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MICRORNA EXPRESSION AND ARTERIAL FUNCTION IN TYPE II DIABETES MELLITUS

by

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Declaration

I, Batsheva Goldfein, declare that this dissertation is my own, except to the extent indicated in the contribution and acknowledgments sections. It is being submitted for the degree of Master of Science in Medicine in the School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this or any other University.

I hereby certify that the studies contained in this dissertation have been approved by the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg. The ethic approval number is M21/05/15.

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Signed on the 5th day of June 2024

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Abstract

Background. Type II diabetes mellitus (T2DM) is a major health concern which significantly contributes to the global cardiovascular disease (CVD) burden. Arterial dysfunction is considered a subclinical marker of CVD and is associated with an increased risk of cardiovascular events. However, treatment outcomes for T2DM patients remain suboptimal, mainly due to a poor understanding and the lack of an early marker for the identification of subclinical CVD. Recently, microRNAs (miRNA), small, non-coding RNA molecules that regulate major signalling pathways through post-transcriptional modification, have been identified as possible epigenetic regulators in the development of many diseases. MiR-146a-5p, in particular, has received considerable attention as a biomarker associated with several disease states including inflammation, T2DM and CVD. However, studies surrounding the role of miR-146a-5p in arterial function and subclinical CVD risk in diabetic populations have yielded contradictory results. Therefore, the aim of this study was to determine the role of miR-146a-5p expression in the development of arterial dysfunction in patients with T2DM.

Methods. This case control study (n=118) included participants with a previous diagnosis of insulin resistance or T2DM (n=67), and a non-DM control group (n=51). Demographic characteristics and CVD risk factors were assessed using standard approaches. Arterial function was measured using applanation tonometry and SphygmoCor software. From the recorded radial and aortic waveforms, central systolic (cSBP) and pulse pressure (cPP), augmentation pressure (AP) and the forward (FWP) and reflected wave pressure (RWP) were derived using a generalised transfer function. The carotid-femoral pulse wave velocity (PWV) was measured as a marker of arterial stiffness. Using a fasting blood sample, serum concentrations of tumour necrosis factor α (TNFα) and matrix metalloproteinase 1 (MMP1) were quantified by ELISA. Real time quantitative PCR was used to determine the relative expression of miR-146a-5p using the comparative CT method relative to an endogenous control miRNA, miR-16-5p. Differences in anthropometric variables, miRNA expression and arterial function between the two groups were determined using unpaired t-tests or Mann Whitney U tests, as appropriate. Associations between miR-146a-5p expression and arterial function were determined using Pearson's correlations. Participants were

further stratified according to CVD risk using the Framingham risk score (FRS), and the associations with miRNA expression analysed using multivariate linear regression.

Results. Participants with DM had significantly higher body mass index (p=0.002), triglyceride levels (p=0.004), and systolic blood pressure (p<0.001) than the control participants. Diabetic participants also had increased CVD risk compared to the control group, as assessed using FRS (P<0.001). Participants with DM also had significantly higher cSBP (p=0.003), mean arterial pressure (p<0.001), peripheral PP (p=0.04), FWP (p=0.045) and PWV (p=0.04). The relative expression of miR-146a-5p was significantly increased in the DM group compared to the control group (p=0.02). Across the study cohort, miR-146a-5p expression was significantly associated with waist-to-hip ratio (partial r=0.29, p = 0.002), triglyceride concentrations (partial r=0.2, p = 0.04), the atherogenic index (partial r=0.20, p=0.04) and TNFα concentrations (partial r=0.23, p=0.02), in age, sex and race adjusted analysis. In multivariate adjusted analysis, miR-146a-5p expression was not associated with any of the arterial function variables (all p>0.05). However, when stratifying participants based on CVD risk, in those with a high risk for CVD (FRS≥20), miR-146a-5p was inversely associated with peripheral PP (Std β=-0.76, p=0.03, cPP (Std β=-0.76, p=0.01), cSBP (Std β=-0.38, p=0.02), FWP (Std β=-0.68, p=0.04) and RWP (Std β=-0.66, p=0.04). When MMP1 was included as a confounder, these associations were no longer significant.

Conclusion. MiR-146a-5p expression was significantly higher in participants with T2DM compared to control participants and was significantly associated with traditional CVD risk factors and inflammation. MiR-146a-5p expression was not association with arterial function measures in the total population. Interestingly, in patients at a high risk for CVD, decreased expression of miR-146a-5p was associated with increased pressure pulsatility and wave reflection. These associations were lost when MMP1, a marker of arterial remodelling, was included as a confounding factor. These results suggest that miR-146a-5p may have a regulatory role in the development of arterial dysfunction through arterial remodelling in persons at high risk for CVD.

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Statement of contribution to data collection and analysis

I declare that I designed this study in conjunction with my supervisors Prof AME Millen and Dr S Gunter. I was part of a team (including Prof Millen, Dr Gunter, Dr Hoosain) responsible for collecting data for a larger study, of which this thesis forms a part. I was responsible for all applanation tonometry assessments of arterial function and for optimising the technique and collecting data for the main outcome variable in this thesis, microRNA expression. I was responsible for all data analysis and wrote this thesis which was reviewed and edited by Prof Millen and Dr Gunter.

Table of Contents

List of Figures

List of Tables

Abbreviations

AIx	Augmentation index
BMI	Body mass index
BP	Blood pressure
cAP	Central augmentation pressure
cPP	Central pulse pressure
cSBP	Central systolic blood pressure
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FPG	Fasting plasma glucose
FRS	Framingham risk score
FWP	Forward wave pressure
HDL	High-density lipoprotein
HO-1	Heme oxygenase 1
HR	Heart rate
IL	Interleukin
IQR	Interquartile range
IRAK1	IL-1 receptor-associated kinase 1
KLF4	Krüppel-like factor 4
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAP	Mean arterial pressure

MDA	Malondialdehyde
miRNA	microRNA
MMP1	Matrix metalloproteinase 1
NFkB	Nuclear factor kappa beta
NO	Nitric oxide
NOX4	NADPH oxidase 4
oxLDL	Oxidised low-density lipoprotein
PPA	Pulse pressure amplification
pPP	Peripheral pulse pressure
PWV (cf-, ba-)	Pulse wave velocity (carotid femoral-, brachial ankle-)
RM	Reflection magnitude
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative PCR
RWP	Reflected wave pressure
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SOD	Superoxide dismutase
Std β	Standardised beta
T2DM	Type II diabetes mellitus
TC	Total cholesterol
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRAF6	TNFα receptor-associated factor 6
VSMC	Vascular smooth muscle cell

Chapter 1: Introduction

Type II diabetes mellitus (T2DM) is a major global health concern (Ong *et al.*, 2023). In South Africa, the prevalence of T2DM has more than doubled in the last decade and affects approximately 10.8% of the population (International Diabetes Federation, 2021). Patients with T2DM have a notably increased risk for the development of cardiovascular disease (CVD), which is the leading cause of morbidity and mortality in this population (Johnson *et al.*, 2020). Despite the significant burden of CVD in the diabetic population, treatment outcomes for these patients remain suboptimal (Gulsin *et al.*, 2019). Moreover, the management of T2DM is further complicated by the presence of multiple traditional CVD risk factors associated with T2DM, which compounds the independent contribution of T2DM to CVD (Marti *et al.*, 2012; Martín-Timón *et al.*, 2014; Ramzan *et al.*, 2021). Therefore, early CVD risk stratification in diabetic populations is crucial to limit the significant health burden of non-communicable diseases, especially in resource limited settings such as South Africa.

Arterial dysfunction plays a central role in the development of CVD and is a significant predictor of cardiovascular events (Laurent *et al.*, 2006; Kullo & Malik, 2007; Townsend *et al.*, 2015). Several traditional risk factors have shown a strong association with the development of arterial dysfunction, as measured by arterial stiffness, increased pressure pulsatility and wave reflection (Townsend *et al.*, 2015). Although traditional risk factor exposure accelerates the development of arterial dysfunction, T2DM is considered an independent risk factor for the development of arterial dysfunction (Martín-Timón *et al.*, 2014). Indeed, in patients with T2DM increased arterial stiffness, pressure pulsatility and wave reflection are independently associated with cardiovascular events (Sharif *et al.*, 2019; Kim *et al.*, 2020; Tran *et al.*, 2021). However, the exact mechanisms of arterial dysfunction in patients with T2DM is currently under investigation. Therefore, identifying molecular biomarkers of these early arterial function changes may improve CVD risk stratification in patients with T2DM and increase our understanding of the molecular mechanisms underlying the pathogenesis of CVD in diabetic patients.

Epigenetic modifications, including gene regulation by microRNAs (miRNAs), have previously been associated with CVD pathogenesis (Sun *et al.*, 2019; Churov *et al.*, 2019). Considering the strong involvement of genetic and lifestyle factors in the development of diabetes, it is not surprising that various epigenetic modifications have received considerable attention in the regulation of T2DM-induced CVD (Churov *et al.*,

2019; Metere & Graves, 2020). Indeed, altered expression of various miRNAs, a measure of epigenetic changes, have been reported in individuals with T2DM. However, the relationship of these epigenetic changes and the development of CVD in T2DM require elucidation (Weale *et al.*, 2021).

With regards to epigenetic changes, miRNA expression has received considerable attention in the last decade, as it has shown to influence several disease processes. MiRNAs are short, non-coding sequences of RNA that suppress gene expression through post-transcriptional modifications (Churov *et al.*, 2019; Lopez-Pedrera *et al.*, 2020). Several miRNAs reportedly influence molecular pathways involved in arterial dysfunction (Sun *et al.*, 2019; Ramzan *et al.*, 2021). In this regard, miR-146a-5p has been associated with several processes underlying the development of arterial dysfunction. The molecular targets of miR-146a-5p have been associated with inflammation, oxidative stress, fibrosis, hyperglycaemia, and endothelial dysfunction, which are all pathways that are central to the development of arterial dysfunction (Lo *et al.*, 2017; Mann *et al.*, 2017; Xie *et al.*, 2018; Liu *et al.*, 2018; Runtsch *et al.*, 2019). Despite being extensively associated with these molecular pathways that contribute to vascular dysfunction and CVD, limited research has been performed on the direct associations between miR-146a-5p and arterial dysfunction, with only one previous study assessing the relationship between miR-146a-5p expression and arterial stiffness measures in a diabetic population (Guo *et al.*, 2020). Furthermore, these findings have not been corroborated, highlighting the need for further research to determine the direct relationship between miR-146a-5p and arterial dysfunction.

MiR-146a-5p expression in diseases characterised by systemic inflammation, is conflicting. Studies in inflammatory diseases such as rheumatoid arthritis and atherosclerosis, consistently report that miR-146a-5p expression is upregulated (Nakasa *et al.*, 2008; Cao *et al.*, 2015; Anaparti *et al.*, 2017). However, contradictory findings have been reported regarding miR-146a-5p expression patterns in T2DM. Although several studies have reported an increased miR-146a-5p expression (Kong *et al.*, 2011; Rong *et al.*, 2013; García-Jacobo *et al.*, 2019), others report a decreased miR-146a-5p expression (Balasubramanyam *et al.*, 2011; Lo *et al.*, 2017; Xie *et al.*, 2018; Zeinali *et al.*, 2021). Moreover, currently the association between changes in miR-146a-5p expression and arterial dysfunction in T2DM is uncertain. Therefore, further research is required to understand the changes in miR-146a-5p expression in

T2DM, and the potential risk factors that contribute to the altered expression. Furthermore, whether miR-146a-5p expression is associated with arterial dysfunction in T2DM warrant further investigation.

This thesis is structured into 5 chapters. Following the introduction in chapter 1, chapter 2 will give a literature review that summarises the current understanding of the role of miR-146a-5p in the pathogenesis of arterial dysfunction and its involvement in T2DM-related vascular injury and will also highlight the contradictory findings regarding the expression and function of miR-146a-5p in T2DM and early CVD. Chapter 3 will outline the study design and methods used in this research project. Chapter 4 will present the results of the study and chapter 5 will put our findings in the context of the current knowledge of miR-146a-5p.

Chapter 2: Literature Review

2.1 Introduction

Type II diabetes mellitus (T2DM) is a highly prevalent lifestyle disease, affecting approximately 6.1% of the global population (Ong *et al.*, 2023). The incidence of diabetes is increasing at an alarming rate and is projected to increase by 61% from 2021 to 2050 (Ong *et al.,* 2023). Patients with T2DM have a 2- to 2.5-fold increased risk of developing cardiovascular disease (CVD), compared to the general population (Gulsin *et al.*, 2019). While diabetes itself is considered one of the main risk factors for the development of CVD, diabetic patients commonly also have other traditional CVD risk factors, including hypertension, dyslipidaemia and obesity (Martín-Timón *et al.*, 2014; Ramzan *et al.*, 2021). Importantly, a high proportion of mortality in the diabetic population does not result from diabetes *per se* but rather from associated vascular complications (Johnson *et al.*, 2020). Indeed, diabetic populations are particularly prone to the development of vascular dysfunction (Dhananjayan *et al.*, 2016; Kim *et al.*, 2020). Changes in vascular function are considered one of the earliest predictors of CVD (Deanfield *et al.*, 2007; Dhananjayan *et al.*, 2016). Therefore, early detection of changes in vascular function may improve risk stratification amongst diabetic patients, which may improve disease management and prevention of cardiovascular morbidity and mortality. Hence, it is not surprising that the mechanisms associated with vascular dysfunction in persons with diabetes are currently under investigation.

2.2 Arterial dysfunction in T2DM

Arterial remodelling is a universal consequence of numerous diseases (van Varik *et al.*, 2012). Arterial dysfunction is an independent predictor of adverse cardiovascular events and all-cause mortality in both the general population and in T2DM (Cardoso *et al.*, 2013; Prenner & Chirinos, 2015; Kim *et al.*, 2020). Although several non-invasive measures of arterial function exist, arterial function measured by applanation tonometry is considered the non-invasive gold standard method (Townsend *et al.*, 2015; Barroso *et al.*, 2020). Measures of arterial function by applanation tonometry including arterial stiffness as measured by pulse wave velocity, pressure pulsatility and wave reflection, have all shown to be adversely affected in diabetic populations (Gajdova *et al.*, 2017). Several associations between these arterial function measures and diabetic disease characteristics have been reported (Gunarathne *et al.*, 2009; Park *et al.*, 2016; Guo *et al.*, 2020). In this regard, individuals with T2DM typically demonstrated arterial stiffness values equivalent to their non-DM counterparts that

were 15 years older (Cameron *et al.*, 2003). Although arterial stiffness increases with age, these results suggest the slope of the increase is steeper in T2DM patients compared to controls (Cameron *et al.*, 2003). Others have reported that independent of the systolic BP level, arterial stiffness is greater in T2DM than in their non-DM counterparts (Cruickshank *et al.*, 2002; Safar *et al.*, 2006; Wu *et al.*, 2023). Similarly, in a large population of middle-aged participants, T2DM was associated with increased aortic stiffening (Chirinos *et al.*, 2013). Changes in arterial stiffness and vessel thickness have also been observed in patients even with a short T2DM duration of less than 5 years, suggesting that many of the vascular changes occur early in the disease or even during the prediabetic state (Antoniou *et al.*, 2021). The measurement of arterial function could therefore provide clinically useful information that could assist with risk stratification and disease management. Nevertheless, despite being a focal point in the pathogenesis of both T2DM and CVD, arterial dysfunction has received little attention in clinical practice and hence further investigations on arterial function in clinical populations of T2DM are required.

*2.3 Measures of a*rterial function and its relation to subclinical CVD

Arterial remodelling is a natural process associated with ageing, termed arteriosclerosis, which can be accelerated in several disease states (Spinetti *et al.*, 2008; van Varik *et al.*, 2012). Arteriosclerosis is characterised by increases in large vessel stiffness, which involves partial destruction of elastic tissue, hypertrophy and hyperplasia of underlying vascular smooth muscle cells (VSMCs) and accelerated collagen deposition in the vessel interstitium (Wang *et al.*, 2010; Vlachopoulos *et al.*, 2010*b*; Barodka *et al.*, 2011; Cecelja & Chowienczyk, 2012). This remodelling process alters the mechanical properties of the blood vessels, resulting in decreased compliance and distensibility (McVeigh *et al.*, 2002). Functional large vessels expand during systole to act as a reservoir that accommodates the additional volume of blood, and recoil during diastole to ensure continuous flow of blood. During systole, the pressure generated by ventricular ejection results in the formation of an aortic pressure wave that travels down the arterial tree. The ability of the large vessels to dampen pulsatile energy created by cardiac contraction, as described by the Windkessel effect, becomes impaired when vessel compliance and distensibility is decreased (Nichols *et al.*, 2011). Although invasive hemodynamic measures obtained by catheterization are considered the gold standard for quantifying arterial function, several measures of

arterial stiffness, pressure pulsatility and wave reflection obtained by applanation tonometry have been shown to be strongly associated with invasive hemodynamic measures (Benetos *et al.*, 2010; Nelson *et al.*, 2010; Parragh *et al.*, 2015; Goodman & Kitchen, 2023). Moreover, these arterial function measures obtained by applanation tonometry have shown to predict cardiovascular events beyond brachial blood pressure (Roman *et al.*, 2007; Pini *et al.*, 2008; Wilkinson *et al.*, 2020). In this section, I will therefore provide a brief overview of the different hemodynamic measures obtained by applanation tonometry and how they relate to arterial function and CVD.

Carotid femoral pulse wave velocity (cfPWV) measures the rate of wave transmission and is considered the 'gold standard' measurement of aortic stiffness (Laurent *et al.*, 2006; Townsend *et al.*, 2015). PWV is obtained by measuring the time taken for a pulse wave to travel a specified distance, divided by the time to travel the distance. PWV relates the velocity by which the pulse wave travels within a vessel and the distensibility of that vessel, and therefore a stiffer vessel will have a faster PWV. It is well accepted the PWV increases with age (Diaz *et al.*, 2018). Increased PWV has been strongly associated with adverse cardiovascular events in several clinical population, independent of traditional risk factors including brachial BP (Bonarjee, 2018).

With arterial remodelling, the impedance against which the heart has to pump is also increased. This results in a substantial increase in central systolic pressure (Roman *et al.*, 2007; Subherwal *et al.*, 2010). This increase in central aortic pressure in turn increases central pulse pressure and reduces coronary blood flow. Importantly, increases in central systolic pressure and central pulse pressure have been associated with cardiovascular events (Vlachopoulos *et al.*, 2010*b*; McEniery *et al.*, 2014; Zuo *et al.*, 2020). While peripheral pulse pressure provides an indication of the elastic capacity of larger arteries, it is well established that central aortic pressure and central pulse pressure are superior in predicting CVD risk beyond that of brachial blood pressure and brachial pulse pressure (Roman *et al.*, 2007; Pini *et al.*, 2008; Zuo *et al.*, 2020).

When progressing distally along the arterial tree, there is a modification in the elastic properties of the vessels, leading to a gradual amplification of the pressure waveform (Wilkinson *et al.*, 2001; Laurent *et al.*, 2006; Nichols *et al.*, 2008; Avolio *et al.*, 2009; Mitchell *et al.*, 2010; Barodka *et al.*, 2011). This results in a higher pulse pressure

observed in the smaller to medium vessels compared to the larger central arteries, termed as central-to-peripheral pulse pressure amplification. When central (aortic) systolic and pulse pressure increase, as a result of remodelling of the large elastic vessels, the central-to-peripheral pulse pressure amplification is reduced. A decreased pulse pressure amplification is associated with an increased cardiac workload and hence may result in adverse cardiac remodelling (Benetos *et al.*, 2010). Indeed, decreased pulse pressure amplification has been associated with adverse cardiovascular outcomes (Benetos *et al.*, 2010, 2012; Regnault *et al.*, 2012).

Besides the increased workload to the heart, arterial remodelling may also contribute to cardiovascular events by facilitating the transmission of pulsatile energy into end-organ circulatory systems (Mitchell *et al.*, 2011). Therefore, an alternative large artery change that has been hypothesised to be of importance with respect to the pathogenesis of cardiovascular events is increased wave reflection. Pressure waves traveling through the arterial tree are reflected off medium and small vessels back to the central aorta (Mitchell *et al.*, 2011). This results in the aortic pressure pulse waveform comprising both incident (forward) and reflected waves (Nichols *et al.*, 2008; Avolio *et al.*, 2009; Mitchell *et al.*, 2010; Barodka *et al.*, 2011). Changes in blood vessel composition, diameter, and branching points can alter wave reflection back to the aorta. Consequently, the faster traveling reflected wave reaches the heart during systole, augmenting aortic systolic pressures and reducing diastolic pressure, which can limit coronary perfusion. Increased wave reflection leads to increased central pulse pressure, which in turn contributes to a reduced pulse pressure amplification. The reduced central-to-peripheral pressure amplification contributes to a significantly heightened risk of cardiovascular events (Dart & Kingwell, 2001; Belfiore *et al.*, 2022). In addition to the reflected wave, the forward wave is considered a marker of proximal arterial function and peak aortic flow (Mitchell *et al.*, 2008; Mitchell, 2015). Although the reflected wave has been associated with increased CVD risk, the forward wave has shown to contribute significantly to elevated pulse pressure with ageing (Mitchell *et al.*, 2010).

The augmentation index is calculated as the increment in pressure after the first systolic shoulder to the peak of the aortic pressure wave, which is expressed as a percentage of the pulse pressure (Kelly *et al.*, 1989), and has been used as a surrogate marker of wave reflection (Wang *et al.*, 2010; Chirinos *et al.*, 2012). While

some studies have reported significant associations between augmentation index and cardiovascular events (London *et al.*, 2001; Ueda *et al.*, 2004; Chirinos *et al.*, 2005; Weber *et al.*, 2005; Vlachopoulos *et al.*, 2010*a*), not all studies have replicated these findings (Mitchell *et al.*, 2010; Hayashi *et al.*, 2014). Several reports have criticized augmentation index as a measure of wave reflection, as it incorporates a significant proportion of the forward wave (Heusinkveld *et al.*, 2019). Recent data suggest stronger relationships between indices of wave reflection as determined by wave separation analysis, into the component forward and reflected waves, and cardiovascular outcomes (Wang *et al.*, 2010; Chirinos *et al.*, 2012).

Taken together, the above-mentioned measures of aortic stiffness and arterial function obtained by applanation tonometry provides insight into physiological and functional changes due to adverse arterial remodelling. These arterial function measures may provide useful information for CVD risk stratification in T2DM and potential markers of monitoring disease progression, beyond that predicted by brachial blood pressure. However, to aid the interpretation of these measures, it is important to understand the mechanisms underlying altered arterial function in T2DM.

2.4 Mechanisms underlying the development of arterial dysfunction in T2DM

While arterial remodelling and dysfunction occurs as a part of the natural ageing process, cardiovascular risk exposure accelerates this process (Antonini *et al.*, 2007; Castellon & Bogdanova, 2016). Although several traditional risk factors have been associated with arterial dysfunction, it is suggested that systemic inflammation and oxidative stress may be the primary mechanisms that drive the process of arterial remodelling and hence, the development of arterial dysfunction (Lavi *et al.*, 2008; Wenzel *et al.*, 2008; Nazari-Jahantigh *et al.*, 2012; Kattoor *et al.*, 2017; Petrie *et al.*, 2018). Indeed, several studies have shown a strong, independent relationship between markers of low-grade inflammation and arterial dysfunction, including PWV (Jain *et al.*, 2014; Ambrosino *et al.*, 2015; Muhammad *et al.*, 2017; Aminuddin *et al.*, 2020; Agbaje *et al.*, 2023), wave reflection (Schroeder *et al.*, 2019), augmentation index (Mahmud & Feely, 2005; Ambrosino *et al.*, 2015), and pulse pressure (Abramson *et al.*, 2002; Amar *et al.*, 2004) and pulse pressure amplification (Mozos *et al.*, 2019). Considering that T2DM is characterized by low-grade inflammation, it is not surprising that similar associations between inflammatory cytokines, oxidative stress and

measures of arterial dysfunction have been reported in diabetic populations (Kotani & Yamada, 2014; Mozos *et al.*, 2017; Reddy *et al.*, 2017; Zhang *et al.*, 2021).

T2DM contributes to the development of arterial dysfunction by promoting endothelial dysfunction, VSMC proliferation and extracellular matrix (ECM) alterations (Kolluru *et al*., 2012; Reddy *et al*., 2015). This development is summarised in Figure 2.1. Endothelial dysfunction is considered one of the earliest manifestations of vascular pathology (Gimbrone & García-Cardeña, 2016), and has been shown to occur as a consequence of hyperglycaemia, impaired insulin signalling and inflammation, which are characteristic features of diabetes (Sena *et al.*, 2013). Endothelial cells are uniquely targeted in T2DM, due to their incapacity to regulate glucose influx (Spinetti *et al.*, 2008). Hyperglycaemia promotes protein glycation and excessive generation of reactive oxygen species (ROS) within endothelial cells, that negatively impacts on cell function. Increased production of ROS suppresses the expression of endothelial nitric oxide synthase (eNOS) (Marti *et al*., 2012; Paulus & Tschöpe, 2013). Reductions in eNOS ultimately result in decreased nitric oxide (NO) bioavailability, which is required for appropriate vasodilation (Liao, 2013; Kattoor *et al*., 2017). A hallmark feature of endothelial dysfunction is therefore excessive ROS production, with associated impairments in vascular relaxation (Steyers & Miller, 2014).

Endothelial dysfunction further impacts negatively on arterial function. Reduced endothelial derived nitric oxide and increased activity of opposing vasoconstrictive substances such as endothelin-1 results in an increased contractile tone of the underlying VSMCs. The net result is a functional stiffening of the large vessels (McEniery & Wilkinson, 2005). Moreover, in diabetic patients, hyperglycaemia and oxidative stress also cause the VSMCs to become resistant to nitric oxide (Manea *et al.*, 2013). These functional alterations cause increased vessel stiffness in the early stages of T2DM.

The endothelium may also be directly activated by circulating inflammatory cytokines, that are released as a consequence of hyperglycaemia-associated ROS production. Many studies have shown that inflammation is the central to the development of endothelial dysfunction and exacerbates oxidative stress and vascular remodelling (Paulus & Tschöpe, 2013; Cheng *et al.*, 2013; Sena *et al.*, 2013). Once the endothelium is activated, several processes may ensue that result in remodelling of the vessel following endothelial dysfunction.

In addition to functional arterial stiffness, inflammation-induced endothelial dysfunction triggers various modifications within the extracellular matrix, including VSMC proliferation and increased production of structural proteins, notably collagen (Lacolley *et al.*, 2018). Inflammatory cytokines activate matrix metalloproteinases (MMPs) that are involved in ECM remodelling (Park & Lakatta, 2012), particularly elastin degradation and increased collagen production. The resultant increase in collagen/elastin ratio increases vessel stiffness (Intengan & Schiffrin, 2001). Indeed, molecular studies have demonstrated that arterial stiffness is mediated by increased gene expression of MMPs and collagen, in animal models of T2DM (Song & Ergul, 2006).

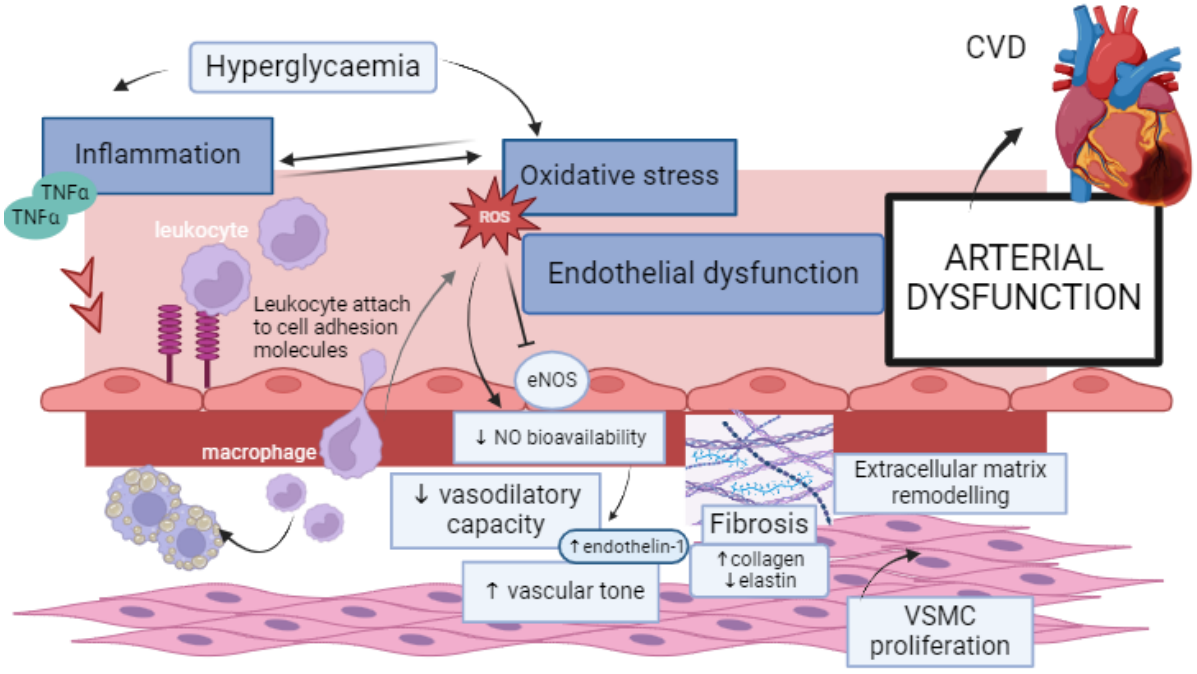

Figure 2.1 A summary of the development of arterial dysfunction in T2DM. Inflammatory stimulation and oxidative stress, induced in diabetes, are key features of endothelial dysfunction. Excessive ROS production impairs vasodilatory functions. This is exacerbated by the stimulation of arterial remodelling pathways such as ECM remodelling, VSMC proliferation and fibrosis which increase vascular tone and further lead to arterial dysfunction. Created with BioRender.com.

Taken together, hyperglycaemia and the resultant increases in oxidative stress and inflammation are believed to drive the development of endothelial dysfunction. Endothelial dysfunction, the earliest evidence of vessel remodelling, is characterised

by impaired vasodilatory capacity and changes in the structural vessel components which may lead to vessel stiffness and impaired vessel function. Although our understanding of the molecular mechanisms underlying the development arterial dysfunction has improved significantly over the past decade, the prevention and management of T2DM-induced functional vessel changes remain suboptimal (Gulsin *et al.*, 2019; Palanca *et al.*, 2019). Therefore, identifying biomarkers that may predict early arterial changes have received considerable attention. Although changes in gene expression of markers associated with endothelial dysfunction have been related to arterial dysfunction, most of these candidate markers detect endothelial dysfunction in the advanced stages, when cardiovascular complications are already apparent (Gimbrone & García-Cardeña, 2016; Chia *et al*., 2020; Kumric *et al*., 2021). Considering the strong impact of both genetic and lifestyle factors in the development of T2DM, identifying genetic markers that detect early changes in arterial dysfunction may improve risk stratification in an at-risk diabetic population.

2.5 Epigenetic modifications and CVD pathogenesis

DNA methylation, histone modifications and non-coding RNA regulation are epigenetic modifications that affect gene expression without changing the DNA sequence (Lopez-Pedrera *et al.*, 2020). These modifications occur as a result of lifestyle factors and underpin the influence of the environment on the genome. MicroRNAs (miRNAs) have received considerable attention due to the altered expression patterns noted in various lifestyle diseases and CVD (Churov *et al.*, 2019; Sun *et al.*, 2019; Weale *et al.*, 2021; García-Jacobo *et al.*, 2019).

MiRNAs are short, non-coding, single-stranded molecules of RNA that post-transcriptionally modify major signalling pathways by either promoting the degradation of mRNA strands or by inhibiting their translation into protein (Churov *et al.*, 2019; Lopez-Pedrera *et al.*, 2020). MiRNAs precisely regulate cell signalling pathways by binding to the 3’ untranslated region (3’ UTR) of specific mRNA sequences (Churov *et al.*, 2019). Due to their short length, a single miRNA can bind directly to multiple targets, thereby regulating various physiological processes simultaneously (Taganov *et al.*, 2006). In addition, miRNAs are stable molecules in different physiological environments, which make them promisingly suitable biomarker candidates (Li & Zhang, 2015). Several miRNAs reportedly influence molecular pathways involved in

the pathogenesis of endothelial dysfunction and arterial dysfunction (Sun *et al.*, 2019; Ramzan *et al.*, 2021). Furthermore, the expression of several miRNAs is altered in T2DM patients (Weale *et al.*, 2021). However, whether altered expression of microRNAs in T2DM is associated with endothelial and/or arterial dysfunction remains uncertain.

2.6 MiR-146a-5p as a potential biomarker for arterial dysfunction

One of the miRNAs that has received considerable attention in several disease processes related to vascular dysfunction is miR-146a-5p. MiR-146a-5p has been implicated in the regulation of inflammatory signalling cascades, oxidative stress and endothelial function (Lopez-Pedrera *et al.*, 2020). Furthermore, miR-146a-5p has consistently been reported to be dysregulated in T2DM (Wang *et al.*, 2014; Sun *et al.*, 2019). Given the importance of diabetes-induced endothelial dysfunction in the development of arterial stiffness, the regulation of these key processes by miR-146a-5p may be useful to better understand the pathogenesis of arterial dysfunction in T2DM. Therefore, further research into the role of miR-146a-5p in the involvement of diabetes-induced arterial dysfunction is warranted.

2.6.1 Inflammation as a stimulus for increased miR-146a-5p expression

A testament to its central role in the inflammatory response, miR-146a-5p expression is stimulated by multiple proinflammatory factors. In this regard, increased miR-146a-5p expression has shown to be induced by proinflammatory cytokines including tumour necrosis factor alpha (TNFα), and interleukins (IL)-1β and IL-8, in multiple *in vitro* and *in vivo* studies (Nakasa *et al.*, 2008; Li *et al.*, 2012; Cheng *et al.*, 2013). *In vitro* models of inflammation including lipopolysaccharide (LPS)-stimulated cells, used to initiate a high-grade inflammatory environment, and oxidised low-density lipoprotein (oxLDL)-stimulated cells, a model of the chronic inflammatory disease mediated atherosclerosis, have both been shown to induce an increased expression of miR-146a-5p (Taganov *et al.*, 2006; Li *et al.*, 2016). *In vivo* murine models, characterised by systemic inflammation, also showed increased miR-146a-5p expression (Cheng *et al.*, 2017; Mann *et al.*, 2017).

In keeping with the theory that miR-146a-5p expression is linked to inflammation, it has been shown that miR-146a-5p expression is stimulated through toll-like receptor (TLR) signalling (Taganov *et al.*, 2006). The TLR family are important mediators of

inflammatory pathways (Taganov *et al.*, 2006; El-Zayat *et al.*, 2019). The activation of the transcription factor nuclear factor kappa beta (NFκB) is pivotal to the TLR-mediated inflammatory response, with NFκB considered the 'master regulator' of all TLR responses (Carmody & Chen, 2007). Accordingly, several studies have shown that stimulation of miR-146a-5p expression occurs in a NFκB-dependent manner (Taganov *et al.*, 2006; Li *et al.*, 2016; Mann *et al.*, 2017). Evidently, the expression of miR-146a-5p is tightly linked to inflammation, as depicted in Figure 2.2.

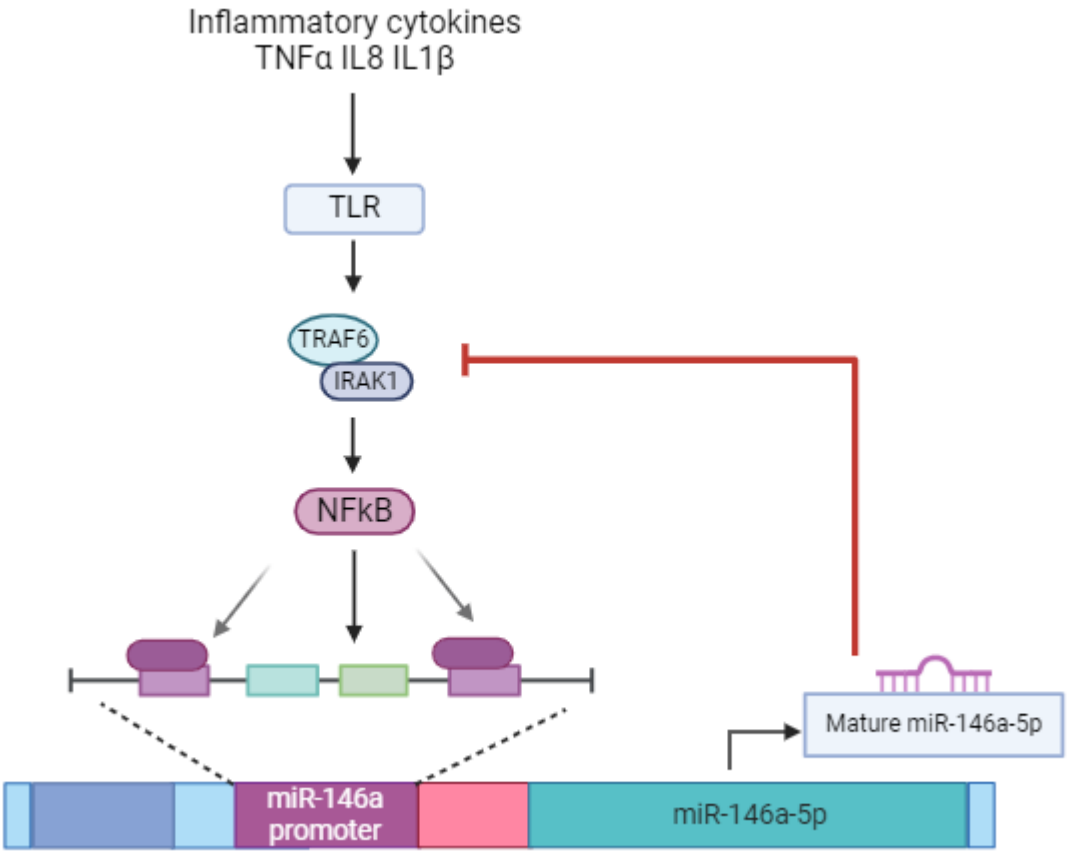

Figure 2.2 A summary of the relationship between miR-146a-5p and inflammation. The expression of miR-146a-5p is stimulated through inflammatory pathways and miR-146a-5p is involved in a negative feedback loop that inhibits inflammation. Created with BioRender.com.

2.6.2 Anti-inflammatory effects of miR-146a-5p upregulation

The upregulation of miR-146a-5p has been shown to have anti-inflammatory effects in the endothelium (Taganov *et al.*, 2006; Lo *et al.*, 2017). However, the regulatory ability of miR-146a-5p is complex and has far-reaching impact beyond is direct targets.

Importantly, miR-146a-5p is considered to regulate NFκB-mediated inflammation in a negative feedback manner, as its expression is dependent on the activation of NFκB, as previously stated (section 2.6.1), and in turn, increased miR-146a-5p expression inhibits the activation of NFκB (Mann *et al.*, 2017; Xie *et al.,* 2018). In this regard, miR-146a-5p binds to and inhibits TNFα receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), two important upstream adaptors of the NFκB signalling pathway, and therefore suppresses NFκB activity which results in anti-inflammatory effects (Taganov *et al.*, 2006). The suppression of TLR signalling through this inhibition of TRAF6/IRAK1 is an essential regulatory action that is thought to aid in 'fine-tuning' the inflammatory immune response and prevent unimpeded NFκB stimulation, thereby attenuating inflammation (Lo *et al.*, 2017; Hung *et al.*, 2019). The anti-inflammatory role of miR-146a-5p is depicted in Figure 2.2 above. Indeed, an increased expression of miR-146a-5p has been associated with a decrease in the circulating levels of TNFα, IL-6, IL-8, and IL-1β, demonstrating that cytokine production is halted, along with the progression of inflammation (Balasubramanyam *et al.*, 2011; Wang *et al.*, 2014; Xie *et al.*, 2018). Furthermore, the increased expression of miR-146a-5p was observed to enhance the polarisation of macrophages to the anti-inflammatory "M2" phenotype, as well as opposing the activation of proinflammatory "M1" macrophages (Huang *et al*., 2016). M2 macrophages express anti-inflammatory cytokines and stimulate tissue repair, which further highlights the role of miR-146a-5p in the process of inflammation.

Considering the strong association between chronic inflammation, endothelial dysfunction and CVD it is not surprising that the anti-inflammatory effects of miR-146a-5p expression have been suggested a biomarker of interest involved in the protection against the early stages of subclinical CVD (Cheng *et al.*, 2013).

2.6.3 The role of miR-146a-5p in endothelial dysfunction

The involvement of miR-146a-5p in attenuating endothelial dysfunction is multifactorial. First, it is well known that the activation of the NFκB pathway is central to the development endothelial dysfunction (Patel & Santani, 2009). Therefore, the miR-146a-5p-mediated inhibition of NFκB may prevent the progression of endothelial dysfunction. Second, treatment with miR-146a-5p mimics in both T2DM mice models and glucose-stimulated endothelial cells has shown to downregulate the expression of cell adhesion molecules and reduce adhesion of monocytes to endothelial cells,

both of which are associated with endothelial dysfunction progression (Wang *et al.*, 2014; Lo *et al.*, 2017). Third, miR-146a-5p has been shown to downregulate the expression of CXCL16, which functions both as a chemokine and an adhesion molecule in the endothelium associated with the pathogenesis of endothelial dysfunction (Xiao *et al.,* 2019). Lastly, miR-146a-5p attenuates oxidative stress, a key component of endothelial dysfunction. In this regard, miR-146a-5p combats oxidative stress and restores redox balance by directly binding and inhibiting the ROS-producing enzyme, NADPH oxidase 4 (NOX4), which reduces superoxide anion generation in the endothelium (Wang *et al.*, 2014; Xie *et al.*, 2018). MiR-146a-5p expression was also shown to be positively correlated to superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) levels, which are antioxidant proteins, and negatively correlated to levels of malondialdehyde (MDA) and a subunit of NOX, p22phox, which are pro-oxidant indicators, demonstrating its involvement in oxidative balance (Xie *et al.*, 2018).

Taken together, miR-146a-5p opposes the progression of endothelial dysfunction by limiting the molecular processes involved in endothelial activation, reducing inflammation, and combatting oxidative stress. Considering that endothelial dysfunction is one of the earliest prognostic markers and often progress to the development of arterial dysfunction, what is the evidence that miR-146a-5p is directly related to arterial dysfunction?

2.6.4 The association of miR-146a-5p with arterial function

Despite such a strong association between miR-146a-5p and the molecular mechanisms of endothelial dysfunction, the evidence supporting an association between miR-146a-5p and arterial function variables in human studies is severely lacking. In the general population, to our knowledge, there have been no direct reports of associations between miR-146a-5p and markers of arterial function. Although some studies have suggested that miR-146a-5p may be associated with the development and progression of hypertension, it has been proposed based on the link via endothelial dysfunction (Nemecz *et al.,* 2016). The only studies that have reported a relationship between miR-146a-5p and arterial remodelling were in populations characterised by systemic inflammation. In this regard, in patients with rheumatoid arthritis, miR-146a-5p was significantly associated with carotid intima media thickness, a marker that represents vascular remodelling in response to increased blood

pressure (Llop *et al.,* 2023). These authors further showed that miR-146a-5p is associated with carotid plaque presence, but not with pulse wave velocity (Llop *et al.,* 2023). Similarly, in patients with carotid plaque, miR-146a-5p was associated with more severe coronary artery stenosis (Huang *et al.*, 2020). In T2DM patients, to our knowledge, only one study has reported that increased miR-146a-5p was associated with arterial stiffness (Guo *et al.,* 2020). Furthermore, miR-146a-5p has demonstrated to prevent and/or alleviate both diabetic retinopathy and nephropathy, further suggesting its role in vascular remodelling in diabetes (Chen *et al.,* 2017; Zhuang *et al.,* 2017; Wan & Li, 2018). Clearly, the low abundance of evidence showing associations (or the lack thereof) between miR-146a-5p and a comprehensive set of arterial function markers warrant investigation.

Although direct associations between miR-146a-5p and markers of arterial remodelling are lacking, mechanistic studies in animals and cell models support a role for miR-146a-5p in the process of arterial remodelling, summarised in Figure 2.3 below, beyond its role in inflammation-induced endothelial dysfunction. In this regard, similar to the human studies, miR-146a-5p was associated with the atherosclerotic process, driven by inflammation (Li *et al.*, 2015). Moreover, in our laboratory we recently showed a significant association between miR-146a-5p and markers of vessel inflammation, pentraxin-3, and vessel reactivity in a collagen-induced arthritis model (Gunter *et al.,* 2022). Indeed, it has been suggested that miR-146a-5p may play a role in vascular remodelling via changes in smooth muscle proliferation and fibrosis, which may further contribute to arterial dysfunction.

Dannert *et al.* (2018) showed that miR-146a-5p downregulates the expression and activity of matrix metalloproteinase (MMP)-2 and MMP-9. Additionally, miR-146a-5p expression has also been associated with decreased levels of MMP-3, an enzyme involved in degrading several ECM components (Liu *et al.*, 2018; Wan *et al.*, 2021). Furthermore, miR-146a-5p has also been associated with decreased fibronectin expression (Feng *et al.*, 2011). Fibronectin is an ECM protein that, when deposited, stimulates endothelial remodelling by forming a network between cell-surface receptors and ECM compounds, such as collagen, as well as leading to basement-membrane thickening (Feng *et al.*, 2011; Halper, 2021), and hence a key component of increased arterial stiffness (Marti *et al.*, 2012; Al-Yafeai *et al.*, 2018).

MiR-146a-5p has also been demonstrated to promote VSMC proliferation and inhibit the apoptosis of these cells, by targeting and inhibiting the pro-apoptotic factor p53, and two anti-proliferative regulators, Notch2 and Krüppel-like factor 4 (KLF4) (Sun *et al.*, 2011; Cao *et al.*, 2015; Luo *et al.,* 2017). Increased proliferation of VSMCs augments their accumulation in the subendothelial space, affecting the structure and thickness of the vessel wall, which can increase vascular tone and arterial stiffness (Townsend *et al.*, 2015; Lacolley *et al.*, 2017). Although VSMCs are beneficially involved in the attempt to repair the damaged endothelium in the early stages of arterial dysfunction (Cao *et al.,* 2022), when their proliferation is dysregulated, they contribute adversely to arterial dysfunction and vascular remodelling changes (Lacolley *et al.,* 2017; Cao *et al.,* 2022). Hence, whether the relationship between miR-146a-5p and VSMC proliferation accelerates or protects against arterial dysfunction warrant further investigation.

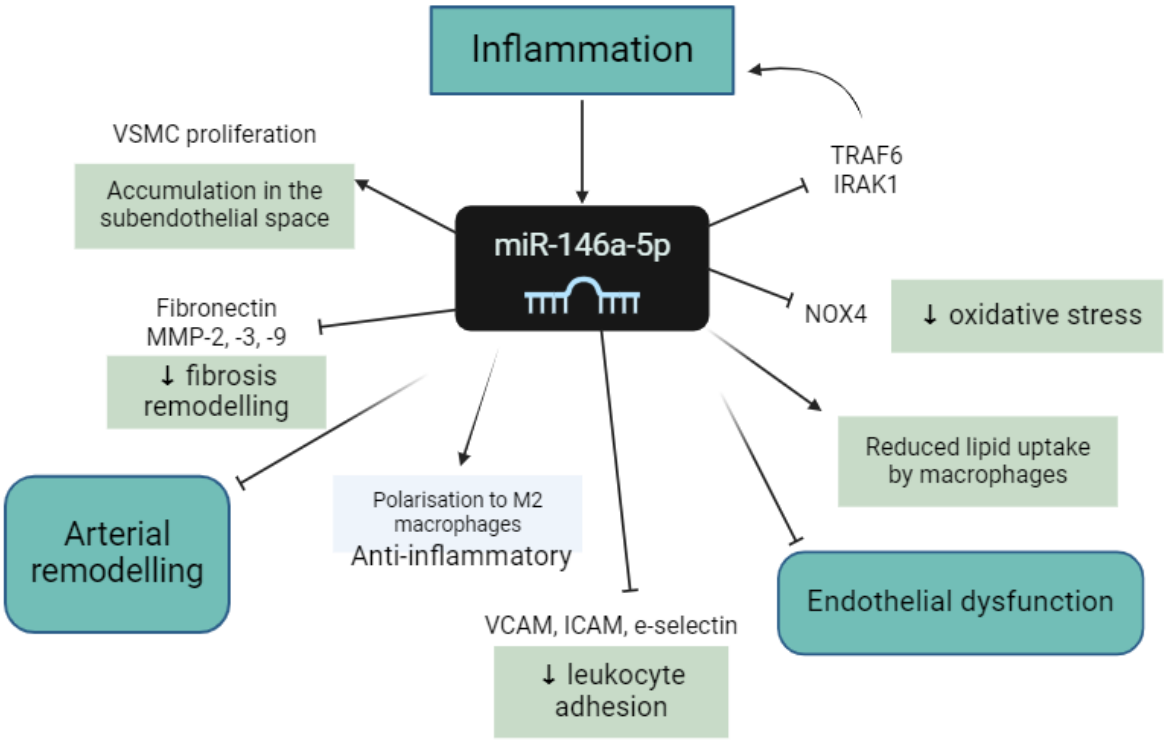

Figure 2.3 A Summary of the role of miR-146a-5p in arterial function. The upregulation of miR-146a-5p has been associated with multiple pathways that contribute to arterial dysfunction, including arterial remodelling, inflammation and endothelial dysfunction. Created with BioRender.com.

Taken together, there is a lack of evidence supporting the association between miR-146a-5p and arterial function. However, mechanistic studies suggest a protective role of miR-146a-5p and the processes involved in the development of arterial dysfunction, including endothelial dysfunction, extracellular matrix remodelling and VSMC proliferation. However, the translational aspects of these mechanisms require investigation.

2.7 Dysregulation of miR-146a-5p in different diseases states

Considering the expression of miR-146a-5p is stimulated by inflammation, it is not surprising that majority of the studies reporting dysregulated expression of miR-146a-5p levels have been reported in chronic systemic inflammatory disorders. Increased miR-146a-5p expression has been reported in whole blood samples and synovial fibroblasts in rheumatoid arthritis patients; in atherosclerotic plaques; in macrophages from patients with cystic fibrosis; in serum samples from systemic lupus erythramatosus patients and obese compared to lean individuals (Nakasa *et al.*, 2008; Cao *et al.*, 2015; Anaparti *et al.*, 2017; Nunez Lopez *et al.*, 2017; Luly *et al.*, 2019; Labib *et al.*, 2020).

Despite diabetes being a disease characterised by low-grade systemic inflammation (Furman *et al.*, 2019; Okdahl *et al.*, 2022), the research regarding the expression of miR-146a-5p in T2DM populations is contradictory. Many studies investigating miR-146a-5p levels in T2DM populations showed that miR-146a-5p is downregulated in animal and cell culture models, as well as in T2DM patients (Balasubramanyam *et al.*, 2011; Yang *et al.*, 2014; Lo *et al.*, 2017; Xie *et al.*, 2018; Zeinali *et al.*, 2021). In contrast, others have shown an upregulation of miR-146a-5p expression in diabetic rats, and in T2DM patients as compared to healthy controls or prediabetic patients (Kong *et al.*, 2011; Rong *et al.*, 2013; Alipour *et al.*, 2020; Helal *et al.*, 2021). Some have suggested that differences in disease duration and progression may explain the differential miR-146a-5p expression in diabetic patients. Nevertheless, the contradictions between the relationship between systemic inflammation, diabetes and miR-146a-5p expression require elucidation.

2.8 Problem statement

The prevalence of T2DM is rising globally. Patients with T2DM have a disproportionally increased risk of developing CVD compared to the general population. Patients with

T2DM are particularly prone to arterial dysfunction, which is mainly driven by systemic inflammation induced by insulin resistance. Arterial dysfunction is an early sign of CVD and an independent predictor of cardiovascular events. Despite this, arterial function changes have been overlooked in clinical practice, and the prevention of arterial dysfunction in T2DM remains suboptimal. Current markers are generally not sensitive enough to detect early alterations in arterial function. Therefore, further investigations are required to increase our understanding of the mechanisms underlying in the development of arterial dysfunction, which may help to identify potential biomarkers that will allow better monitoring of CVD progression and improve CVD risk stratification in diabetic populations.

Considering that both lifestyle and genetic predispositions are associated with T2DM, epigenetic markers may be relevant to predict early changes in an at-risk diabetic population. In this regard, miR-146a-5p is of particular interest as its expression has been found to be inflammation-dependent. Furthermore, miR-146a-5p is suggested to have a protective role in arterial dysfunction, where it downregulates inflammation, oxidative stress, and arterial remodelling pathways. Regardless of the strong associations between miR-146a-5p and these molecular mechanisms associated with arterial function, minimal research has been performed on the direct relationship of miR-146a-5p with arterial function variables in human studies.

Moreover, dysregulated expression of miR-146a-5p has repeatedly been observed in diabetic populations, and despite T2DM being characterised by systemic inflammation, research on miR-146a-5p expression in T2DM is contradictory. Whether miR-146a-5p expression is associated with arterial function is currently unknown, and the contradictory relationship between diabetes and miR-146a-5p and the effect it may have on arterial dysfunction in T2DM populations warrants further investigation.

2.9 Aim

To investigate the changes in expression of miR-146a-5p in type II diabetes mellitus and to determine its association with arterial function.

2.10 Study Objectives

The specific objectives were to determine

- the difference in relative miR-146a-5p expression between non-diabetic and diabetic participants.

- whether the expression of miR-146a-5p is associated with traditional risk factors and markers of inflammation.
- the association between miR-146a-5p expression and measures of arterial function, including pressure pulsatility, wave reflection and arterial stiffness in a T2DM population.

Chapter 3: Methods

3.1 Ethical approval

This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (clearance number: M21/05/15, Appendix A) and adheres to the principles of the Declaration of Helsinki. All participants provided informed, written consent prior to participation in the study.

3.2 Study design and participants

This case-control study included 118 participants, categorised into two groups. Participants with a previous diagnosis of T2DM and/or insulin resistance comprised the DM group (n=67). Antidiabetic drugs recorded in this group included metformin, insulin, vildagliptin and glimepiride. The remaining participants were categorised into the non-DM control group (n=51). Participants were excluded if they had any history of cardiovascular events or CVD, were pregnant, had acute liver or kidney disease, or autonomic dysfunction, such as orthostatic hypotension or peripheral neuropathy. Participants were categorised as hypertensive if they had an average systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg, employed anti-hypertensive medications, or if they had a previous diagnosis of hypertension. Antihypertensive drugs recorded included hydrochlorothiazide, amlodipine, enalapril, perindopril, amiloride, atenolol, valsartan, losartan and bisoprolol. Dyslipidaemia was defined using total cholesterol (TC) and high-density lipoprotein (HDL) levels when the TC/HDL ratio > 4 (Agca *et al.*, 2017) or if lipid-lowering medications were in use. Lipid-lowering drugs included atorvastatin, simvastatin, bezafibrate, and rosuvastatin.

During a single visit to the School of Physiology at the University of the Witwatersrand, the participants' cardiometabolic risk characteristics and arterial function measures were recorded, and a 30 ml blood sample was collected and stored for later analyses of biomarkers and miRNAs.

3.3 Participant characteristics

Demographic and lifestyle variables were recorded using a standardised general health questionnaire, and included exercise, alcohol use, and smoking which were recorded as "yes" or "no". Anthropometric variables were obtained, including height, weight, waist and hip circumference. Obesity was defined as body mass index (BMI) > 30 kg/m^2. Increased waist circumference was defined as a waist circumference > 88 cm for females and > 102 cm for males. Standard protocols were used to measure

blood pressure. Fasting plasma glucose (FPG) levels and lipid profiles, including TC, HDL and triglycerides, were measured using a CardioChek Plus analyser at the point-of-care (Polymer Technology Systems, Inc., Indianapolis, IN, USA). Low-density lipoprotein (LDL) levels were estimated/calculated using the Friedewald equation (Friedewald *et al.*, 1972). A 30 ml blood sample was obtained in one EDTA-coated blood collection tube for plasma and four clot activator collection tubes for serum, centrifuged at 3000 RPM for 12 minutes, and stored separately as serum and plasma at -80°C.

Participants were stratified according to their ten-year CVD risk using the Framingham risk score (FRS), which was selected due its improved risk stratification compared to other prediction models (Selvarajah *et al.*, 2014). The FRS incorporates age, sex, SBP, use of antihypertensive drugs, smoking, diabetes and either BMI or HDL and TC levels to estimate a 10-year CVD risk score. Due to the limitations of using BMI in an African population (Manne-Goehler *et al.,* 2020), in the present study, FRS was calculated with both BMI and the lipid markers, and the two scores averaged. The use of either variable has been found to have equivalent performance in risk prediction (Selvarajah *et al.,* 2014), and the inclusion of both was done to add to the comprehensiveness of the risk assessment by incorporating as many risk factors as possible. A 10-year CVD risk of less than 10% is considered low risk, between 10% and 20% as moderate risk, and high risk as greater than or equal to 20% (Jahangiry *et al.*, 2017).

3.4 Arterial function

Radial applanation tonometry was used to evaluate central haemodynamic parameters using SphygmoCor software version 9.0 (AtCor Medical Pty. Ltd., West Ryde, New South Wales, Australia). After the participant had rested in a supine position for 15 minutes, a radial waveform was recorded using a high-fidelity SPC-301 micromanometer (Millar Instrument, Inc., Houston, TX, USA), interfaced with SphygmoCor software. Ten high-quality consecutive waveforms were recorded with the micromanometer. Where systolic or diastolic variability exceeded 5% in consecutive waveforms or if the pulse wave signal amplitude was below 80 mV, the measurement was excluded. The pulse waves were calibrated by manual measurement of brachial blood pressure that was performed immediately before the waveform recordings. A central aortic waveform was derived from the peripheral waveform using a validated generalised transfer function incorporated in the

SphygmoCor software, from which central blood pressures were derived. The following arterial function measures were calculated as previously reported (Libhaber *et al.*, 2015). Central pulse pressure (cPP) was determined as the difference between central SBP (cSBP) and DBP and pulse pressure amplification (PPA) as the ratio of peripheral pulse pressure to central pulse pressure. The central augmentation pressure (cAP) was determined as the difference between the second and first systolic shoulders of the central waveform and the augmentation index (AIx) was then calculated as the augmentation pressure divided by the central pulse pressure, given as a percentage.

The central waveform was separated by the SphygmoCor software into its forward and reflected wave components using a modified triangular waveform. Reflection magnitude (RM) was determined as the ratio of reflected wave pressure (RWP) to forward wave pressure (FWP), as a percentage. The carotid-femoral (aortic) pulse wave velocity (PWV) was determined using ten consecutive carotid and femoral waveforms. PWV was calculated as the distance between the carotid and femoral sampling sites divided by the transit time (Libhaber *et al.*, 2015). Technically sound arterial wave forms could be obtained in 108 participants and pulse wave velocity measurements in 116 participants.

3.5 Enzyme-Linked Immunosorbent Assays (ELISA)

Sandwich ELISAs were used to determine the serum concentrations of TNFα and MMP1 (ABclonal Technology Co., Ltd., Wuhan, China). The lower detection limit was 15.6 pg/ml for TNFα and 156.25 pg/ml for MMP1 and the intra- and inter-assay coefficients of variation were < 10% and < 15%, respectively. Samples were undiluted. A subset (n=43) of samples were assayed in duplicate for technical replicates. A Labsystems Multiskan Ascent microplate reader was used to read the ELISA plates at a wavelength of 450 nm. Concentrations of samples were interpolated from a four-parameter logistic curve fit using GraphPad Prism software (version 9.1.2).

3.6 Quantification of miR-146a-5p

3.6.1 RNA extraction

RNA was isolated from serum using the NucleoSpin® miRNA Plasma kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol with the addition of the recommended DNAse digestion step to prevent the interference of DNA during

later miRNA quantification steps. RNA extracts were eluted with 30 µl of RNase-free water.

3.6.2 cDNA Synthesis

cDNA templates were synthesised from the RNA extracts using TaqMan™ Advanced miRNA cDNA Synthesis kits (Thermo Fisher Scientific, Waltham, MA, USA). A poly(A) tailing step and an amplification step were included, in addition to the standard reverse transcription step, to facilitate adequate cDNA synthesis. cDNA production (>1000 ng/µl) was confirmed using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.6.3 Real-time quantitative PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was then performed using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems, Life Technologies, Waltham, MA, USA). Each reaction well contained cDNA (2 µl), Taqman Fast Advanced Master Mix (5 µl), pre-designed VIC-labelled probe mixes for the miRNA of interest (0.5 µl, Taqman Advanced miRNA Assay) or a FAM-labelled probe for the reference miRNA (0.25 µl, Taqman assay), and was made up to a total volume of 10 µl with RNase-free water. The reference miRNA used was miR-16-5p (Taqman assay ID: 477860_mir). According to the manufacturer's instructions, the following cycling conditions were performed for a total of 40 cycles: 20 seconds of enzyme activation at 95˚C, 1 second of denaturation at 95˚C, followed by 20 seconds of annealing and extension at 60˚C. For each sample, the endogenous control and the target were run on the same plate to avoid inter-plate variation.

3.6.4 Analysis of relative miRNA expression data using the ΔΔCT method

The expression of miR-146a-5p (Taqman assay ID: 478399_mir) was quantified using the Comparative CT method relative to an endogenous control miRNA, miR-16-5p (Livak & Schmittgen, 2001). We evaluated three potential endogenous controls, SnU6, miR-191, and miR-16-5p. MiR-16-5p was found to be most stable expressed and was therefore used as the control miRNA in this study. Samples with average Ct values ≥ 35 were removed (Dakterzada *et al.*, 2021). ΔCT values were determined by subtracting the Ct value for the control miR-16-5p from the Ct value for the target miR-146a-5p for each sample. The average ΔCT for the control group was then subtracted from each sample's ΔCT to determine its ΔΔCT. The fold-change in relative miR-146a-

5p expression was then calculated as $2^{-\Delta\Delta Ct}$. Due to the high number of CVD risk factors present in the non-diabetic control group, we identified 18 healthy participants with no CVD risk factors, that were used to determine the fold changes for the remainder of the participants. These participants did not have diabetes, insulin resistance or hypertension, did not smoke, had a TC/HDL ratio < 4, BMI < 30 kg/m^2 and SBP < 140 mmHg.

3.7 Data Analysis

Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc., USA). To achieve statistical power at 80% with a two-sided α value of <0.05 and medium effect size (0.15), a minimum sample size of 92 was required. Statistical significance was considered when $p<0.05$. Normality of variables was determined using Shapiro-Wilk tests. Normally distributed continuous data are presented as mean (standard deviation (SD)) and non-normally distributed data as median (interquartile range (IQR)). Non-normally distributed variables were logarithmically, or square root transformed prior to statistical analysis to improve normality. Categorical variables are presented as percentages or proportions. Differences between the DM and non-DM control groups were determined using Student's unpaired t-tests or Mann Whitney U tests, as appropriate, and Chi-squared tests for group differences in categorical variables. Differences between CVD risk groups were determined using a one-way ANOVA, followed by a Tukey's *post hoc* test.

The associations between patient characteristics and relative miR-146a-5p expression were determined using Pearson's correlation analysis, in age, sex and race adjusted models. Characteristics that had $p<0.05$ for associations with miR-146a-5p expression were included as potential confounders in subsequent analyses. Additionally, confounders of arterial function variables, determined similarly with Pearson's correlations, were included in fully adjusted multivariate models. The independent associations of miR-146a-5p expression with arterial function measures were then assessed in univariate and multivariate correlation models with the adjustment for potential confounders, identified as described.

There is an increased risk of cardiovascular disease in patients with T2DM, in addition to other traditional CVD risk factors. Therefore, we determined the impact of CVD risk profiles on the relationship between miR-146a-5p expression and arterial function

variables in interaction analysis. Interaction terms were determined based on results from multivariate regressions and stratified analysis was performed when appropriate (interaction terms $p<0.05$).

Chapter 4: Results

4.1 Participant characteristics

Of the total sample (n=118), fifty-one participants did not have a diagnosis of DM, while 67 participants were previously diagnosed with T2DM or insulin resistance by their primary care physician and were matched for age and sex (Table 4.1). The mean (SD) age was 47.7 (9.5) years in the non-DM group and 51.0 (8.3) years in the DM group (p=0.05). Of the total sample, 25% were men and there was no difference in sex distribution between the two groups (p=0.40). The DM group had a significantly greater proportion of black participants and a lower proportion of white participants compared to the non-DM group (both p<0.001).

Of the total sample, 51% was overweight or obese, and there was a significantly higher proportion of obese participants in the DM group compared to the non-DM group (p=0.001). There was no difference in lifestyle factors between the groups, including exercise (p=0.28), alcohol use (p=0.06) and smoking (p=0.31).

Table 4.1 Recorded characteristics of the study population.

Variable	Total (n=118)	Non-DM (n=51)	DM (n=67)	*p*
Demographic characteristics				
Age (years)	49.6 (8.9)	47.7 (9.5)	51.0 (8.3)	0.05
Male, n (%)	30 (25.4)	11 (9.3)	19 (16.1)	0.40
Race				
Black, n (%)	57 (48.3)	11 (9.3)	46 (39.0)	**<0.001**
White, n (%)	33 (28.0)	27 (22.9)	6 (5.1)	**<0.001**
Asian, n (%)	21 (17.8)	10 (8.5)	11 (9.3)	0.97
Mixed, n (%)	7 (5.9)	3 (2.5)	4 (3.4)	0.98
Anthropometry				
Height (m)	1.65 (0.09)	1.66 (0.09)	1.64 (0.09)	0.26
Weight (kg)	84.2 (17.8)	79.8 (20.1)	87.5 (15.2)	**0.02**
BMI (kg/m^2)	30.91 (6.35)	28.85 (6.93)	32.47 (5.42)	**0.002**
% Obese, n (%)	60 (50.9)	17 (14.4)	43 (36.4)	**0.001**
Waist circumference (cm)	94 (13)	89 (13)	98 (11)	**<0.001**
% Increased waist circumference, n (%)	62 (53.9)	21 (18.3)	41 (35.7)	**0.01**
Waist-hip ratio	0.87 (0.09)	0.83 (0.10)	0.90 (0.07)	**<0.001**
Lifestyle factors				
Exercise, n (%)	58 (49.2)	28 (23.7)	30 (25.4)	0.28
Alcohol use, n (%)	31 (26.3)	18 (15.3)	13 (11.0)	0.06
Current smoking, n (%)	21 (17.8)	7 (5.9)	14 (11.9)	0.31

Continuous variables are expressed as mean (SD) or median (IQR), as appropriate, and categorical variables as proportions or percentages. Significant group differences between non-DM and DM participants are shown in bold. P-values were determined for differences between the non-DM and DM groups using Student's unpaired t-tests, Mann Whitney U tests or Chi-squared tests, as appropriate. BMI: body mass index.

4.2 Cardiovascular disease risk factors

The DM participants had higher FPG levels compared to the control non-DM group (non-DM: median (IQR) 5.8 (5.5-6.1) mmol/l; DM: 6.9 (5.8-9.2) mmol/l, p<0.001, Table 4.2). The DM group had significantly higher triglyceride levels than the non-DM group (non-DM: median (IQR) 1.07 (0.75-1.55) mmol/l; DM: 1.41 (1.00-2.01) mmol/l, p=0.004), while the non-DM group had higher TC and LDL levels (respectively: p=0.045 and p=0.01). In the DM group, 91% of participants were on metformin and 48% were on insulin.

Of the total population, 49% were considered hypertensive, and the proportion of participants with hypertension was higher in the DM compared to non-DM groups (p=0.01). Additionally, there was a greater proportion of antihypertensive drug use in the DM group compared to non-DM group (p<0.001).

In the total population, 45% had dyslipidaemia. There were no differences in the prevalence of dyslipidaemia between the non-DM and DM groups (p=0.07) nor in the use of lipid-lowering drugs (p=0.76).

There was no difference in TNFα concentrations between the groups (non-DM: median (IQR) 7.5 (4.6-10.8) pg/ml; DM: 8.7 (4.5-12.0) pg/ml, p=0.19). MMP1 levels were increased in the DM group compared to the non-DM group (non-DM: median (IQR) 0.24 (0.09-0.59) ng/ml; DM: 0.45 (0.17-0.97) ng/ml, p=0.02).

The Framingham risk score was significantly higher in the DM group with a median (IQR) FRS of 13.2 (8.65-23.9) in the DM group and 3.5 (2.05-7.55) in the non-DM group (p<0.001). Of the 21 participants in the high CVD risk group (FRS≥20), 20 of those participants were in the DM group (95%). 80% of the non-DM participants were in the low CVD risk (FRS<10), compared to only 27% of the DM group (p<0.001).

Table 4.2 Recorded CVD risk factors of the study population.

Variable	Total (n=118)	Non-DM (n=51)	DM (n=67)	*P*
Traditional risk factors				
FPG (mmol/l)	6.1 (5.6-7.3)	5.8 (5.5-6.1)	6.9 (5.8-9.2)	**<0.001**
TC (mmol/l)	3.31 (2.79-4.01)	3.56 (3.01-4.16)	3.18 (2.63-3.95)	**0.045**
LDL (mmol/l)	1.47 (1.24-2.03)	1.70 (1.38-2.12)	1.34 (1.14-1.87)	**0.01**
HDL (mmol/l)	1.08 (0.94-1.25)	1.14 (1.02-1.32)	1.03 (0.89-1.19)	**0.003**
Triglycerides (mmol/l)	1.30 (0.93-1.76)	1.07 (0.75-1.55)	1.41 (1.00-2.01)	**0.004**
TC/HDL ratio	2.98 (2.57-3.61)	3.02 (2.48-3.43)	2.96 (2.67-3.66)	0.39
TC/HDL ratio > 4, n (%)	21 (17.8)	9 (7.6)	12 (10.2)	0.97
SBP (mm Hg)	129 (19)	122 (13)	134 (21)	**<0.001**
DBP (mm Hg)	81 (9)	77 (8)	84 (9)	**<0.001**
Hypertension, n (%)	54 (48.7)	17 (15.3)	37 (33.3)	**0.01**
Dyslipidaemia, n (%)	53 (44.9)	18 (15.3)	35 (29.7)	0.07
Inflammatory & fibrotic markers				
TNFα (pg/ml)	8.3 (4.6-11.5)	7.5 (4.6-10.8)	8.7 (4.5-12.0)	0.19
MMP1 (ng/ml)	0.33 (0.12-0.96)	0.24 (0.09-0.59)	0.45 (0.17-0.97)	**0.02**
Current pharmacological treatments				
Antidiabetic drugs, n (%)	65 (55.1)	0 (0)	65 (55.1)	**<0.001**
Antihypertensive drugs, n (%)	43 (36.4)	9 (7.6)	34 (28.8)	**<0.001**
Lipid-lowering drugs, n (%)	20 (17.0)	8 (6.8)	12 (10.2)	0.76
Cardiovascular disease risk				
Family history of CVD, n (%)	44 (37.3)	27 (22.8)	17 (14.4)	**0.002**
Framingham risk score	9.7 (3.55-17.30)	3.5 (2.05-7.55)	13.2 (8.65-23.9)	**<0.001**
CVD risk stratification*, n (%)				
Low (FRS <10)	59 (50)	41 (34.75)	18 (15.3)	**<0.001**
Moderate (10 ≤FRS <20)	38 (32.2)	9 (7.63)	29 (24.6)	**<0.001**
High (FRS ≥ 20)	21 (17.8)	1 (0.85)	20 (16.9)	**<0.001**

Continuous variables are expressed as mean (SD) or median (IQR), as appropriate, and categorical variables as proportions or percentages. Significant group differences between non-DM and DM participants are shown in bold. P-values were determined for differences between the non-DM and DM groups using Student's unpaired t-tests, Mann Whitney U tests or Chi-squared tests, as appropriate. FPG: fasting plasma glucose; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; SBP: systolic blood pressure; DBP: diastolic blood pressure; TNFα: tumour necrosis factor alpha; MMP1: matrix metalloproteinase 1; CVD: cardiovascular disease; FRS: Framingham risk score. * CVD risk stratification was based on the Framingham score.

4.3 Relative miR-146a-5p expression

Figure 4.1A shows that the relative expression of miR-146a-5p was significantly higher in the DM group (mean (SD) 0.47 (0.72)) compared to relative expression in the non-DM group (0.17 (0.67), p=0.02). Additionally, when stratified according to CVD risk, as shown in Figure 4.1B, miR-146a-5p was significantly increased in the moderate CVD risk group compared to the low-risk group (p<0.01) but miR-146a-5p expression did not differ between the low-risk group and the high-risk group.

4.4 Arterial function measures

Several pressure pulsatility variables including peripheral PP, central SBP and FWP were higher in the DM group than in the non-DM group (p=0.04, p=0.003, and p=0.045, respectively, Table 4.3). However, there were no differences in central PP (p=0.28) or PPA (p=0.45) between the groups.

Wave reflection properties did not differ between the groups (all p>0.05).

Arterial stiffness, as measured by carotid femoral PWV, was higher in the DM group compared to the non-DM group (non-DM: 7.2 (1.8) m/s; DM: 8.5 (4.3) m/s, p=0.04).

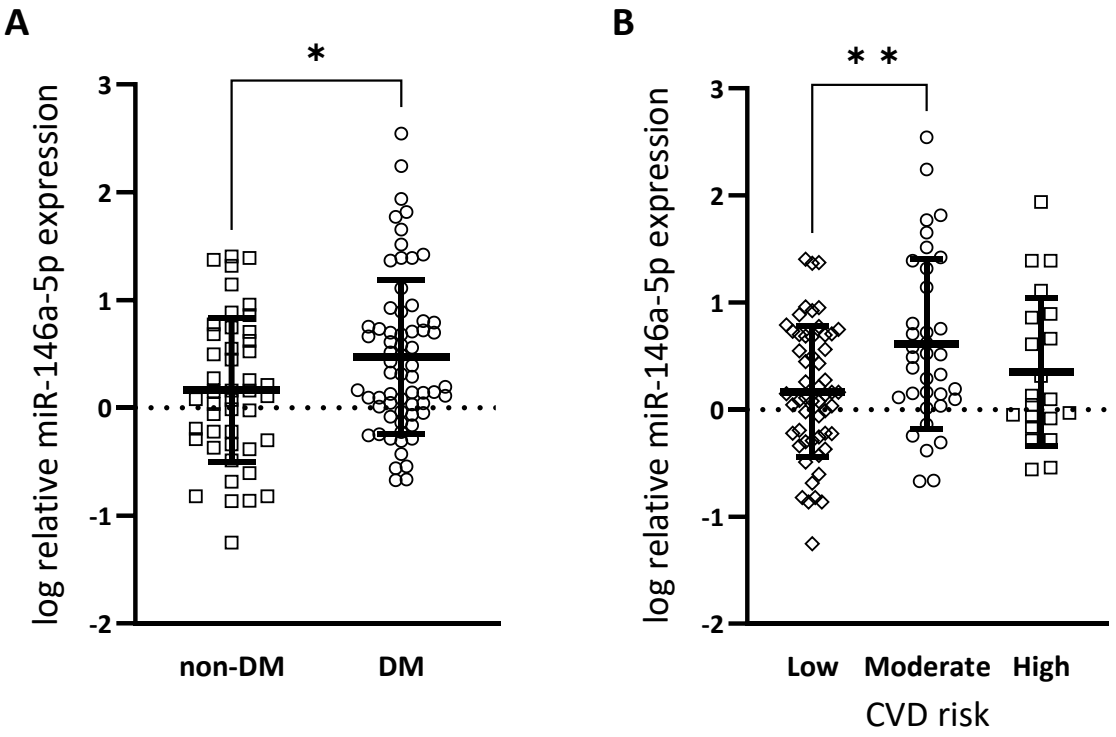

Figure 4.1 The relative expression of miR-146a-5p in (A) non-diabetic (non-DM) and diabetic (DM) groups and (B) in low-, moderate-, and high CVD risk groups. Relative miR-146a-5p expression was determined using RT-PCR in non-DM (n=46) and DM (n=66) participants, and in low-risk (n=59), moderate-risk (n=38) and high CVD risk (n=21) participants. Relative expression was log transformed to improve normality and data is presented as mean (SD), with the log-fold miR-146a-5p change relative to miR-16-5p. Differences between the non-DM and DM groups were determined using Student's unpaired t-test and between CVD risk groups using a one-way ANOVA followed by Tukey's *post hoc* test. *p<0.05; **p<0.01.

Table 4.3 Measures of arterial function in the study population.

Variable	Total (n=118)	Non-DM (n=51)	DM (n=67)	*p*
Heart rate (bpm)	73 (13)	69 (11)	76 (13)	**0.003**
MAP (mm Hg)	97 (12)	92 (9)	101 (12)	**<0.001**
Pressure pulsatility				
pPP (mm Hg)	46 (41-54)	44 (38-51)	47 (42-55)	**0.04**
cPP (mm Hg)	35 (28-41)	35 (28-39)	35 (28-43)	0.28
cSBP (mm Hg)	117 (107-128)	111 (102-124)	119 (111-133)	**0.003**
cDBP (mm Hg)	82 (9)	78 (9)	85 (9)	**<0.001**
PPA	1.3 (1.2-1.5)	1.3 (1.2-1.4)	1.3 (1.2-1.6)	0.45
FWP (mm Hg)	26 (22-30)	26 (21-28)	27 (23-32)	**0.045**
Wave reflection				
cAP	10 (7)	9 (5)	10 (8)	0.37
AIx (%)	25 (12)	26 (11)	24 (14)	0.56
RWP (mm Hg)	16 (13-21)	15 (13-20)	16 (13-22)	0.32
RM (%)	65 (18)	66 (17)	65 (19)	0.76
Arterial stiffness				
PWV (m/s)	7.9 (3.5)	7.2 (1.8)	8.5 (4.3)	**0.04**

Continuous variables are expressed as mean (SD) or median (IQR), as appropriate. Significant group differences between non-DM and DM participants are shown in bold. P-values were determined using Student's unpaired t-tests and Mann Whitney U tests, as appropriate. MAP: mean arterial pressure; pPP: peripheral pulse pressure; cPP: central pulse pressure; cSBP: central systolic blood pressure; cDBP: central diastolic blood pressure; PPA: pulse pressure amplification; FWP: forward wave pressure; AP: augmentation pressure; AIx: augmentation index; RWP: reflected wave pressure; RM: reflection magnitude; PWV: pulse wave velocity.

4.5 Associations between demographic characteristics and miR-146a-5p expression

Appropriate confounding factors for multivariate analysis were determined as participant characteristics that were significantly associated with miR-146a-5p expression. Because age, sex, and race are established confounders in the context of arterial function, these variables were included in the multivariate analysis to determine the appropriate confounding factors.

The traditional risk factors that were significantly associated with miR-146a-5p in univariate and adjusted multivariate analysis, shown in Table 4.4, included waist-hip ratio (partial r=0.29, p=0.002), triglyceride levels (partial r=0.20, p=0.04), TC/HDL ratio (partial r=0.20, p=0.04), TNFα concentrations (partial r=0.23, p=0.02), and antidiabetic (partial r=0.20, p=0.03) and antihypertensive drug use (partial r=0.25, p=0.01). These characteristics were thus included as potential confounders in subsequent regression analysis. In age, sex, and race adjusted analysis, MMP1 and miR-146a-5p expression were also significantly associated (partial r (95%CI) 0.21 (0.01-0.39), p=0.04).

Table 4.4 Associations between participant characteristics and relative miR-146a-5p expression.

	miR-146a-5p*			
	Univariate model		Adjusted model†	
	r	***P***	**Partial r**	***p***
Age	0.02	0.81	0.02	0.88
Sex	0.02	0.87	0.02	0.84
Race	0.07	0.48	0.07	0.51
Height	0.04	0.67	0.04	0.66
Weight	-0.02	0.88	0.01	0.88
BMI	-0.03	0.72	0.00	0.98
Waist circumference	0.17	0.08	0.17	0.08
Waist-Hip ratio	0.25	**0.01**	0.29	**0.002**
FPG*	0.13	0.16	0.14	0.16
TC*	0.11	0.27	0.10	0.32
LDL*	0.01	0.94	0.01	0.94
HDL*	-0.12	0.19	-0.13	0.20
Triglycerides*	0.22	**0.02**	0.20	**0.04**
SBP	0.04	0.66	0.07	0.47
DBP	0.05	0.58	0.05	0.61
TC/HDL ratio	0.21	**0.03**	0.20	**0.04**
TNFα#	0.22	**0.03**	0.23	**0.02**
Antidiabetic drugs	0.18	0.06	0.20	**0.03**
Antihypertensive drugs	0.22	**0.02**	0.25	**0.01**

Associations were determined using Pearsons' correlation analysis. † Adjusted for age, sex, and race. Significant relationships are shown in bold. *: Logarithmically transformed. #: square-rooted. BMI: body mass index; FPG: fasting plasma glucose; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; SBP: systolic blood pressure; DBP: diastolic blood pressure; TNFα: tumour necrosis factor alpha.

4.6 Associations between demographic characteristics and arterial function measures

Similarly, to determine the confounding factors of arterial function, we determined the univariate and age, sex, and race-adjusted associations of participant characteristics with pressure pulsatility measures (Table 4.5A) and wave reflection and arterial stiffness measures (Table 4.5B). Confounders of arterial function, not already identified as confounders due to their associations with miR-146a-5p, were then included in fully adjusted multivariate models. These included SBP, DBP, heart rate and weight (all $p<0.05$). Systolic and diastolic blood pressure were replaced by mean arterial pressure (MAP) in subsequent models.

Based on the associations between demographic variables and arterial function variables and miR-146a-5p expression, the following variables were included as confounders in multivariate models shown in Table 4.6: Multivariate Model 1 – age, sex, and race; Multivariate Model 2 – additionally adjusted for waist-hip ratio, triglyceride levels, TC-HDL ratio, TNFα levels, antidiabetic drug use, and antihypertensive drug use (Table 4.4); Multivariate Model 3 – further adjusted for MAP, heart rate, and weight (Tables 4.5A and 4.5B).

4.7 Associations of miR-146a-5p expression and arterial function variables

Table 4.6 shows that relative miR-146a-5p expression was associated with a higher pulse pressure amplification (r (95% CI) 0.21 (0.02-0.38), p=0.03) in univariate analysis. However, when age, sex, and race were adjusted for, the association was no longer significant (partial r (95% CI) 0.19 (-0.01-0.38), p=0.06, Model 1). In the fully adjusted models 2 and 3, the association between miR-146a-5p and PPA remained insignificant (both $p>0.05$). Additionally, relative miR-146a-5p expression was not associated with any of the other markers of arterial function in either the univariate or multivariate models.

Table 4.5A Associations between participant characteristics and pressure pulsatility measures.

	pPP*		cPP*		cSBP		PPA*		FWP*	
	Partial r	***P***	**Partial r**	***p***	**Partial r**	***p***	**Partial r**	***p***	**Partial r**	***p***
Age	0.20	**0.03**	0.37	**<0.001**	0.30	**0.002**	-0.38	**<0.001**	0.15	0.14
Sex	0.10	0.33	0.04	0.68	0.17	0.09	0.10	0.34	0.12	0.25
Race	-0.27	**0.01**	-0.27	**0.01**	-0.31	**0.002**	0.07	0.46	-0.26	**0.01**
Height	0.06	0.58	0.00	0.98	0.07	0.52	0.11	0.27	0.01	0.96
Weight	0.28	**0.01**	0.20	**0.04**	0.36	**<0.001**	0.09	0.39	0.26	**0.01**
BMI	0.27	**0.01**	0.21	**0.03**	0.36	**<0.001**	0.05	0.60	0.27	**0.01**
Waist circumference	0.29	**0.003**	0.18	0.07	0.35	**<0.001**	0.16	0.12	0.28	**0.01**
Waist/Hip ratio	0.07	0.49	-0.01	0.96	0.10	0.31	0.14	0.16	0.03	0.78
FPG*	0.18	0.08	0.07	0.49	0.17	0.10	0.19	0.06	0.14	0.17
TC*	0.03	0.78	0.04	0.70	0.01	0.95	-0.03	0.78	0.01	0.93
LDL*	-0.02	0.82	-0.01	0.92	-0.02	0.84	-0.02	0.83	-0.05	0.60
HDL*	-0.21	**0.04**	-0.22	**0.03**	-0.27	**0.01**	0.08	0.45	-0.16	0.12
Triglycerides*	0.26	**0.01**	0.26	**0.01**	0.26	**0.01**	-0.06	0.58	0.24	**0.02**
SBP	0.84	**<0.001**	0.76	**<0.001**	0.92	**<0.001**	-0.04	0.73	0.75	**<0.001**
DBP	0.34	**0.001**	0.35	**<0.001**	0.81	**<0.001**	-0.11	0.30	0.30	**0.002**
Heart rate	-0.06	0.53	-0.35	**<0.001**	-0.05	0.59	0.61	**<0.001**	-0.02	0.82
TC/HDL ratio	0.15	0.15	0.17	0.10	0.16	0.11	-0.07	0.49	0.10	0.32
TNFα #	-0.05	0.61	-0.12	0.24	-0.05	0.64	0.15	0.13	-0.05	0.60

Associations were determined using Pearsons' correlation analysis. Known confounders were adjusted for and included age, sex, and race. Significant relationships are shown in bold. *: Logarithmically transformed. #: square-rooted. BMI: body mass index; FPG: fasting plasma glucose; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; SBP: systolic blood pressure; DBP: diastolic blood pressure; TNFα: tumour necrosis factor alpha; pPP: peripheral pulse pressure; cPP: central pulse pressure; cSBP: central systolic blood pressure; PPA: pulse pressure amplification; FWP: forward wave pressure.

Table 4.5B Associations between participant characteristics and wave reflection and arterial stiffness measures.

	cAP		AIx		RWP*		RM		PWV	
	Partial r	***P***	**Partial r**	***p***	**Partial r**	***P***	**Partial r**	***p***	**Partial r**	***p***
Age	0.43	**<0.001**	0.37	**<0.001**	0.39	**<0.001**	0.37	**<0.001**	0.06	0.56
Sex	0.00	0.97	-0.13	0.19	0.04	0.72	-0.06	0.53	0.02	0.87
Race	-0.18	0.07	-0.03	0.76	-0.27	**0.01**	-0.09	0.36	-0.17	0.08
Height	-0.06	0.55	-0.12	0.22	-0.01	0.93	0.00	0.99	-0.06	0.59
Weight	-0.04	0.68	-0.14	0.15	0.15	0.13	-0.04	0.67	0.04	0.68
BMI	-0.02	0.87	-0.10	0.30	0.16	0.11	-0.05	0.61	0.07	0.47
Waist circumference	-0.07	0.50	-0.22	**0.03**	0.11	0.28	-0.12	0.22	0.14	0.15
Waist/Hip ratio	-0.06	0.56	-0.11	0.26	-0.04	0.67	-0.08	0.41	0.15	0.15
FPG*	-0.03	0.75	-0.11	0.29	0.03	0.79	-0.10	0.31	0.03	0.75
TC*	0.06	0.59	0.03	0.78	0.02	0.86	0.02	0.83	0.05	0.63
LDL*	0.00	0.99	0.03	0.77	0.00	0.99	0.06	0.57	-0.01	0.96
HDL*	-0.11	0.28	-0.05	0.60	-0.20	**0.04**	-0.11	0.29	-0.11	0.26
Triglycerides*	0.18	0.07	0.05	0.64	0.17	0.09	-0.01	0.89	0.20	**0.045**
SBP	0.48	**<0.001**	0.04	0.73	0.67	**<0.001**	0.13	0.20	0.40	**<0.001**
DBP	0.30	**0.003**	0.14	0.17	0.33	**0.001**	0.14	0.17	0.28	**0.004**
Heart rate	-0.47	**<0.001**	-0.49	**<0.001**	-0.49	**<0.001**	-0.61	**<0.001**	0.01	0.94
TC/HDL ratio	0.10	0.31	0.06	0.57	0.13	0.19	0.08	0.45	0.08	0.45
TNFα #	-0.08	0.41	-0.11	0.27	-0.12	0.22	-0.10	0.32	-0.16	0.111

Associations were determined using Pearsons' correlation analysis. Known confounders were adjusted for and included age, sex, and race. Significant relationships are shown in bold. *: Logarithmically transformed. #: square-rooted. BMI: body mass index; FPG: fasting plasma glucose; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; SBP: systolic blood pressure; DBP: diastolic blood pressure; TNFα: tumour necrosis factor alpha; cAP: central augmentation pressure; AIx: augmentation index; RWP: reflected wave pressure; RM: reflection magnitude; PWV: pulse wave velocity.

Table 4.6 Univariate and multivariate adjusted associations between arterial function measures and relative miR-146a-5p expression.

	miR-146a-5p*							
	Univariate model		Multivariate Model 1		Multivariate Model 2		Multivariate Model 3	
	r (95% CI)	***p***	**Partial r (95% CI)**	***p***	**Partial r (95% CI)**	***p***	**Partial r (95% CI)**	***p***
pPP*	0.04 (-0.15-0.22)	0.70	0.06 (-0.14-0.26)	0.54	0.06 (-0.15-0.27)	0.59	0.10 (-0.12-0.30)	0.39
cPP*	-0.07 (-0.25-0.12)	0.49	-0.04 (-0.23-0.16)	0.72	-0.03 (-0.23-0.19)	0.82	0.01 (-0.21-0.22)	0.95
cSBP*	-0.02 (-0.20-0.17)	0.85	0.01 (-0.19-0.21)	0.95	-0.04 (-0.25-0.17)	0.73	-0.06 (-0.27-0.16)	0.60
PPA*	0.21 (0.02-0.38)	**0.03**	0.19 (-0.01-0.38)	0.06	0.17 (-0.04-0.37)	0.11	0.16 (-0.05-0.36)	0.14
FWP*	0.02 (-0.17-0.22)	0.81	0.02 (-0.18-0.22)	0.86	0.01 (-0.20-0.22)	0.91	0.03 (-0.19-0.24)	0.78
cAP	-0.09 (-0.27-0.10)	0.36	-0.09 (-0.28-0.11)	0.38	-0.09 (-0.30-0.12)	0.38	-0.08 (-0.29-0.14)	0.48
AIx	-0.09 (-0.27-0.10)	0.35	-0.11 (-0.30-0.09)	0.30	-0.10 (-0.30-0.12)	0.37	-0.08 (-0.29-0.14)	0.49
RWP*	-0.04 (-0.23-0.15)	0.66	-0.08 (-0.27-0.12)	0.45	-0.06 (-0.27-0.15)	0.59	-0.03 (-0.24-0.18)	0.77
RM	-0.08 (-0.27-0.12)	0.43	-0.11 (-0.30-0.09)	0.27	-0.08 (-0.29-0.13)	0.46	-0.05 (-0.26-0.16)	0.63
PWV	0.02 (-0.17-0.20)	0.86	0.01 (-0.19-0.21)	0.91	0.02 (-0.19-0.23)	0.87	0.03 (-0.18-0.24)	0.78

Associations were determined using Pearsons' correlation analysis. Multivariate Model 1: age, sex, race adjusted. Multivariate Model 2: additionally adjusted for waist-hip ratio, triglycerides*, TC-HDL ratio, TNFα#, antidiabetic drug use, antihypertensive drug use (See Table 4.4). Multivariate Model 3: additionally adjusted for MAP, HR, and weight (See Tables 4.5A and B). *Logarithmically transformed. #: square-rooted to normality. pPP: peripheral pulse pressure; cPP: central pulse pressure; cSBP: central systolic blood pressure; PPA: pulse pressure amplification; FWP: forward wave pressure; cAP: central augmentation pressure; AIx: augmentation index; RWP: reflected wave pressure; RM: reflection magnitude; PWV: pulse wave velocity. MAP: mean arterial pressure; HR: heart rate.

4.8 Independent relationship of miR-146a-5p expression with arterial function measures in stratified analysis

Table 4.7 shows that in participants with a high risk of CVD (FRS≥20), a higher relative miR-146a-5p expression was associated with a decreased peripheral pulse pressure (Std β (SE) -0.76 (0.23), p=0.03), central pulse pressure (Std β (SE) -0.76 (0.16), p=0.01), central SBP (Std β (SE) -0.38 (0.09), p=0.02), forward wave pressure (Std β (SE) -0.68 (0.19), p=0.04), and reflected wave pressure (Std β (SE) -0.66 (0.18), p=0.04). However, no significant associations existed between miR-146a-5p expression and arterial function measures in the low and moderate CVD risk groups (all p>0.05). When additionally including MMP1 as a confounder, the associations were no longer significant.

Table 4.7 Stratified analysis for associations between miR-146a-5p expression and arterial function measures.

		CARDIOVASCULAR DISEASE RISK					
		Low (n=59)		Moderate (n=38)		High (n=21)	
		Std β (SE)	***p***	**Std β (SE)**	***p***	**Std β (SE)**	***p***
Model 1	**pPP**	0.08 (0.12)	0.53	0.13 (0.14)	0.38	-0.76 (0.23)	**0.03**
	cPP	0.04 (0.09)	0.67	0.07 (0.12)	0.56	-0.76 (0.16)	**0.01**
	cSBP	0.01 (0.04)	0.79	0.01 (0.05)	0.79	-0.38 (0.09)	**0.02**
	FWP	0.05 (0.15)	0.72	-0.05 (0.16)	0.76	-0.68 (0.19)	**0.04**
	RWP	0.10 (0.09)	0.29	0.07 (0.13)	0.60	-0.66 (0.18)	**0.04**
Model 2	**pPP**	0.03 (0.15)	0.85	0.14 (0.14)	0.36	-0.68 (0.25)	0.11
	cPP	0.03 (0.11)	0.78	0.07 (0.13)	0.58	-0.73 (0.20)	0.07
	cSBP	0.03 (0.06)	0.56	0.01 (0.06)	0.84	-0.37 (0.13)	0.11
	FWP	-0.02 (0.18)	0.90	-0.02 (0.16)	0.88	-0.63 (0.30)	0.29
	RWP	0.12 (0.11)	0.29	0.06 (0.13)	0.68	-0.75 (0.16)	0.13

Std β: standardised beta, SE: standard error. Significant associations shown in bold. Model 1: Fully adjusted for age, sex, race, waist-hip ratio, triglycerides, TC/HDL ratio, TNFα, antidiabetic drug use, antihypertensive drug use, MAP, HR, and weight. Model 2: Included adjustment for MMP1. pPP: peripheral pulse pressure; cPP: central pulse pressure; cSBP: central systolic blood pressure; FWP: forward wave pressure; RWP: reflected wave pressure.

Chapter 5: Discussion

The expression of miR-146a-5p and its relationship with measures of arterial dysfunction was investigated in diabetic patients. The main findings of this study are that the relative miR-146a-5p expression was increased in diabetic participants when compared to non-diabetic control participants. Although several traditional risk factors including markers of obesity, dyslipidaemia and TNFα were strongly associated with miR-146a-5p expression, it was not related to measures of arterial function in the total population. However, when stratifying participants according to CVD risk, in the group with a high risk for CVD, a lower miR-146a-5p expression was associated with increased pressure pulsatility and wave reflection, independent of traditional risk factors. When including MMP1, a marker of arterial remodelling, as a confounder in the model, the association between miR-146a-5p and arterial function was no longer significant. These results suggest that miR-146a-5p may be involved in the development of arterial dysfunction, via its role in arterial remodelling. In summary, this study showed that traditional risk factors and inflammation may drive the upregulation of miR-146a-5p in diabetes, however, in those patients with the highest risk, when miR-146a-5p is downregulated, it may lose its protective effect against the development of arterial dysfunction.

5.1 Changes in miR-146a-5p expression in diabetic populations

It is well known that miR-146a-5p expression is dysregulated in T2DM. In the present study, miR-146a-5p expression was significantly upregulated in diabetic participants compared to non-diabetic participants. This study confirms previous reports that have observed an upregulation of miR-146a-5p in diabetic groups (Kong *et al*., 2011; Rong *et al*., 2013; García-Jacobo *et al*., 2019). In contrast, several others have reported a decreased miR-146a-5p expression in diabetic populations (Balasubramanyam *et al.*, 2011; Baldeón *et al.*, 2014; Zeinali *et al.*, 2021). This lack of consensus regarding miR-146a-5p expression may be as a result of differences in age, sex, race, disease duration and cardiometabolic profiles between study populations. However, the exact reasons for these discrepancies in expression of miR-146a-5p in T2DM remain uncertain.

Considering that other comorbidities commonly develop in parallel to diabetes, it is plausible that traditional and non-traditional risk factors may contribute to aberrant miRNA expression. Indeed, in this study, miR-146a-5p expression was associated with several traditional risk factors associated with T2DM, including the waist-hip ratio,

triglyceride levels, and the atherogenic TC/HDL index. It is well established that these markers of obesity and dyslipidaemia are strongly associated with miR-146a-5p dysregulation, and also with CVD risk in T2DM. Increased central adiposity could, at least partially, explain the differences in miR-146a-5p expression patterns in this study, as the DM group had a higher average BMI compared to the non-DM group. Previous studies have also shown miR-146a-5p expression is increased in obesity and that its expression is associated with dyslipidaemia (Wu *et al.,* 2014; Nunez Lopez *et al.*, 2018). Similarly, others have shown an increased expression of miR-146a-5p in dyslipidaemia in both animal models (Vickers *et al.,* 2011; Barbalata *et al.,* 2020) and in humans (Simionescu *et al.,* 2014).

In addition to the direct effect of these traditional risk factors on miRNA expression, it is well known that these risk factors induce a state of systemic inflammation (Pachori *et al.*, 2017; Nunez Lopez *et al.*, 2018; Hijmans *et al.*, 2018). Indeed, inflammation is the most well characterised stimulus for increased miR-146a-5p expression (Taganov *et al.*, 2006; Nakasa *et al.*, 2008; Mann *et al.*, 2017). Similar to previous reports, in the present study, I show that miR-146a-5p expression was strongly associated with TNFα concentrations, a marker of systemic inflammation.

The upregulation of miR-146a-5p is induced through NFkB signalling, which is a key inflammatory pathway (Taganov *et al.*, 2006; Li *et al.*, 2016; Mann *et al.*, 2017). NFkB is a transcription factor that is activated in response to stimulation of TLR signalling pathways by inflammatory cytokines, and its activation is considered a key development in both T2DM and CVD pathogenesis (Cai *et al.*, 2005; Patel & Santani, 2009). The activation of NFkB has been shown in response to the proinflammatory cytokines, IL-1B and TNFα, both of which have also been associated with an increase in miR-146a-5p expression in different cell culture models (Taganov *et al.*, 2006; Nakasa *et al.*, 2008; Li *et al.*, 2012; Cheng *et al.*, 2013). The inflammatory stimulation of miR-146a-5p expression has been further demonstrated in LPS, IL-1B, and TNFα-stimulated endothelial cells and oxLDL-stimulated macrophages, as well as in murine models of chronic inflammation (Cheng *et al.*, 2013, 2017; Li *et al.*, 2016; Lo *et al.*, 2017; Xiao *et al.*, 2019). Moreover, the promoter sequence of miR-146a-5p was found to contain two binding sites for the transcription factor NFkB, confirming that miR-146a-5p is a direct target of NFkB and that its expression is dependent on this

transcription factor's binding, depicted in Figure 2.2 above (Taganov *et al.*, 2006; Li *et al.*, 2016).

Indeed, chronic inflammatory diseases including rheumatoid arthritis, systemic lupus erythematosus and cystic fibrosis have all been observed to involve a congruent upregulation of miR-146a-5p expression (Carlsen *et al.*, 2013; Anaparti *et al.*, 2017; Luly *et al.*, 2019). Considering that systemic inflammation is strongly associated with T2DM (Furman *et al.*, 2019), it is not surprising that miR-146a-5p was upregulated in diabetes in the present study.

Another factor that may impact miR-146a-5p expression in T2DM is the disease duration. The majority of studies reporting elevated miR-146a-5p in T2DM included newly diagnosed diabetics only (Kong *et al.*, 2011; Rong *et al.*, 2013). While the advantages of using early-stage diabetics without complications are clear, the expression of miR-146a-5p may be influenced by disease duration as well as by the impact of comorbid conditions and diabetic complications (García-Jacobo *et al.*, 2019; Helal *et al.*, 2021). In the present study, disease duration was not recorded, which may have impacted the interpretability of the results. Nevertheless, the present study showed similar increases in miR-146a-5p expression in previously diagnosed T2DM participants who were already employing medication.

In this regard, the influence of medication on miRNA expression should also be considered. In this study, miR-146a-5p expression was positively associated with the use of antidiabetic drugs. Similarly, when comparing diabetic participants treated with metformin to those not treated with metformin, one study reported increased miR-146a-5p expression (Mensà *et al.*, 2019), which has been suggested to be involved in the anti-inflammatory effect of metformin (Alimoradi *et al.*, 2021). Notably, in this study, 61 of the 67 DM participants were using metformin and hence the expression of miR-146a-5p may have been impacted. However, others report decreased miR-146a-5p expression even with antidiabetic treatment (Balasubramanyam *et al.*, 2011; Demirsoy *et al.*, 2018; García-Jacobo *et al.*, 2019). While the effects of different pharmacological agents on microRNA expression is beyond the scope of this study, it is clear that further research is required to ascertain the impact of the duration of diabetes and whether treatment benefits extend to improvements in miRNA profiles.

Taken together, increased miR-146a-5p expression in T2DM may be impacted by several confounding factors including disease duration, age, obesity, inflammation, antidiabetic drug use, and the presence of complications. As such, it is likely that the pattern of miR-146a-5p expression in T2DM is influenced by both the natural pathogenesis of diabetes as well as the effect of interventions or treatments. Nevertheless, dysregulated miR-146a-5p expression in T2DM may be an important prognostic marker for risk stratification.

5.2 Associations between miR-146a-5p and arterial function in T2DM

Arterial dysfunction is a significant predictive factor for the development of CVD (Deanfield *et al.*, 2007; Dhananjayan *et al.*, 2016). In T2DM, the development of arterial dysfunction is significantly accelerated compared to their healthy counterparts (Cruickshank *et al.*, 2002; Safar *et al.*, 2006; Wu *et al.*, 2023). Indeed, the present study showed that measures of pressure pulsatility and arterial stiffness, but not wave reflection, were significantly higher in DM compared to non-DM participants.

Traditional risk factors including age, race, weight, height, waist circumference, HDL cholesterol, triglycerides and blood pressure were associated with measures of pressure pulsatility, while only age, blood pressure and heart rate were consistently associated with measures of wave reflection. Triglycerides and blood pressure were the only markers associated with arterial stiffness. Traditional risk factors, such as obesity, hypertension, and dyslipidaemia, are well known to drive arterial remodelling, which leads to increased vascular stiffness and arterial dysfunction (Sena *et al.*, 2013; Gimbrone & García-Cardeña, 2016).

Despite evidence that traditional risk factors induce a state of chronic, low-grade inflammation, this study failed to show an association between TNFα and any measure of arterial function in this study. This may be explained by the lack of differences in TNFα between the DM and non-DM participants in the present study. Nevertheless, several studies in populations characterised by chronic systemic inflammation have shown associations between traditional risk factors and arterial function, independent of inflammatory markers (Gunter *et al.*, 2017; Mokotedi *et al.*, 2019; Kilic *et al.*, 2021). Similarly, previous reports in diabetics also showed independent associations between traditional risk factors and arterial function measures, but not with markers of systemic inflammation (Guo *et al.,* 2020; Staef *et al.,* 2023). However, these results should be

interpreted with caution as majority of these studies, including the present study, reported only one marker of inflammation representing overall inflammation. Other more vascular specific markers of inflammation should be included in future human studies. Indeed, our lab has previously shown that in a rat model of inflammation, markers specifically associated with vascular inflammation such as pentraxin-3 are better indicators of arterial remodelling compared to markers of general inflammation, such as TNFα (Gunter *et al.*, 2022).

Considering the close association between miR-146a-5p expression and inflammation, also demonstrated in the present study, it was surprising that no associations between miR-146a-5p expression and arterial function measures were shown in this study. Even when stratifying the participants by the presence of T2DM, there were no associations between miR-146a-5p and any measures of arterial function. These findings are in contrast with the only other study that showed that increased miR-146a-5p expression was associated with brachial ankle (ba) PWV, in a diabetic population (Guo *et al.*, 2020). However, in the aforementioned study, the researchers did not report measures of pressure pulsatility or wave reflection. Moreover, the researchers used the baPWV as a measure of arterial stiffness. It has been shown that the cfPWV is more closely associated with hypertensive target organ damage compared to the brachial ankle index (Lu *et al.,* 2017). Therefore, further studies investigating the relationship between miR-146a-5p and a comprehensive assessment of arterial dysfunction are warranted.

Nevertheless, Guo and colleagues (2020) suggested that the association between miR-146a-5p and baPWV may be driven by inflammation. Indeed, miR-146a-5p expression is consistently associated with inflammatory signalling pathways involved in endothelial dysfunction (Wang *et al.*, 2014; Lo *et al.*, 2017; Mann *et al.*, 2017; Xie *et al.*, 2018; Lopez-Pedrera *et al.*, 2020). Inflammation and oxidative stress associated with diabetes impair the vasodilatory capacity of vessels and play a considerable role in the development of arterial dysfunction (Mozos *et al.*, 2017; Furman *et al.*, 2019; Ramesh *et al.*, 2022). While miR-146a-5p has been shown to be upregulated in various *in vitro* and *in vivo* models of inflammation (Taganov *et al.*, 2006; Li *et al.*, 2016; Cheng *et al.*, 2017; Mann *et al.*, 2017), it is important to note that miR-146a-5p plays a compensatory role in that it functions to inhibit the activation of inflammatory response pathways. MiR-146a-5p deactivates NFkB by inhibiting the upstream

activators TRAF6 and IRAK1 (Wang *et al.*, 2014; Mann *et al.*, 2017; Xie *et al.*, 2018). MiR-146a-5p also directly targets and inhibits ROS production by the enzyme NOX4 (Wang *et al.*, 2014; Xie *et al.*, 2018). The latter is particularly significant because of the role oxidative stress has in reducing vasodilatory capacity and increasing arterial stiffness (Marti *et al.*, 2012; Mozos *et al.*, 2017). The protective effect of miR-146a-5p in reduced vessel compliance is further evident by the inhibitory effect it has on markers of fibrosis and arterial remodelling, including fibronectin and MMPs (Feng *et al.*, 2011; Liu *et al.*, 2018; Dannert *et al.*, 2018). Indeed, in the present study, despite the lack of association between miR-146a-5p and arterial dysfunction, a significant association with MMP1 was shown. Thus, by modulating inflammation, oxidative stress, and endothelial remodelling, miR-146a-5p likely has a protective role against the development of arterial dysfunction. Nevertheless, the translational impact of these molecular pathways in the development of arterial dysfunction warrant investigation.

Lastly, in the present study, the lack of associations between miR-146a-5p and arterial dysfunction may be a function of the relatively homogenous study population. In this regard, despite T2DM being a significant risk factor for CVD and the inclusion of two seemingly distinct groups, relatively high CVD risk factors were present across both the non-DM control and DM groups. Indeed, the non-DM control group had significantly higher total cholesterol, LDL levels and family history of CVD compared to the DM group. Additionally, lifestyle risk factors such as alcohol use and smoking, and metabolic risk factors such as dyslipidaemia were similar between the groups. Therefore, in the non-DM group, these traditional risk factors may have contributed to increases in systemic inflammation, as there were no differences in TNFα concentrations between the two groups. The latter is particularly important considering the role of inflammation in both miR-146a-5p expression and arterial dysfunction. Nevertheless, although several previous studies have shown higher inflammatory cytokines in T2DM compared to controls (Balasubramanyam *et al.,* 2011; Mensa *et al.,* 2019; Zeinali *et al.,* 2021), the adverse CVD risk profiles in the present study may have impacted circulating inflammatory markers in both groups, and hence it is likely that the traditional CVD risk factors may be a stronger mediator of the relationship between miR-146a-5p and arterial dysfunction. Based on these observations, participants were stratified into CVD risk groups, where the association of miR-146a-5p with arterial function variables was further explored.

5.3 The impact of CVD risk on the relationship between miR-146a-5p and arterial function

In the present study, when stratifying the participants based on the Framingham score for CVD risk, miR-146a-5p expression was increased in the moderate CVD risk group (Framingham score between 10 and 20) compared to the low CVD risk group (Framingham score < 10). Interestingly, in the high CVD risk group (Framingham score ≥ 20), miR-146a-5p expression was not different from the low-risk CVD group. Moreover, TNFα showed a similar distribution pattern across the CVD risk groups (data not shown). Despite this unexpected finding, arterial dysfunction markers increased across the CVD stratification groups (data not shown). Considering these interesting findings, perhaps the most clinically relevant finding in the present study was the significant inverse associations of miR-146a-5p expression with pressure pulsatility and wave reflection measures, that existed in the high CVD risk group only. Despite adjusting for all relevant traditional risk factors and TNFα, this association remained significant. Considering that in the high CVD risk group arterial dysfunction was predictably worst, it is likely that the protective effect of miR-146a-5p was lost in this group. In the moderate-risk CVD group, the upregulation of miR-146a-5p expression was perhaps protective against adverse arterial remodelling.

This pattern of miR-146a-5p expression might also relate to disease duration, progression, and severity. Typically, increased inflammation is observed in the early stages of a disease or in tissue damage, and it has been shown that the inflammatory cytokine profile changes in the later stages of disease (Chen *et al.,* 2018; Furman *et al.,* 2019; Henein *et al.,* 2022). Indeed, in the present study in the high CVD risk group, TNFα concentrations were lower compared to the moderate risk CVD group. The lower inflammation in the high CVD risk group may also be attributed to insulin treatment. In the high CVD risk group, insulin treatment was higher compared to the other groups, with 67% of the high-risk group employing insulin compared to 32% of the moderate-risk group, and 10% of the low-risk group. Insulin treatment has been shown to have anti-inflammatory effects (Sun *et al.*, 2014). Similarly, others have shown that diabetics treated with insulin, compared to diabetics not treated with insulin, had lower levels of miR-146a-5p (García-Jacobo *et al.*, 2019).

As previously discussed, increased inflammatory cytokines stimulate the expression of miR-146a-5p. This suggests that in the high CVD risk group the lower level of TNFα

may have limited the stimulation of miR-146a-5p expression and hence resulted in the loss of its protective effect. Indeed, it has been previously noted that miR-146a-5p downregulation results in a loss of protective effect in the vasculature, which could play a significant role in arterial remodelling in T2DM (Cheng *et al.*, 2013; Wang *et al.*, 2014, 2017; Lo *et al.*, 2017; Xie *et al.*, 2018; Runtsch *et al.*, 2019).

Interestingly, in the present study, when including MMP1 as an additional confounder in the regression model, miR-146a-5p expression was no longer associated with any measure of arterial dysfunction. Moreover, I showed that miR-146a-5p is positively associated with MMP1 expression, an enzyme involved in arterial matrix remodelling (Parente *et al.,* 2018). These results suggest that perhaps the relationship between miR-146a-5p and arterial dysfunction is mediated by extracellular matrix remodelling. In this regard, remodelling of the extracellular space contributes to changes in arterial function and is pivotal to the development of arterial dysfunction (Wan *et al.*, 2021). MMP1 is associated with endothelial cell senescence, VSMC proliferation, vasoconstriction and increased vascular tone, all of which contribute to arterial dysfunction and stiffness (Struewing *et al.*, 2009; Kalogeris & Korthuis, 2010; Rodrigues *et al.*, 2010; Austin *et al.*, 2013).

It is well accepted that during the early stages of disease progression, functional impairments in endothelial vasodilatory capacity contribute to arterial dysfunction (McEniery and Wilkinson, 2005). In this regard, coupled with the development of endothelial dysfunction, an imbalance in nitric oxide and endothelin-1 ensues, resulting in impaired vasodilation and hence functional stiffening of the vasculature (McEniery and Wilkinson, 2005; Mozos *et al.,* 2017). However, in the longer term, other pathophysiological processes contribute to arterial dysfunction. The primary contributor to long term arterial dysfunction is driven by extracellular matrix remodelling and VSMC proliferation (McEniery and Wilkinson, 2005; Cao *et al.,* 2022). These structural changes are a natural occurrence with vascular aging but may be accelerated in certain disease states (Lacolley *et al.,* 2017). Hence, structural remodelling reflects the changes that develop with prolonged exposure of the vasculature to these pathological signals, as would occur in populations at high risk of CVD. Indeed, miR-146a-5p has previously also been associated with increased VSMC proliferation (Sun *et al.*, 2011; Cao *et al.*, 2015). The interesting relationship in this study between miR-146a-5p and remodelling pathways warrants further investigation

in more mechanistic studies to fully understand the regulatory role of miR-146a-5p in arterial remodelling.

Taken together, the association of miR-146a-5p with arterial dysfunction is likely mediated via extracellular matrix remodelling. In the high CVD risk group, it is possible that the endothelial stimulation and the protective effect of miR-146a-5p is lost when structural remodelling pathways, rather than endothelial dysfunction, predominantly drive arterial dysfunction. These results are depicted in Figure 5.1 below.

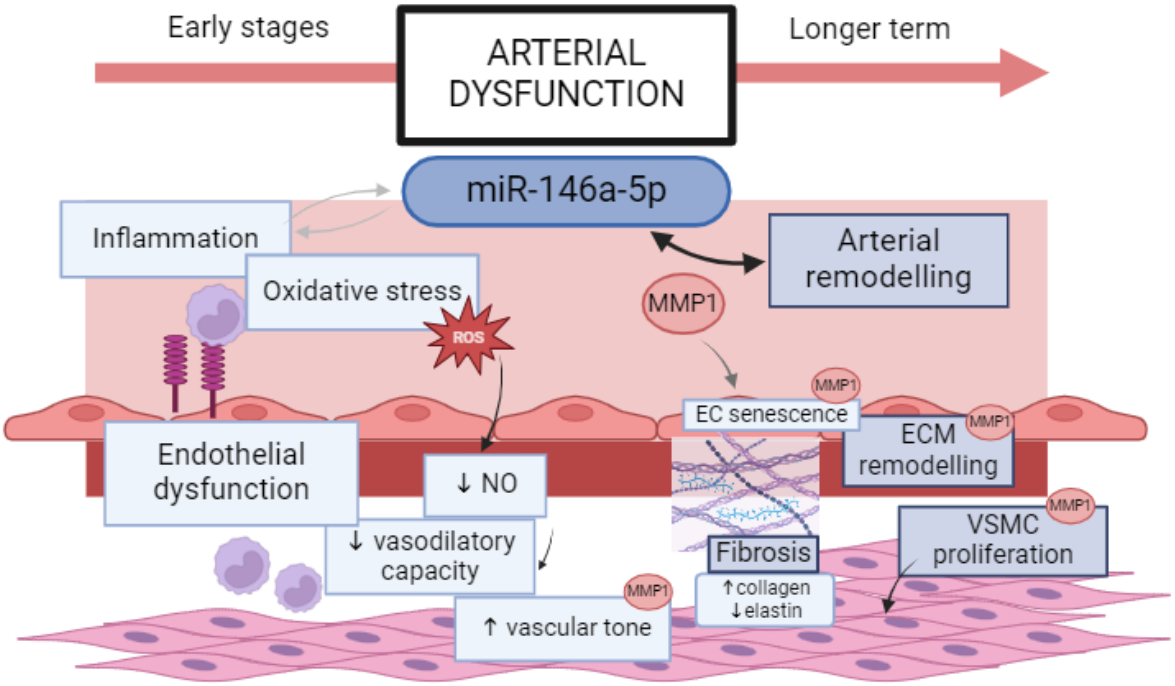

Figure 5.1 Summary of the role of miR-146a-5p in arterial remodelling in high CVD risk groups observed in the present study**.** Created with BioRender.com.

5.4 Limitations and future perspectives

This study has further limitations. The cross-sectional nature of this study precludes making conclusions about cause and effect. Future studies with a longitudinal study design would better characterise the changes in miR-146a-5p expression that occur over time, in addition to the changes that occur with the influence of traditional risk factors. In the present study, the control group had a high prevalence of CVD risk factors, and hence a more heterogenous population may improve the generalisability of the results.

Although measuring miRNA expression using RT-PCR is a well-accepted technique, the upstream regulation of miR-146a-5p is not well defined and the impact of its aberrant expression during T2DM-induced CVD is unknown. While it has not yet been confirmed whether genomic variations are responsible for impaired miR-146a-5p regulation in diabetes, polymorphisms in the immature pre-miRNA sequence have been hypothesised to affect mature miR-146a-5p expression and function. However, research on the topic has yielded inconsistent results. Nonetheless, future studies should determine if the presence or absence of polymorphisms in the miR-146a-5p sequence in a diabetic population account for differences in outcome variables.

Results regarding the association between inflammatory markers and miR-146a-5p should be interpreted with caution, as I only measured TNFα as a marker of inflammation, which a non-specific marker of inflammation and thus perhaps not sensitive to changes in arterial inflammation. Furthermore, although a comprehensive profile of arterial function measures was examined, I did not measure molecular markers of arterial function. Moreover, atherosclerosis was not investigated, which has a major contribution to vascular disease in T2DM. Future studies should include a more extensive battery of inflammatory markers related to vascular function, including the NFkB pathway; vessel-specific expression of markers associated with endothelial and arterial dysfunction such as cell adhesion molecules, and markers of oxidative stress and ECM remodelling; as well as measures of atherosclerosis to provide a more comprehensive overview of vascular dysfunction. This comprehensive investigation would further improve our understanding of the precise role of miR-146a-5p in the different processes involved in arterial dysfunction.

5.5 Conclusion

In conclusion, this study showed that the expression of miR-146a-5p differed between the diabetic and non-diabetic groups, but across these groups, miR-146a-5p expression was not related to arterial function variables. Interestingly, miR-146a-5p appeared to be more strongly impacted by CVD risk and by inflammation. In this regard, miR-146a-5p was associated with measures of pressure pulsatility and wave reflection in high CVD risk participants. The relationship between miR-146a-5p and these aspects of arterial function was investigated for the first time in the present study and provides new insights into the association between miR-146a-5p and early CVD in a diabetic population. This study proposes that miR-146a-5p has a protective role

in arterial function, as demonstrated by the loss of protective effects in the high CVD risk group that had notable arterial dysfunction and where miR-146a-5p expression was no longer elevated. The present study demonstrated a novel relationship between miR-146a-5p and arterial matrix remodelling through MMP1 expression. Further research is required to understand the molecular mechanisms through which miR-146a-5p protects against arterial dysfunction and its exact targets during the pathogenesis of early CVD. As such, while miR-146a-5p may not be an effective biomarker of arterial dysfunction in the general population with its expression influenced by many diverse factors, in CVD risk stratification, its inclusion may improve the prediction of arterial function in high-risk populations.

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Appendix A

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Drs S Gunter and A Hoosain; Professor A Milne
School of Physiology
Medical School
University

E-mail: Sule.Gunter@wits.ac.za

CC: Supervisor: Not applicable
<>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2021/11/10

REF: R14/49

PROTOCOL NO: **M210515** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Molecular markers associated with the pathogenesis and progression of diabetic cardiomyopathy*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Drs S Gunter and A Hoosain; Professor A Milne

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210515

NAME: (Principal Investigator)	Drs S Gunter and A Hoosain; Professor A Milne
DEPARTMENT:	School of Physiology Medical School University
PROJECT TITLE:	*Molecular markers associated with the pathogenesis and progression of diabetic cardiomyopathy*
DATE CONSIDERED:	2021/05/28
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Not applicable
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/11/10

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in May and therefore reports and re-certification will be due in the month of **May** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator _____ Date _____

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2022/05/17

Drs S Gunter and A Hoosain; Professor A Milne
School of Physiology
Medical School
University

Sent by e-mail to: Sule.Gunter@wits.ac.za

Dear Dr Gunter

Re: **Protocol Ref No**: M21/05/15
Protocol Title: *Molecular markers associated with the pathogenesis and progression of diabetic cardiomyopathy*
Principal Investigator: Drs S Gunter and A Hoosain; Professor A Milne

Thank you for your e-mail of 2022/05/12.

I confirm that we have noted and approve of the addition of Ms B Goldfein (s/n 1984023) as an additional investigator on this study. We further note that she will be making an independent application for ethics clearance for her MSc study in due course, this study being a component of M21/05/15.

Thank you for keeping us informed.

Yours Sincerely

I. Burns
.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

CB Penny
.....
Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)