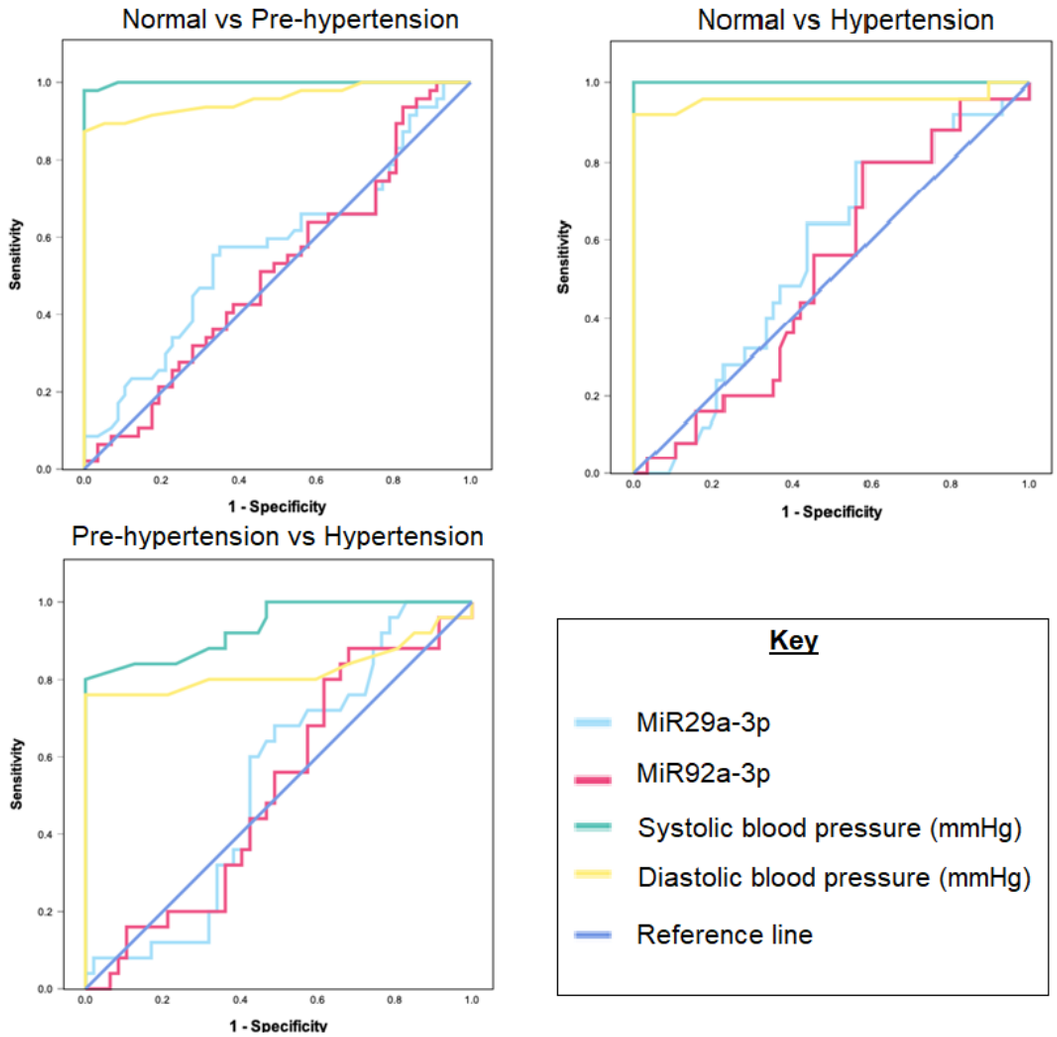

Figure 3.4: ROC curves for miR-92a-3p and miR-29a-3p between hypertension groups.

3.3.5 MicroRNA expression analysis according to diabetes status

As shown in Figure 3.5 and 3.6 there were no significant expression differences of miR-92a-3p and miR-29a-3p between glycaemic groups ($p \geq 0.564$). However, miR-92a-3p had the highest expression levels within the normal glycaemic state and lowest within the prediabetes state. On the other hand, miR-29a-3p expression shows a "J" shape trend with the expression decreasing from normal to prediabetes before rising past both other groups within the diabetes group.

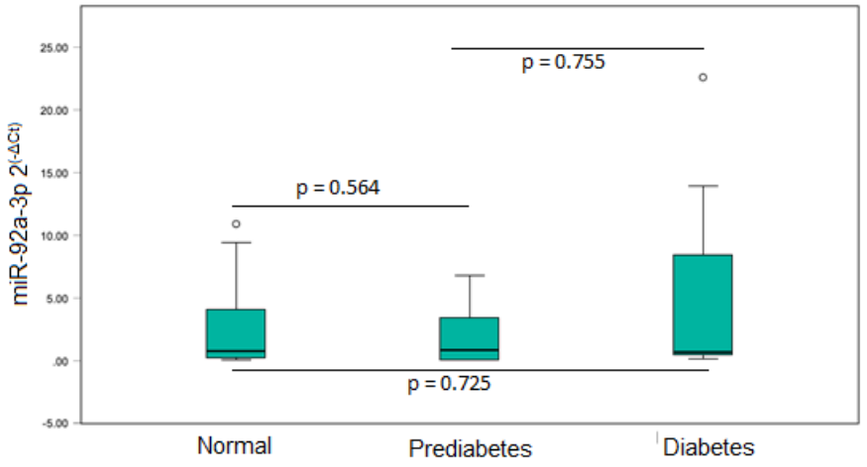

Figure 3.5: miR-92a-3p $2^{(-\Delta Ct)}$ according to diabetes status.

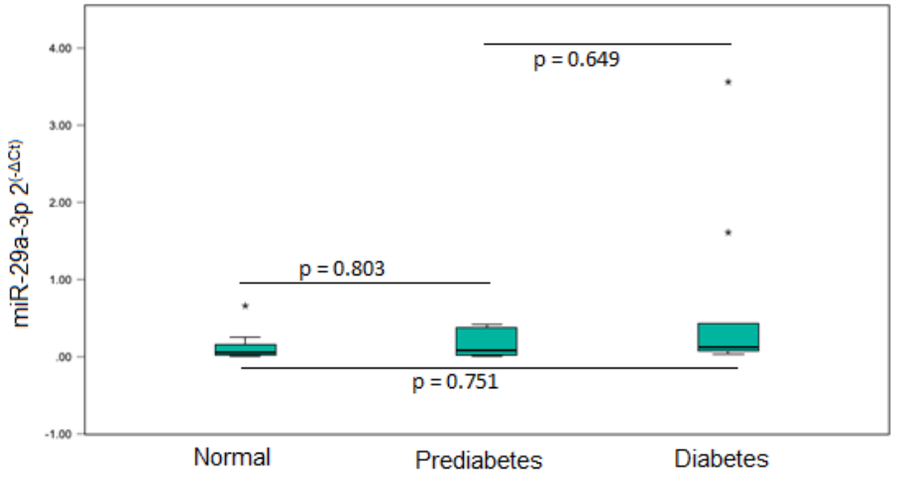

Figure 3.6: miR-29a-3p $2^{(-\Delta Ct)}$ according to diabetes status.

3.3.6 Spearman correlations between microRNA expression and clinical and anthropometric parameters according to glycaemic status

Spearman correlations adjusted for age were conducted in order to assess the relationships between the expression of the target miRNAs and various anthropometric and biochemical parameters (Tables 3.6 and 3.7). The expression of miR-92a-3p in Table 3.6 showed a strong positive correlation with that of miR-29a-3p, throughout all glycaemic groups ($r = 0.843$, $p < 0.001$). Moreover, when investigating relationships with blood pressure parameters, miR-92a-3p expression demonstrated an overall weak positive correlation with DBP ($r = 0.189$), as well as within the normoglycaemia group ($r = 0.277$) and prediabetes group ($r = 0.311$) (all $p \leq 0.047$).

When assessing associations with glycaemic markers, only a weak positive correlation was observed between miR-92a-3p expression and Fasting Blood Glucose in the normal group ($r = 0.311$, $p = 0.025$). Lastly, an overall weak inverse relationship was observed between miR-92a-3p expression and Creatinine ($r = -0.181$) as well as in prediabetes ($r = -0.302$, all $p \leq 0.031$).

Similar observations were identified with miR-29a-3p expression, with weak positive correlations seen with Fasting Blood Glucose across all groups ($r = 0.191$), as well as within the normoglycaemic group ($r = 0.395$, all $p \leq 0.03$) (Table 3.7). Furthermore, a weak negative correlation was determined between only miR-29a-3p and Insulin 120 Minutes in the prediabetes group ($r = -0.297$, $p = 0.045$).

Table 3.6: Correlation 92a-3p $2^{(-\Delta CT)}$ adjusted for age according to diabetes status

	Overall		**Normal**		**Prediabetes**		**Screened diabetes**	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{(-\Delta Ct)}$	1.000		1.000		1.000		1.000	
miR-29a-3p $2^{(-\Delta Ct)}$	**0.843**	**<0.001****	**0.85**	**<0.001****	**0.900**	**<0.001****	**0.662**	**<0.001****
Weight (kg)	-0.110	0.189	-0.042	0.767	-0.162	0.256	-0.165	0.302
Height (cm)	-0.034	0.687	-0.071	0.622	-0.080	0.578	0.081	0.620
BMI	-0.107	0.201	-0.058	0.686	-0.180	0.206	-0.187	0.248
Ave Waist (cm)	-0.061	0.465	0.001	0.996	-0.089	0.535	-0.173	0.279
Ave Hip (cm)	-0.083	0.323	-0.057	0.693	-0.075	0.601	-0.213	0.181
SBP (mmHg)	0.089	0.287	0.173	0.219	0.107	0.453	-0.070	0.666
DBP (mmHg)	**0.189**	**0.022**	**0.277**	**0.047**	**0.375**	**0.007**	-0.004	0.980
Glucose 2 HRs (mmol/L)	0.111	0.185	0.144	0.319	0.078	0.588	0.024	0.880
Fasting Blood Glucose(mmol/L)	0.010	0.902	**0.311**	**0.025**	-0.106	0.462	-0.171	0.286
HbA1c (%)	-0.013	0.877	0.001	0.994	-0.075	0.602	-0.064	0.694
Insulin 120 Minutes (mIU/L)	-0.026	0.757	0.066	0.651	-0.186	0.204	0.059	0.720
Insulin Fasting (mIU/L)	0.013	0.873	0.200	0.160	-0.073	0.609	-0.118	0.462
Triglycerides (mmol/L)	0.036	0.673	0.002	0.988	0.030	0.836	0.110	0.501
Cholesterol LDL (mmol/L)	-0.092	0.273	-0.028	0.843	-0.269	0.056	0.041	0.802
Cholesterol HDL-S(mmol/L)	0.015	0.862	0.068	0.633	0.141	0.323	-0.198	0.220
Cholesterol (mmol/L)	-0.064	0.444	0.035	0.808	-0.170	0.234	-0.022	0.890
CRP Ultrasensitive (mg/L)	-0.127	0.127	-0.096	0.499	-0.380	0.006	0.075	0.642
Cotinine Serum (ng/mL)	-0.051	0.550	0.117	0.422	-0.155	0.277	-0.104	0.528
ALT (IU/L)	0.046	0.578	0.235	0.094	-0.123	0.389	0.055	0.733
AST (IU/L)	0.087	0.298	0.149	0.292	-0.036	0.800	0.175	0.280
GGT (IU/L)	0.049	0.560	0.164	0.247	-0.245	0.083	0.164	0.307
Creatinine (umol/L)	**-0.181**	**0.029**	-0.185	0.190	**-0.302**	**0.031**	-0.044	0.783

Table 3.7: Correlation 29a-3p $2^{(-\Delta CT)}$ adjusted for age according to diabetes status

	Overall		Normal		Prediabetes		Screened diabetes	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{(-\Delta Ct)}$	**0.843**	**<0.001****	**0.850**	**<0.001****	**0.900**	**<0.001****	**0.662**	**<0.001****
miR-29a-3p $2^{(-\Delta Ct)}$	1.000		1.000		1.000		1.000	
Weight (kg)	-0.046	0.604	0.074	0.640	-0.234	0.106	0.049	0.775
Height (cm)	0.008	0.925	-0.034	0.828	-0.158	0.278	0.307	0.072
BMI	-0.054	0.542	0.058	0.714	-0.236	0.103	-0.011	0.949
Ave Waist (cm)	-0.001	0.994	0.154	0.330	-0.193	0.184	0.064	0.711
Ave Hip (cm)	-0.009	0.918	0.072	0.649	-0.096	0.513	-0.032	0.855
SBP (mmHg)	-0.044	0.621	0.094	0.549	-0.078	0.592	-0.226	0.185
DBP (mmHg)	0.096	0.276	0.203	0.192	0.171	0.239	-0.031	0.856
Glucose 2 HRs (mmol/L)	0.150	0.091	0.180	0.261	0.101	0.492	0.106	0.539
Fasting Blood Glucose(mmol/L)	**0.191**	**0.030**	**0.395**	**0.009**	0.006	0.966	0.262	0.122
HbA1c (%)	0.040	0.654	0.033	0.835	-0.081	0.579	0.146	0.402
Insulin 120 Minutes (mIU/L)	-0.175	0.053	-0.057	0.724	**-0.297**	**0.045**	-0.238	0.176
Insulin Fasting (mIU/L)	-0.024	0.785	0.155	0.327	-0.221	0.127	-0.080	0.643
Triglycerides (mmol/L)	0.079	0.374	0.111	0.484	0.055	0.707	0.144	0.411
Cholesterol LDL (mmol/L)	-0.050	0.570	0.073	0.640	-0.163	0.263	0.018	0.918
Cholesterol HDL-S(mmol/L)	0.046	0.607	0.149	0.340	0.187	0.199	-0.222	0.193
Cholesterol (mmol/L)	-0.013	0.881	0.113	0.470	-0.075	0.611	-0.018	0.916
CRP Ultrasensitive (mg/L)	-0.090	0.313	-0.125	0.426	-0.277	0.057	0.233	0.171
Cotinine Serum (ng/mL)	-0.040	0.654	0.128	0.433	-0.153	0.295	-0.002	0.992
ALT (IU/L)	0.073	0.411	0.127	0.416	-0.072	0.625	0.185	0.280
AST (IU/L)	0.087	0.329	0.072	0.647	0.009	0.950	0.186	0.286
GGT (IU/L)	0.073	0.410	0.128	0.413	-0.144	0.324	0.141	0.411
Creatinine (umol/L)	-0.102	0.250	-0.038	0.808	-0.229	0.113	0.041	0.813

3.3.7 Logistic regression

Logistic regression analysis was performed in order to assess the associations between the expression levels of miR-92a-3p and miR-29a-3p and diabetes. The normal glycaemic state group was used as the reference group. Subsequent findings demonstrated that the expressions of both target miRNAs were not significantly associated with risk of diabetes in the crude or adjusted models used ($p \geq 0.161$).

Table 3.8: Logistic regression for miR-92a-3p for Normal vs Diabetes

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.982	0.896	1.076	0.693
Model 2	1.028	0.921	1.147	0.624
Model 3	1.042	0.922	1.178	0.511

Model 1: Crude, Model 2: Crude + Age, Model 3: Crude + Age +Cholesterol LDL + CRP Ultrasensitive + Creatinine

Table 3.9: Logistic regression for miR-29a-3p for Normal vs Diabetes

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	1.472	0.531	4.079	0.457
Model 2	2.217	0.695	7.075	0.179
Model 3	2.349	0.712	7.751	0.161

Model 1: Crude, Model 2: Crude + Age, Model 3:Crude + Age + Height

3.3.8 Receiver-operating characteristic curves by diabetes status

Receiver operating characteristic (ROC) curves analysis was conducted in order to investigate the diagnostic capabilities of the target miRNAs for diabetes (Figure 3.7). The resultant findings were that established glycaemic clinical parameters of diabetes such as Glucose 2 HRs, Fasting Blood Glucose and HbA1c had higher discriminatory capabilities for prediabetes and diabetes (AUC ≥ 0.707, p ≤ 0.001), compared to that of miR-92a-3p and miR-29a-3p (AUC ≤ 0.544, p = 0.480).

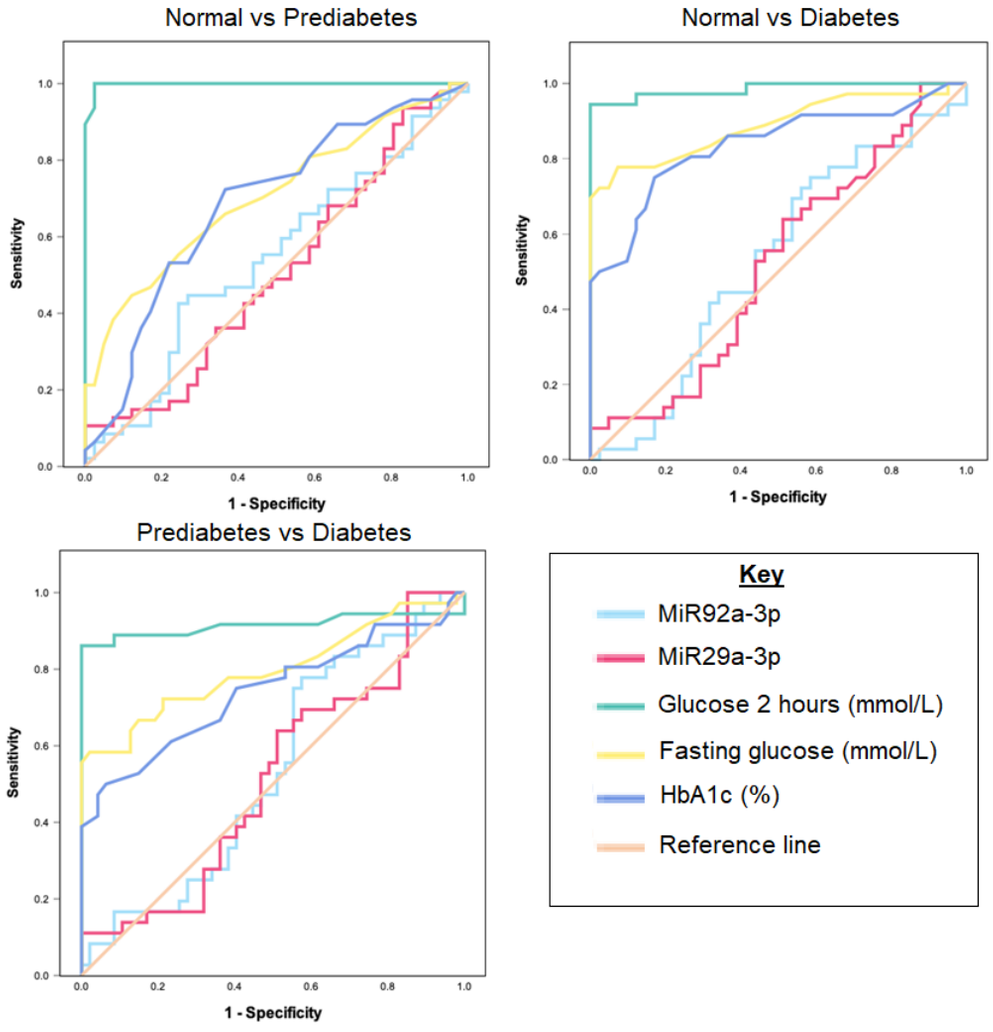

Figure 3.7: ROC curves by diabetes status using clinical glycaemic markers and miRNAs.

3.4 Secondary analysis: cytokine profiling according to participant hypertension and diabetes statuses

Cytokine profiling was done on a subgroup of 36 participants of the total 150. The cytokine profiles were assessed in the following categories: normotension, pre-hypertension, hypertension, normoglycaemia, prediabetes and screen-diabetes. There were six participants in each of the categories

3.4.1 Study participant characteristics according to hypertension status

As shown in Table 3.10 below, the cytokine analysis was performed in a cohort with an overall mean age of 51 years and 26 (72.2%) of the participants were female. There was a significant difference in the SBP and DBP across all groups ($p < 0.001$). There was also a significant difference in the monocytes % between the normotensives and hypertensives ($p = 0.013$). Additionally, HbA1c differed significantly between the pre-hypertension and hypertension groups ($p = 0.034$). There was no significant difference in the expression of Hu TNF-α across the three blood pressure groups ($p = 0.344$).

Table 3.10: Multiplex study participant characteristics according to hypertension status

	Overall (n=36) Median (25th; 75th percentile)	Normal (N) (n=18) Median (25th; 75th percentile)	Pre-hypertension (PreHPT) (n=12) Median (25th; 75th percentile)	Hypertension (HPT) (n=6) Median (25th; 75th percentile)	p-value			
					Overall	N vs PreHPT	N vs HPT	P vs HPT
Age (years)*	51.3 ± 14.4	51.7 ± 15.1	45.8 ± 11.7	61.5 ± 13.3	0.089	0.491	0.299	0.073
Sex					0.758	0.745	0.586	0.457
Female, %(n)	72.2 (26)	72.2 (13)	66.7 (8)	83.3 (5)				
Male, %(n)	27.8 (10)	27.8 (5)	33.3 (4)	16.7 (1)				
Weight (kg)	70.2 (54.1; 85.3)	70.7 (55.5; 83.8)	66.0 (50.7; 98.9)	70 (51.1; 94)	0.900	0.641	0.894	0.851
Height (cm)*	159.7 ± 8.8	160.4 ± 8.2	159.4 ± 10.6	158.2 ± 7.5	0.868	0.957	0.863	0.958
BMI	27.9 (20.6; 34.3)	27.9 (21.5; 32.8)	25.8 (20.4; 38.5)	27.7 (20.5; 35.8)	0.961	0.832	0.947	0.779
BMI status					0.714	0.427	0.744	0.755
Normal, %(n)	41.7 (15)	38.9 (7)	50 (6)	33.3 (2)				
Overweight, %(n)	19.4 (7)	27.8 (5)	8.3 (1)	16.7 (1)				
Obese, %(n)	38.9 (14)	33.3 (6)	41.7 (5)	50 (3)				
Ave Waist (cm)*	91.9 ± 16.5	92.3 ± 17.4	90.0 ± 16.6	94.9 ± 16.1	0.838	0.929	0.942	0.829
Ave Hip (cm)	100.0 (89.2; 121.3)	100.0 (88.5; 113.6)	98.7 (89.7; 124.9)	102.5 (91.8; 118.3)	0.853	0.799	0.594	0.708
SBP (mmHg)*	121.6 ± 17.9	108.9 ± 9.2	126.9 ± 7.0	149.2 ± 16.8	**<0.001****	**<0.001****	**<0.001****	**<0.001****
DBP (mmHg)*	78 ± 12.4	68.7 ± 7.5	82.4 ± 5.4	97.2 ± 3.2	**<0.001****	**<0.001****	**<0.001****	**<0.001****
Glucose 2 HRs (mmol/L)	8.8 (6.9; 12.3)	8.4 (7.1; 12.3)	7.5 (5.3; 9.5)	13.0 (11.9; 25.0)	**0.003**	0.126	**0.014**	**0.001**
Fasting Blood Glucose (mmol/L)	5.0 (4.3; 6.1)	5.2 (4.4; 6.4)	4.6 (4.2; 5.5)	6.5 (4.5; 15.4)	0.168	0.144	0.350	0.122
HbA1c (%)	5.8 (5.5; 6.4)	6.0 (5.4; 6.4)	5.6 (5.3; 6.3)	6.5 (5.7; 11.2)	0.101	0.248	0.194	**0.034**
Insulin 120 Minutes (mIU/L)	54.6 (22.9; 81.2)	55.1 (18.6; 78.6)	57.9 (25.6; 78.6)	29.4 (23.1; 126.2)	0.934	0.894	0.726	0.779
Insulin Fasting (mIU/L)	6.7 (3.1; 11.2)	8.1 (2.7; 13.5)	7.1 (3.8; 9.5)	4.9 (3.2; 7.2)	0.534	0.735	0.386	0.223
Triglycerides (mmol/L)	1.1 (0.8; 1.4)	1.1 (0.8; 1.4)	1.0 (0.9; 1.5)	1.3 (0.9; 2.2)	0.523	0.912	0.327	0.261
Cholesterol LDL (mmol/L)*	3.3 ± 1.1	3.1 ± 0.8	3.3 ± 0.8	3.7 ± 1.9	0.541	0.848	0.521	0.806
Cholesterol HDL (mmol/L)	1.2 (1.1; 1.5)	1.3 (1.1; 1.5)	1.2 (1.0; 1.6)	1.3 (1.1; 1.5)	0.786	0.508	0.841	0.671
Cholesterol (mmol/L)*	5.2 ± 1.2	5.0 ± 0.8	5.2 ± 0.9	5.6 ± 2.2	0.542	0.908	0.511	0.742
CRP Ultrasensitive (mg/L)	7.8 (2.8; 17)	9.7 (3.9; 23.1)	6.8 (2.4; 12.2)	4.8 (3.0; 20.5)	0.622	0.409	0.463	0.851
Cotinine Serum (ng/mL)	232.0 (181.5; 311.0)	232.0 (171.0; 318.0)	241.0 (191.5; 326.5)	218.0 (218.0; 218.0)	0.840	0.571	0.885	0.770
ALT (IU/L)	18.0 (13.0; 24.0)	19.0 (15.3; 34.3)	15.5 (13.3; 22.5)	17.0 (9.3; 41.0)	0.323	0.122	0.483	0.925
AST (IU/L)	21.0 (19.0; 24.0)	23.0 (20.0; 33.5)	19.5 (19; 23.5)	20.5 (16.8; 57.8)	0.310	0.136	0.379	0.962
GGT (IU/L)	3.0 5(23.5; 64.3)	37.5 (24.3; 58.8)	30.5 (23.8; 53)	50.5 (18.8; 84.5)	0.794	0.597	0.815	0.542
Creatinine (umol/L)	56.0 (50.0; 65.0)	59.0 (51.8; 81.5)	56.0 (47.3; 62.8)	52.5 (48.3; 87)	0.395	0.182	0.526	0.672
White Cell Count (x10E9/L)*	8.0 ± 1.9	8.6 ± 1.9	8.0 ± 1.6	6.6 ± 1.8	0.073	0.684	0.059	0.247
Lymphocytes %*	27.1 ± 9.3	26.5 ± 12.2	26.6 ± 5	29.8 ± 6.1	0.745	0.999	0.743	0.743
Lymphocytes ABS (x10E9/L)	2.1 (1.4; 2.6)	1.8 (1.3; 3.1)	2.1 (2.0; 2.4)	2.0 (1.3; 2.6)	0.856	0.756	0.806	0.538
Monocytes %	6.4 (4.9; 7.2)	5.1 (4.6; 6.8)	6.6 (5.7; 8.5)	6.9 (6.5; 10.3)	**0.027**	0.080	**0.013**	0.302
Monocytes ABS (x10E9/L)	0.5 (0.4; 0.6)	0.5 (0.4; 0.5)	0.6 (0.4; 0.7)	0.6 (0.4; 0.7)	0.179	0.074	0.268	0.773
Neutrophils %*	64.1 ± 10.5	65.1 ± 14.2	64.8 ± 5.1	60.2 ± 5.6	0.611	0.996	0.600	0.673
Neutrophils ABS (x10E9/L)*	5.2 ± 1.7	5.6 ± 1.9	5.2 ± 1.3	4.0 ± 1.2	0.102	0.773	0.084	0.267
Basophils %	0.4 (0.3; 0.5)	0.4 (0.2; 0.5)	0.4 (0.4; 0.5)	0.35 (0.28; 0.53)	0.524	0.297	0.887	0.386
Basophils ABS (x10E9/L)	0.01 (0.01; 0.01)	0.01 (0.01; 0.01)	0.01 (0.01; 0.01)	0.01 (0.01; 0.03)	0.857	0.876	0.618	0.606
Eosinophils %	1.3 (0.9; 2.6)	1.7 (0.8; 3.7)	1.3 (0.8; 1.8)	1.1 (0.8; 2)	0.412	0.288	0.277	0.778
Eosinophils ABS (x10E9/L)	0.1 (0.1; 0.2)	0.2 (0.08; 0.315)	0.1 (0.1; 0.2)	0.1 (0.01; 0.125)	0.285	0.468	0.155	0.213
Platelet Count (x10E9/L)	277 (210; 320)	277 (204; 322.5)	297 (216; 342.5)	252 (202; 311)	0.672	0.507	0.649	0.454
miR-92a-3p $2^{(-\Delta Ct)}$	0.683 (0.227; 4.822)	0.503 (0.102; 3.968)	1.376 (0.253; 4.822)	0.862 (0.486; 11.719)	0.535	0.735	0.257	0.454
miR-29a-3p $2^{(-\Delta Ct)}$	0.103 (0.027; 0.357)	0.108 (0.025; 0.299)	0.098 (0.016; 0.381)	0.103 (0.059; 1.164)	0.832	0.839	0.661	0.546
Hu TNF-α	34.6 (31.7; 42.0)	34.6 (31.7; 42.0)	34.6 (31.7; 42.0)	34.6 (31.7; 42.0)	0.344	0.196	0.867	0.223

*mean ± standard deviation, SBP: systolic blood pressure, DBP: diastolic blood pressure, Cholesterol LDL: Low density lipoprotein cholesterol, Cholesterol HDL: High density lipoprotein cholesterol, BMI: Body mass index, ALT: Alanine transaminase, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl Transferase.

3.4.2 TNF-α profiling scatterplot according to hypertension status

The scatterplots to show the relationships between TNF-α and miRNA expression levels in the blood pressure groups are illustrated in Figure 3.8 and 3.9. However, no relationship was observed between the expression of TNF-α and either of miR-92a-3p and miR-29a-3p.

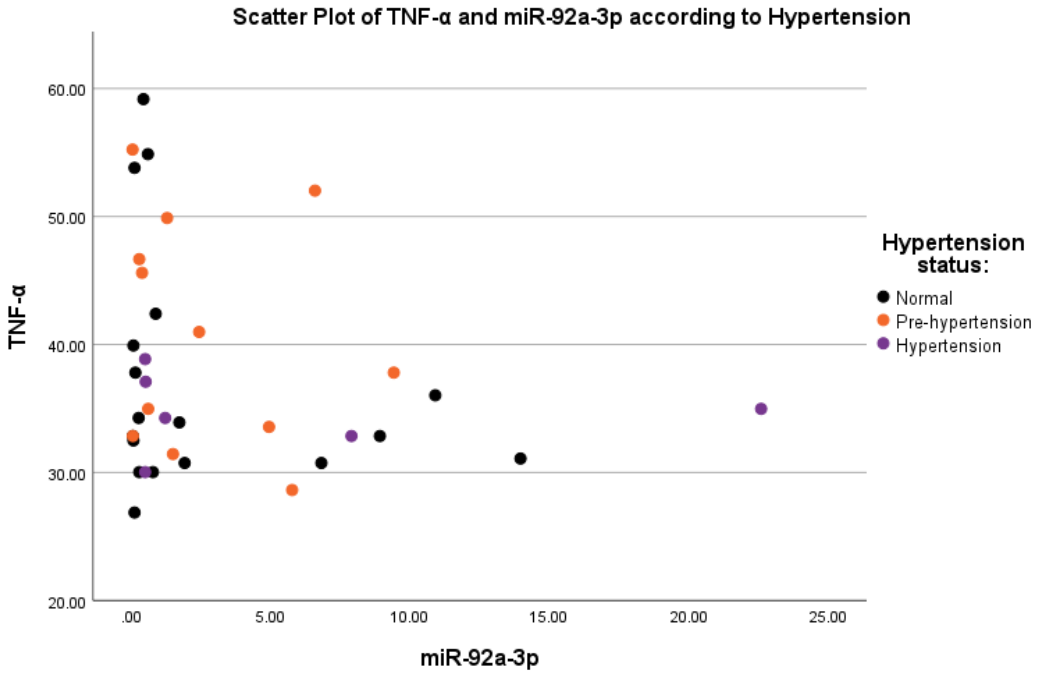

Figure 3.8: Scatterplot of miR-92a-3p $2^{(-\Delta CT)}$ against TNF-α according to blood pressure group.

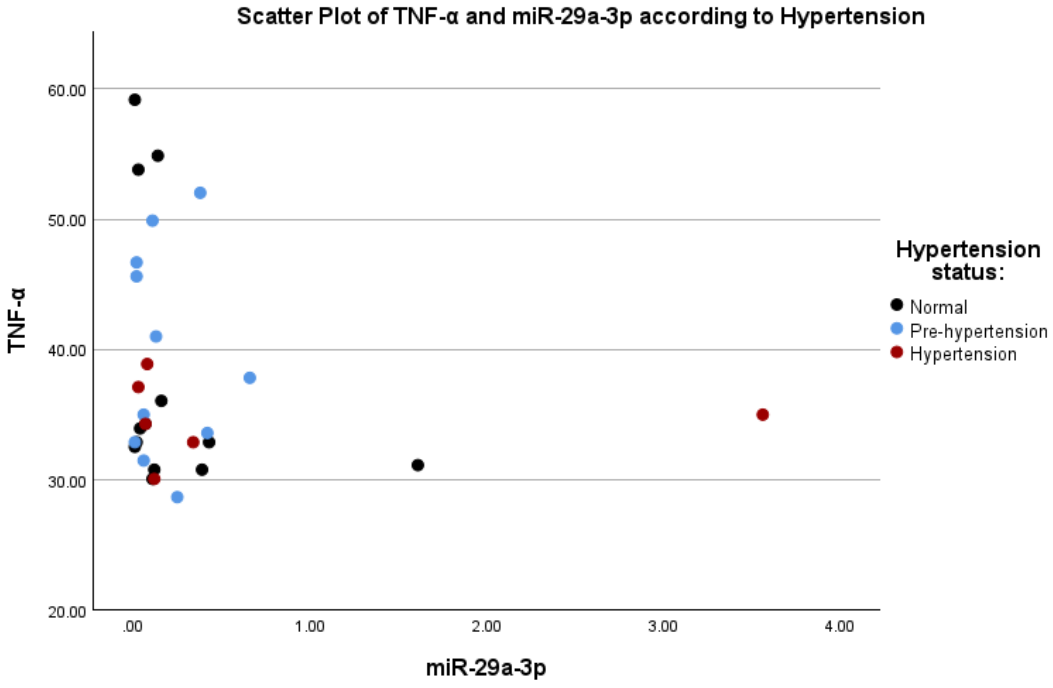

Figure 3.9: Scatterplot of miR-29a-3p $2^{(-\Delta CT)}$ against TNF-α according to blood pressure group.

3.4.3 Spearman correlations between TNF-α expression and clinical parameters according to hypertension status.

Only a few clinical and biochemical markers correlated significantly with TNF-α in a preliminary unadjusted Spearman correlation. Afterwards adjusted correlation was conducted. adjusted for age and waist circumference. TNF-α had a medium and positive correlation with absolute monocyte counts (Monocytes ABS) in the pre-hypertensive group, (r =0.649, p = 0.042).

Table 3.11: Spearman correlations between TNF-α expression and clinical parameters according to hypertension status

	Overall		Normal		Pre-hypertension		Hypertension	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
Hu TNF-α	1,000		1,000		1,000		1,000	
Glucose 2 HRs (mmol/L)	-0,070	0,704	-0,218	0,455	0,179	0,620	0,916	0,084
Monocytes ABS (x10E9/L)	0,326	0,068	0,220	0,450	**0,649**	**0,042**	-0,942	0,058
Platelet Count (x10E9/L)	0,333	0,062	0,425	0,130	0,296	0,406	0,166	0,834

3.4.4 TNF-α profiling scatterplot according to diabetes status

Both miR-92a-3p and miR-29a-3p in figures 3.10 & 3.11 shows similar grouping for the diabetic groups being grouped up in the lower end of the expression while normal and prediabetes are scattered across all ranges.

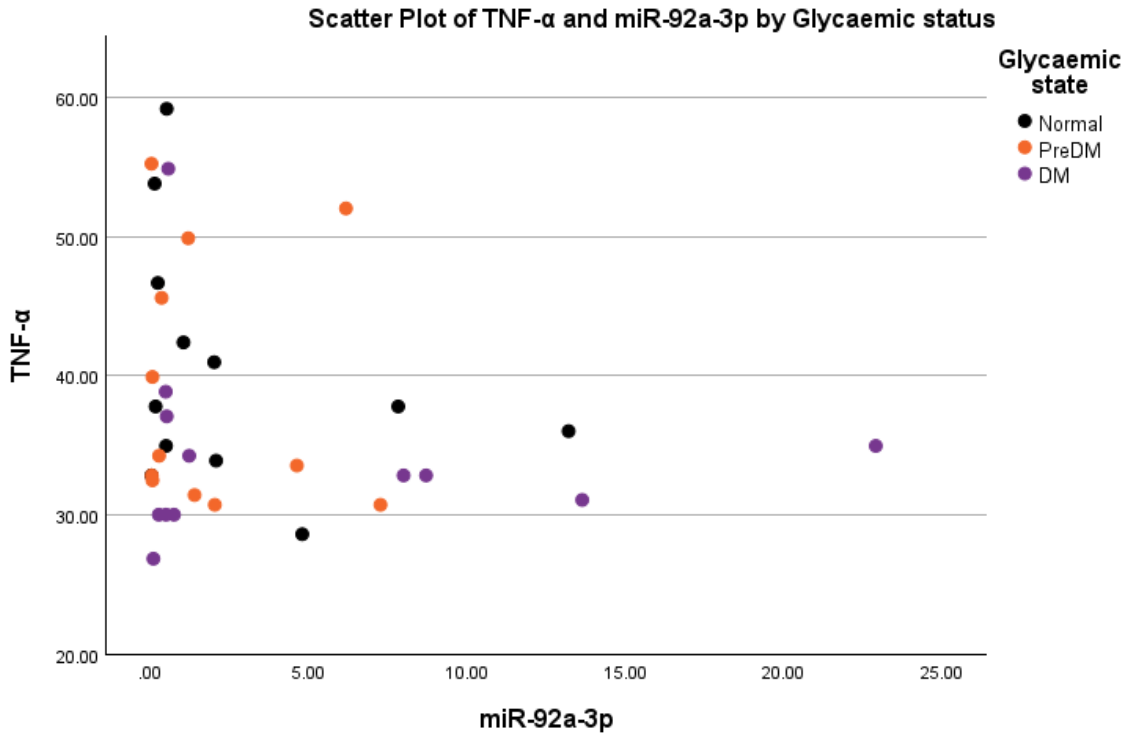

Figure 3.10: Scatterplot of miR-92a-3p $2^{(-\Delta CT)}$ against TNF-α according to glycaemic state.

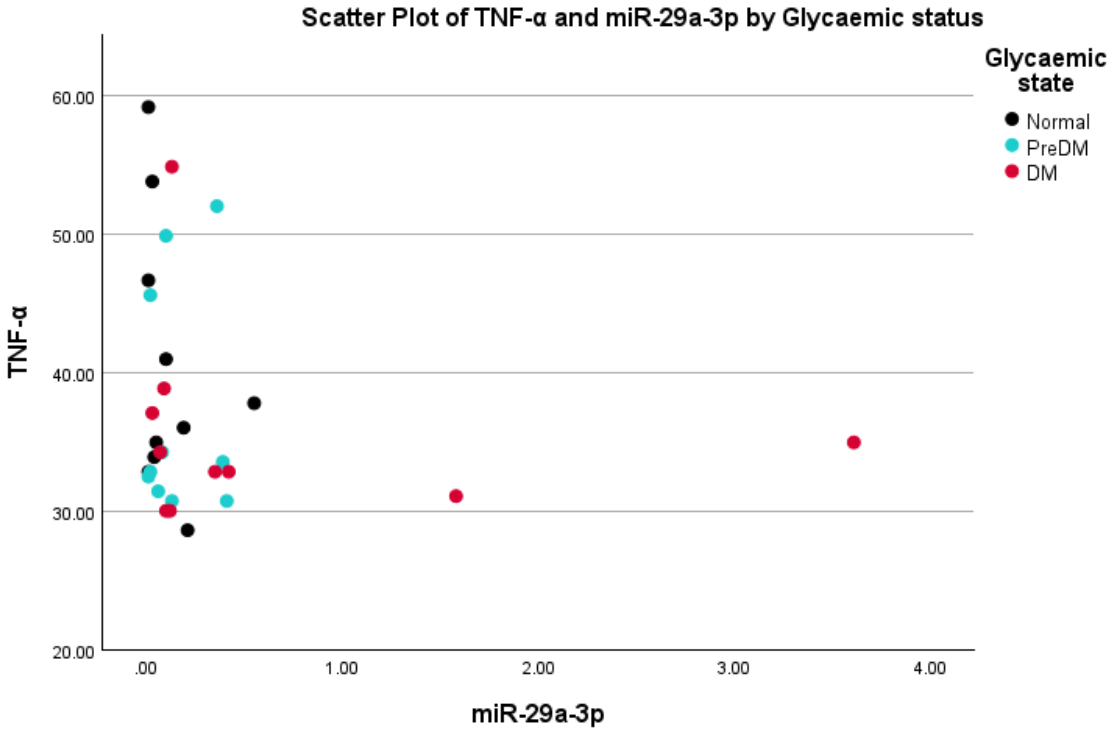

Figure 3.11: Scatterplot of miR-29a-3p $2^{(-\Delta CT)}$ against TNF-α according to glycaemic state.

3.4.5 Spearman correlations between TNF-α expression and clinical parameters according to glycaemic status.

Unadjusted Spearman correlations were performed between TNFα and clinical and biochemical markers which are in the appendix according to glycaemic status under Table 6.14. This was followed by adjusted correlations, adjusted for age and waist circumference. TNF-α had a strong positive correlation with AST (r = 0.717, p = 0.03).

Table 3.12: Spearman correlations between TNF-α expression and clinical parameters according to glycaemic status

	Overall		Normal		Prediabetes		Diabetes	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
Hu TNF-α	1,000		1,000		1,000		1,000	
AST (SGOT)(IU/L)	-0,095	0,606	**0,717**	**0,030**	0,218	0,546	-0,221	0,568
White Cell Count (x10E9/L)	0,226	0,214	-0,033	0,933	0,596	0,069	0,122	0,754

CHAPTER FOUR: DISCUSSION

This study aimed to isolate and quantify serum extracellular vesicle-derived miR-92a-3p and miR-29a-3p expression levels and compare their expression levels between different glycaemic, as well as blood pressure states, as well as their diagnostic capabilities for both. A further aim was to measure and explore cytokine profiles, in a small subset from the same sample, across the varied glycaemic and blood pressure statuses, to investigate any associations between both the target miRNAs and cytokines with diabetes and hypertension. Overall, there was no significant difference in the expression of miR-92a-3p and miR-29a-3p across various glycaemiac or blood pressure groups. However, the expression of miR-92a tended to increase with as the blood pressure increased. Although the expression of miR-92a-3p and miR-29a-3p correlated with DBP, AST, 2 Hour glucose, Fasting Blood Glucose, Insulin and Creatinine, upon assessing the discriminatory capabilities of the miRNAs for disease, findings revealed that these transcripts did not perform as well as traditional markers of glycaemia, such as 2-hour glucose, fasting blood glucose and HbA1c. Similarly, SBP and DBP outperformed the miRNAs in distinguishing pre-hypertension and hypertension. Subsequently TNF-α did not indicate any significant difference in expression levels between different glycaemic or hypertensive states. However, TNF-α levels significantly correlated with monocyte levels in the pre-hypertensive group and AST levels in normoglycaemic individuals. Overall, the expression patterns of both miRNAs in this study did not support their use as biomarkers for the diagnosis of either dysglycaemia or hypertension.

4.1 MiR-92a-3p and miR-29a-3p expression in hypertension

MiRNAs play an important role in regulating gene expression through determining the amount of protein a cell would produce with a downstream effect (Shang et al., 2023). MiR-92a-3p expression in this study increased incrementally as blood pressure increased. In contrast, a study by Kwon where exosomes were isolated from urine found that those who are healthy had the highest expression of miR-92a compared to those who had essential and renovascular hypertension (Kwon et al., 2016). Although the outcome of their findings opposed the findings of this study, this indicates the importance of sample type, organs already affected and medication altering organ functioning when interpreting miRNA expression. In hypertension, miR-92a-3p has been reported to significantly promote oxidized low-density lipoprotein (ox-LDL) induced-apoptosis by activating MAPK signalling pathway. As well as a strong association with increased blood pressure and regulating angiogenesis within the retina, a common development with onset of diabetes, via targeting serum and glucocorticoid inducible kinase 3 (SGK3) (Xu et al., 2021; Cui et al., 2022; Li et al., 2024). Moreover, a separate study conducted by Li and colleagues remarked an association between miR-92a-3p and DBP in a hypertensive population, these findings corroborated ours, whereby a positive relationship was

determined between miR-92a-3p expression and DBP (Li et al., 2024). Another study investigating circulating plasma miR-92a expression showed positive correlation with DBP ($r = 0.649$, $p < 0.001$). Elevated serum miR-92a levels in those living with hypertension is associated with accepted clinical target end organ damage markers which correlates with both DBP, and organ functioning (Huang et al., 2017).

Both miR-92a-3p and miR-29-3p had correlations with AST in the pre-hypertensive group. Other studies that investigate the association between AST and hypertension found that AST was significantly higher expressed in their hypertensive populations (Gupta et al., 2012; Rahman et al., 2020; Liu et al., 2021). This will be further discussed in the lower section where cytokines are involved.

The use of miR-92a-3p as a diagnostic marker has seen more value in being indicative to events such as predicting acute coronary syndrome in diabetic patients or asymptomatic carotid artery stenosis patients and cerebrovascular events in those being hypertensive (Wang et al., 2019; Chen et al., 2020). Furthermore, postulations surrounding the use of miRNAs as diagnostic biomarkers for hypertension have demonstrated promising efficiencies (Tan et al., 2021; Sharma et al., 2022). For instance, in a study conducted by Zhang and colleagues, the researchers demonstrated that better discriminatory capabilities were observed when combining a panel of miRNA markers (miR-199a-3p, miR-208a-3p, miR-122-5p, and miR-223-3p), as opposed to their individual use (Zhang et al., 2018). Although there are limited direct studies focused solely on miR-92a-3p as a diagnostic marker, research has been indicative as to the potential involvement of the transcript in pathogenesis of hypertension and cardiovascular complications (Wiese et al., 2019). As such, although in the current study the diagnostic performance of miR-92a-3p for hypertension was poor, this investigation was the first of its kind in South Africa. Moreover, for future endeavours it might be worth exploring other miRNAs in conjunction with miR-92a-3p and assess their combined use as diagnostic markers for hypertension.

MiR-29a-3p has been poised as a myokine, molecules responsible for auto-, para- and/or endocrine functions, that are secreted from skeletal muscle cells relating to energy metabolism (Landrier et al., 2019). Our findings revealed that the expression of miR-29a-3p was highest in the normotensives and lowest in the pre-hypertensives, while expression levels in the hypertensive group was between the two. A study by Huang has found upregulated expression of miR-29a and a positive correlation with DBP in their hypertensive patients which concur with this study's finding as well (Huang et al., 2017). Although there are not a lot of studies on the diagnostic capabilities of miR-29a-3p and hypertension there is a study that provides insight in the potential involvement in this cardiovascular complication. In pulmonary arterial hypertension (PAH), miR-29a-3p acts as a messenger in PAH-induced cardiac fibrosis through potential binding sites on thrombospondin-2 (THBS2) thus regulating cardiac

fibroblast and similar to our study they had reduce expression of miR-29a-3p with the onset of hypertension (Hsu et al., 2021). As such, although the diagnostic performance of miR-29a-3p in the present study was not notable, this was the first investigation exploring this in South Africa, and further investigations are warranted to fully elucidate the role of miR-29a-3p in hypertension.

4.2 MiR-92a-3p and miR-29a-3p expression in type 2 diabetes mellitus

MiRNAs have long since been associated with T2DM as studies were focused on regulation of glucose metabolism, the role in diabetes itself and glucose homeostasis (Gauthier & Wollheim, 2006; Poy et al., 2007; Tang et al., 2008). In this study, miR-92a-3p expression was the lowest expressed in the prediabetes group. But not enough to be considered significant. Other studies have demonstrated otherwise – for instance, in a longitudinal study, Lewis et al showed that the expression of miR-92a-3p reduced in individuals who progressed to T2DM (Lewis et al., 2024). Furthermore, miR-92a-3p has also been significantly downregulated in EV in response to pioglitazone treatment in a T2DM study within the proliferative diabetic retinopathy group (Solis-Vivanco et al., 2022). In contrast, another study found that their circulating expression of miR-92a-3p remained unchanged when 14 T2DM patients which were on metformin took part in a hypoglycaemia clamp experiment (Eyileten et al., 2022).There was also correlation found between miR-92-3p and creatinine in the prediabetes group, where creatinine is normally used to test kidney function and is a waste product from muscle tissue (Mora et al., 2008; Kashani et al., 2020). Studies have long investigated the back-and-forth effect of kidney disease and increase risk for T2DM and T2DM leading to kidney disorders and CKD (Afkarian et al., 2013; Koye et al., 2018; Bakris et al., 2020). The literature is limited on any investigations looking at miR-92-3p and creatine. However, creatinine serum levels aid in tracking progression in diabetic retinopathy but lacks in clinical sensitivity, but maybe in the future with the right miRNA the diagnostic performance could increase (Wang et al., 2019). In both the hypertensive and diabetes context miR-92a-3p expression decreased in the pathological affected groups in the studies relating to retinopathy and pioglitazone treatment (Cui et al., 2022; Solis-Vivanco et al., 2022). Limited literature exists regarding the diagnostic capabilities of miR-92a-3p for T2DM, as such, although miR-92a-3p did not exhibit significant diagnostic performance in this study, it represents a unique finding within the South African context.

In this study, the expression trend for miR-29a-3p according to glycaemic status was highest in the normal and lowest in the prediabetes group. MiR-29a-3p had a “J” shape trend with the normal being expressed above the prediabetes but below the diabetes group. This trend is supported by other studies indicating the increase expression of miR-29a in tissue and circulation in states of obesity, metabolic syndrome and T2DM (Dalgaard et al. 2022). Similar trends of heightened expression of miR-29a-3p in various tissue and cell types and organs such as pancreatic β-cells, liver, skeletal

muscle and adipocytes have been recorded (van de Bunt et al., 2013; Ludwig et al., 2016; Li et al., 2021). Likewise, to our study, miR-29a-3p was highest expressed in the diabetes group. There is limited evidence in the literature as for miR-29-3p diagnostic value yet even more in the South African environment. Furthermore, in this study miR-29a-3p inversely correlated with Insulin in the prediabetes group. A study by Bagge and colleagues found similar findings that when they overexpress miR-29a there was a decrease in protein levels of Syntaxin-1a (Stx-1a) which impairs insulin secretion (Bagge et al., 2013).

MiR-92a-3p, miR-29a-3p and other miRNAs are touted as potential biomarkers as they point towards specific tissue, organ and disease state outcomes and holds aptitude for diagnosis (Huang et al., 2017; Dalgaard et al., 2022). Below Figure 4.1 gives a holistic overview how a miRNA family can be indicative towards metabolic outcome from different samples. The target miRNAs (miR-92a-3p, miR-29a-3p) have various contributions within hypertension and diabetes, either by continuing regulation of their current expression levels, or events that cause them to be dysregulated. This study however did not find conclusive evidence for a holistic view as using these miRNAs as significant predictive biomarkers to be indicative of the current glycaemic state: normal, prediabetes or diabetic as well as blood pressure state: normal, pre-hypertensive or hypertensive. However, correlations may indicate that miR-92a-3p and miR-29a-3p could be further investigated to shed light on the contributing role they play within these different glycaemic and blood pressure states.

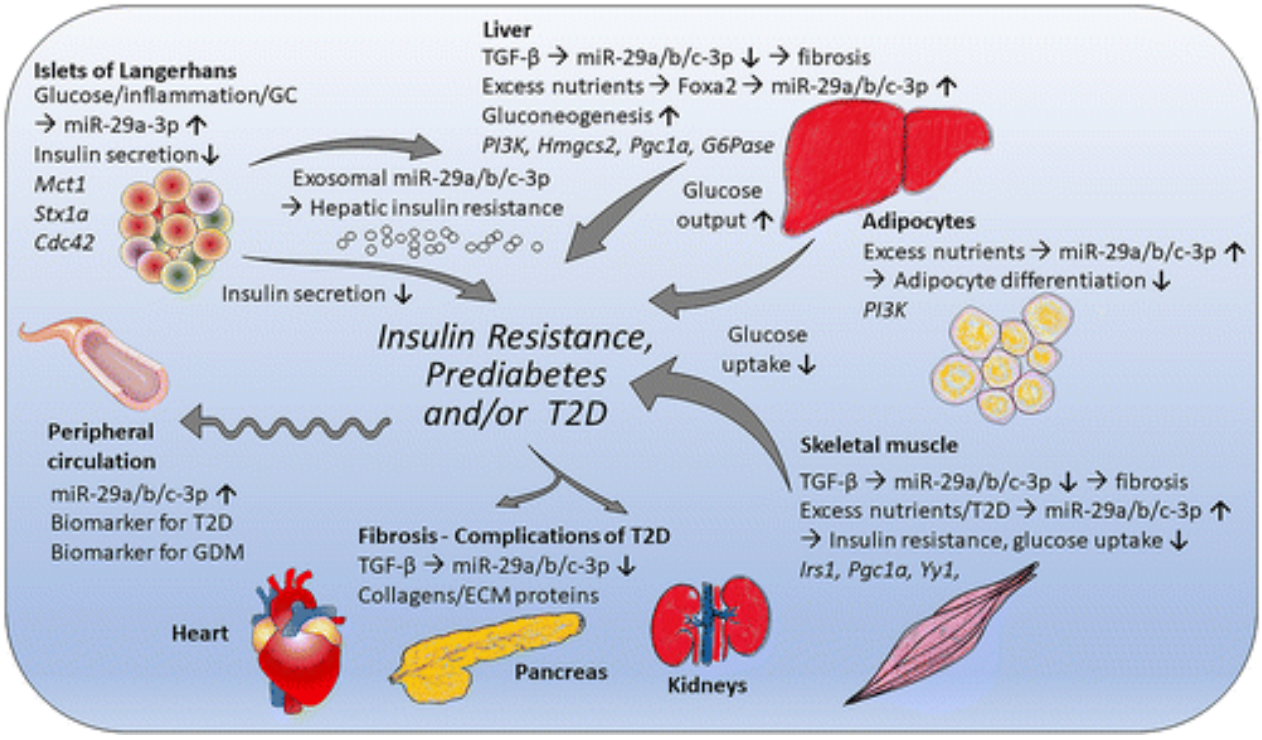

Figure 4.1: MiR-29 family expression change within different tissues and how the glycaemic control is affected from it (Dalgaard et al., 2022).

4.3 TNF-α, miR-92a-3p and miR-29a-3p in cardiometabolic inflammation

These events mentioned above, relates to another pathophysiological development such as retinopathy, fatty liver disease or CKD. Both miR-92a-3p and miR-29a-3p had positive correlation towards AST, a liver enzyme/protein made by liver cells, which normally is found upregulated when there is liver damage (Gowda et al., 2009). The correlation may be indicative of potential links between the miRNAs (miR-92a-3p and -29a-3p) and chronic inflammation in the liver, which subsequently leads to the increase in AST. TNF-α strongly correlated with AST in the normal group investigating participants according to their glycaemic status. Most other studies found correlations between TNF-α and ALT when investigating diabetes and insulin resistance (De Luis et al., 2009; De Luis et al 2013; Lin et al., 2015). ALT is more relevant when it specifically comes to the liver functioning as to AST which is expressed in mitochondria of the liver and cytosol of red blood cells (Panteghini, 1990; Lala et al., 2023). This means that AST can be indicative of liver function but not exclusively as it may indicate other organ functioning such as the cardiac muscle (Panteghini, 1990; Weng et al., 2015 Ndrepepa, 2021).

With the addition of the cytokine profile a significant positive correlation was indicated between miR-29a-3p and monocyte percentage within the hypertension group. Additionally, there was a strong correlation between TNF-α and Monocytes ABS in the pre-hypertension group. MiR-92-3p had shown

a greater variety of significant correlation towards lymphocytes, monocytes and basophils across the different glycaemic states. It should however be noted that the sample size of the cytokine profile was a lot more condensed compared to the miRNA sample and calls for further investigation with a larger size to truly validate the weight the significance holds.

Various studies have alluded to the involvement of cytokines and miRNAs in mediating chronic inflammation, arterial remodelling and endothelial dysfunction (Wieser et al., 2013; Sinah & Haque, 2022). Although global proinflammatory cytokines like TNF-α and IL-6 are overexpressed in diseases like hypertension and T2DM, and there are some discrepancies with IL-10, an anti-inflammatory cytokine, being up and down regulated in T2DM population (van Excel et al., 2002; Randeria et al., 2019). In this study, only TNF-α was successfully measured and assessed, as the other pro-and-anti-inflammatory markers fell outside standards minimum and maximum values. This may be due to several factors such as the age of the sample, being agitated with temperature changes, reacting with the vial itself or shipment (Betensky et al., 2000; Aziz et al 2016; Martikainen et al., 2020). The present study aimed to shed light on the expression patterns of TNF-α in accordance with participant hypertension status, in a smaller subset of the cohort. Moreover, to investigate the potential links between TNF-α and other clinical parameters. With regards to TNF-α levels, these were similar for all blood pressure states, with no statistically significant differences achieved. However different from this study, research done in Makurdi, Nigeria showed a significant increase in serum TNF-α expression in a pre-hypertensive group compared to normotensive (Agbecha & Gberindyer, 2018). These differences may be attributed to sample size difference, as the previous study had a considerably larger sample in comparison to the present study. Although mentioned above that only TNF-α was successfully measured and a point of limitation it also opens room for discussion, as there were not any strong variations between the expressions of TNF-α across the different states of hypertension and T2DM. It is imperative to have an all-inclusive view of cytokines as it has been shown that IL-6 can stimulate or counter regulate TNF-α (Tilg et al., 1997; Woods et al., 2000). A study demonstrated that after 3 hours of strenuous exercise only protein release of IL-6 increased and not for TNF-α which supported their hypothesis that TNF-α is associated with impair glucose uptake in skeletal muscles and not IL-6 (Steenberg et al., 2002). Additionally, another study's finding suggests that TNF-α is an inducer of IL-10 in human monocytes which translates to the molecule provides negative feedback on its own production (Wanidworanun & Strober, 1993).

MiRNAs have been poised as regulators of cytokine expression over the years and how they influence the immune response (Cobb et al., 2006; Asirvatham et al., 2008; Khokhar et al., 2022). Although no significant associations were uncovered between the target miRNAs and TNF-α, strong correlations were observed between miR-92a-3p and miR-29a-3p expression and percentage of monocytes within hypertensive participants. Monocytes are known to mediate both pro-and-anti-inflammatory cascades

(Patrick et al., 2021; Austermann et al., 2022). This is because monocytes contribute with recruitment of other leukocytes and thus initiating inflammation, continuing through with clearance of cell debris and furthermore resolving it through anti-inflammatory cytokines such as IL-10 (Suttles et al., 1999; Verma et al., 2016; Austermann et al., 2022). M1 macrophage polarization and inflammation in lipopolysaccharide (LPS) stimulated RAW264.7 cells mediated by miR-92a serves as evidence how miRNAs could drive renal injury and fibrosis showing a strong link between the miR-92a expression and macrophages (Mingzhi et al., 2024). Other miRNAs such as MiR-155 and miR-125b have been shown to play a central role in TNF-α expression in mouse RAW264.7 macrophage cells stimulated by LPS (Tili et al., 2007). This again indicates that a multitude of miRNAs would collectively be better when investigating disease state compared to mechanisms. In view of these findings, despite the relatively small sample size, relevant links have been identified between the expression of the miRNAs and inflammatory markers. As such, this could be better investigated in a larger population as an intertwined means of predicting disease state or current momentum of disease progression.

This study may have been the first in Africa to investigate a mix ancestry population using serum EV-derived miRNAs (miR-92a-3p & miR-29a-3p) expression patterns with cytokines to determine if there was significance worth investigating further base on literature and what other studies have found in different parts of the world. The overall findings of this study warrant further investigation but have laid out the foundation to continue expanding on miRNAs and cytokines with those living with T2DM and hypertension. Previous studies predominately used animal-based models, different human cell culture and circulation, whole blood, plasma and serum to investigate miRNAs (Huang et al, 2019; Huang et al, 2017; Niu et al., 2023). The parent study from this study investigated miRNAs in whole blood and this pilot study explored serum exosome isolated miRNAs to determine the predictive value. Variables such as gene expression of different populations, environments or other variables may influence the expression of this miRNA and should extensively be tested. South Africa is rich in terms of genetic variance/diversity with different ancestral backgrounds and population groupings making it a prime region to do this type of analysis (Choudhury et al., 2017). It becomes clear that an epigenetic approach would benefit South Africans and the world as medication has been shown to work differently on different ancestral backgrounds (Huang et al., 2009; Malandrino & Smith, 2011). Hence personalised medication could increase the success of treatment, however having a personalised method of diagnosing or predicting the progress of a disease state such as diabetes or hypertension could prevent extreme interventions more accurately (Pearson 2016).

CHAPTER FIVE: LIMITATIONS, FUTURE PERSPECTIVES AND CONCLUSION

The development of both T2DM and hypertension becomes complex as they have multiple variables contributing to their onset like dysfunctional epithelial cells affecting the vascular and hormone regulation deteriorating glycaemic control. Both these diseases have prolonged inflammation that plays a critical role in their development and progression. This all disrupts gene expression and cascades towards miRNAs acting to compensate for change, however with prolonged cytokine activity miRNAs may contribute towards disease progression as their expression deviates. MiRNAs and cytokines in combination could be useful in mapping the progress of T2DM and hypertension, however, would need excessive research done to accurately account for the large scale of variables that make up for these diseases.

5.1 Limitations of this study

As this was a cross-sectional study it only reflects on a given point in time of the population instead of over time change. This study could not make definite indication towards using miRNA expression levels to concretely differentiate between glycaemic or blood pressure states from participants who had T2DM and hypertension. A small population size makes it difficult to attest to true findings and even smaller subgroup for the cytokine profile. Then to consider those with a combination of T2DM and hypertension and prediabetes and pre-hypertension and so forth and further dividing them in even smaller groups would only give weight to potential outliers and further away from true population reflections in sample size of 150 participants. Time from sample collection to when analysis was done also creates room to question more variables such as definitive population variance, strict temperature storage and handling of samples. Only TNF-α was used after the cytokine profile was done as the other pro-and-anti- inflammatory markers did have too many discrepancies surrounding them. Missing data from different data fields diluted the possibility to fully evaluate potential biomarkers with the miRNAs and cytokine profile. There is also the case of disparities of miRNA expression from different samples such as whole blood, serum, plasma, saliva, tissue and urine all which are countering for different potential variables as well as the effectiveness of endogenous controls. Then some studies do not isolate from exosomes or total RNA. Taking all of this into account for the limits to cover the aims of the investigation of miRNAs and cytokine profile to paint a transparent picture of the value they hold.

5.2 Future Perspectives

MiRNAs hold such vast potential as predictive markers or even within therapeutic applications when investigating cardiometabolic diseases such as hypertension and T2DM. They form part of a very intricate and complex system of regulation of homeostasis and response towards pathological development. This can be achieved by increasing the population size to create enough weight for being inclusive to different progressive states within a disease. Incorporating longitudinal approaches will further add value to track the case with far better accuracy and explore causation. Including more biomarkers to support observation could all be considered when exploring to do a similar study. Another important inclusion for future research regarding exosomes would be exploring exosomal miRNA expression from different sources such as whole blood, plasma, serum and specific cells like muscle cells or organ cells, to thoroughly determine what significance the miRNAs hold. The reason behind using miRNAs is to determine if they can be used as a better and less invasive predictor of glycaemic or hypertensive state. Certain miRNAs should maybe be combined to form an array in means of diagnosing a metabolic or vascular state rather than one (Ivanovska & Cleary, 2008; Balaga et al., 2012). But this also means that greater measurement should be taken to effectively test variables and to justify the reasoning behind the expression levels of a miRNA. To validate them further and scrutinise their functional roles within T2DM and hypertension. Furthermore, linking miRNAs functional roles with pathways relating to inflammation.

5.3 Conclusion

Although this study did not identify statistically significant differences in the expression of extracellular vesicle-derived miR-92a-3p, miR-29a-3p, or cytokines across varying glycaemic and hypertensive states within a mixed-ancestry population, the observed correlational patterns suggest potential biological relevance worth further investigation. The findings highlight the complexity of biomarker behaviour in genetically diverse cohorts and point to the need for larger sample sizes and more refined methodologies. Importantly, this study supports the growing body of evidence suggesting that miRNAs, particularly when analysed alongside cytokines, may offer valuable insights into the regulation of chronic inflammation and the co-progression of metabolic and cardiovascular diseases. With continued research, such combined biomarker approaches may contribute not only to improved diagnostic strategies but also to the development of targeted epigenetic therapies.

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APPENDICES

APPENDIX A: Results

Hypertension:

Table 6.1: ROC curves according to hypertension.

	Area under the curve	95% Confidence Interval		p-value
		Lower	Upper	
Normal vs Pre-hypertension				
SBP(mmHg)	0.999	0.996	1.002	<0.001
DBP(mmHg)	0.956	0.914	0.998	<0.001
Inverse miR-92a-3p $2^{(-\Delta CT)}$	0.511	0.399	0.623	0.842
Inverse miR-29a-3p $2^{(-\Delta CT)}$	0.567	0.454	0.680	0.243
Normal vs Hypertension				
SBP(mmHg)	1.000	1.000	1.000	<0.001
DBP(mmHg)	0.959	0.889	1.028	<0.001
Inverse miR-92a-3p $2^{(-\Delta CT)}$	0.523	0.394	0.652	0.725
Inverse miR-29a-3p $2^{(-\Delta CT)}$	0.556	0.426	0.686	0.400
Pre-hypertension vs Hypertension				
SBP(mmHg)	0.935	0.873	0.997	<0.001
DBP(mmHg)	0.825	0.693	0.957	<0.001
Inverse miR-92a-3p $2^{(-\Delta CT)}$	0.516	0.379	0.653	0.822
miR-29a-3p $2^{(-\Delta CT)}$	0.538	0.403	0.672	0.581

Table 4.2: Logistic regression for miR-92a-3p for Normal vs Pre-hypertension.

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.973	0.894	1.060	0.538
Model 2	0.978	0.896	1.067	0.620
Model 3	0.985	0.897	1.082	0.749
Model 4	0.953	0.867	1.049	0.326
Model 5	0.978	0.888	1.077	0.655

Model 1: Crude, Model 2: Crude + age + waist, Model 3: Crude + age+ waist + creatinine + AST, Model 4: Crude + age + waist + creatinine + CRP, Model 5: Crude + age + waist + creatinine + AST + CRP

Table 6.3: Logistic regression for miR-92a-3p for Pre-hypertension vs Hypertension

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.983	0.865	1.116	0.789
Model 2	1.048	0.914	1.201	0.501
Model 3	0.904	0.741	1.104	0.322
Model 4	1.070	0.928	1.234	0.350
Model 5	0.445	0.760	1.128	0.445

Model 1: Crude, Model 2: Crude + age + waist, Model 3: Crude + age + waist + AST, Model 4: Crude + age + waist + CRP+ creatinine + total cholesterol, Model 5:Crude + age + waist + AST + CRP + creatinine + total cholesterol

Table 6.4: Logistic regression for miR-29a-3p for Normal vs Pre-hypertension

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.504	0.108	2.349	0.383
Model 2	0.486	0.107	2.215	0.351
Model 3	1.128	0.180	7.054	0.898
Model 4	0.924	0.141	6.069	0.934
Model 5	1.233	0.176	8.614	0.833

Model 1: Crude, Model 2: Crude + age + waist, Model 3: Crude + age + waist + glucose 2hr + fasting blood glucose, Model 4: Crude + age + waist + ALT + AST+ Gamma GT, Model 5: Crude + age + waist + glucose 2hr+fasting blood glucose + ALT + AST + Gamma GT

Table 6.5: Logistic regression for miR-29a-3p for Pre-hypertension vs Hypertension

		Confidence interval		
	Odds ratio	Lower	Upper	p-value
Model 1	1.553	0.509	4.740	0.439
Model 2	2.527	0.689	9.272	0.162
Model 3	1.517	0.117	19.623	0.750

Model 1: Crude, Model 2: Crude + age + waist, Model 3: Crude + age + waist + ALT + AST + Gamma GT

Table 6.6: Correlation 92a-3p $2^{(-\Delta Ct)}$ per hypertension status

	Overall		Normal		Pre-hypertension		Hypertension	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{(-\Delta Ct)}$	1.000		1.000		1.000		1.000	
miR-29a-3p $2^{(-\Delta Ct)}$	**0.895**	**<0.001****	**0.874**	**<0.001****	**0.964**	**<0.001****	0.543	0.266
Hu TNF-α	-0.179	0.295	-0.106	0.674	-0.231	0.471	-0.257	0.623
Age (years)	-0.030	0.861	0.044	0.861	-0.245	0.443	-0.314	0.544
Weight (kg)	-0.099	0.567	-0.091	0.720	0.098	0.762	-0.429	0.397
Height (cm)	-0.056	0.744	0.116	0.648	-0.270	0.397	-0.058	0.913
BMI	-0.080	0.642	-0.100	0.693	0.133	0.681	-0.771	0.072
Ave Waist (cm)	0.015	0.930	0.094	0.711	0.217	0.499	-0.600	0.208
Ave Hip (cm)	-0.008	0.963	-0.022	0.932	0.182	0.571	-0.600	0.208
SBP (mmHg)	0.126	0.464	-0.151	0.549	0.207	0.519	-0.029	0.957
DBP (mmHg)	0.109	0.527	-0.124	0.623	-0.056	0.862	0.314	0.544
Glucose 2 HRs (mmol/L)	0.239	0.166	0.130	0.618	0.217	0.499	-0.143	0.787
Fasting Blood Glucose (mmol/L)	0.006	0.972	0.207	0.410	-0.056	0.863	-0.714	0.111
HbA1c (%)	-0.130	0.458	-0.010	0.970	-0.243	0.448	**-0.829**	**0.042**
Insulin 120 Minutes (mIU/L)	-0.061	0.726	-0.191	0.462	-0.007	0.983	0.600	0.208
Insulin Fasting (mIU/L)	-0.075	0.664	0.090	0.723	-0.123	0.704	-0.486	0.329
Triglycerides (mmol/L)	-0.082	0.640	0.027	0.918	-0.154	0.632	-0.600	0.208
Cholesterol LDL (mmol/L)	-0.092	0.595	0.086	0.735	-0.126	0.696	-0.429	0.397
Cholesterol HDL (mmol/L)	-0.021	0.903	-0.187	0.456	0.283	0.373	0.334	0.518
Cholesterol (mmol/L)	-0.028	0.871	0.060	0.813	0.102	0.753	-0.429	0.397
CRP Ultrasensitiv (mg/L)	**-0.356**	**0.033**	-0.286	0.250	-0.476	0.118	-0.200	0.704
Cotinine Serum (ng/mL)	-0.313	0.222	-0.109	0.750	-0.800	0.104		
ALT (IU/L)	0.023	0.895	0.218	0.386	-0.119	0.712	0.200	0.704
AST (IU/L)	0.025	0.885	-0.110	0.675	-0.032	0.921	**0.829**	**0.042**
GGT (IU/L)	0.044	0.799	0.019	0.942	-0.147	0.649	0.314	0.544
Creatinine (umol/L)	**-0.368**	**0.027**	-0.399	0.101	-0.317	0.315	0.257	0.623
White Cell Count (x10E9/L)	0.173	0.321	0.331	0.194	0.081	0.803	0.319	0.538
Lymphocytes %	0.243	0.159	0.413	0.099	0.473	0.121	-0.600	0.208
Lymphocytes ABS (x10E9/L)	0.286	0.096	0.476	0.053	0.260	0.415	-0.143	0.787
Monocytes %	0.040	0.821	0.155	0.554	-0.350	0.265	0.725	0.103
Monocytes ABS (x10E9/L)	0.286	0.096	**0.611**	**0.009**	-0.244	0.444	**0.928**	**0.008**
Neutrophils %	-0.277	0.108	-0.402	0.110	-0.280	0.378	0.371	0.468
Neutrophils ABS(x10E9/L)	-0.021	0.904	0.037	0.889	-0.067	0.837	0.429	0.397
Basophils %	0.071	0.685	0.163	0.532	-0.080	0.804	0.290	0.577
Basophils ABS(x10E9/L)	**0.397**	**0.018**	**0.667**	**0.003***	-0.044	0.893	0.131	0.805
Eosinophils %	0.033	0.850	0.134	0.609	0.231	0.470	-0.232	0.658
Eosinophils ABS(x10E9/L)	0.115	0.512	0.249	0.335	0.118	0.715	0.216	0.681
Platelet Count(x10E9/L)	-0.224	0.195	-0.239	0.355	-0.336	0.286	-0.086	0.872

Table 6.7: Correlation miR-29a-3p $2^{(-\Delta CT)}$ per hypertension status

	Overall		Normal		Pre-hypertension		Hypertension	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{(-\Delta Ct)}$	**0.895**	**<0.001****	**0.874**	**<0.001****	**0.964**	**<0.001****	0.543	0.266
miR-29a-3p $2^{(-\Delta Ct)}$	1.000		1.000		1.000		1.000	
Hu TNF-α	-0.265	0.157	-0.427	0.146	0.036	0.915	-0.371	0.468
Age (years)	0.199	0.291	0.528	0.064	-0.027	0.937	-0.429	0.397
Weight (kg)	0.082	0.665	0.171	0.577	0.073	0.832	0.029	0.957
Height (cm)	-0.114	0.548	-0.093	0.762	-0.087	0.800	-0.116	0.827
BMI	0.111	0.558	0.313	0.297	0.073	0.832	-0.200	0.704
Ave Waist (cm)	0.235	0.211	0.429	0.144	0.145	0.670	0.086	0.872
Ave Hip (cm)	0.194	0.304	0.360	0.226	0.118	0.729	0.086	0.872
SBP (mmHg)	0.109	0.565	0.338	0.258	0.205	0.544	-0.714	0.111
DBP (mmHg)	0.070	0.712	0.232	0.446	-0.178	0.600	0.429	0.397
Glucose 2 HRs (mmol/L)	0.415	0.025	**0.627**	**0.029**	0.355	0.285	-0.257	0.623
Fasting Blood Glucose (mmol/L)	0.286	0.125	**0.710**	**0.007**	0.182	0.593	-0.371	0.468
HbA1c (%)	-0.013	0.945	0.161	0.616	0.000	1.000	-0.600	0.208
Insulin 120 Minutes (mIU/L)	-0.064	0.741	-0.112	0.729	-0.100	0.770	-0.086	0.872
Insulin Fasting (mIU/L)	0.014	0.941	0.390	0.188	-0.328	0.325	-0.371	0.468
Triglycerides (mmol/L)	0.016	0.933	0.343	0.276	-0.077	0.821	-0.600	0.208
Cholesterol LDL (mmol/L)	-0.116	0.540	0.140	0.647	-0.059	0.863	-0.657	0.156
Cholesterol HDL (mmol/L)	0.144	0.448	-0.025	0.935	0.318	0.341	0.091	0.864
Cholesterol (mmol/L)	0.007	0.969	0.278	0.357	0.247	0.465	-0.657	0.156
CRP Ultrasensitiv (mg/L)	-0.343	0.063	-0.549	0.052	-0.245	0.467	-0.200	0.704
Cotinine Serum (ng/mL)	-0.374	0.208	-0.143	0.736	-0.800	0.200		
ALT (IU/L)	0.120	0.529	0.218	0.474	0.082	0.811	0.200	0.704
AST (IU/L)	0.051	0.792	-0.186	0.563	-0.064	0.851	**0.829**	**0.042**
GGT (IU/L)	0.136	0.474	0.143	0.642	-0.200	0.555	0.543	0.266
Creatinine (umol/L)	-0.295	0.114	-0.456	0.117	-0.046	0.893	-0.543	0.266
White Cell Count (x10E9/L)	0.265	0.165	0.491	0.105	0.264	0.432	0.029	0.957
Lymphocytes %	0.008	0.967	0.084	0.795	0.323	0.332	-0.600	0.208
Lymphocytes ABS (x10E9/L)	0.049	0.800	0.211	0.511	0.220	0.515	-0.486	0.329
Monocytes %	-0.078	0.686	-0.067	0.837	-0.273	0.417	**0.841**	**0.036**
Monocytes ABS (x10E9/L)	0.218	0.256	0.433	0.160	-0.066	0.847	0.638	0.173
Neutrophils %	-0.051	0.794	-0.077	0.812	-0.210	0.536	0.486	0.329
Neutrophils ABS(x10E9/L)	0.186	0.333	0.350	0.265	0.096	0.780	-0.029	0.957
Basophils %	-0.114	0.557	-0.093	0.774	0.005	0.989	-0.290	0.577
Basophils ABS(x10E9/L)	0.283	0.138	**0.652**	**0.022**	0.100	0.770	-0.393	0.441
Eosinophils %	-0.057	0.770	0.014	0.966	0.023	0.947	-0.754	0.084
Eosinophils ABS(x10E9/L)	0.017	0.932	0.088	0.786	-0.005	0.988	-0.339	0.510
Platelet Count(x10E9/L)	-0.189	0.326	-0.259	0.417	-0.200	0.555	0.257	0.623

Diabetes:

Table 6.8: Logistic regression for miR-92a-3p for Normal vs Prediabetes

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.970	0.882	1.068	0.539
Model 2	0.994	0.898	1.100	0.906
Model 3	1.004	0.901	1.119	0.942

Model 1: Crude, Model 2: Crude + age, Model 3: Crude + age +LDL cholesterol + CRP + creatinine

Table 6.9: Logistic regression for miR-92a-3p for Prediabetes vs Screened-Diabetes

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	1.006	0.916	1.106	0.896
Model 2	1.028	0.928	1.139	0.595
Model 3	1.042	0.938	1.158	0.445

Model 1: Crude, Model 2: Crude + age, Model 3: Crude + age +LDL cholesterol + CRP + creatinine

Table 6.10: Logistic regression for miR-29a-3p for Normal vs Prediabetes

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	0.491	0.077	3.122	0.451
Model 2	0.644	0.093	4.461	0.656
Model 3	0.992	0.122	8.040	0.994

Model 1: Crude, Model 2: Crude + age, Model 3:Crude + age + Insulin 120 minutes + CRP + creatinine

Table 6.11: Logistic regression for miR-29a-3p for Prediabetes vs Screened-Diabetes

	Odds ratio	Confidence interval		p-value
		Lower	Upper	
Model 1	1.982	0.573	6.858	0.280
Model 2	2.352	0.714	7.740	0.159
Model 3	2.193	0.625	7.691	0.220

Model 1: Crude, Model 2: Crude + age, Model 3:Crude + age + insulin 120 minutes + CRP + creatinine + height

Table 5.12: ROC curves according to diabetes status

	Area Under the Curve	Confidence Interval Lower	Confidence Interval Upper	p-value
Normal vs Prediabetes				
Inverse miR-92a-3p $2^{(-\Delta Ct)}$	0.544	0.422	0.666	0.480
Inverse miR-29a-3p $2^{(-\Delta Ct)}$	0.506	0.383	0.629	0.924
Glucose 2 HRs (mmol/L)	0.998	0.993	1.003	0.000
Fasting Blood Glucose(mmol/L)	0.707	0.600	0.814	<0.001
HbA1c(%)	0.688	0.576	0.800	0.001
Normal vs Screened-Diabetes				
Inverse miR-92a-3p $2^{(-\Delta Ct)}$	0.530	0.400	0.661	0.648
miR-29a-3p $2^{(-\Delta Ct)}$	0.521	0.390	0.651	0.756
Glucose 2 HRs (mmol/L)	0.985	0.961	1.009	<0.001
Fasting Blood Glucose(mmol/L)	0.888	0.809	0.967	<0.001
HbA1c(%)	0.836	0.741	0.930	<0.001
Prediabetes vs Screened-Diabetes				
miR-29a-3p $2^{(-\Delta Ct)}$	0.514	0.388	0.641	0.826
miR-92a-3p $2^{(-\Delta Ct)}$	0.535	0.410	0.661	0.580
Glucose 2 HRs(mmol/L)	0.915	0.833	0.998	<0.001
Fasting Blood Glucose(mmol/L)	0.799	0.695	0.903	<0.001
HbA1c(%)	0.741	0.627	0.856	<0.001

Table 6.13: Correlation 92a-3p $2^{(-\Delta CT)}$ per diabetes status.

	Overall		Normal		Prediabetes		Screened diabetes	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{(-\Delta Ct)}$	1.000		1.000		1.000		1.000	
miR-29a-3p $2^{(-\Delta Ct)}$	**0.887**	**<0.001****	**0.888**	**0.001***	**0.875**	**0.001***	**0.760**	**0.011**
Hu TNF-α	-0.164	0.339	-0.273	0.390	-0.333	0.290	0.300	0.343
Age (years)	-0.047	0.788	-0.182	0.571	-0.137	0.672	-0.141	0.662
Weight (kg)	-0.104	0.544	0.350	0.265	-0.179	0.579	-0.277	0.383
Height (cm)	-0.029	0.866	0.305	0.335	-0.416	0.179	0.072	0.824
BMI	-0.100	0.562	0.175	0.587	0.000	1.000	-0.357	0.254
Ave Waist (cm)	0.001	0.998	0.112	0.729	-0.063	0.846	-0.356	0.256
Ave Hip (cm)	-0.020	0.907	0.231	0.471	0.025	0.940	-0.224	0.484
SBP (mmHg)	0.159	0.355	0.109	0.737	0.196	0.540	-0.105	0.745
DBP (mmHg)	0.140	0.414	-0.049	0.879	0.347	0.269	0.081	0.803
Glucose 2 HRs (mmol/L)	0.193	0.266	0.227	0.502	0.030	0.926	0.200	0.534
Fasting Blood Glucose (mmol/L)	-0.023	0.894	0.326	0.301	0.056	0.862	-0.392	0.207
HbA1c (%)	-0.170	0.328	0.064	0.851	-0.430	0.163	-0.302	0.340
Insulin 120 Minutes (mIU/L)	-0.071	0.683	0.118	0.729	-0.291	0.359	-0.042	0.897
Insulin Fasting (mIU/L)	-0.084	0.627	0.182	0.572	-0.105	0.745	-0.364	0.244
Triglycerides (mmol/L)	-0.062	0.723	0.345	0.298	-0.567	0.054	-0.221	0.491
Cholesterol LDL (mmol/L)	-0.114	0.509	0.165	0.608	-0.315	0.318	-0.137	0.672
Cholesterol HDL (mmol/L)	-0.028	0.872	-0.317	0.316	0.450	0.142	-0.150	0.641
Cholesterol (mmol/L)	-0.052	0.764	0.176	0.585	-0.035	0.913	-0.072	0.824
CRP Ultrasensitive (mg/L)	**-0.367**	**0.028**	-0.399	0.199	**-0.781**	**0.003***	0.172	0.594
Cotinine Serum (ng/mL)	-0.264	0.307	0.107	0.819	**-0.928**	**0.008**	-0.800	0.200
ALT (IU/L)	0.016	0.928	0.028	0.931	-0.265	0.405	0.202	0.529
AST (IU/L)	0.033	0.851	-0.177	0.582	-0.205	0.524	0.336	0.312
GGT (IU/L)	0.053	0.757	-0.025	0.940	-0.494	0.103	**0.613**	**0.034**
Creatinine (umol/L)	**-0.353**	**0.035**	-0.116	0.721	**-0.662**	**0.019***	-0.336	0.285
White Cell Count (x10E9/L)	0.149	0.394	**0.682**	**0.021**	-0.296	0.349	0.505	0.094
Lymphocytes %	0.220	0.205	0.387	0.239	0.420	0.174	-0.165	0.609
Lymphocytes ABS (x10E9/L)	0.259	0.133	**0.712**	**0.014**	0.306	0.334	-0.051	0.875
Monocytes %	0.055	0.754	-0.424	0.194	0.378	0.225	0.026	0.935
Monocytes ABS (x10E9/L)	0.296	0.085	0.106	0.757	0.158	0.624	**0.707**	**0.010**
Neutrophils %	-0.259	0.134	-0.282	0.401	-0.445	0.147	0.025	0.940
Neutrophils ABS (x10E9/L)	-0.035	0.841	0.391	0.235	-0.462	0.130	0.389	0.211
Basophils %	0.067	0.704	-0.455	0.159	**0.607**	**0.036**	0.004	0.991
Basophils ABS (x10E9/L)	**0.391**	**0.020**	0.000	1.000	0.404	0.193	0.497	0.101
Eosinophils %	0.010	0.955	-0.173	0.612	0.557	0.060	-0.125	0.699
Eosinophils ABS (x10E9/L)	0.096	0.583	-0.106	0.757	0.504	0.095	0.107	0.741
Platelet Count (x10E9/L)	-0.213	0.219	-0.105	0.759	**-0.588**	**0.044**	0.365	0.243

Table 6.14: Correlation miR-29a-3p $2^{(-\Delta CT)}$ per diabetes status.

	Overall		Normal		Prediabetes		Diabetes	
	r-value	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**	**r-value**	**p-value**
miR-92a-3p $2^{(-\Delta Ct)}$	**0.887**	**<0.001****	**0.888**	**0.001***	**0.875**	**0.001***	**0.760**	**0.011**
miR-29a-3p $2^{(-\Delta Ct)}$	1.000		1.000		1.000		1.000	
Hu TNF-α	-0.206	0.275	-0.334	0.345	-0.012	0.973	-0.213	0.554
Age (years)	0.190	0.315	0.098	0.789	-0.070	0.847	-0.232	0.519
Weight (kg)	0.105	0.583	0.450	0.192	0.055	0.881	-0.122	0.738
Height (cm)	-0.113	0.554	0.308	0.387	-0.456	0.185	-0.109	0.763
BMI	0.129	0.495	0.201	0.578	0.128	0.725	-0.176	0.627
Ave Waist (cm)	0.246	0.189	0.334	0.345	0.036	0.920	0.030	0.934
Ave Hip (cm)	0.212	0.261	0.377	0.283	0.292	0.413	0.018	0.960
SBP (mmHg)	0.133	0.483	0.424	0.222	0.140	0.699	-0.564	0.090
DBP (mmHg)	0.125	0.512	0.067	0.853	0.474	0.166	-0.103	0.777
Glucose 2 HRs (mmol/L)	**0.404**	**0.030**	0.285	0.458	0.088	0.809	-0.091	0.803
Fasting Blood Glucose (mmol/L)	0.283	0.129	0.399	0.253	0.315	0.375	-0.236	0.511
HbA1c (%)	-0.009	0.963	0.042	0.915	-0.248	0.490	-0.370	0.293
Insulin 120 Minutes (mIU/L)	-0.053	0.785	0.025	0.949	-0.237	0.510	-0.006	0.987
Insulin Fasting (mIU/L)	0.053	0.781	0.195	0.590	-0.158	0.663	0.006	0.987
Triglycerides (mmol/L)	0.052	0.789	0.267	0.488	-0.304	0.393	-0.248	0.489
Cholesterol LDL (mmol/L)	-0.113	0.553	0.155	0.668	-0.229	0.525	-0.462	0.179
Cholesterol HDL (mmol/L)	0.118	0.534	-0.197	0.586	0.629	0.051	-0.092	0.800
Cholesterol (mmol/L)	0.001	0.998	0.245	0.496	0.080	0.826	-0.353	0.318
CRP Ultrasensitive (mg/L)	-0.348	0.059	-0.407	0.243	-0.541	0.106	0.139	0.701
Cotinine Serum (ng/mL)	-0.408	0.166	0.200	0.747	**-0.975**	**0.005***	-0.500	0.667
ALT (IU/L)	0.120	0.528	-0.159	0.661	-0.223	0.537	0.535	0.111
AST (IU/L)	0.011	0.956	-0.198	0.584	-0.486	0.154	**0.763**	**0.017**
GGT (IU/L)	0.148	0.434	-0.109	0.763	-0.462	0.179	0.612	0.060
Creatinine (umol/L)	-0.339	0.067	0.052	0.887	-0.584	0.077	-0.503	0.138
White Cell Count (x10E9/L)	0.239	0.212	**0.667**	**0.050**	-0.311	0.382	0.500	0.141
Lymphocytes %	0.030	0.876	0.218	0.574	0.310	0.383	-0.345	0.328
Lymphocytes ABS (x10E9/L)	0.049	0.800	0.403	0.282	0.003	0.993	-0.176	0.626
Monocytes %	-0.044	0.822	-0.350	0.356	**0.736**	**0.015**	-0.079	0.828
Monocytes ABS (x10E9/L)	0.257	0.178	0.017	0.965	0.363	0.302	0.514	0.129
Neutrophils %	-0.080	0.681	-0.167	0.668	-0.432	0.213	0.292	0.413
Neutrophils ABS (x10E9/L)	0.146	0.450	0.433	0.244	-0.401	0.250	0.576	0.082
Basophils %	-0.120	0.534	-0.501	0.170	0.513	0.130	-0.396	0.257
Basophils ABS (x10E9/L)	0.291	0.125			0.273	0.445	0.082	0.822
Eosinophils %	-0.059	0.761	-0.133	0.732	0.146	0.688	-0.353	0.318
Eosinophils ABS (x10E9/L)	0.012	0.949	-0.156	0.689	0.084	0.817	-0.093	0.799
Platelet Count (x10E9/L)	-0.148	0.444	-0.083	0.831	**-0.638**	**0.047**	0.358	0.310

Table 6.15: Secondary analysis: TNF-α

	Overall		Normal HPT		Pre-hypertension		Hypertension	
	r-value	p-value	r-value	p-value	r-value	p-value	r-value	p-value
Hu TNF-a	1,000		1,000		1,000		1,000	
Ave Waist (cm)	**-0,170**	**0,322**	**-0,214**	**0,394**	0,077	0,812	-0,371	0,468
Ave Hip (cm)	-0,035	0,840	-0,112	0,658	0,214	0,505	-0,371	0,468
SBP (mmHg)	0,005	0,979	-0,420	0,082	0,158	0,624	-0,200	0,704
DBP(mmHg)	0,189	0,270	0,070	0,782	0,046	0,888	0,257	0,623
Glucose 2 HRs (mmol/L)	-0,320	0,061	.-.606**	0,010	0,091	0,779	0,771	0,072
Glucose Fasting Blood (mmol/L)	-0,253	0,137	-0,438	0,069	0,392	0,208	0,086	0,872
HbA1c (%)	-0,156	0,370	-0,358	0,158	0,264	0,408	0,257	0,623
Insulin 120 Minutes (mIU/L)	-0,078	0,657	-0,236	0,362	0,322	0,308	-0,257	0,623
Insulin Fasting (mIU/L)	0,054	0,755	**-0,119**	0,639	0,333	0,291	-0,371	0,468
Triglycerides-S (mmol/L)	-0,221	0,201	-0,436	0,080	0,116	0,721	-0,200	0,704
LDL Cholesterol (Measured) (mmol/L)	-0,131	0,446	-0,209	0,406	-0,091	0,778	-0,143	0,787
Cholesterol HDL-S (mmol/L)	0,264	0,120	0,272	0,275	0,290	0,361	0,030	0,954
Cholesterol-S (mmol/L)	-0,091	0,599	-0,161	0,523	0,021	0,948	-0,143	0,787
CRP Ultrasensitive-Cardiac (mg/L)	0,108	0,532	0,351	0,153	-0,161	0,618	0,086	0,872
Cotinine Serum (ng/mL)	-0,008	0,976	-0,228	0,501	0,500	0,391		
ALT (SGPT) (IU/L)	0,004	0,980	-0,223	0,375	0,218	0,497	0,257	0,623
AST (SGOT)(IU/L)	0,015	0,930	0,039	0,882	0,260	0,414	-0,600	0,208
Creatinine-S (umol/L)	-0,222	0,193	-0,356	0,147	-0,025	0,939	-0,314	0,544
Gamma GT-S (IU/L)	-0,028	0,870	-0,101	0,690	-0,119	0,713	0,314	0,544
White Cell Count (x10E9/L)	0,172	0,323	0,146	0,577	0,550	0,064	-0,116	0,827
Lymphocytes %	0,057	0,747	0,259	0,315	-0,095	0,770	-0,371	0,468
Lymphocytes ABS (x10E9/L)	0,203	0,242	0,229	0,376	0,349	0,266	-0,257	0,623
Monocytes %	0,187	0,283	-0,018	0,946	0,350	0,265	-0,696	0,125
Monocytes ABS (x10E9/L)	0,321	0,060	0,393	0,119	0.621*	0,031	-0,464	0,354
Neutrophils %	-0,042	0,809	-0,244	0,345	0,123	0,704	0,600	0,208
Neutrophils ABS (x10E9/L)	0,069	0,694	-0,123	0,639	0,487	0,108	0,143	0,787
Basophils %	0,087	0,620	0,028	0,915	-0,146	0,650	0,232	0,658
Basophils ABS (x10E9/L)	0,051	0,773	-0,011	0,965	**0,306**	**0,334**	-0,131	0,805
Eosinophils %	-0,042	0,812	0,051	0,846	-0,158	0,625	0,029	0,957
Eosinophils ABS (x10E9/L)	0,001	0,996	0,119	0,650	0,000	1,000	-0,216	0,681
Platelet Count (x10E9/L)	0,331	0,052	.520*	0,033	0,140	0,665	-0,200	0,704
Weight (kg)	-0,072	0,676	-0,009	0,971	0,000	1,000	-0,543	0,266
Height (cm)	0,018	0,916	-0,037	0,885	-0,147	0,648	0,261	0,618
BMI	-0,034	0,845	-0,048	0,851	0,210	0,513	-0,314	0,544
Age (years)	.-0.351*	0,036	**-.613***	**0,007**	0,105	0,746	-0,086	0,872
miR-92a-3p $2^{\wedge(-\Delta Ct)}$	-0,164	0,339	-0,106	0,674	-0,231	0,471	-0,257	0,623
miR-29a-3p $2^{\wedge(-\Delta Ct)}$	-0,206	0,275	-0,427	0,146	0,036	0,915	-0,371	0,468

Updated ethics clearance certificate

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

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5 March 2025
HWS-REC Approval Reference No:
CPUT/HWS-REC 2024/H3 (Renewal)

Faculty of Health and Wellness Sciences

Dear Mr. Daniel Johannes Botes - 230986536

Re: APPLICATION TO THE HWS-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to **Mr. DJ Botes** for ethical clearance. This approval is for research activities related to research for **Mr. DJ Botes** at Cape Peninsula University of Technology.

TITLE: Predictive value of microRNAs and cytokines in development of type 2 diabetes mellitus and hypertension in a South African mixed ancestry population.

Supervisor: Dr. D Matshazi and Dr. C Weale

Comment:
Approval will not extend beyond 7 March 2026. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the CPUT HWS-REC in December of that particular year, for the CPUT HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr. Samantha Meyer
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences