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Understanding the *in vitro* effect of TNF-α and hyperglycaemia on endothelial activation

Joel Amoni

1685003

A dissertation to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

Johannesburg, South Africa

2023

DECLARATION

I, Joel Amoni, declare that this dissertation is my own, except to the extent indicated in the contributions and acknowledgement section. It is being submitted for the degree of Master of Science 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.



Joel Amoni

Signed on 23rd November 2023







Dr Sulè Gunter Dr Ashmeetha Manilall Prof Aletta Millen

ABSTRACT

Background. Inflammation is one of the main underlying mechanisms in the development of cardiovascular disease (CVD). Indeed, in diseases characterised by high-grade systemic inflammation and in comorbid conditions, such as diabetes mellitus, it has been suggested that inflammation, at least in part, contribute to the increased risk of CVD. One of the earliest signs of inflammation-induced CVD is endothelial dysfunction. However, whether inflammation promotes endothelial dysfunction via the same signalling pathways in different pathological conditions characterised by systemic inflammation is not well understood. Inflammation-induced aberrant expression of microRNA (miRNA), small non-coding RNAs that function to regulate gene expression post-transcriptionally, have been linked to impaired endothelial function. However, the mechanisms whereby miRNAs may mediate endothelial activation requires further investigation. Therefore, the aim of this study was to investigate the molecular mechanisms involved in the regulation of endothelial function in different models of inflammation.

Methods. *EA.hy926* immortalized endothelial cells were cultured in Dulbecco's modified eagle's medium (DMEM) + HAM's F12 nutrient mix supplemented with 10% foetal bovine serum (FBS). *EA.hy926* cells were exposed to tumour necrosis factor-alpha (TNF-α) at a concentration of 10ng/ml for 24 hours to induce an inflammatory response while the controls were cells exposed to plain media for 24 hours. *EA.hy926* cells were also exposed to either 5mM or 30mM glucose for 72 hours, as a model of glycemia-induced inflammation, while the control cells were exposed to plain media for 72 hours. Total RNA was extracted from the cell pellets and subsequently reverse transcribed to miRNA cDNA and mRNA cDNA. Quantitative real time PCR was used to determine the relative expression of interleukin-6 (*IL-6*), vascular cell adhesion molecule 1 (*VCAM-1*), *miRNA-155-5p,* endothelial nitric oxide synthase (*eNOS*) and superoxide dismutase 2 (*SOD-2*). Additionally, an ELISA assay was used to determine the ratio of phosphorylated p65/total p65 in cells exposed to TNF-α (10ng/ml for 24 hours), glucose (30mM for 72 hours) and plain media controls.

Results. Compared to control cells, the relative mRNA expression of the inflammatory marker *IL-6* was significantly increased in the cells exposed to TNF-α ($p=0.002$) and 5mM ($p=0.002$) and 30mM ($p = 0.0001$) glucose, respectively. In addition, the relative mRNA expression of *VCAM-1* was increased in the cells exposed to TNF-α ($p <0.0001$) and 30mM glucose ($p =$

0.03) when compared to their respective controls. Interestingly, miRNA-*155-5p* expression was also increased in the cells exposed to TNF-α (p= 0.04), 5 mM glucose (p = 0.007) and 30 mM glucose (p = 0.02). Exposure to TNF-α, and 5 mM and 30 mM glucose resulted in increased *eNOS* expression compared to the control cells (p = 0.04, p=0.002 and p = 0.0002, respectively). The ratio of phosphorylated-to-total NF-κB p65 were not different in either the TNF-α exposed or glucose exposed cells compared to control cells (all p>0.05).

Conclusion. These findings suggest that TNF-α and hyperglycaemia exposure resulted in endothelial dysfunction. However, hyperglycaemia caused much greater oxidative stress (increased *eNOS*) most likely due to glucose scavenging of nitric oxide (NO). This suggests that the underlying mechanisms of endothelial dysfunction occurring due to hyperglycaemia and inflammation may be driven by different mechanisms. This study highlights the need for further investigation into the mechanisms whereby *miRNA-155-5p* regulate endothelial dysfunction.

STATEMENT OF CONTRIBUTIONS

This study was designed in conjunction with my supervisors Prof A Millen, Dr S Gunter and Dr A Manilall. I was responsible for technique optimisation and data collection, with the help of Dr Manilall. I performed the data analysis for this dissertation with the help of Prof A Millen and Dr A Manilall. I wrote this thesis which was reviewed by Prof A Millen, Dr S Gunter and Dr A Manilall.

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ABBREVIATIONS

ADMA	asymmetric dimethylarginine
BH_4	Tetrahydrobiopterin
CVD	cardiovascular disease
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle's medium
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	intercellular cell adhesion molecule-1
IL-6	interleukin-6
LDL	low density lipoprotein
miRNA	microRNA
NF-κB	nuclear factor kappa B
NO	nitric oxide
NIK	NF-κB inducing kinase
oxLDL	oxidized low density lipoprotein
PBS-EDTA	phosphate-buffered saline-ethylenediaminetetraacetic acid
pri-miRNA	primary microRNA

pre-miRNA	precursor microRNA
RA	rheumatoid arthritis
RASF	rheumatoid synoviocytes
ROS	reactive oxygen species
SLE	systemic lupus erythematosus
SOD-2	superoxide dismutase-2
TNF-α	tumour necrosis factor-alpha
TNFR	tumour necrosis factor receptor
VCAM-1	vascular cellular adhesion molecule-1
VEGF	vascular endothelial growth factor
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of global mortality and a major contributor to the ever-rising cost of public healthcare (Roth *et al.*, 2020). The global prevalence of CVD, including coronary heart disease, stroke, peripheral arterial disease, and aortic disease, increased from an estimated 271 million in 1990 to 523 million in 2019 (Roth *et al.*, 2020; WHO, 2023). According to the current World Health Organisation (WHO) report in 2019, CVDs accounted for 17.9 million deaths annually, with 85% of them attributed to myocardial infarctions and strokes (WHO, 2023). It is well accepted that traditional risk factors including age, hypertension, dyslipidaemia, obesity, diabetes mellitus (DM) and cigarette smoking contribute substantially to the development of CVD (de Goma *et al.*, 2012). Of these risk factors, DM remains one of the main causes of cardiovascular mortality and cardiac events (Yusuf *et al.*, 2020).

DM is a metabolic disorder that is characterized by hyperglycaemia and compromised glucose tolerance (Tsalamandris *et al.*, 2019). Individuals with DM are two to three times more likely to develop CVD compared to the general population (Marks & Raskin, 2000; Zegkos *et al.*, 2016). Furthermore, diabetes-associated vascular complications are responsible for 75% of deaths in diabetic populations, a two-to-four-fold increase in incidence of coronary artery disease and a 10-fold increase in peripheral vascular diseases (Einarson *et al.*, 2018; Grundy *et al.*, 2002; Strain & Paldánius, 2018). The DM-induced vascular dysfunction has been suggested to be largely driven by systemic inflammation as a result of hyperglycaemia (Lumeng & Saltiel, 2011). It has been proposed that hyperglycaemia increase causes the production of ROS, which induces an inflammatory response (Naudi *et al.*, 2012). The pathogenesis of diabetes-induced CVD is therefore believed to be initiated by chronic systemic inflammation (Shoelson *et al.*, 2006).

Similar to individuals with DM, the risk of developing CVD is higher in individuals with highgrade systemic inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and vasculitis (Mason & Libby, 2015; Zegkos *et al.*, 2016)). In these conditions, inflammatory mediators play a central role in the development of endothelial activation and dysfunction (Steyers & Miller, 2014) and is considered a key initiator of atherogenesis (Katsiari *et al.*, 2019). Indeed inflammation-induced atherosclerosis accounts for the highest prevalence of CVD incidence in patients suffering from several inflammatory diseases (Einarson *et al.*, 2018; Urman *et al.*, 2018). Hence it is not surprising that inflammation have been strongly implicated in the development of CVD in these systemic inflammatory conditions (Aletaha & Smolen, 2018).

Systemic inflammation is an important non-traditional risk factor for the development of CVD and has been considered one of the main underlying mechanisms of co-morbid disease induced CVD (Katsiari *et al.*, 2019). Inflammation-induced atherosclerosis accounts for the greatest portion of CVD incidence in patients with DM (Einarson *et al.*, 2018).

It is widely accepted that the earliest signs of atherosclerosis are endothelial activation and endothelial dysfunction (Gimbrone & García-Cardeña, 2016; Mudau *et al.*, 2012). Endothelial activation is an independent prognostic marker for the risk of future CV events (Gonzalez & Selwyn, 2003; Y. Xu *et al.*, 2014). Congruently, the development of atherosclerosis in DM is commonly preceded by endothelial activation and dysfunction (Low Wang *et al.*, 2016; Ye *et al.*, 2022). Endothelial activation occurs when proinflammatory cytokines trigger the expression of cell surface adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Liao, 2013). Expression of cell adhesion molecules promotes the recruitment, attachment and trans-endothelial migration of leukocytes (Gerhardt & Ley, 2015; Liao, 2013). The activated cell produces reactive oxygen species (ROS) that decrease nitric oxide (NO) bioavailability through suppression of the enzyme endothelial nitric oxide synthase (eNOS) resulting in endothelial dysfunction (Kattoor *et al.*, 2017; Liao, 2013).

However, the role of inflammation in the mechanisms underlying the development of endothelial dysfunction in DM is not well understood. A greater understanding of the role of inflammation in the development of endothelial dysfunction in DM will aid in the treatment and management of CVD risk in patients with DM. Therefore, mechanistic studies investigating the role of inflammation in hyperglycaemia-induced endothelial activation is warranted.

In this regard, dysregulated expression of miRNAs has been implicated in the pathogenesis of multiple diseases, including inflammatory and diabetic diseases. This theory is further supported by several *in vitro* and *in vivo* experiments (Pashangzadeh *et al.*, 2021; Papadopoulos, *et al.*, 2023; Zárate-Neira *et al.*, 2017) which have shown that miRNAs are implicated in several pathways associated with disease pathogenesis. Studies have shown that miRNA-155-5p might play a role in the development of endothelial activation and dysfunction (Wu *et al.*, 2014; Sun *et al.*, 2012). However, the mechanisms whereby miRNA-155-5p may contribute to endothelial activation especially in inflammatory conditions require further investigation.

This dissertation is structured into five chapters. After this introductory chapter, the literature is reviewed, and the rationale for conducting this study is highlighted in Chapter 2. Chapter 3 provides an outline of the study design and methods. The results are presented in Chapter 4 and interpreted in the context of the current literature in Chapter 5.

CHAPTER 2: LITERATURE REVIEW

2.1 Endothelial activation as a risk marker for cardiovascular disease

Blood vessels consist of three layers (Figure 2.1), namely the outer layer known as the adventitia, the middle layer known as the tunica media and the innermost layer known as the tunica intima (Martinez-Lemus, 2012). The innermost layer, composed of the endothelium, lines the vasculature and consists of a single layer of epithelial cells, which is vital to the function of the vasculature (Martinez-Lemus, 2012; Tse & Stan, 2010). The endothelium mediates vascular homeostasis by controlling the movement of hormones, fluid, solutes, macromolecules and blood cells (Krüger-Genge *et al.*, 2019). Importantly, the endothelium actively mediates vascular tone through the production and secretion of vasoconstrictors and vasodilators. Factors that result in endothelial contraction include endothelin I, angiotensin II, uridine adenosine tetraphosphate and ROS (Cau *et al.*, 2018; Krüger-Genge *et al.*, 2019). Factors that modulate relaxation including endothelial-derived relaxing factors (EDRF) such as NO, endothelial-derived hyperpolarizing factors (EDHF), bradykinin and prostacyclin. A fine balance between vasorelaxation and vasocontraction is required to maintain optimal perfusion to all parts of the body (Cau *et al.*, 2018; Deanfield *et al.*, 2007). In addition, this balance between vasorelaxation and vasoconstriction is also a marker of the function and health of the vascular system.

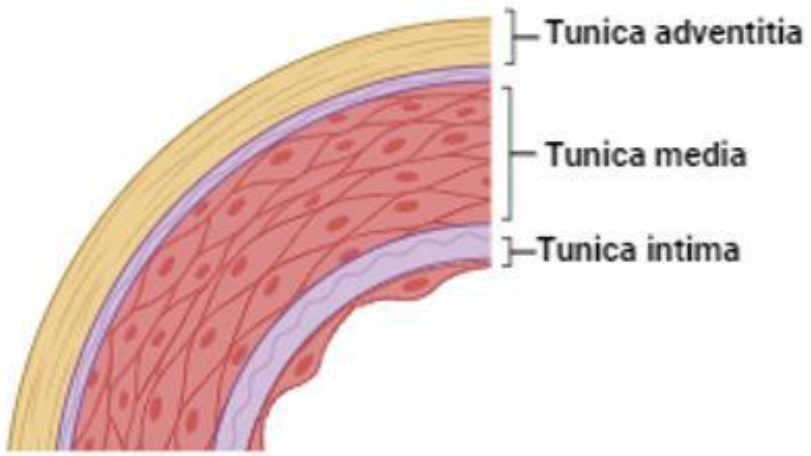


Figure 2.1. Layered structure of a typical blood vessel (from "Chronic allograft rejection: A significant hurdle to transplant success", by Kloc & Ghobrial., 2014, Burns Trauma *2,*3-10. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4994504/pdf/41038_2014_Article_20010003.pdf).

Indeed, endothelial dysfunction is described by an increase in endothelium-derived vasoconstrictors and decreased availability and functionality of endothelial vasodilators,

especially NO (Hadi *et al.*, 2005). Endothelial dysfunction is an independent prognostic marker for the risk of future CV events (Gonzalez & Selwyn, 2003; Y. Xu *et al.*, 2014).

However, what are the factors that could result in endothelial dysfunction?

2.2 Risk factors for endothelial activation

The endothelium, being the innermost layer of the blood vessel, is exposed to various circulating biomolecules which may alter its function. In several illnesses and disease states, the production of certain circulating molecules may adversely affect the functioning of endothelial cells. In this regard, exposure to several traditional risk factors including smoking, ageing, hypertension, hypercholesterolemia and hyperglycaemia have been shown to significantly affect the endothelium (Liao, 2013). However, it has been suggested that increased circulating systemic inflammatory markers in these comorbid conditions may be the underlying mechanism that alter endothelial function (Theofilis *et al.*, 2021). Independent of comorbid conditions, inflammation has been shown to affect endothelial function.

Acute phase proteins, such as C-reactive protein (CRP), and inflammatory cytokines have been strongly implicated in alterations in endothelium function (Carnevale *et al.*, 2014; Clapp *et al.*, 2004). Some cytokines interact with endothelial cells to express specific receptors called selectins (Duś *et al.*, 2003). Circulating neutrophils then react with these selectins, which ultimately results in them "rolling along" the endothelium (Williams *et al.*, 2011). These inflammatory signals cause these neutrophils to express integrins on their surface, which bind onto cell surface adhesion molecules expressed on the endothelial surface (Williams *et al.*, 2011). Therefore, circulating cytokines induce and upregulate the endothelial expression of various soluble adhesion molecules including VCAM-1, intracellular adhesion molecule (ICAM-1) and E-selectin (Liao, 2013; Szekanecz & Koch, 2000). The inflammatory cytokines also cause the release of bradykinin which loosens the tight junctions between endothelial cells (Claesson-Welsh *et al.*, 2021). Together, the expression of adhesion molecules and the loosening of tight junctions allows circulating neutrophils and monocytes to infiltrate the arterial wall intima where they differentiate to become macrophages, which produce more inflammatory cytokines (Dos Santos *et al.*, 2021). This process of increased expression of the adhesion molecules is known as endothelial activation.

2.3 Consequences of endothelial activation

In a state of endothelial activation, the production of inflammatory cytokines leads to a cascade of events that alters vessel function in several ways. First, increased pro-inflammatory cytokines reduces the bioavailability of NO and failure to secrete mediators that regulate vascular homeostasis (Hadi *et al.*, 2005). This failure of the endothelium to perform its vasoactive function is termed endothelial dysfunction, which limits the ability of the vessels to vasodilate and hence to regulate perfusion to target organs (Deanfield *et al.*, 2007). Endothelial dysfunction is frequently used as a marker of risk stratification to predict cardiovascular events (Bonetti *et al.*, 2003; Lerman & Zeiher, 2005).

Second, endothelial activation and the subsequent development of endothelial dysfunction is one of the earliest pathophysiological changes that occurs prior to the development of atherosclerosis (Mudau *et al.*, 2012). In this regard, with endothelial activation, when monocytes adhere to cell adhesion molecules, infiltration of the endothelial lining and the arterial wall intima results in these monocytes differentiating into phagocytes that engulf lipoproteins (Gerhardt & Ley, 2015). When macrophages engulf lipoproteins, they are transformed into cholesterol-filled foam cells (Moore *et al.*, 2013; Schumski *et al.*, 2018). The formation of foam cells induces the development of atherosclerotic plaque.

Finally, endothelial dysfunction has also been linked to the development and progression of remodelling and stiffening of the vessel, i.e., arteriosclerosis. Arteriosclerosis is characterised by thickening and hardening of the arterial intima-media that usually develops with age (Park & Lakatta, 2012). However, endothelial activation drives the process of increased collagen production and increased elastin degradation (Park & Lakatta, 2012). Ultimately, endothelial activation drives the process of arterial stiffening, which is characterised by an altered structure and reduced functional capabilities of the arteries. Arterial stiffness has been linked to and is considered an independent predictor of cardiovascular events and mortality (Cecelja & Chowienczyk, 2012).

Taken together, markers of endothelial activation have been strongly associated with the development of CVDs (Camici *et al.*, 2015; Shirwany & Zou, 2010). Figure 2.2 shows that the development of endothelial activation may initiate a cascade of events that can contribute to adverse cardiovascular outcomes. In this regard, once the endothelium is activated, inflammatory cytokines are produced, which ultimately results in endothelial dysfunction that is characterised by an imbalance in vasoactive substances and hence, vessel tone. Furthermore,

endothelial activation and the subsequent production of inflammatory cytokines drive the progression of smooth muscle proliferation that leads to vessel wall stiffening (arteriosclerosis) and the development of atherosclerotic plaque (atherosclerosis) (Park & Lakatta, 2012). In order to develop effective therapeutic targets for the treatment of endothelial activation, it is important to understand the mechanisms whereby endothelial dysfunction develops and the factors that are involved in the regulation of endothelial dysfunction.

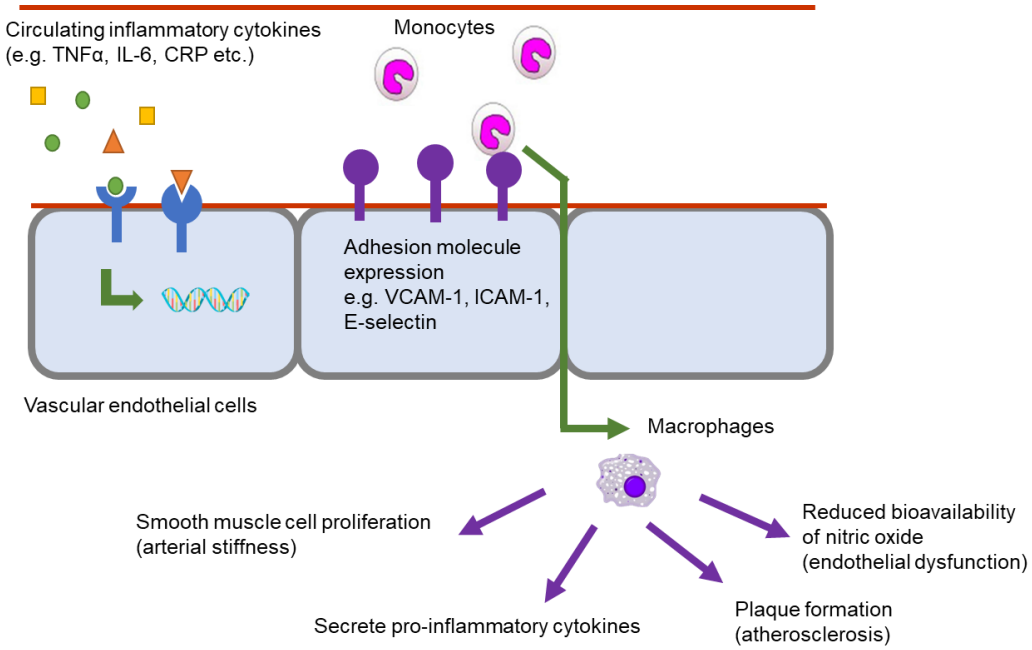


Figure 2.2. A summary of the process of endothelial activation and the consequence of endothelial activation. The presence of inflammatory cytokines triggers the expression of cell surface adhesion molecules such as VCAM-1, resulting in endothelial activation. Binding of leukocytes to the adhesion molecules initiates a series of processes that may result in endothelial dysfunction, arteriosclerosis and atherosclerosis.

2.4 The role of hyperglycaemia and inflammation in the molecular mechanisms of endothelial activation and dysfunction

Prolonged exposure to high levels of glucose can lead to endothelial dysfunction, primarily through oxidative stress and inflammation. Hyperglycaemia triggers the production of ROS and activates various inflammatory pathways. In this regard, ROS is produced in various locations as byproducts of aerobic respiration. Under normal physiological conditions ROS are maintained at low levels, as cellular anti-oxidants mechanisms are in place to counteract ROS production. However, in the presence of exogeneous stimuli such as increased inflammatory

cytokines or prolonged exposure to high levels of glucose, ROS production increases to a level which surpasses the capacity of cellular antioxidants to neutralize ROS, known as oxidative stress (Bae *et al.*, 2011; Ray *et al.*, 2012). Increased ROS disrupts NO signalling in endothelial cells, which contributes to endothelial dysfunction. ROS production in endothelial cells alters the expression of endothelial nitric oxide (eNOS). Endothelial NOS is an enzyme involved in the production of NO (Liao, 2013). Majority of studies have shown that changes in eNOS expression and activity ultimately results in decreased NO bioavailability, which impairs the vasodilatory capacity of the vessel. In this regard, NO scavenges ROS, and hence increased ROS decreases the bioavailability of NO. Some studies have shown that ROS decreases eNOS production and hence decreases NO production (An *et al.*, 2023; Meza *et al.*, 2019). In contrast, others have shown that ROS increases eNOS production (Förstermann & Li, 2011). In this scenario, through a process known as uncoupling, ROS-induced increased eNOS production causes eNOS to produce superoxide rather than NO (Förstermann & Li, 2011). The production of superoxide results in the formation of peroxynitrite, which inactivates the antioxidant superoxide dismutase (SOD) (Meza *et al.*, 2019). Increased ROS and decreased bioavailability of NO enhances leukocyte adhesion (Kattoor *et al.*, 2017; Liao, 2013), and induces the expression of adhesion molecules, including ICAM-1 and VCAM-1. These adhesion molecules further facilitate the attachment of circulating immune cells to the endothelium, promoting a proinflammatory state within the blood vessel walls. ROS also promotes the oxidation of low-density lipoprotein (LDL) to oxidised low density lipoprotein (oxLDL) that advances plaque formation (Li *et al.*, 2016; Liao, 2013). Altogether, these effects of hyperglycaemia on endothelial activation are key contributors to the development and progression of vascular complications associated with DM.

Recent research has focused on understanding the molecular pathways involved in the development of endothelial dysfunction. Although several pathways have been implicated in the development of endothelial dysfunction, nuclear factor-kappa B (NF-κB) signalling pathways have received particular attention in both hyperglycaemia and inflammation-induced endothelial dysfunction. NF-κB is a proinflammatory transcription factor that promotes the expression of molecules that regulate endothelial function, including cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin (Li *et al.*, 2016; Zhong *et al.*, 2018). Increased circulating pro-inflammatory cytokine concentrations activates NF-κB signalling, thereby initiating endothelial dysfunction (Yang *et al.*, 2016). Figure 2.3 shows the activation of the NF-κB occurs through either the canonical pathway or noncanonical pathway (Liu *et al.*, 2017).

The canonical NF-κB pathway is stimulated by a diverse group of ligands where activation of the pathway occurs through site-specific phosphorylation of two N-terminal serine residues of inhibitor of nuclear factor kappa B(IκB) proteins (Karin & Delhase, 2000). The IκB proteins then undergo ubiquitin-dependent degradation in the proteosome that leads to the nuclear translocation of the released NF-κB (Israël, 2010). Once the canonical pathway is activated, it has been linked to increased inflammatory cytokine production, proliferation, apoptosis and matrix remodelling.

In contrast, the noncanonical pathway is only activated by a specific group of ligands that belong to a subset of the tumour necrosis factor receptor (TNFR) family (Liu *et al.*, 2017). This pathway is activated when NF-κB-inducing kinase (NIK) activates IKKα and in conjunction with IKKα mediates the phosphorylation of p100 (a precursor for NF-κB2), which leads to ubiquitination and processing of p100 (Liu *et al.*, 2017; Xiao *et al.*, 2001). During the processing of p100, its C-terminal structure is degraded, and this results in production of the mature NF-κB2 (p52) that is subsequently translocated to the nucleus (Sun, 2012; Zhang & Sun, 2015). Activation of the noncanonical pathway has been linked to adaptive immune responses and cell survival.

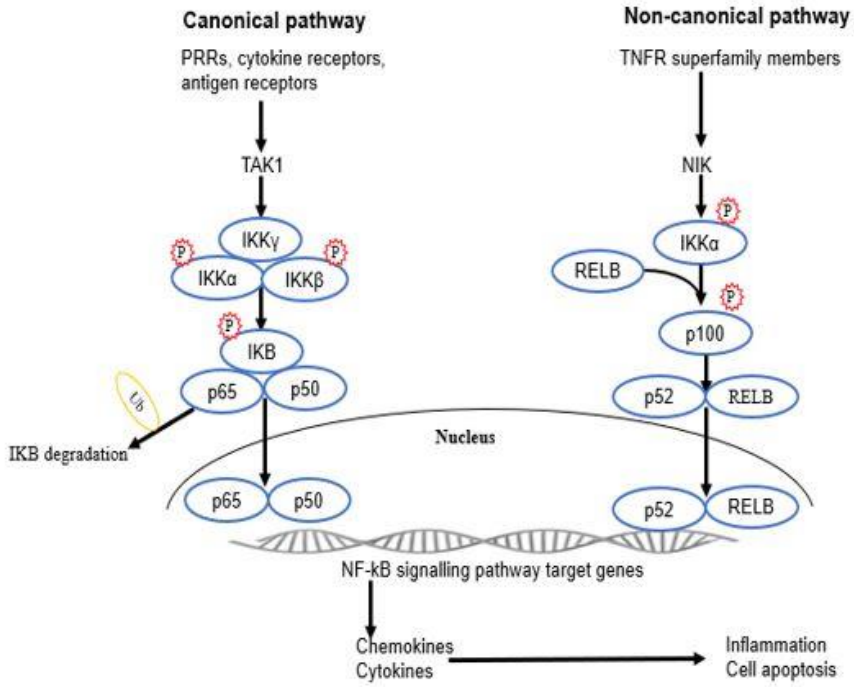


Figure 2.3. Canonical and non-canonical pathways of NF-κB activation. The canonical pathway is triggered by a diverse group of stimuli while the non-canonical pathway is only triggered by a group of stimuli belonging to a subset of the TNFR family. (Adapted from https://www.mdpi.com/1422-0067/20/17/4185).

Although several factors have been shown to initiate the activation of the NF-κB pathways, for the purpose of this dissertation, I will focus only on the effects of hyperglycaemia and inflammation. Hyperglycaemia causes oxidative stress in cells through increased production of ROS, which cause a redox imbalance (Evans *et al.*, 2002). Hyperglycaemia-induced oxidative stress in the cells leads to activation of the canonical NF-κB pathway leading to a proinflammatory state (Evans *et al.*, 2002). Hyperglycaemia causes oxidative stress in various ways, some of these include: 1) triggering an increase in the production of mitochondrial ROS (Brownlee, 2001); 2) formation of advanced glycation end products (AGEs) by forming covalent links with plasma proteins (Brownlee, 2000; Brownlee & Cerami, 1981); 3) glucose autoxidation (Wolff *et al.*, 1991); 4) production of ROS from mitochondrial uncoupling and β-oxidation due to elevated levels of free fatty acids (FFAs) (Carlsson *et al.*, 1999; Rao & Reddy, 2001; Wojtczak & Schönfeld, 1993; Yamagishi *et al.*, 2001). The resulting oxidative stress activates various signalling pathways including the NF-κB pathway (Evans *et al.*, 2002). Once the NF-κB pathway is activated it modulates the expression of a host of genes including those for TNF-α and VCAM-1 resulting in inflammation and endothelial activation (Evans *et al.*, 2002). Furthermore, the oxidative stress causes eNOS uncoupling such that even though its expression increases there is still a reduction in the bioavailability of NO thus causing endothelial dysfunction (Förstermann & Li, 2011), as previously explained.

Besides hyperglycaemia, it is well known that systemic inflammation causes the activation of the NF-κB pathways in endothelial cells. In this regard, tumour necrosis factor alpha (TNF-α) a cytokine that is crucial in the inflammatory cascade, has been shown to activate the NF-κB pathway (Liu *et al.*, 2017). The increased concentrations of TNF-α in the inflammatory process activate the NF-κB canonical pathway resulting in a proinflammatory state that causes oxidative stress and endothelial activation (Liu *et al.*,2017; Evans *et al.*, 2002; Mussbacher *et al.*, 2019). TNF-α signalling causes oxidative stress through increased ROS production (Daiber *et al.*, 2019; Kim *et al.*, 2010). Similarly, the TNF-α mediated increased ROS causes eNOS uncoupling, which ultimately leads to endothelial dysfunction (Förstermann & Münzel, 2006).

Taken together, the NF-κB pathway is integral to the development of endothelial dysfunction. However, the activation and regulation of the canonical and noncanonical NFκB pathways in various disease states is currently under investigation. More evidence is required to understand the role of the different NF-κB pathways in states of systemic inflammation and in hyperglycaemia. Moreover, the upstream regulation of the NF-κB pathway activation is currently uncertain. In recent literature, several mechanisms for the regulation of the NF-κB

pathway have been described, including a mechanism involving microRNA (miRNA). miRNAs regulate protein expression of specific mRNA targets, leading to mRNA degradation or translational repression, which is a crucial post-transcriptional mechanism that regulates normal gene expression in various biological processes. The aberrant expression of certain miRNAs has been linked to inflammation and vascular integrity; therefore, miRNAs have the potential to be novel biomarkers of endothelial dysfunction and targets for therapeutics (Hosen *et al.*, 2020).

2.5 The role of miRNAs in the regulation of endothelial function

Several biomarkers of endothelial activation and dysfunction including ICAM-1, VCAM-1, E-selectin, pentraxin-3 (PTX-3), asymmetric dimethylarginine (ADMA) and vascular endothelial growth factor (VEGF) have been identified (Constans & Conri, 2006; Page & Liles, 2013; Sibal *et al.*, 2010). These biomarkers are frequently evaluated in research as predictors of the development of endothelial activation and dysfunction (Choudhury & Devagourou, 2017).

Although the molecules and growth factors that modulate these markers of endothelial function have received considerable attention, little is known about the upstream factors that modulate gene expression to determine vascular integrity (Urbich *et al.*, 2008). In this regard, recent literature points to miRNAs as potential upstream regulators of endothelial function (Urbich *et al.*, 2008).

miRNAs are small noncoding RNAs that are, on average, 22 nucleotides and are transcribed from DNA and function to modulate gene expression (O'Brien *et al.*, 2018). In the canonical pathway of miRNA biogenesis, DNA sequences are transcribed into primary miRNAs (primiRNAs), by RNA polymerase II in the cytoplasm (Denli *et al.*, 2004). The miRNAs are then processed by microprocessor complex of the RNase III enzyme Drosha and two DGCR8 molecules in the nucleus to give the precursor miRNAs (pre-miRNAs) (Han *et al.*, 2004). The pre-miRNA is cleaved near its terminal loop by another RNase III enzyme, Dicer, to produce double stranded miRNA that interacts with an Argonaute (Ago) protein (Denli *et al.*, 2004; Yoda *et al.*, 2010). One of the strands in the double stranded miRNA, the passenger strand, is subsequently discarded and this leaves the final mature miRNA (O'Brien *et al.*, 2018). Alternative miRNA biogenesis pathways exist that are either dicer-independent and/or drosha independent and these are known as non-canonical pathways. miRNAs usually bind to the 3′ UTR of target mRNAs to regulate gene expression. Modulation occurs via induction of mRNA

degradation or through translational inhibition and mRNA capping and degradation (Ipsaro & Joshua-Tor, 2015).

Aberrant expression of certain miRNAs has been linked to inflammation (Zhong *et al.*, 2018), diabetes mellitus (Kim & Zhang, 2019) and impaired vascular integrity (Hosen *et al.*, 2020). miRNAs may serve as novel biomarkers of endothelial dysfunction and a potential therapeutic target for the treatment of endothelial dysfunction (Hosen *et al.*, 2020), with several miRNAs being identified in the mediation of NF-κB-associated inflammation and by extension, endothelial function. Specifically, miR-155-5p has received considerable attention in the regulation of endothelial function and integrity as it has been implicated in the inflammatory response, the expression of cellular adhesion molecules, and in atherogenesis (Zhong *et al.*, 2018). The section below briefly highlights the current evidence of the involvement of miRNA 155-5p in endothelial dysfunction.

MiR155-5p is considered an important regulator of inflammation (Mahesh and Biswas, 2019). Growing evidence suggests that miR155-5p is aberrantly expressed in a variety of activated immune cells (Moran-Moguel *et al.*, 2018). MiR-155-5p is particularly responsive to inflammatory stimuli, including TNF-α, interleukin-1β and interferons (O'Connell *et al.*, 2007), and its expression significantly increases upon stimulation by these inflammatory cytokines. However, the pathways involved in increased production of miR-155-5p is controversial. In this regard, some have suggested that miR-155-5p is upregulated in response to NF-κB activation. In turn, miR155-5p expression activates NF-κB signalling via positive feedback loop which increases IL-6 and TNF-α production (Mann *et al.*, 2017) and hence the inflammatory response is either maintained or increased (Filkova *et al.*, 2012). Indeed, an *in vitro* study found that inhibition of miR-155-5p prevented phosphorylation of IκB proteins thus suppressing NF-κB activation (Zhang *et al.*, 2017). In contrast, others have shown that increased miR-155-5p expression may limit NF-κB activity or downregulate its expression by controlling the expression of various genes along the pathway (Costinean *et al.*, 2006; Ma *et al.*, 2011). Although there is a strong link between the miR-155-5p and the NF-κB pathways, the exact relationship requires further investigation.

With regards to the effects of upregulated MiR-155-5p, it has been linked to changes in endothelial function under inflammatory conditions. In this regard some studies suggested that miR-155-5p downregulates eNOS and hence leads to a decrease in production and bioavailability of NO. (Jankauskas *et al.*, 2021; Majid *et al.*, 2020; Prattichizzo *et al.*, 2021; Sun *et al.*, 2012). In contrast, others have shown that eNOS expression may increase in response

to miR-155-5p, but that due to uncoupling of the enzyme there is decreased NO bioavailability as the enzyme instead produces more ROS and less NO (Kalinowski *et al.*, 2016). Nevertheless, increased miR-155-5p expression is consistently associated with a decreased NO bioavailability. However, the exact mechanism whereby miR-155-5p contribute to endothelial dysfunction requires further investigation.

MiR-155-5p has been implicated in the pathogenesis of hyperglycaemic disease states such as diabetes mellitus. Yang *et al.* (2015) reported that miR-155-5p expression was upregulated in patients with DM compared to their healthy counterparts (Yang *et al.*, 2015). Two clinical trials also found that patients with diabetic nephropathy displayed higher serum levels of miR155-5p when compared to healthy control participants (Bai *et al.*, 2020; Wang *et al.*, 2020). This suggests that under high glucose conditions, miR-155-5p may be involved in the progress endothelial injury. However, the exact mechanisms whereby miR-155-5p is involved in endothelial dysfunction in diabetes mellitus warrant investigation.

In summary, the involvement miR-155-5p in the inflammatory response has been well documented. Recent evidence further indicates that this miRNA is also involved in the regulation of endothelial function. However, the role of miR-155-5p in endothelial function in hyperglycaemia is uncertain. In this regard the current controversies surrounding the mechanisms whereby miR-155-5p influence the NF-κB pathway and contribute to endothelial dysfunction requires further investigation.

2.6 Problem statement

It is widely accepted that inflammation is implicated in the development of CVD. Endothelial dysfunction is considered an early prognostic marker of CVD. In this regard it is uncertain whether inflammation promotes endothelial dysfunction via the same signalling pathways in different pathological conditions characterised by systemic inflammation, such as RA and DM. It is currently uncertain whether the mechanisms and cellular pathways that initiate endothelial activation are the same for comorbid disease-induced inflammation and diseases of systemic inflammation. Furthermore, the role of aberrant expression of miR-155-5p in the development of endothelial activation in inflammatory conditions and in hyperglycaemia requires elucidation. Therefore, further investigation is required to determine the role of miR-155-5p in the signalling pathways of endothelial activation in different disease states characterised by systemic inflammation and to determine whether they hold potential as therapeutic strategies

against the development of inflammation-induced endothelial dysfunction, atherosclerosis and ultimately CVD.

2.7 Aim and objectives

The aim of this study was to investigate the *in vitro* effects of TNF-α and hyperglycaemia in *EA.hy926* cells and their roles in the development of endothelial activation.

The specific objectives were to:

1. assess the relative expression of markers of inflammation *(IL-6)*, endothelial activation (*VCAM-1)* and vascular integrity *(eNOS)* in *EA.hy926* cells exposed to TNF-α and hyperglycaemia using comparative gene expression real-time PCR.
2. determine a suitable reference gene for the analysis of miRNA expression in an endothelial cell line.
3. assess the relative expression of miR-155-5p in *EAhy926* cells exposed to TNF-α and hyperglycaemia using comparative gene expression real-time PCR.
4. investigate the role of the NF-κB signalling pathway inflammation-induced vascular dysfunction by measuring the NF-κB p65 molecule using an ELISA.
5. determine the association between miR-155-5p and markers of inflammation and endothelial activation.

CHAPTER 3: METHODS

3.1 Cell culture models of endothelial function

In vivo models are commonly used to investigate a wide range of physiological mechanisms. However, it is not possible to elucidate the independent effects of inflammation on specific pathways using *in vivo* models, due to the presence of multiple confounding factors (MedinaLeyte *et al.*, 2020). The human umbilical vein endothelial cell (HUVEC) line is an *in vitro* model that closely represents the vascular endothelium in humans (Medina-Leyte *et al.*, 2020). Cell culture studies using HUVECs allow researchers to study the independent effect of inflammation on miRNA expression and endothelial dysfunction. The specific contribution of inflammatory cytokines can be studied in a confounder-controlled environment using endothelial cell lines, providing a possible advantage over the use of *in vivo* models for this application. The *EA.hy926* is a human endothelial/-somatic hybrid cell line created by fusing HUVECs with cells of the human lung carcinoma cell line *A549* (Lu *et al.*, 2009). The *EA.hy926* cell line is a popular *in vitro* model in cardiovascular disease research (Ahn *et al.*, 1995; Lu *et al.*, 2009).

3.2 Ethical clearance

An ethics waiver was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (W-CBP-220926-01) as no humans or animals were used in this study (Appendix A). The cell line used in the study is a commercially available immortalized human vascular endothelial cell line (*EA.hy926*, (ATCC CRL-2922, Manassas, Virginia, USA).

3.3 Cell culture

EA.hy926 cells (ATCC CRL-2922, Manassas, Virginia, USA), initially derived by fusion of human umbilical vein endothelial cells (HUVEC) with continuous human lung carcinoma cell line A549 (Edgell *et al.*,1983), were cultured in Dulbecco's modified Eagle's medium (DMEM) + HAM's F12 nutrient mix (Gibco; Thermo Fisher Scientific, Waltham, USA)) supplemented with 10% foetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Waltham, USA)) and Hank's balanced salt solution (HBSS 1X, Gibco; Thermo Fisher Scientific, Waltham, USA) in T75 flasks. Flasks were maintained in a humidified incubator at 37°C with 5% CO_2. The *EA.hy926* cells were seeded into 12 well plates in serum-free DMEM + Ham's F-12 supplemented with 10% Penicillin-Streptomycin and allowed to reach ~85% confluence.

3.4 Cell treatment with TNF-α

EA.hy926 cells were incubated with or without tumour necrosis factor alpha (TNF-α, 10ng/ml; Sigma Aldrich, St. Louis, MO) for 8 hours (h) or 24 h, with 6 wells per treatment and each experiment was performed in duplicate. The 10 ng/ml TNF-α was added to the plates at 1 ml/well (King *et al.*, 2019). To determine the optimal treatment period, we evaluated the mRNA expression of *IL6*, the gene that codes for interleukin 6. TNF-α incubation was performed to induce an inflammatory response, which was assessed by mRNA expression of *IL-6*, a known marker of inflammation (Hirano, 2021). mRNA expression of *IL-6* was increased following incubation of the cells with TNF-α at both 8 h (mean ± SD; 4.28 ± 0.26; $p<0.05$) and at 24 h (3.49 ± 1.04; $p <0.05$) when compared to controls. There was no significant difference between the mRNA expressions of *IL-6* following incubation of the cells with TNFα at 8 h when compared to 24 h ($p >0.05$). Therefore, the 24h incubation was used for all subsequent experiments.

3.5 Cell treatment with glucose

EA.hy926 cells were incubated with glucose to model a hyperglycaemic state. Cells were treated as follows: 1) 0 mM glucose representing an untreated control, 2) 5 mM glucose represent moderate glycaemia and 3) 30 mM glucose representing hyperglycaemia and incubated for 72 h. The concentrations for the glucose exposure were selected based on previous literature, which showed significant hyperglycaemic effects in endothelial cells (Chiechanowska *et al.*, 2021). The treatment period of 72 h was selected based on a previous study, which showed that 72 h was the optimal time to investigate vascular effects of hyperglycaemia-induced metabolic dysfunction, which mimics diabetic conditions in endothelial cells (Irshad *et al.*, 2019).

3.6 Total RNA extraction

Once the treatment periods were completed, the cell culture media was discarded from the culture plates and 2 ml/well phosphate buffered saline-ethylenediaminetetraacetic acid (PBSEDTA;1 x PBS, 10 mM EDTA) was added to detach the cells from the plate. Once cells were detached, each cell suspension was transferred to a respective 15 ml tube and cells were pelleted by centrifugation (1500 rpm for 5 minutes; Hettich benchtop centrifuge, Universal 320, Berlin, Germany). The supernatant was discarded, and lysis buffer was added to the cell pellet. Total mRNA was extracted from the cell pellets using an Illustra™ Mini Spin RNA

extraction kit (GE Healthcare, Buckinghamshire, UK) and total miRNA was extracted using the MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturers' instructions. A Nanodrop OneC spectrophotometer (Thermo Fischer Scientific, Waltham, USA) was used to quantify and confirm the presence of high-quality RNA (concentration of >20 ng/ul with a 260/280 reading of ~1.8), prior to cDNA synthesis.

3.7 cDNA synthesis for relative miRNA expression

Total miRNA was reverse transcribed to produce cDNA templates using the TaqMan® Advanced miRNA cDNA synthesis kit, (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Prior to reverse transcription, the miRNA was extended at the 3'-end by poly(A) tailing and subsequently at the 5'-end by ligation of an adaptor sequence to produce cDNA which was then uniformly amplified. The cDNA constructs were then stored at -20°C prior to performing the relative miRNA expression assays.

3.8 cDNA synthesis for relative mRNA expression

Total mRNA was reverse transcribed to produce cDNA templates using SuperScript™ IV VILO™ cDNA synthesis master mix (Thermo Fisher Scientific, Waltham, USA), according to manufacturer's instructions. Cycling conditions consisted of incubation at 25ºC for 10 minutes to allow the primers to bind to RNA, followed by incubation at an increased temperature of 42ºC for 60 minutes to allow for DNA polymerization and finally a cycle at 85ºC for 5 minutes to inactivate the enzyme.

3.9 Determination of a stable reference gene for miRNA expression

For determination of a stable reference gene for miRNA expression analysis in the current study, three endogenous reference genes, *miR-191-5p* (TaqMan® assay ID: 002299), *miR-165p* (TaqMan® assay ID: 000391), and *SNU6* (TaqMan® assay ID: 001973) were selected based on the literature and recommendations made by the manufacturer of the TaqMan probes (Thermo Fischer Scientific, Waltham, USA), as these have been reported to have stable expression across most tissues, serum and plasma (Chamorro-Jorganes *et al*., 2011; Fortner *et al*., 1994; Nagpal & Kulshreshtha, 2014; Rinnerthaler *et al*., 2016). These reference genes were evaluated using a subset of cDNA samples from all representative groups (n=4 per group, n=24 total). *miR155-5p* (TaqMan® assay ID: 002623) was used as the target probe in duplex

reactions with each specified reference gene. Each reaction well contained cDNA (1 µl) and the *miR-155-5p* target gene probe (1 µl,) or each of the endogenous reference gene probes (*miR-191-5p*, miR*16-5p* or *SNU6*) with TaqMan® fast advanced PCR master mix (5 µl) in a final reaction volume of 10 µl. Cycling conditions were as follows; 95°C for 20 seconds, followed by 40 cycles at 95°C for 1 second and 60°C for 20 seconds.

Table 2.1 miRNA candidate reference genes

Full Gene Name	**Gene Function/Reported Gene function**
microRNA 191-5p	Regulate gene expression and stably and consistently expressed. It can be detected in human serum or saliva (Nagpal & Kulshreshtha, 2014)
microRNA 16-5p	Regulate gene expression of some angiogenic mediators in endothelial cells (Chamorro-Jorganes *et al.*, 2011; Rinnerthaler *et al.*, 2016)
Small nuclear RNA U6	Enables the U6 small nuclear ribonucleoproteins (snRNP) to bind tightly to the U4 small nuclear RNA (snRNA) and loosely to the U5 snRNA of a triple-snRNP during the initial phase of the splicing reaction (Fortner *et al.*, 1994)

To calculate the mean ΔCt, the Ct value of the reference gene was subtracted from the Ct value of the target gene. The ΔΔCt was then calculated by subtracting the ΔCt mean for each sample from the mean of all control samples within the same experiment. The fold-change in relative expression was then calculated as $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). In order to select the most stable endogenous reference control gene, as determined by the lowest standard deviation across groups, the data was entered into RefFinder software (RefFinder;RRID:SCR_000472). This software tool uses a single data input to compute multiple algorithms equivalent to the most popular stability testing programs available such as BestKeeper, NormFinder, geNorm and the comparative ΔCt method. The software automatically determines the most stable and appropriate endogenous control (Panina *et al.*, 2018).

3.10 Comparative miRNA expression using RT-PCR

Comparative miRNA gene expression was performed using real time PCR with a StepOne Plus thermocycler (Thermo Fisher Scientific, Waltham, USA) with miRNA cDNA (1 µl) and pre-designed TaqMan® probe mixes. Reactions were performed in duplicate. Each reaction contained cDNA (1 µl, ~1 µg), the miRNA target probe (1 µl, *miR-155-5p* (TaqMan® assay ID: 002623) or *miR-let-7g* (TaqMan® assay ID: 002282) or the selected reference gene (*miR-16-5p*) and TaqMan® fast advanced PCR master mix (5 µl), made up to a final reaction volume of 10 µl with nuclease-free water. The following thermal cycling conditions were used; 95ºC for 20 seconds and 40 cycles at 95ºC for 1 second and 60ºC for 20 seconds. Cycle threshold (Ct) values were obtained from the instrument software. The $2^{-\Delta\Delta Ct}$ method was then used to calculate the fold change in miRNA expression (Livak & Schmittgen, 2001). Despite optimisation of the method and performing several repeat experiments, results for *miR-let-7g* were undetermined for all samples, and hence was not reported in this dissertation.

3.11 Comparative mRNA expression using RT-PCR

Comparative mRNA gene expression RT-PCR was performed in duplex reactions using a StepOne Plus thermocycler (Thermo Fisher Scientific, Waltham, USA) with mRNA cDNA (1µl), pre-designed TaqMan® probe mixes for the reference gene *GAPDH* (0.25 µl, TaqMan® assay ID: Hs02786624_g1, Thermo Fisher Scientific, Life Technologies, Carlsbad, USA) (Keratibumrungpong *et al.*, 2023), the gene of interest (0.5 µl, TaqMan® gene expression assay) and TaqMan® Fast Advanced PCR master mix (5 µl) made up to a final volume of 10 µl with nuclease free water. The following thermal cycling conditions were used: initial hold at 50°C for 2 minutes, another hold at an increased temperature of 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute using a StepOne Plus Real-Time PCR System. Comparative gene expression of *eNOS* (TaqMan® assay ID: Hs00427620_m1), *VCAM-1* (TaqMan® assay ID: Hs01003372_m1), *SOD-2* (TaqMan® assay ID: Hs00167309_m1) and *IL-6* (TaqMan® assay ID: Hs00174131_m1) were determined with the *GAPDH* endogenous control probe. The relative expression of each gene of interest was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

3.12 ELISA detection of NF-κB p65

EA.hy926 cells were incubated with or without tumour necrosis factor alpha (TNF-α, 10ng/ml; Sigma Aldrich, St. Louis, MO) for 24 hours with 6 wells per treatment. For the glucose

treatment, *EA.hy926* cells were incubated with or without 30mM glucose for 72 hours with 6 wells per treatment

Following incubation as described above, the media was discarded and 1ml/well PBS-EDTA was added to the plate to detach the cells. Once cells were detached, each cell suspension was transferred to a respective 15ml tube and cells were pelleted by centrifugation (1500rpm for 5 minutes; Hettich benchtop centrifuge, Universal 320, Berlin, Germany). The supernatant was discarded and a cell lysis buffer mix (1X) was added to the cell pellets to lyse the cells. Cell lysates were used to assess phosphorylation of NF-κB by using the NF-κB p65 (Total/Phospho) Human InstantOne™ ELISA Kit (Catalogue number: 85-86083-11, Thermo Fisher Scientific, Waltham, MA, USA), as per the manufacturer's instructions. Equal volumes of the detection antibody reagent and the capture antibody reagent were prepared prior to the experiment. To each well of the 96 well ELISA reaction plate, 50 µL of whole cell lysate, a cell lysis mix (negative control) made from cell lysis buffer and enhancer solution that were supplied with the ELISA kit, and a positive control cell lysate (supplied with the ELISA kit) were added individually onto the ELISA plate. The two capture antibody reagents (total-NF-κB p65 antibody and phospho-NF-κB p65 antibody), were incubated in separate wells of the pre-coated plate containing sample lysates for 1 hour at room temperature, while shaking at 300rpm on an orbital shaker. Following this incubation, the plate was washed with 200µl of wash buffer three times before adding a detection reagent and allowing the plate to stand at room temperature for 30 minutes. The reaction was terminated by addition of the stop solution (100 µL/well). The absorbances were read at 450 nm using a Multiskan Ascent 354 microplate reader (Thermo Fisher Scientific, Waltham, USA).

3.13 Statistical analysis

Statistical analyses were performed using SAS software, version 9.4 (SAS institute Inc., USA). Data are expressed as mean ± standard deviation (SD). Differences in relative miRNA expression, and relative mRNA expression of genes involved in inflammation, endothelial activation and endothelial function between cells incubated with TNF-α and the untreated control cells were assessed by unpaired t-test. Differences in relative miRNA expression and relative mRNA expression of markers of inflammation endothelial activation and endothelial function between groups in the glucose model were assessed using one-way analysis of variance (ANOVA) test followed by Tukey's *post hoc* tests. Associations were assessed using Pearson's correlation analyses. Target genes measured for the current study that yielded gene

expression levels outside the detection limits of the gene expression assays were excluded from those analyses. Statistical significance was considered if $p < 0.05$.

CHAPTER 4: RESULTS

4.1 Reference gene selection

Using RefFinder software, as described in the methods – (section 3.9), miR-16-5p was selected as the most stable miRNA to be use as a reference gene as it had a lower geometric mean ranking value than miR-191-5p. SNU6 was excluded from all comparison as it was undetected in our samples despite multiple PCR runs.

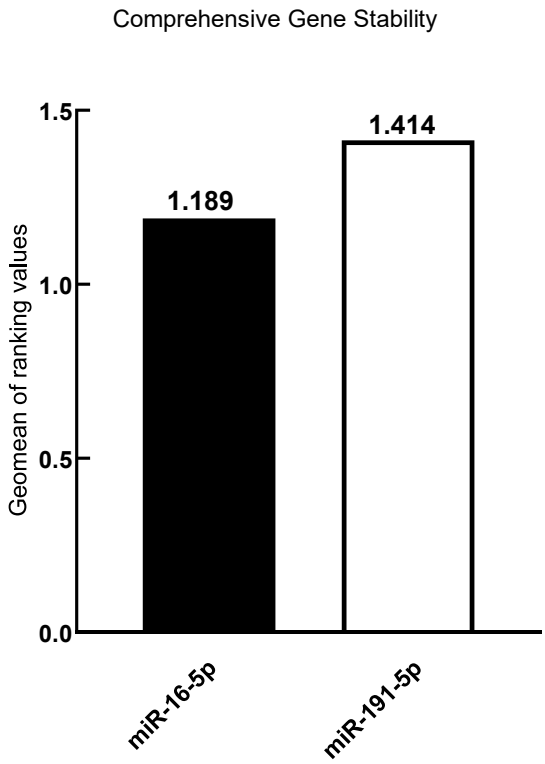


Figure 4.1. Selection of the most stable reference gene for miRNA PCR analysis. This figure represents the results generated from ΔCT values (n=12) input into Reffinder software that evaluates and screens reference genes from an extensive list of datasets to select the most stable gene from the given experimental values. *SNU6* was undetected in our samples and therefore no comparison could be made. Data are in arbitrary units represented as mean ± SD

4.2 Treatment with TNF-α and hyperglycaemia predisposes *EA.hy926* cells to inflammation

Figure 4.2A shows an increased relative mRNA expression of *IL6* in cells exposed to TNF-α compared to controls (mean ±SD: 9.46 ± 4.88; p = 0.002). Similarly, in cells exposed to 30mM glucose relative mRNA expression of *IL-6* was increased compared to controls (30mM glucose 3.38 ± 091; p = 0.001), and in cells exposed to 5mM glucose (3.23 ± 1.29; p = 0.002; Figure 4.2B). The increase in *IL-6* relative mRNA expression confirmed that we were able to induce inflammation via exposure to TNF-α and glucose.

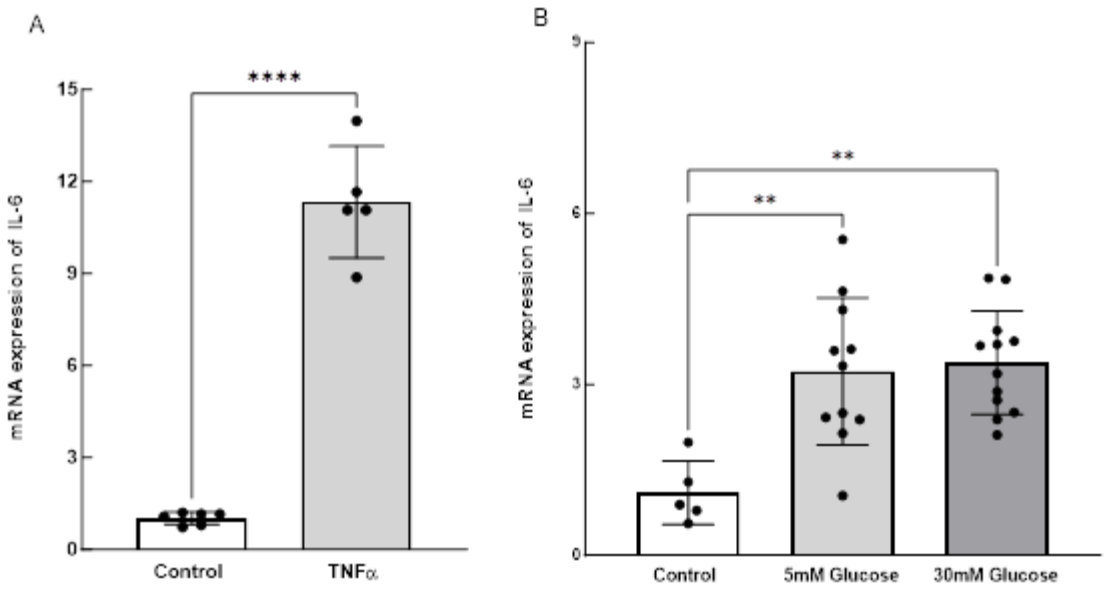


Figure 4.2. Relative mRNA expression of *IL-6* in *EA.hy926* cells. Cells were exposed to plain media for 24 hours (control, n=6) and to 10 ug/µl TNFα for 24 hours (TNF-α, n=6) (A) and plain media for 72 hours (control, n=5) and to 5mM glucose (n=11) or 30mM glucose (n=12) for 72 hours (B) before relative mRNA expression of *IL-6* was measured. Data are in arbitrary units represented as mean ± SD. **p<0.01 vs control, ****p<0.0001 vs control.

4.3 Exposure to TNF-α and hyperglycaemia increases endothelial activation in *EA.hy926* cells

Exposure to TNF-α increased the relative mRNA expression of *VCAM-1* compared to controls (mean ±SD: control 1.09 ± 0.50; TNFα 9.77 ± 2.65; p < 0.0001; Figure 4.3 A). Compared to controls (1.01 ± 0.15), exposure to 30mM glucose resulted in an increased *VCAM-1* relative mRNA expression (7.60 ± 6.68; p = 0.03) but not in cells exposed to 5mM glucose (3.49 ± 1.11; p = 0.66) (Figure 4.3 B). These results suggest that exposure of *EA.hy926* cells to TNFα and hyperglycaemia results in endothelial activation.

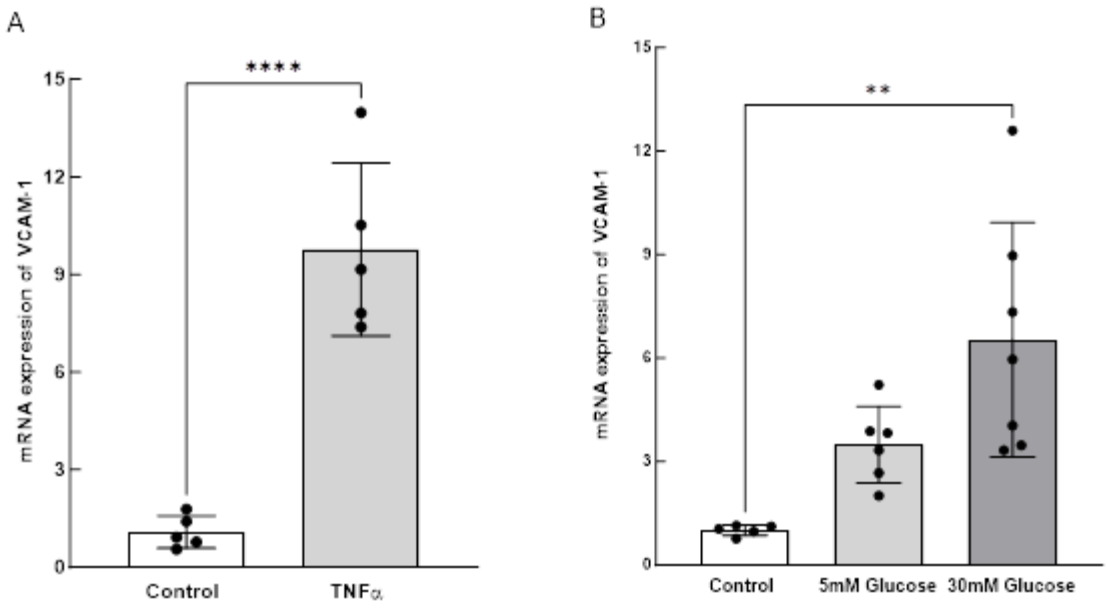


Figure 4.3. Relative mRNA expression of *VCAM-1* in *EA.hy926* cells. Cells were exposed to plain media for 24 hours (control, n=5) and to 10 ug/µl TNF-α for 24 hours (TNF-α n=5) (A) and plain media for 72 hours (control, n = 5) and to 5mM glucose (n=6) or 30mM glucose (n=7) for 72 hours (B) before relative mRNA expression was measured. Data are arbitrary units represented as mean ± SD. **p<0.01 vs control, ****p<0.0001 vs control.

4.4 Exposure to TNF-α and hyperglycaemia increases relative expression of *miR-155-5p* in *EA.hy926* cells

Exposure to TNF-α resulted in an upregulation in the relative expression of *miR-155-5p* (mean ±SD: 2.72 ± 1.15) compared to control cells (1.25 ± 0.77; p = 0.04; figure 4.4A). In Figure 4.4B, compared to control cells (1.23 ± 0.58), exposure to both 5mM (3.32 ± 0.81; p = 0.007) and 30 mM glucose (2.91 ± 0.73; p = 0.02) resulted in increased *miR155-5p* expression. These results suggest that exposure to either inflammation or glucose results in an upregulation of *miR-155-5p* expression in *EA.hy926* endothelial cells.

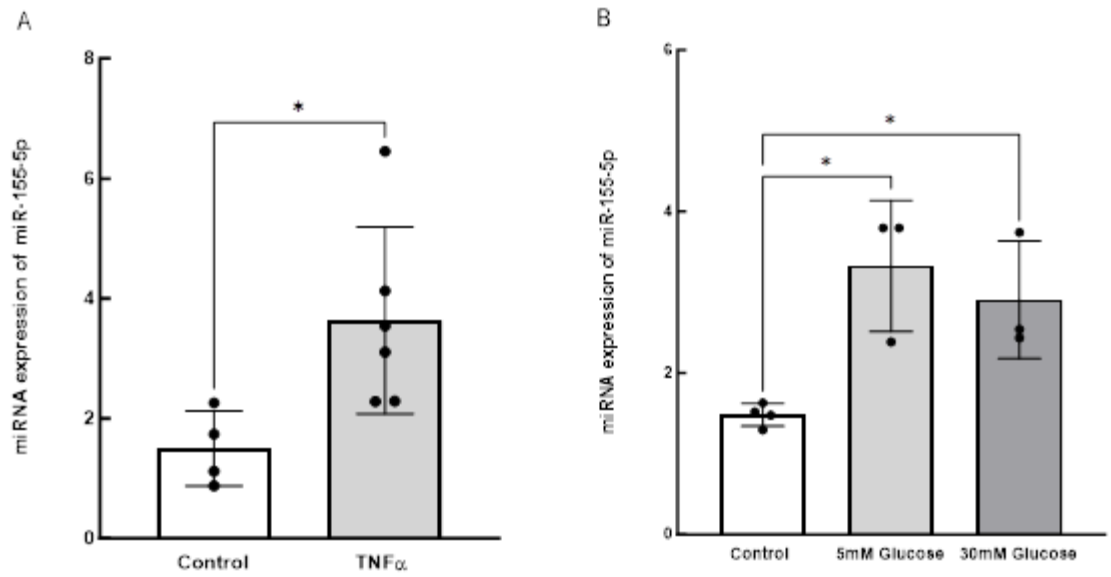


Figure 4.4. Relative miR-155-5p expression in *EA.hy926* cells. Cells were exposed to plain media for 24 hours (Control, n=4 and to 10 ug/µl TNF-α for 24 hours (TNF-α n=6) (A) and plain media for 72 hours (n = 4) and to 5mM glucose (n=3) or 30mM glucose (n=3) for 72 hours (B) before relative miR-155-5p expression was measured. Data are arbitrary units represented as mean ± SD. *p<0.05 vs control.

4.5 Exposure to TNF-α and hyperglycaemia on total and phosphorylated p65

Neither total nor phosphorylated p65 expressions were affected by either TNF-α (Figure 4.5 A and B) or glucose exposure (Figure 4.5 C and D) (all p>0.05). There were no differences in the ratios of phosphorylated-to-total p65 in either TNF-α (Figure 4.5 C) or glucose exposure (Figure 4.5E) compared to controls (both p > 0.05).

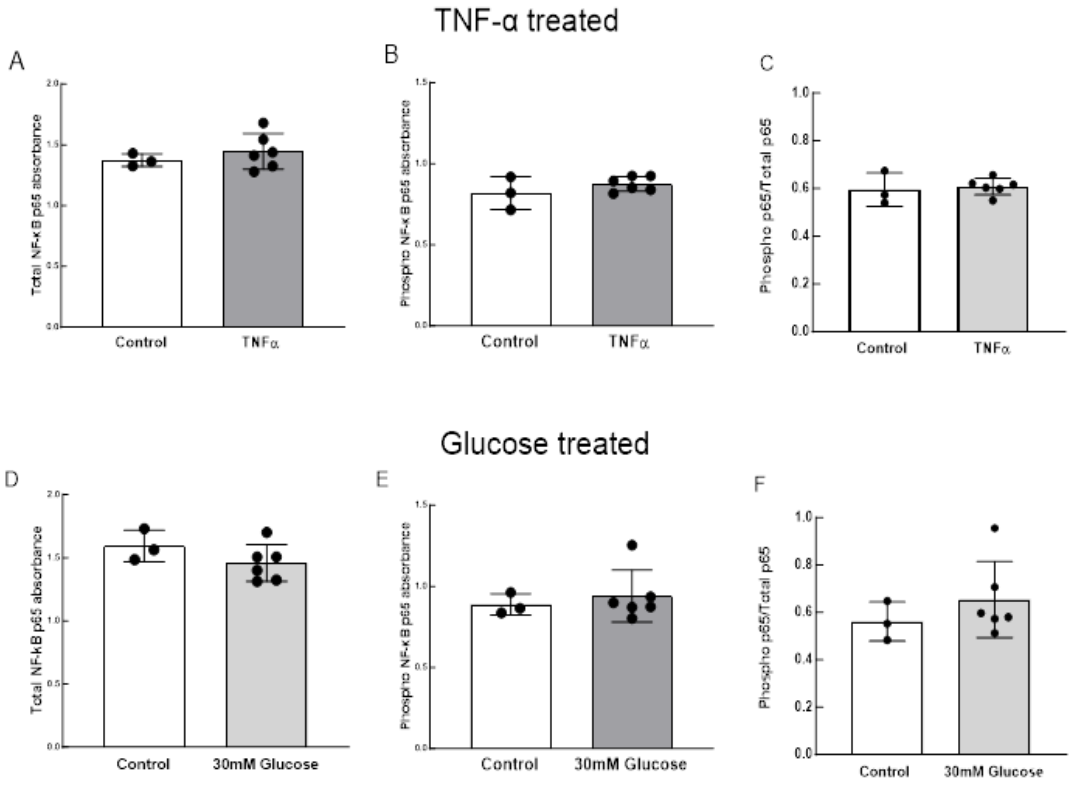


Figure 4.5. Total NF-κB p65, phosphorylated NF-κB p65 and the ratio of phosphorylated NF-κB p65 to total p65 in *EA.hy926* cells. The expression of phosphorylated NF-κB p65 and total p65 was measured at 24 h in cells exposed to plain media and to 10 ng/µl TNF-α (A, B and C) and after 72 h in cells exposed to plain media and 30mM glucose (D, E and F). Data are arbitrary units of absorbance (A, B, D and E) at 450nm or a ratio of phosphorylated to total p65 (C and F) and are presented as mean ± SD.

4.6 TNF-α induced inflammation increases the mRNA expression of *SOD2* in *EA.hy926* cells

Exposure to TNF-α resulted in an increased relative expression of *SOD-2* (mean ± SD: 8.41 ± 4.17) compared to controls (1.02 ± 0.23; p = 0.04; Figure 4.6). Upregulation of *SOD-2* expression suggests that there was a compensatory increase in antioxidant expression, in response to inflammation-induced oxidative stress.

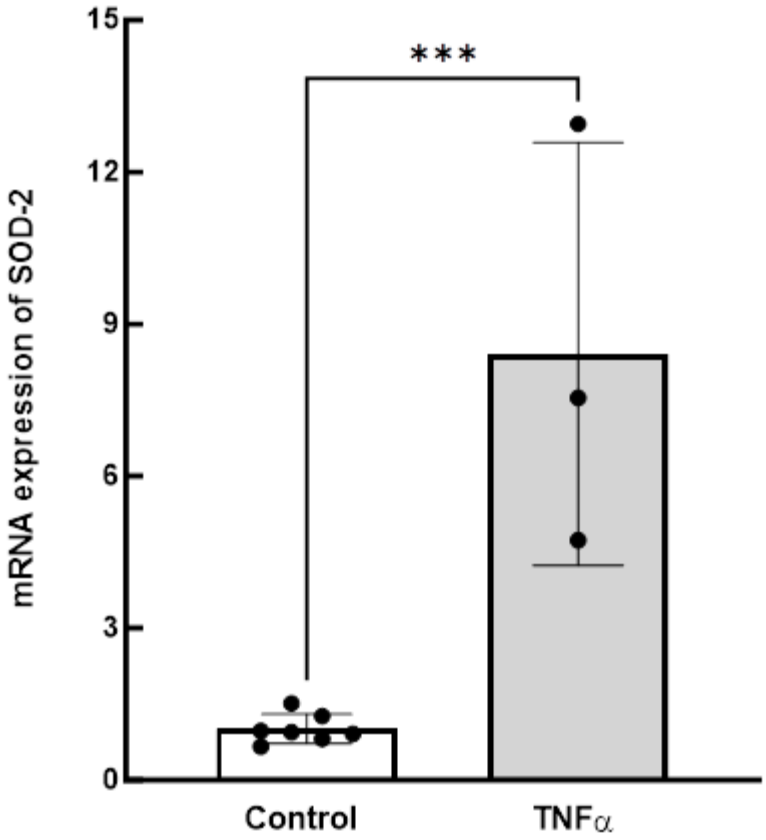


Figure 4.6. Relative mRNA expression of *SOD-2* in *EA.hy926* cells. Cells were exposed to plain media for 24 hours (control, n=3) and to 10 ug/µl TNFα for 24 hours (TNFα, n=3) before relative mRNA expression of *SOD-2* was measured. Data are arbitrary units represented as mean ± SD. ***p<0.001 vs control.

4.7 The effect of TNFα and hyperglycaemia exposure on *eNOS* mRNA expression in *EA.hy926* cells

Figure 4.7A shows that the relative mRNA expression of *eNOS* was increased in cells exposed to TNF-α (mean ±SD: 1.89 ± 0.51), compared to the control cells (1.08 ± 0.15, p = 0.04). Cells exposed to both 5mM glucose (2.91 ± 0.94; p = 0.002) and 30mM glucose (3.26 ± 0.92; p = 0.0002) showed an increased relative mRNA expression of *eNOS* compared to control cells (1.06 ± 0.44; Figure 4.7B).

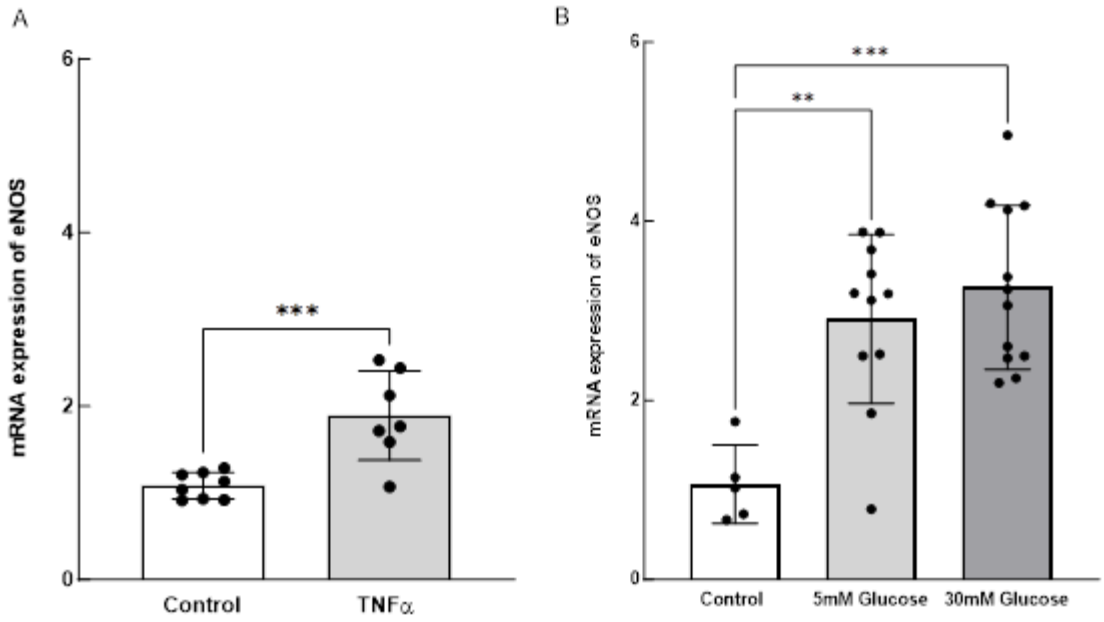


Figure 4.7. Relative mRNA expression of *eNOS* in *EA.hy926* cells. Cells were exposed to plain media for 24 hours (control, n=9) and to 10 ug/µl TNFα for 24 hours (TNFα, n=8) (A), and to plain media for 72 hours (control, n=5) and to 5mM glucose (n=11) or 30mM glucose (n=12) for 72 hours (B) before relative mRNA expression of *eNOS* was measured. Data are arbitrary units and represented as mean ± SD. **p<0.01 vs control, ***p<0.001 vs control.

4.8 Associations between markers of inflammation and endothelial function

Table 4.1 and table 4.2 show the correlation matrices between inflammatory markers and endothelial function markers in the cells exposed to TNF-α and glucose, respectively. In the model where cells were exposed to TNF-α, *IL6* expression was associated with an increased expression of *SOD2* (r = 0.84, p = 0.03), confirming that cytokine stimulation increased antioxidant activity, likely in response to increased reactive oxygen species. The increased *eNOS* expression was associated with increased *VCAM-1* expression (r = 0.73, p = 0.02).

In the glucose exposed cell model, *IL-6* expression was associated with increased *miRNA-155-5p* expression (r = 0.76, p = 0.006). In the hyperglycemia model, eNOS expression was associated with *IL-6* (r=0.85, p<0.0001), *miR-155-5p* (r=0.66, p=0.02) and with *VCAM-1* (r=0.75, p=0.0001) expression. *miRNA 155-5p* expression was associated with *IL6* expression (r=0.76, p=0.006), whereas *IL6* expression was associated with *VCAM-1* (r=0.48, p=0.03) expression, suggesting that *miR-155-5p* may play a role in mediating the association between oxidative stress-induced inflammation and endothelial activation.

Table 4.1. Pearsons correlation coefficients showing associations between inflammatory markers and markers of endothelial function in *EA.hy926* cells exposed to plain media or TNFα

		miR-155-5p	***SOD-2***	***eNOS***	***VCAM-1***
IL-6	r	0.30	**0.84**	-0.005	0.66
	p	0.36	**0.03**	0.99	0.22
	n	11	**6**	9	5
miR-155-5p	r		0.16	-0.35	0.29
	p		0.77	0.35	0.64
	n		6	9	5
SOD-2	r			0.19	0.12
	p			0.71	0.92
	n			6	3
eNOS	r				**0.73**
	p				**0.02**
	n				**10**

Statistical significance was considered if $p < 0.05$. Significant values are shown in bold.

Table 4.2. Pearsons correlation coefficients showing associations between inflammatory markers and markers of endothelial function in *EA.hy926* cells exposed to plain media or glucose

		miR-155-5p	***eNOS***	***VCAM-1***
IL-6	r	**0.76**	**0.85**	**0.48**
	p	**0.006**	**<.001**	**0.03**
	n	**11**	**28**	**20**
miR-155-5p	r		**0.66**	0.28
	p		**0.02**	0.46
	n		**11**	9
eNOS	r			**0.75**
	p			**0.0001**
	n			**20**

Statistical significance was considered if $p < 0.05$. Significant values are shown in bold.

CHAPTER 5: DISCUSSION

It is widely accepted that DM is associated with an increased risk for CVD, particularly due to atherosclerotic coronary artery disease (Aronson & Edelman, 2014). In the present study, we investigated whether *EA.hy926* cell exposure to hyperglycaemia induced oxidative stress and endothelial activation, to a similar extent as in TNF-α-induced inflammation. This study further set out to determine whether hyperglycaemia-induced inflammation was associated with changes in *miRNA-155-5p* expression.

The main findings of this study were that *in vitro* exposure of endothelial cells to hyperglycaemia induced significant oxidative stress, as evidenced by increased relative *eNOS* mRNA expression. The oxidative stress associated with hyperglycaemia further resulted in significant inflammation, as shown by an increase in relative *IL-6* mRNA expression. At high glucose concentrations (30 mM) but not low glucose concentrations (5 mM), inflammation-induced endothelial activation was present, as indicated by significant increases in *VCAM-1* mRNA expression. Moreover, *miR-155-5p* was significantly upregulated in cells exposed to high glucose concentrations, whereby relative *miR-155-5p* expression was associated with markers of oxidative stress and inflammation. The findings from this study confirm previous reports that hyperglycaemia-induced oxidative stress and inflammation result in endothelial activation (Meza et al., 2019; Zhang et al., 2021). The present study further implicates miR-155-5p in the development of oxidative stress and inflammation-induced endothelial activation, and sheds light on the potential of miRNA-155-5p for use as a biomarker of oxidative stress and early endothelial activation.

5.1 Reference gene selection

The selection of an appropriate normalization method is fundamental in the analysis of relative miRNA expression RT-PCR data. An effective technique for standardizing samples involves concurrently measuring endogenous reference genes. This approach efficiently compensates for variations arising from pre-PCR and PCR processing steps (Vandesompele *et al.*, 2002). In the past, studies have made use of SNU6 or RNU6 as endogenous controls, particularly for miRNA profiles measured in the plasma as potential diagnostic markers for the detection cancer (Adam et al., 2013; Chen et al., 2012; Tsujiura et al., 2010). However, in recent years multiple studies have shown that these genes are not as stably expressed as initially reported, and may therefore, not be suitable for use as reference genes (Lim *et al.*, 2011; Schwarzenbach *et al.*, 2015; Xiang *et al.*, 2014). SNU6 and RNU6 do not reflect the biological nature of miRNAs in terms of their transcription, posttranscriptional processing and expression profiles, therefore,

they might respond differently during extraction, reverse transcription PCR and comparative PCR amplification steps (Schwarzenbach *et al.*, 2015; Serafin *et al.*, 2014). However, in biological systems, no endogenous reference genes are consistently expressed at a steady level across different tissues (De Spiegelaere *et al.*, 2015). The stability of a reference gene can also fluctuate within a cell type under different conditions (Ceelen *et al.*, 2011). Therefore, the most effective strategy for normalizing raw RT-PCR data is to initially evaluate a group of reference genes that are independently regulated, to identify the most stable ones in each specific experiment or biological context (De Spiegelaere *et al.*, 2015). A normalization factor is calculated using the geometric mean of the most stable reference genes from the subset of stably expressed genes (Ceelen *et al.*, 2011; De Spiegelaere *et al.*, 2015). Based on the results from the present study, miR-16-5p was selected as the most stable miRNA to be use as a reference gene, as it had a lower geometric mean ranking value than miR-191-5p and it showed the smallest within sample deviation.

5.2 Inflammation status in cells exposed to TNF-α and glucose

In the present study, relative *IL-6* mRNA expression was upregulated in response to TNF-α and glucose exposure, indicating that both interventions induced an inflammatory response. IL-6 production at the site of inflammation plays a critical role in the acute phase response and mediates the transition from acute to chronic inflammation (Gabay, 2006). These results are in agreement with previous *in vitro* studies where exposure to TNF-α significantly increased the expression of *IL-6* in HUVECs (Zhou *et al.*, 2017) and human urothelial cells (Wang & Bjorling, 2011). TNF-α induces inflammation through the NF-κB pathway. TNF-α activates NF-κB canonical pathway resulting in a proinflammatory state that causes oxidative stress and endothelial activation (Liu *et al*.,2017; Evans *et al*., 2002; Mussbacher *et al*., 2019).

In our model, exposure of *EA.hy926* to glucose concentrations of 5mM and 30mM resulted in significant increases in relative *IL-6* mRNA expression. Our findings are consistent with previous studies reporting an association between hyperglycaemia and inflammation both *in vivo* (Ye *et al.*, 2022) and *in vitro* models (Zhang *et al.*, 2021).

Interestingly, even moderate glucose levels (5mM) induced a significant increase in *IL-6* gene expression compared to control cells, suggesting that glucose may be a potent inducer of inflammation, even at low to moderate concentrations. The mechanisms whereby glucose drives inflammatory responses are currently under investigation. However, one primary mechanism suggested to be involved in hyperglycaemia-induced inflammation, is excessive

oxidative stress. Prolonged exposure to high levels of glucose increases the production of ROS to a level that surpasses the capacity of cellular antioxidants to neutralize ROS, this causes oxidative stress that leads to inflammation (Bae *et al*., 2011; Ray *et al*., 2012). These two different models of inflammation have different mechanisms of action that the subsequent results will address further.

5.3 TNF-α induced inflammation increases the expression of antioxidants

In the present study, cells exposed to TNF-α demonstrated an increase in the relative mRNA expression of *SOD-2*, a gene that codes for superoxide dismutase 2, an antioxidant enzyme that protects cells from the harmful effects of oxidative stress by scavenging ROS. This result suggests that there is an increase in intracellular superoxide ions which may be driven by NFκB-induced activation of NADPH oxidases (Wu *et al*., 2021). Our findings confirm that exposure to inflammatory cytokines, such as TNF-α, induced oxidative stress, whereby a greater requirement for antioxidant measures were necessitated. Indeed, increased *SOD-2* expression has consistently been reported in inflammatory disorders (Li & Zhou, 2011). Although in the present study, we did not measure *SOD-2* mRNA expression in the glucose treated cells, it is well accepted that states of oxidative stress, including diabetes (GómezMarcos *et al.*, 2016), coronary artery disease (Peng *et al.*, 2016) and hypertension (GómezMarcos *et al.*, 2016) antioxidant gene expression is increased.

Indeed, inflammation and oxidative stress are interrelated processes that augment each other. While oxidative stress can activate immune cells to elicit an inflammatory response, inflammatory cytokines also generate ROS, to result in a positive feedback cycle, eventually causing endothelial injury (Chanda *et al.*, 2023). Nevertheless, what are the effects of the increased oxidative stress in these cells? In this regard, oxidative stress leads to endothelial dysfunction due to decreased NO bioavailability, an increase in inflammation and leukocyte adhesion as well as damage to the mitochondria (Scioli et al., 2020), which will be elaborated on below.

5.4 Endothelial function in response to exposure to TNF-α and glucose

Several studies have reported that in response to increased oxidative stress induced by hyperglycaemia, the production of eNOS is reduced, which limits the production and bioavailability of NO (An *et al.*, 2023; Meza *et al.*, 2019). In contrast, in the present study, the relative *eNOS* mRNA expression was significantly increased in cells exposed to moderate and

high concentrations of glucose compared to the control cells. Similar findings have been reported in human glomerular endothelial cells, where *eNOS* expression increased in parallel with increased free radical production (Cosentino *et al.*, 1997) and in human glomerular endothelial cells where *eNOS* expression increased despite reduced NO bioavailability (Hoshiyama *et al.*, 2004).

It is widely accepted that eNOS is the major producer of NO, which is a critical vasoprotective molecule in the healthy vasculature (Kemp-Harper *et al.*, 2021). Besides the vasodilatory function of NO through NO•-sGC/cGMP signalling, NO also potently inhibits platelet aggregation and leukocyte adhesion (Förstermann & Münzel, 2006). However, in states of oxidative stress, there is excess production of superoxide, which reacts with NO to form peroxynitrite, whereby vascular protection is lost. Over time, the enzymology of eNOS is disturbed, whereby eNOS begins to produce superoxide itself, instead of NO (Łuczak *et al.*, 2020). This process whereby the bioavailability of NO is reduced, despite increased eNOS production is referred to as eNOS uncoupling (Łuczak *et al.*, 2020).

Multiple sources of oxidative stress exist in hyperglycaemia associated with diabetes, including enzymatic, non-enzymatic and mitochondrial pathways (Johansen *et al.*, 2005). Nonenzymatic sources of oxidative stress in hyperglycaemia include direct production of ROS by autoxidation of glucose to produce hydroxyl free radicals (•OH) (Turko *et al.*, 2001). Enzymatic sources of augmented generation of reactive species in diabetes include NOS, NAD(P)H oxidase and xanthine oxidase (Ceriello *et al.*, 2001; Guzik *et al.*, 2000; Guzik *et al.*, 2002). The mitochondrial respiratory chain also produces ROS when electrons are transferred between electron carriers during ATP production (Green *et al.*, 2004). Under normal, conditions, $O^{\bullet}_{2-}$ is immediately eliminated by natural antioxidant mechanisms, such as SOD-2.

When antioxidant mechanisms are diminished, increased hydrogen peroxide (H_2O_2) concentrations upregulate the expression of uncoupled eNOS through transcriptional and posttranscriptional mechanisms (Cai, 2005). Accelerated degradation of the remaining NO occurs through a reaction with superoxide to produce peroxynitrite (ONOO), which uncouples additional eNOS enzymes in a vicious cycle (Förstermann *et al.*, 2006). Cardiovascular risk factors, including inflammation and diabetes, are therefore associated with decreased NO bioavailability and a simultaneous increase in uncoupled eNOS expression (Li *et al.*, 2002). These reported mechanisms are consistent with our findings where *eNOS* expression was increased in cells exposed to TNF-α and glucose.

Interestingly, in the present study, exposure to moderate glucose concentrations resulted in similar degrees of eNOS uncoupling and upregulation, compared to high glucose concentrations. This indicates that hyperglycaemia exerts potent destabilizing effects on eNOS. Hyperglycaemia exerts damaging effects on the endothelium, as glucose exists in equilibrium with its enediol, which can spontaneously undergo auto-oxidation to produce superoxide radicals (Khan *et al.*, 2012). These radicals need to be rapidly scavenged by endothelial antioxidant mechanisms, including NO. However, hyperglycaemia also reduces NO availability by inhibiting synthesis of the eNOS cofactor tetrahydrobiopterin (BH_4) (Xu *et al.*, 2007), leading to impaired endothelium-dependent vasodilation (Heitzer *et al.* 2000; Okon *et al.* 2005). Pronounced eNOS uncoupling, in conjunction with glucose scavenging of NO (Funk *et al.*, 2012), results in pronounced endothelial dysfunction in states of hyperglycaemia (Brodsky *et al.*, 2001). This endothelial dysfunction indicates a loss in NO responsiveness, termed NO resistance, commonly reported in diabetic mellitus (Kemp-Harper *et al.*, 2021). These reports are consistent with our findings where eNOS uncoupling occurred to a greater extent in cells exposed to hyperglycaemia, compared to cells exposed to inflammation. Nevertheless, cells exposed to TNF-α still displayed a degree of oxidative stress and eNOS uncoupling. As previously described, proinflammatory cytokines increase oxidative stress, eNOS uncoupling and decrease NO bioavailability (Łuczak *et al.*, 2020; Förstermann & Li, 2011).

Taken together, the relative mRNA expression of *eNOS* was significantly increased in cells exposed to both the 5mM and the 30mM glucose compared to the control cells. Glucose is an essential energy source, but at increased levels exerts deleterious effects on vascular function. In the present study, the upregulated *eNOS* expression that was seen in response to high glucose levels suggests that there might be a compensatory mechanism to improve free radical scavenging, in response to oxidative stress associated with glucose metabolism.

5.5 Endothelial activation in cells exposed to TNF-α and glucose

In the present study, *EA.hy926* cells exposed to TNF-α demonstrated an increased mean relative mRNA expression of *VCAM-1* compared to the control group, indicating endothelial activation. This finding is supported by a HUVEC cell culture study where TNF-a induced VCAM-1 upregulation was attenuated by TNF-a receptor blockade (Sawa *et al.*, 2007). Another study also showed that in human cardiac fibroblasts incubated with different concentrations of TNF-α, VCAM-1 expression increased in a dose- and time-dependent manner

(Lin *et al.*, 2015). In this context, VCAM-1 plays a role in immune cell recruitment, adhesion and trans-endothelial migration following an inflammatory response (Hannoodee & Nasuruddin, 2023). VCAM-1 is consistently upregulated during inflammation (Hannoodee & Nasuruddin, 2023). Therefore, it is widely accepted that, TNF-α induced inflammation leads to endothelial activation, as evidenced by upregulated VCAM-1 expression.

In the present study, exposure of *EA.hy926* cells to glucose concentrations of 30mM glucose, but not 5mM glucose resulted in increased relative mRNA expression of *VCAM-1* compared to the control group. Similar findings were reported by a study that utilized HUVECs obtained from women with gestational diabetes and exposed the cells to hyperglycaemia and found that VCAM- 1 expression was upregulated, and this led to an increase in the adhesion of monocytes to the endothelium (Zhang et al., 2021). Another study found that human endothelial cells exposed to sera from type 1 diabetic patients had a much higher expression of VCAM-1 than those expose to sera from non-diabetic patients, as well as an increased cellular response to the inflammatory TNF-α (Rasmussen *et al*., 2002). Another study using human diabetic endothelial cells showed that exposure of the cells to hyperglycaemia increased VCAM-1's expression, which was not seen in the non-diabetic endothelial cell controls (Haubner *et al.*, 2007).

In the present study, the differential response in *VCAM-1* expression following exposure to moderate versus high glucose concentrations was notable. We speculate that, while acute exposure to glucose causes oxidative stress and reductions in NO availability, compensatory antioxidant mechanisms are still present to prevent endothelial activation. Only once the antioxidant capacity of the endothelial cell is exceeded, will an inflammatory response be elicited that will upregulate *VCAM-1* expression. Previous studies have also supported the notion that the degree of inflammation present determines the pro-adhesive effect of glucose on the vasculature (Azcutia *et al.*, 2010). While NO serves a vital anti-adhesive role within the vasculature (Li & Förstermann, 2000), upregulation of cellular adhesive molecules remain the key determinants of endothelial activation. Alternatively, it may be that the exposure time of 72h in the low glucose concentrations may have been insufficient to sufficiently activate the cascade of events leading to upregulation of adhesion molecules.

In the present study, we found that *VCAM-1* expression was directly related to *eNOS* and *IL-6,* indicating that the oxidative stress present was likely only sufficiently high to elicit an inflammatory response upon exposure to high glucose concentrations. This finding from the present study may have some significance for understanding the relationship between

hyperglycaemia, as seen in diabetes, and endothelial dysfunction, which is one of the earliest predictors of the development of CVD.

5.6 MiR-155-5p expression after exposure to TNF-α and glucose. A potential biomarker?

The relative expression of *miR-155-5p* was significantly upregulated compared to control cells after exposure to TNF-α. These results are in line with previous reports that suggested miR155-5p is involved in endothelial inflammation. In this regard, a previous study in human aortic smooth muscle cells exposed to TNF-α reported that miR-155-5p biogenesis was increased (Choi *et al.*, 2018). Similarly, a previous study from our laboratory using the collagen induced arthritis model in rats, that induces inflammation, found that miR-155-5p was upregulated compared to controls (Gunter *et al.*, 2022). Another study that used rheumatoid synoviocytes (RASFs) isolated from synovial tissue obtained from RA patients, found that when these RASFs were exposed to TNF-α they showed a higher expression of miR-155-5p compared to the untreated RASFs (Migita *et al.*, 2017). In the current study, TNF-α's ability to upregulate *miR-155-5p* expression suggests that miR-155-5p may play a role in the endothelial response to inflammation and that its upregulation might affect the expression of other genes involved in inflammation and immune function in the endothelium. Nevertheless, we showed no significant association between *miR-155-5p* expression and the expression of endothelial function markers. The lack of association may be as a result of a low sample number. Alternatively, it may suggest that the upregulation miR-155-5p of in inflammation-induced endothelial dysfunction may contribute to endothelial activation via other mechanisms. The exact role of miR-155-5p in the development of endothelial activation in inflammatory conditions should be investigated further.

Exposure of cells to both 5mM and 30mM concentrations of glucose resulted in an upregulation of *miR-155-5p* expression compared to the control group. This finding suggests that miR-1555p may play a role in the cellular response to hyperglycaemia. Similar to our finding, it has been reported that in cultured podocytes and in the serum and kidney tissue of mice with diabetic nephropathy, there was increased expression of miRNA-155 which was accompanied by increased levels of inflammatory molecules (Wang *et al.*, 2021). Another study showed that miR-155 expression was upregulated in endothelial progenitor cells (EPCs) from patients with diabetic foot ulcer compared to normal controls (Gao *et al.*, 2018). In the aforementioned study, the authors also showed that miR-155-5p was upregulated in EPCs from healthy people that were exposed to high glucose concentrations (Gao *et al.*, 2018). In contrast, a clinical study

found that miR-155-5p expression in patients with DM was lower than in normal control patients (Xu *et al.*, 2022). In the context of the current study, elevated glucose levels resulted in increased markers of inflammation and endothelial function and integrity. In the glucose treated cells, the positive correlations between *eNOS* and *IL-6, miRNA 155-5p*, and *VCAM-1* suggest a complex interaction. In this regard, the positive correlation between *IL-6*, *eNOS* and *miR-155-5p* expression suggests that exposure to high glucose levels may lead to ROS-induced endothelial dysfunction and ultimately increased inflammatory cytokine production. Elevated *eNOS* expression, usually associated with endothelial function, is linked to higher levels of endothelial activation (*VCAM-1*) and inflammation (*IL-6*) as well as the higher levels of miR155-5p. This suggests that the oxidative stress experienced by the endothelial cells causes an inflammatory response, which leads to endothelial activation. In addition, the significant associations noted also implicated miR-155-5p in the development of oxidative stress and inflammation-induced endothelial activation. Therefore, this miRNA may be involved in the regulation of genes associated with endothelial and vascular function in hyperglycaemia.

5.7. Potential modulation of NF-κB p65 expression by miR-155-5p in *EA.hy9.26* cells exposed to TNF-α and glucose

The expression of both total p65 and phosphorylated p65 were not affected by either TNF-α or glucose exposure in the present study. There were also no differences in the ratios of phosphorylated-to-total p65 in either TNF-α or glucose models. This indicates that in the *EA.hy926* cell line TNF-α and glucose exposure did not cause a change in p65 protein expression, under these particular experimental conditions. Furthermore, the phosphorylation status that indicates activation of the p65 protein remained unchanged after exposure to TNFα or glucose. The p65 protein is one of the subunits of the transcription factor NF-κB which is known to play a role in the inflammatory responses in cells (Israel, 2010).

These results are in contrast to previous results. Indeed, it is well documented that proinflammatory cytokines such as TNF-α trigger signalling cascades that result in the activation of the NF-κB pathway (O'Brien *et al.*, 2018). This usually involves the activation of various kinases via phosphorylation as well as the recruitment of various proteins (Karin & Delhase, 2000). In contrast to the findings of the present study, several studies have shown that NF-κB upregulation is central to the progression of the inflammatory response and that it is activated by various ligands that include TNF-α (Acar *et al.*, 2018; Liu *et al.*, 2017; Moe *et al.*, 2014).

Similarly, hyperglycaemia has been implicated in the activation of NF-κB. During prolonged periods of hyperglycaemia such as in diabetes, a non-enzymatic process known as glycation occurs in which glucose forms covalent links with plasma proteins (Singh *et al.*, 2014). Advanced glycation end products (AGE)-modified proteins can affect a variety of cells and tissues (Giacco & Brownlee, 2010). It has been found that the AGE receptor (RAGE) mediates signal transduction via the production of ROS and NF-κB activation (Giacco & Brownlee, 2010). A study found that in streptozotocin-induced diabetic RAGE/apoE deficient mice, there was a reduction in the atherosclerotic plaque area and this was associated with a decrease in the diabetes-associated expression of NF-κB p65 subunit (Soro-Paavonen *et al.*, 2008). This suggests that RAGE may trigger the activation and upregulated expression of NF-κB. Additionally, under hyperglycaemic conditions there is increased production of ROS that leads to oxidative stress and activates the NF-κB signalling pathways (Ighodaro, 2018; Pueyo *et al.*, 2000). These findings are in contrast to the present studystudy in which there was no change in NF-κB p65 expression after exposure to glucose.

One possible mechanism that may explain the results in our current study suggests that upon its upregulation, miR-155-5p may target the proteins of the IκB kinase (IKK) complex, IKKα and IKKβ, preventing their phosphorylation and hence the release and translocation of the active NF-κB (Costinean *et al.*, 2006; Ma *et al.*, 2011). Alternatively, in the present study, most markers were measured by miRNA expression using PCR, while p65 expression was measure by ELISA. The ELISA kit used to measure the ratio of total to phosphorylated p65 had a detection range of 0.001 to 100ng/ml. In this regard, the low abundance and yield of phosphorylated p65 in the cell lysate may likely be below the detection limit of the ELISA kit. Therefore, preparing more concentrated cell lysates may address this in future studies.

Taken together, in the present study the effects of inflammation and hyperglycaemia on the activation of the NF-κB pathway are inconclusive. Further investigations are needed to determine the exact role of increased expression of miR-155-5p on the regulation of the NF-κB pathway and its role the development of endothelial function.

5.8 Limitations

The use of mRNA expression analysis might not fully represent the levels of the markers measured in this study as some markers undergo changes post transcriptionally and post translationally. Future studies should include protein expression to improve the interpretability of the mRNA expression data.

With both the miRNAs and NF-κB it might have been useful to measure protein expressions of downstream adaptor proteins and those involved in downstream signalling to strengthen our interpretations.

The *EA.hy926 cell* line used is an immortalized cell line and may have an altered cellular physiology, therefore, this might give a less accurate indication of the endothelial response to these stimuli. Use of the immortalized cell line rather than primary HUVECs could potentially result in muted expression profiles in response to stimuli within the *EA.hy926* cells, compared to HUVECs, likely due to varying levels of stress tolerance between the two cell types (Uruski *et al.*, 2021). It is also important to note that these findings are based on *in vitro* experiments using *EA.hy926* cells and they provide valuable insights into cellular responses. However, two-dimensional cell culture models are limited, and hence any translation to *in vivo* human physiology is challenging and would require further animal studies and clinical studies.

5.9 Conclusions

The findings of this study provide some insights and input into development of a cell model to study inflammation and vascular function. We investigated the effects of TNF-α and glucose exposure on *EA.hy926* cells. We found that miR-155-5p expression was upregulated in *EA.hy926* cells exposed to TNF-α and glucose. MiR-155-5p was also linked to *IL-6* expression in the glucose model suggesting a potential role for this miRNA in the inflammatory process caused by high glucose. We showed that *IL-6, SOD-2, VCAM-1* and *eNOS* expressions were upregulated in *EA.hy926* cells exposed to TNF-α. Similarly, we showed *IL-6, VCAM-1* and *eNOS* expressions were upregulated in *EA.hy926* cells exposed to glucose. The role of the NF-κB pathway in endothelial dysfunction in hyperglycaemia and inflammation in and the regulation of this pathway by miR-155-5p were inconclusive.

This study confirms previous reports of inflammation and hyperglycaemia induced endothelial dysfunction. However, our results suggest that the mechanisms whereby endothelial activation develop in these conditions may be different. Moreover, our results suggest that miR-155-5p may play a significant role in driving endothelial dysfunction in these conditions, however, the exact mechanisms require further investigation. Future studies should investigate the link between miR-155-5p and IL-6 as well as other downstream targets through miR-155-5p knockdown or overexpression to further elucidate its role. Additionally, while we did not observe significant changes in relative expression of NF-κB p65, it might be important to

further investigate the NF-κB pathway's role in inflammation and endothelial dysfunction by modifying experimental conditions or exploring crosstalk with other signalling pathways.

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APPENDIX

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

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

TO: Mr J Amoni, et al
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Medical School
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CC: Supervisor: Dr S Gunter <Sule.Gunter@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

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

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

DATE: 26/09/2021

REF: *R14/49*

PROTOCOL NO: W-CBP-220926-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *Exploring the regulation of endothelial function by microRNA's, in an inflammatory cell culture model*

Please find attached the Ethics Waiver 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 the Government funding of the University.

MSWorks2000/Iain0007/ClearScan\Waiver.wps

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16 August 2023
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TAA

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Dear Mr Joel Amoni

Master of Science in Medicine: Change of title of research

I am pleased to inform you that the following change in the title of your Dissertation for the degree of **Master of Science in Medicine** has been approved:

From:	**Exploring the regulation of endothelial function by microRNAs, in an inflammatory cell culture model**
To:	**Understanding the in vitro effect of TNF-a and hyperglycaemia on endothelial activation**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences