MODULATION OF GUT MICROBIOTA BY AB KEFIR AND ITS EFFECT ON ENDOTHELIAL INSULIN RESISTANCE AND VASCULAR INFLAMMATION IN HIGH FAT DIET-INDUCED OBESE RATS

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
°	Degree
℃	Degree Celsius
γ	Gamma
±	Plus-minus
<	Less than
≤	Less than or equal to
>	Greater than
≥	Greater than or equal to
=	Equal to
µL	Microlitre
mL	Millilitre
cm	Centimetre
kg	Kilograms
g	Gram
mg/mL	Milligrams per millilitre
pg/mL	Picograms per millilitre
pmol/mL	Picomoles per millilitre
mg/kg	Milligrams per kilogram
%	Percentage
mmol/L	Millimoles per litre
mL/kg	Millilitres per kilogram
w/v	Weight per volume
Da	Dalton
kDa	Kilo-Dalton
m	Metre
O_2	Oxygen
CO_2	Carbon dioxide

LIST OF ABBREVIATIONS

ACh	Acetylcholine
Akt	Protein kinase B
AGE	Advanced glycation end products
AI	Atherogenic index
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DAG	Diacylglycerol
DIO	Diet-induced obesity
ED	Endothelial dysfunction
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarization factor
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-receptor kinase
ET-1	endothelin-1
FAD	Flavin adenine dinucleotide
FBG	Fasting blood glucose
FMN	Flavin mononucleotide
GLP-1	Glucagon-like peptide
GRB2	Growth factor receptor-bound 2
HDL	High-density lipoprotein
HFD	High-fat diet
HOMA	Homeostatic model assessment
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukins

IP_3	Inositol 1,4,5-triphosphate
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
JNK	C Jun kinase
KCl	Potassium chloride
L-NAME	L-nitro-arginine methyl ester
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocytes chemoattractant protein-1
MDA	Malondialdehyde
MEK1	Mitogen-activated protein kinase kinase 1
MLC	Myosin light-chain
MLCK	Myosin light chain kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
oxLDL	Oxidized low-density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PDK-1	phosphoinositide-dependent protein kinase-1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGI	Prostacyclin
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
PSS	Physiological saline solution
PVDF	Polyvinylidene difluoride
QUICKI	Quantitative insulin sensitivity check index
RAAS	Renin angiotensin aldosterone system
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids

SEM	Standard error of mean
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
SOS	Son of Sevenless
T2D	Type 2 diabetes mellitus
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline/Tween-20
TC	Total cholesterol
TG	Triglycerides
TLR	Toll-like receptor
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TNF-α	Tumour necrosis factor-alpha
TUDCA	Tauro ursodeoxycholic acid
UDCA	Ursodeoxycholic acid
USM	Universiti Sains Malaysia
VCAM-1	Vascular adhesion molecule-1
VSMC	Vascular smooth muscle cell
WC	Waist circumference
WHO	World Health Organization
WHR	Waist-hip ratio
WHtR	Waist to height ratio

LIST OF APPENDICES

MODULASI MIKROBIOTA USUS OLEH AB KEFIR DAN KESANNYA TERHADAP RINTANGAN INSULIN ENDOTELIAL DAN KERADANGAN DALAM PEMBULUH DARAH TIKUS OBES ARUHAN-DIET TINGGI LEMAK

ABSTRAK

Obesiti dengan ketara meningkatkan risiko penyakit kardiovaskular dan metabolik akibat rintangan insulin yang berkaitan. Rintangan insulin vaskular menyumbang dengan ketara kepada permulaan penyakit kardiovaskular, yang merupakan punca utama kematian dalam kalangan individu yang obes dan yang mempunyai sindrom metabolik. Mikrobiota usus memainkan peranan penting dalam mengekalkan fungsi vaskular dengan mempengaruhi proses metabolik dan keradangan sistemik. Disbiosis mikrobiota usus sering diperhatikan dalam obesiti dan dikaitkan dengan rintangan insulin. Rawatan probiotik boleh mengubah mikrobiota usus dengan berkesan, dan probiotik kefir telah menunjukkan kesan positif dalam mengurangkan risiko beberapa gangguan kardiometabolik. AB kefir ialah probiotik multistrain yang dihasilkan secara industri, terdiri daripada bakteria asid laktik dan *Bifidobacterium*. Walau bagaimanapun, keberkesanan AB kefir dalam memperbaiki rintangan insulin, keradangan dan disbiosis mikrobiota yang berkaitan dengan obesity masih belum jelas. Kajian ini bertujuan untuk menyiasat kesan AB kefir terhadap mikrobiota usus, berat badan, profil lipid, disfungsi endotelium, rintangan insulin metabolik dan vaskular, keradangan, dan tekanan oksidatif dalam tikus obes yang diberi diet tinggi lemak. Dua puluh empat ekor tikus Sprague-Dawley dibahagikan kepada tiga kumpulan: (i) tikus diberi makanan tikus biasa, (ii)

tikus diberi diet tinggi lemak (HFD), dan (iii) tikus diberi HFD dan diberikan AB kefir melalui penyedutan oral selama lapan minggu terakhir kajian. Tikus-tikus dikorbankan pada minggu ke-22, dan berat badan serta indeks jisim badan (BMI) diukur. Sampel darah diambil untuk menilai paras glukosa darah puasa dan insulin. Sampel najis dikumpulkan untuk menentukan komposisi mikrobiota usus menggunakan teknik penjujukan RNA ribosom 16S. Tisu aorta diambil untuk kajian fungsi endotelium dan isyarat insulin serta analisis *Western blot* bagi menentukan ekspresi protein nitrik oksida endotelial (eNOS), substrat reseptor insulin-1 (IRS-1), dan protein kinase protein teraktif mitogen (MAPK). Ujian imunosorben berkaitan enzim (ELISA) dilakukan untuk mengukur penanda keradangan dan tekanan oksidatif. Rawatan AB kefir pada tikus obes telah memodulasi komposisi mikrobiota usus, mengurangkan berat badan, memperbaiki profil lipid, memperbaiki pengeduran-pengantara asetilkolin dan insulin aorta tikus yang terjejas melalui laluan eNOS/IP3K/IRS-1, serta mengurangkan keradangan dan tekanan oksidatif. Oleh itu, AB kefir berpotensi menjadi terapi pelengkap atau tambahan yang berkesan, serta pendekatan pemakanan untuk rawatan dan pencegahan penyakit kardiometabolik berkaitan obesiti.

MODULATION OF GUT MICROBIOTA BY AB KEFIR AND ITS EFFECT ON ENDOTHELIAL INSULIN RESISTANCE AND VASCULAR INFLAMMATION IN HIGH FAT DIET-INDUCED OBESE RATS

ABSTRACT

Obesity significantly increases the risk of cardiovascular and metabolic diseases due to associated insulin resistance. Vascular insulin resistance contributes considerably to the onset of cardiovascular disease which is a primary cause of death among individuals with obesity and metabolic syndrome. Gut microbiota plays a crucial role in maintaining vascular function by influencing metabolic processes and systemic inflammation. Dysbiosis of gut microbiota is commonly observed in obesity and is linked to insulin resistance. Probiotics treatment can effectively modulate intestinal microbiota and kefir probiotics have demonstrated a favourable impact in reducing the risk of several cardiometabolic disorders. AB kefir is an industrially produced multistrain probiotic of lactic acid bacteria and *Bifidobacterium.* It remains unclear whether AB kefir probiotics can effectively improve obesity associated insulin resistance, inflammation and microbiota dysbiosis. This study aimed to investigate the effects of AB kefir on gut microbiota, body weight, lipid profile, endothelial dysfunction, metabolic and vascular insulin resistance, inflammation and oxidative stress in high-fat diet-induced obese rats. Twenty-four Sprague-Dawley rats were divided into three groups: (i) rats fed normal rat chow, (ii) rats fed a high fat diet (HFD), and (iii) rats fed HFD and gavaged with AB kefir for the last eight weeks of the study. The rats were sacrificed at week 22, body weight and body mass index (BMI) were measured. Blood samples were taken to assess fasting blood

glucose and insulin levels. Faecal samples were collected to determine gut microbiota composition using 16S ribosomal RNA sequencing. Aortic tissues were collected for endothelial functional and insulin signalling study as well as Westen blotting to determine the expression of endothelial nitric oxide (eNOS), insulin receptor substrate-1 (IRS-1) and mitogen activated protein kinase (MAPK) protein. Enzyme-linked immunosorbent assay (ELISA) was performed to measure inflammatory and oxidative stress markers. AB kefir treatment in obese rats modulated gut microbiota composition, reduced body weight, improved lipid profiles, improved the impaired acetylcholine- and insulin-mediated relaxation of rat aorta through eNOS/IP3K/IRS-1 pathway, while also reduced inflammation and oxidative stress. Therefore, AB kefir may have the potential to serve as an effective complementary or adjuvant therapy, as well as nutritional approach for the treatment and prevention of cardiometabolic diseases related to obesity.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Obesity is both a disease itself and a risk factor for many life-threatening diseases (Lasker et al., 2019). It usually occurs as a co-morbidity with other cardiovascular and metabolic problems in the form of metabolic syndrome. Other components of metabolic syndrome are hypertension, dyslipidaemia, and insulin resistance (Gunawan et al., 2021). Obesity is widely recognized as a global epidemic with a marked increase in its prevalence and that of its associated conditions like type 2 diabetes (T2D), metabolic syndrome, and cardiovascular diseases (CVDs) (Valenzuela et al., 2023). In addition to being a medical condition, obesity significantly amplifies the risk of cardiovascular and metabolic diseases (Valenzuela et al., 2023). The development of obesity therefore increases the incidence of cardiovascular disease, stroke, T2D, fatty liver, and even cancer (Mballa et al., 2021). Although obesity occurs as a reaction to several factors, such as diet, genetics, and social factors, the main cause underlying its development is a sustained disequilibrium between energy intake and energy expenditure, which causes excess fat accumulation (Valenzuela et al., 2023). The World Health Organization (WHO) predicts the likelihood of obesity and overweight becoming the most significant causes of morbidity surpassing infectious diseases and malnutrition (World Health Organization, 2024). One out of every eight adults globally is living with obesity which is no longer a specific problem of developed nations as it is rapidly becoming a concern in developing countries as well (World Health Organization, 2024). The WHO reported that about 890 million adults and 160 million adolescents and children worldwide were living with obesity in 2022 (World Health Organization, 2024).

Obesity rates among children and adolescents are rising alarmingly; In 1990, just 2% of children and adolescents aged 5–19 were categorised as obese, totaling 31 million people; by 2022, this number increased to 8%, equating to 160 million individuals. Nearly 50% of under 5 obese and overweight children were in Asia as of 2022 (World Health Organization, 2024).

Overweight and obesity are the main causes of morbidity, alongside infectious diseases (Manna Li et al., 2017). About 5 million noncommunicable diseases fatalities such as CVDs, diabetes, chronic respiratory and gastrointestinal disorders, malignancies, and neurological diseases were attributed to obesity in 2019 (GBD 2019 Risk Factor Collaborators, 2020). In terms of economic effects, the worldwide expenses related to overweight, and obesity are anticipated to surpass US$ 3 trillion yearly by 2030 and higher than US$ 18 trillion by 2060 if nothing is done (Okunogbe et al., 2022). Obesity is a serious concern in Malaysia which has the highest prevalence of adult obesity in Southeast Asia (Peng et al., 2018). The National Health and Morbidity Survey (NHMS) established a sharp increase in the occurrence of adult obesity from 4.4% in 1996 to 17.7% in 2015 which is a four-fold increase (Mohd-Sidik et al., 2021). The NHMS 2019 also reported 19.7% prevalence of obesity among Malaysian adults.

Insulin resistance is the primary characteristic of metabolic syndrome development (Janus et al., 2016). Fat accumulation in adipose tissue results in hyperglycaemia and successive reduction in insulin sensitivity (Tiss et al., 2020). Obesity is linked to chronic inflammation, which is pivotal in the aetiology of endothelial insulin resistance (Zand et al., 2017). The accumulation of excess fat in obesity leads to insulin resistance in target tissues precipitating T2D and various

cardiovascular complications including atherosclerosis (Manna Li et al., 2017). Insulin resistance is described as reduced tissue response and/or sensitivity to insulin stimulation, and it underlies obesity, dyslipidaemia, glucose intolerance and diabetes which are all significant cardiovascular risk factors (James et al., 2021). It also contributes to CVDs such as coronary artery disease, atherosclerosis, and hypertension, which are all linked to endothelial dysfunction (Ormazabal et al., 2018).

Insulin exerts its effects on the vasculature by activating two significant signalling pathways: the phosphoinositide-3 kinase/Akt (PI3K/Akt) and the Src/mitogen-activated protein kinase (MAPK) pathway (Boucher et al., 2014). Activation of the PI3K/Akt signalling pathway enhances endothelial nitric oxide (NO) generation leading to vasodilation and an antiatherogenic state (Fu et al., 2021). Conversely, the MAPK signalling pathway activation induces vasoconstriction, vascular cell proliferation, and growth leading to a proatherogenic state (Fu et al., 2021). The overall vascular effect of insulin is contingent upon an equilibrium between these vasodilatory and vasoconstrictive actions (Muniyappa et al., 2020). Under physiological conditions, the vasoprotective PI3K/Akt pathway predominates; however, in the states of insulin resistance, the MAPK pathway predominates (Janus et al., 2016; Jia Liu & Liu, 2019). Obesity, diabetes and other insulin resistance states feature a selective impairment of the PI3K/Akt pathway while maintaining or enhancing MAPK signalling. This imbalance aggravates endothelial dysfunction, atherosclerosis, hypertension, and myocardial dysfunction (Carmichael et al., 2022; Fu et al., 2021). Endothelial dysfunction is an early manifestation of atherosclerosis and an independent predictor of cardiovascular events. It occurs in a complex interplay with the insulin resistance (Muniyappa et al., 2020).

Resistance to insulin's vascular effects contributes to the onset of CVD which is a primary cause of death among individuals with obesity and metabolic syndrome (Carmichael et al., 2022; Kosmas et al., 2023). It is worthy of note that correcting endothelial vascular insulin resistance can significantly decrease the risk of vascular diseases linked with insulin resistance, independent of insulin's effects on other organs (Mather et al., 2013).

The gut and diet play an essential role in several crucial homeostatic activities of the human body. The human gut microbiota plays a significant role, as the gut is occupied by various microbial groups (Blandino et al., 2016). The gut microbiota fulfills structural, functional and crucial metabolic task for health protection (Busnelli et al., 2020; Zsálig et al., 2023). Earlier works revealed that health maintenance is linked to the equilibrium and diversity of the intestinal microbiome, which represents a responsive system that can be influenced (L. Jin et al., 2021). Although gut microbiota is becoming progressively linked with the pathogenesis of insulin resistance, obesity, and metabolic disorders (Liu et al., 2021), the exact mechanisms underlying this interaction remain unclear.

The primary therapeutic approach for obesity and insulin resistance should involve dietary modification to reduce fat and calorie intake, together with an augmentation of energy expenditure via physical exercise (Li et al., 2022). However, the longstanding management of obesity and insulin resistance without complementary pharmacological or surgical interventions remains challenging (Head, 2015). No approved drug is presently available that is specifically designed for the insulin resistance therapy. However, insulin sensitizers and drugs used for other CVDs including renin angiotensin aldosterone system (RAAS) inhibitors and statins, are

being employed to target the insulin resistance associated vasculopathy (Li et al., 2022; Prieto et al., 2014). Despite the likely benefits of these drugs, their cost and side effect profiles limit their use thereby encouraging the exploration of safer and more affordable alternatives for the prevention and treatment of insulin resistance (Lenharo, 2023; Wadden et al., 2023). These alternatives could significantly improve cardiometabolic morbidity and mortality (Campia et al., 2012). In addition, pharmaceuticals for obesity management are also purportedly associated with various adverse effects (Bersoux et al., 2017), highlighting the urgent need for safe and effective alternatives in the treatment of obesity.

Dietary management with probiotics has been proposed as an effective approach to enhance cardiometabolic health by modulating gut microbial flora (Formes & Reinhardt, 2019). Probiotic is defined as a "live microorganism which when administered in adequate amounts confers a health benefit to the host" (Hill et al., 2014). Kefir is a probiotic beverage that contains both bacteria and yeast, obtained via fermentation of water or milk with kefir grains (Talib et al., 2019). Research indicates that kefir consumption leads to decreased body weight and adiposity, along with a reduction in obesity-related comorbidities (Bourrie et al., 2018; Chen et al., 2021; Tiss et al., 2020). Several studies also established the positive health effects of kefir including antiobesity, hypocholesterolaemic, antihypertensive, hypoglycaemic, antioxidant, immunomodulatory, antibacterial, antiallergic, and anti-malignancy effects (Apalowo et al., 2024; Azizi et al., 2021; Bourrie et al., 2020; Culpepper, 2022). These observations indicate that kefir may have the capacity to mitigate excess fat build up in the body. These attributes led to the acceptance of kefir as a functional probiotic meal capable of enhancing health (Azizi et al., 2021).

Research has tried to elucidate which organisms or constituents of kefir are responsible for these positive health actions, and this led to the possibility of commercially manufacturing kefir designed to maximize health benefits (Bourrie et al., 2016). The capacity to combine the optimal strains of beneficial microbes from various kefir sources could yield enhanced benefits beyond those typically noted, while also providing a level of control over these effects that has not been achievable with conventional kefir (Bourrie et al., 2016). The positive health-promoting effects of probiotic interventions could be realized with the use of many strains of probiotic instead of a single strain, as various strains or species seemed to demonstrate varied mechanisms of action (Samah et al., 2016). Many studies established a better effectiveness of selective mixtures of probiotic strains in comparison to single strain in reducing body weight, adiposity and ameliorating metabolic variables including insulin resistance and dyslipidaemia in animal models (Karimi et al., 2017; Kobyliak et al., 2017; Mazloom et al., 2019; Roselli et al., 2018). Meta-analyses have further indicated that multistrain probiotic are more effective compared with a single strain probiotic with a diminished beneficial effect in addressing obesity related parameters (Koutnikova et al., 2019; Z. Wang et al., 2019). *Lactobacillus* and *Bifidobacterium* are the two common genera of probiotic bacteria, which are constantly found in marketed products for human consumption due to their beneficial effects on the intestinal flora and cardiometabolic diseases (Alihosseini et al., 2017; Chen et al., 2021; Ostadrahimi et al., 2015; Williamson et al., 2017).

1.2 Justification for the study

Kefir, a fermented dairy product, is associated with several beneficial cardiovascular and metabolic effects such as cholesterol-lowering abilities, anti-

obesity effects, angiotensin-converting enzyme inhibitory activity, immunomodulatory characteristics, enhanced cardiac function, and anti-hypertensive effect (Bourrie et al., 2018; Friques et al., 2015a; Silva-Cutini et al., 2019).

AB kefir, a probiotic containing six lactic acid bacteria (*Lactobacillus acidophilus* LA1063, *L. helveticus* LH43, *L. fermentum* LF26, *L. rhamnosus* LRH10, *L. paracasei* ssp. *paracasei* LPC12, and *Streptococcus thermophilus* ST30) and one *Bifidobacterium* strain (*Bifidobacterium longum* BL986), has been found to be safe in earlier research (Chen et al., 2021; Hsu et al., 2018). It has also been found to lower body weight and improve glucose tolerance in HFD-induced obese mice (Chen et al., 2021). However, this probiotic is yet to be evaluated for its effectiveness in mitigating obesity-associated metabolic and vascular insulin resistance as well as endothelial dysfunction. The interrelationship of obesity, insulin resistance, and endothelial dysfunction in the onset of CVDs and diabetes has been extensively established in both animal research and clinical studies. Consequently, treatment strategies that convert the vasoconstrictive effect of insulin to its vasodilatory action may serve as an effective approach for addressing both insulin resistance and endothelial dysfunction (Wang et al., 2011).

Since the vasculature plays key roles in the metabolic diseases' progression, we are evaluating for the first time the influence of industrial kefir treatment on metabolic and endothelial insulin resistance in high fat diet (HFD)-induced obesity in animal model. This model will allow to point out molecular mechanisms by which AB kefir might remedy the vascular dysfunction. AB kefir can therefore represent a therapeutic potential to prevent or reverse existing vascular dysfunction.

1.3 Objectives of the study

1.3.1 General objective

To investigate the effects of AB kefir treatment on gut microbiota modulation, endothelial insulin function and signalling pathways, vascular inflammation and oxidative stress in high-fat diet-induced obese rat model.

1.3.2 Specific objectives

1. To investigate whether obese rats exhibit gut dysbiosis compared to normal rats, and to evaluate the effects of kefir treatment on gut microbiota composition.
2. To compare the metabolic profiles (fasting blood sugar, insulin resistance and lipid profile) of kefir-treated obese rats with normal and untreated obese rats.
3. To elucidate the endothelium-dependent relaxation response in the aorta of normal and obese rats and assess the effects of kefir treatment on this response.
4. To evaluate the endothelial insulin relaxation response and associated signalling pathways in the aorta of normal and obese rats, and to determine the effects of kefir treatment on these responses and pathways.
5. To assess the levels of inflammatory markers (TNF-α and IL-6) and oxidative stress markers (SOD activity and MDA level) in the aorta of normal and obese rats, and to evaluate the effects of kefir treatment on these markers.
6. To determine the expression of IRS-1, eNOS and MAPK proteins in the aorta of normal and obese rats, and to investigate the effects of kefir treatment on the expressions of these proteins.

CHAPTER 2

LITERATURE REVIEW

2.1 The vasculature

The vascular system is a complex network of blood vessels responsible for transporting blood throughout the body, with a primary mission of distributing nutrients, cytokines, hormones, and other signalling chemicals to different tissues, while also removing waste products (Pugsley & Tabrizchi, 2000). The architecture of the vascular wall is finely tuned to the vessel's function, with different layers and cellular components contributing to its structural integrity and functionality (Gao, 2022) (Figure 2.1). Understanding this architecture is crucial for comprehending various physiological processes and the aetiopathogenesis of vascular disorders such as hypertension and atherosclerosis.

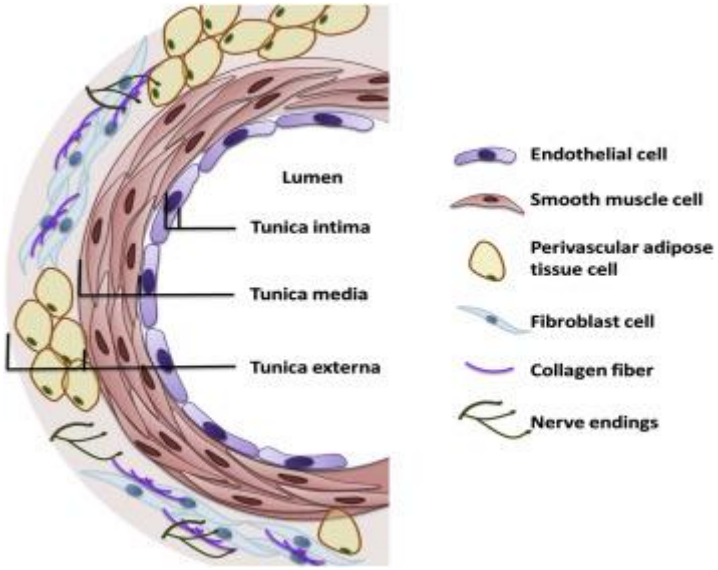

Figure 2.1 Architecture of vascular wall

Adapted from Zhao et al. (2015)

The vascular system comprises arteries, veins, capillaries, and venules, and sometimes also considers the myocardium (Pugsley & Tabrizchi, 2000). The walls of

blood vessels, particularly arteries and veins, are composed of three distinct layers: the tunica intima, tunica media, and tunica adventitia, each with a unique composition and function that influence the vessel's response to hemodynamic forces (Gao, 2022) (Figure 2.1).

The tunica intima is the innermost coat, made of a single layer of endothelium resting on a basement membrane. Beneath this, lies a thin coat of connective tissue, often comprising elastic fibres that provides structural support. The tunica media is the middle layer, mainly made up of elastic fibres and smooth muscle cells. In big elastic arteries, such as aorta, the tunica media is thick and contains multiple layers of smooth muscle cells interspersed with elastic lamellae, allowing these arteries to withstand high-pressure blood flow and maintain vascular tone through vasoconstriction and vasodilation. Veins have a slim tunica media with lesser smooth muscle cells compared to arteries, reflecting their role in low-pressure blood return to the heart.

The outermost layer, the tunica adventitia, is composed of connective tissue containing collagen and elastic fibres, offering structural support and attaching the vessel to adjacent tissues. In larger vessels, the adventitia comprises small blood vessels, termed vasa vasorum, which provide nutrients to the outer layers of the vessel wall. The adventitia also houses nerve fibres that innervate the vessel, contributing to the regulation of vascular tone. The variation in the architecture of blood vessel walls across different types of vessels reflect their specific functions within the circulatory system (Gao, 2022).

2.2 The endothelium and endothelial function

The endothelium is a monolayer of cells lining blood vessels, forming a dynamic interface with all the body organs (Li et al., 2017). In a 70 kg man, it weighs

about 1.5 kg and covers an area of more than 1,000 square metres (Barton et al., 2012). It is situated at the boundary between circulating blood and the vascular wall, serving as the first barrier faced by circulating substances. It functions as both a selective permeable layer, controlling the movement of substances including insulin, and as a transducer, converting biological cues into physiological responses that maintain vascular homeostasis (Fu et al., 2021).

The endothelium plays a pivotal role in vascular tone regulation, inflammation, and maintaining the equilibrium between pro- and anti-thrombotic states (Li et al., 2017). A healthy endothelium acts as a paracrine, endocrine and autocrine organ, secreting diverse vasoactive and trophic factors that affect vasomotion, endothelial and vascular smooth muscle cell growth and proliferation, inflammation, permeability, endothelial-leukocyte interactions, platelet adhesion and coagulation, (Prieto et al., 2014). One of its most essential functions is regulating vascular tone via the liberation of vasodilators such as nitric oxide (NO), cyclooxygenase-derived prostacyclin (PGI2) and various endothelium-derived hyperpolarizing factors (EDHFs), which also have influence on cell growth, coagulation and inflammation (Tousoulis et al., 2012; Vanhoutte et al., 2009). Conversely, endothelial cells also synthesize vasoconstrictors including thromboxane A2 (TXA2), reactive oxygen species (ROS) and endothelin-1 (ET-1), which not only induce vascular contraction but also promote cell proliferation (Vanhoutte et al., 2009). Hence, certain disorders such as obesity and diabetes impair the endothelium's function to maintain vascular health. This impairment, known as endothelial dysfunction, is an independent predictor of CVD (Li et al., 2017; Shankar & Steinberg, 2005). Endothelial dysfunction also promotes atherosclerosis development (Li et al., 2017)

2.3 Endothelium-derived relaxing factor: Nitric oxide (NO)

Nitric oxide is a lipid-permeable free radical that is diminutive, labile, and unstable. A series of research published in the 1980s demonstrated the endogenous production of NO and its physiological significance as an endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980).

2.3.1 The NO synthesis

The synthesis of NO involves the oxidation of L-arginine into L-citrulline and NO, via the action of nitric oxide synthase (NOS) enzymes, which utilizes NADPH and oxygen (O_2) as cofactors. The reaction begins with the conversion of L-arginine into N-hydroxyarginine, which is further oxidized to generate L-citrulline and NO. The overall reaction is as follows:

$$\text{L-arginine} + O_2 + \text{NADPH} \longrightarrow \text{L-citrulline} + \text{NO} + \text{NADP}^+$$

Other cofactors involved in this reaction include flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN), heme and tetrahydrobiopterin (BH_4). Three different NOS isozymes produce NO to perform various biological tasks. These include endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). The primary isozyme regulating vascular function is eNOS. Various stimuli including histamine, bradykinin, acetylcholine, and shear stress can activate eNOS and enhance NO production (Förstermann & Sessa, 2012).

2.3.2 The NO pathway

Substances such as acetylcholine and insulin bind their respective receptors on endothelial cells, activating the NO pathway.

2.3.2(a) Acetylcholine-mediated NO-induced relaxation

Acetylcholine binds to muscarinic receptors (mainly the M_3 subtype) which are G-protein-coupled receptor located on the surface of endothelial cells. This binding leads to the stimulation of phospholipase C, an enzyme that catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers the release of calcium ions (Ca^{2+}) from the endoplasmic reticulum into the cytoplasm of the endothelial cells. The elevated calcium concentration results in calcium binding to calmodulin, which then activates eNOS, which is the enzyme that catalyses the production of NO from amino acid L-arginine.

NO rapidly diffuses from the endothelial cells into the underlying vascular smooth muscle cells where it binds to and activates soluble guanylyl cyclase (sGC), an enzyme that catalyses the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), the second messenger that mediates smooth muscle relaxation. cGMP activates protein kinase G (PKG), which in turn phosphorylates various targets in smooth muscle cells, leading to a diminished intracellular calcium concentration, inhibition of calcium influx into the cells, and activation of myosin light chain phosphatase. This phosphatase dephosphorylates the myosin light chains, reducing actin-myosin interactions and leading to muscle relaxation. The relaxation of vascular smooth muscle cells then results in vasodilation (Förstermann & Sessa, 2012; Leung et al., 2006).

2.3.2(b) Insulin-induced NO-mediated relaxation

Insulin stimulates production of endothelial NO through the activation of phosphoinositide 3-kinase (PI3K) pathway. This pathway phosphorylates and activates protein kinase B (Akt), which in turn phosphorylates and activates eNOS,

leading to NO generation (Steinberg et al., 1994) (insulin signalling is further explained in section 2.4).

2.4 Insulin

Besides its crucial role in the metabolism of major nutrients, insulin also performs important non-metabolic actions via modulation of vasomotion, haemostasis, thrombosis, atherosclerosis and vascular remodelling. It is therefore regarded as a "vascular hormone", due to its capacity to influence vascular system and is suggested to have vascular therapeutical application (Anfossi et al., 2007). Insulin exerts both direct and indirect actions on vascular cells, which are intimately related to the tissues they are supplying. The indirect actions of insulin on the vascular system via its control of lipids and atherosclerosis development has been documented (Fu et al., 2021). However, the non-metabolic insulin actions differ between insulin-sensitive and insulin-resistant conditions including type 2 diabetes mellitus and obesity, where the vascular response to the hormone is often compromised (Anfossi et al., 2007).

2.4.1 Vascular actions of insulin

Insulin has crucial vascular actions that can either protect or damage the vasculature (Figure 2.2). Insulin activates two main signal transduction pathways in vascular endothelium: the PI3K and the mitogen-activated protein kinase (MAPK) pathways (Fu et al., 2021). One of the most significant cardiovascular actions of insulin is activation of NO generation via the PI3K pathway, which protects the vasculature. Conversely, insulin induces detrimental vascular effects by activating different growth factors via the MAPK pathway, resulting in vasoconstriction and proatherogenic consequences (Schulman & Zhou, 2009; Steinberg et al., 1994).

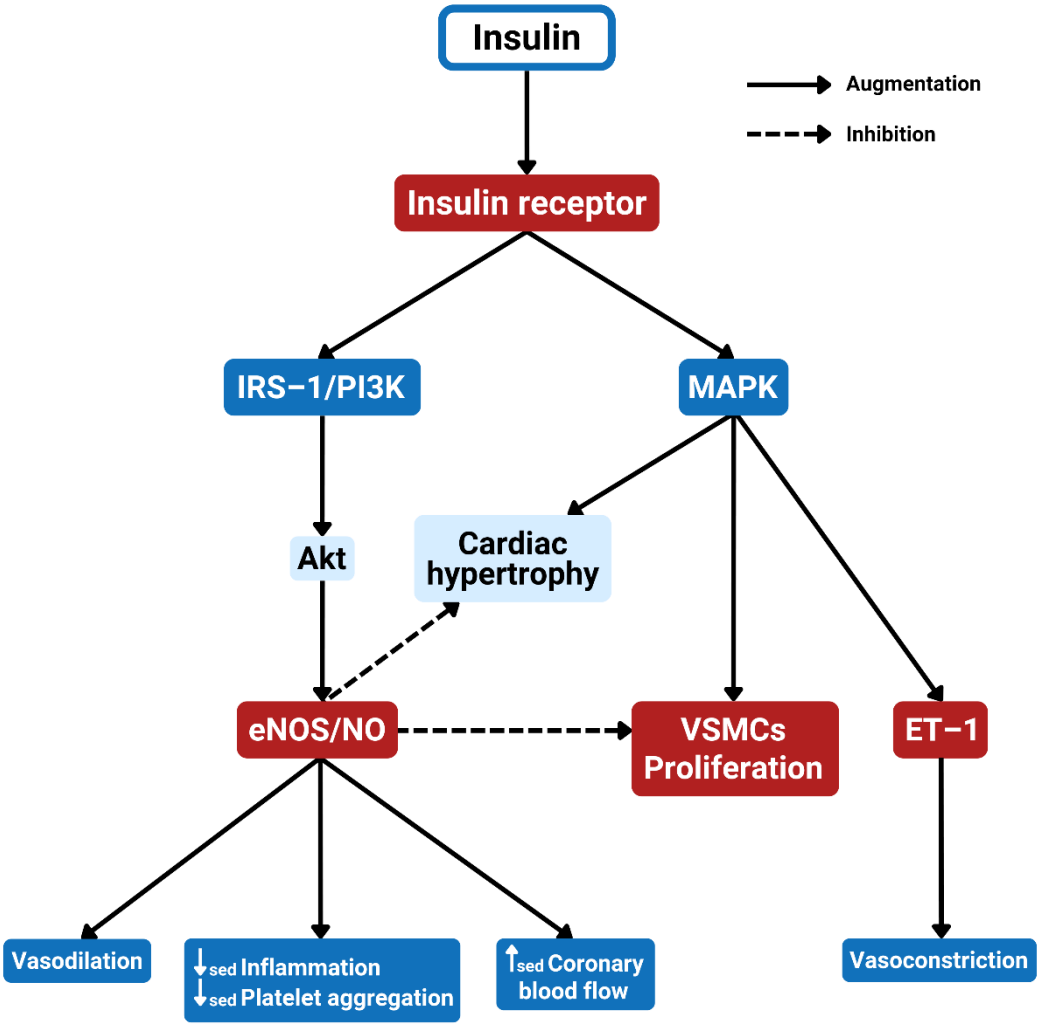

Figure 2.2 Cardiovascular insulin signalling pathways and actions.

IRS-1; Insulin receptor substrate-1, PI3K; phosphatidylinositol 3-kinase, MAPK; mitogen-activated protein kinase. eNOS; endothelial nitric oxide synthase, NO; nitric oxide, VSMC; vascular smooth muscle cell, ET-1; endothelin-1. Adapted from Zhou et al. (2012)

2.4.2 Insulin signalling pathway in vascular endothelium

Figure 2.3 below illustrates the fundamental characteristics insulin signal transduction pathways. Insulin predominantly impacts the endothelium via binding to the insulin receptor, a ligand-activated tyrosine kinase receptor. The insulin receptor on the vascular cells is made of an α-chain that binds insulin and a β-chain containing tyrosine kinase, similar to insulin receptors in other tissues. Insulin binding to the α-

subunit results in conformational changes that stimulate the intrinsic tyrosine kinase activity of β-subunit, triggering the signalling cascades (Fu et al., 2021). This activated insulin receptors phosphorylate intracellular substrates, such as insulin receptor substrate (IRS) family members and Shc, which function as docking proteins for downstream signalling molecules. Insulin receptor signalling in vascular cells takes place primarily through two major pathways: the IRS1/2 PI3 kinase/Akt (IRS1/2/PI3K/Akt) pathway and the MAPK pathway (Fu et al., 2021). These pathways mediate various vasotropic effects of insulin in the endothelium (King et al., 2016).

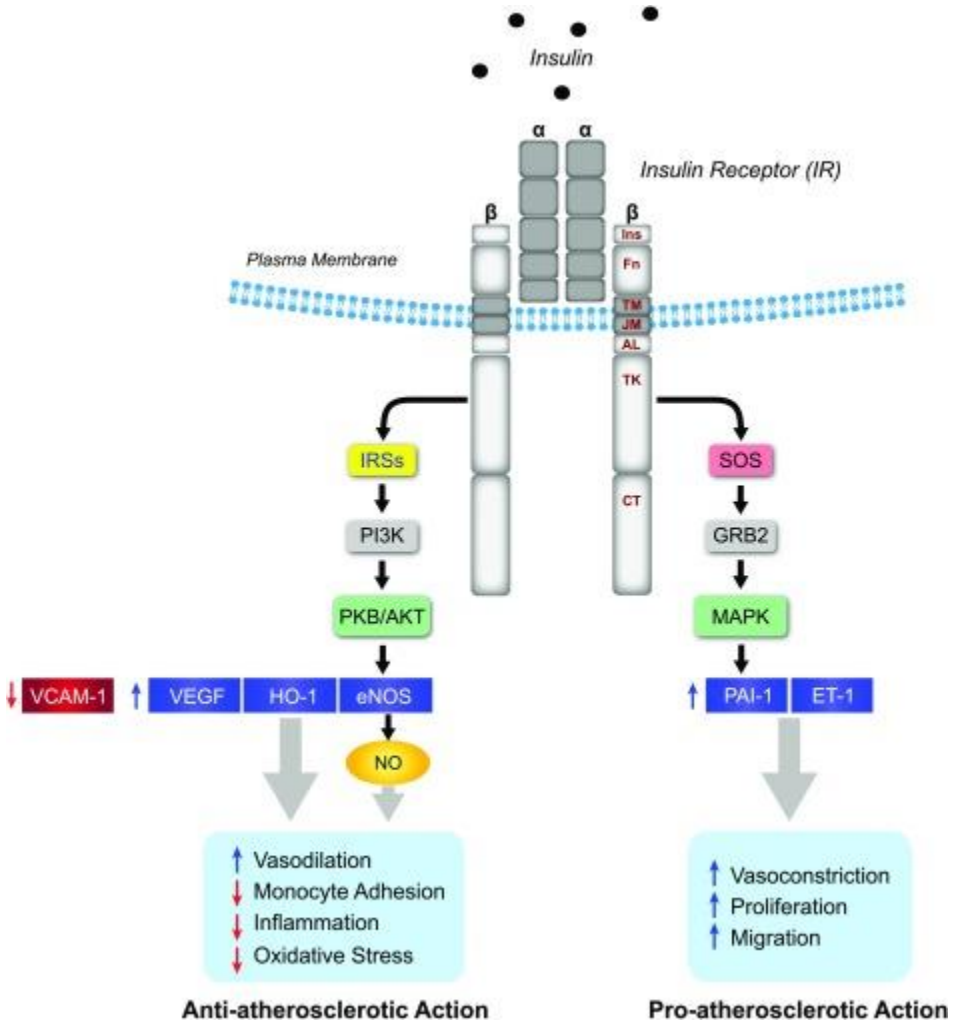

Figure 2.3 Insulin receptor structure and insulin signalling pathways in vascular cells.

Adapted from King et al. (2016)

2.4.2(a) PI3K/Akt pathway

The PI3K/Akt pathway is the most studied in the context of insulin signalling in endothelial cells. Among many intracellular proteins phosphorylated by the insulin receptor in the endothelial cells, IRS-1 and IRS-2 are the best-studied in relation to the vascular actions of insulin (Kim et al., 2006). Binding of insulin to its receptor leads to phosphorylation of IRS-1, which binds and activates PI3K, resulting in phosphorylation and activation of phosphoinositide-dependent protein kinase-1 (PDK-1) through increases in phosphatidylinositol-3,4,5-triphosphate. PDK-1, in turn, phosphorylates and activates Akt, which directly phosphorylates eNOS at Ser1177, leading to augmented eNOS activity (Manrique et al., 2014; Muniyappa & Quon, 2007). eNOS catalyzes the generation of NO and L-citrulline from L-arginine and oxygen (Manrique et al., 2014).

NO diffuses into adjacent vascular VSMC where it activates guanylate cyclase, converting GTP to cGMP. Increased levels of cGMP lower intracellular calcium, promoting vasodilatation, inhibiting vascular VSMC proliferation and regulating angiogenesis (Turco & Folli, 2012). Moreover, Insulin-induced nitric oxide production in endothelial cells fosters an anti-inflammatory and anti-thrombotic phenotype, reducing the secretion of pro-inflammatory cytokines and the expression of adhesion molecules, such as E-Selectin, vascular adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), (Muniyappa & Quon, 2007).

Dysregulation of this pathway, often seen in insulin resistance, results in reduced NO bioavailability, contributing to endothelial dysfunction and atherosclerosis (Turco & Folli, 2012). Insulin signalling in the endothelium is integral to its role in glucose homeostasis and affects various tissues, including the vascular

endothelium. Dysfunction in this signalling is closely linked to vascular complications in metabolic disorders including obesity and diabetes mellitus (Kim et al., 2006).

2.4.2(b) MAPK pathway

The MAPK pathway involves tyrosine phosphorylation of IRS proteins and/or Shc, which further interact with growth receptor-bound 2 (GRB2), and Son of Sevenless (SOS). This interaction activates G protein Ras, which triggers a phosphorylation cascade involving Raf and MAPKs such as mitogen-activated protein kinase kinase 1 (MEK1) and extracellular signal-receptor kinase (ERK) (Anfossi et al., 2007; Muniyappa et al., 2020). MEK1 and ERK induce mitogenic effects, such as expression of endothelin-1 (ET-1) and plasminogen activator-1 (PAI-1), as well as the proliferation and migration of VSMCs (Manrique et al., 2014; Muniyappa & Quon, 2007).

Insulin stimulates ET-1 production via the MAPK pathway but not through PI3K pathway, as shown in the endothelial and mesenteric vessels (Muniyappa & Quon, 2007). ET-1, a potent vasoconstrictor, opposes the vasodilator actions of NO and participates in the onset of cardiovascular diseases like hypertension (Muniyappa & Quon, 2007). Moreover, insulin-induced ET-1 release is mediated through the MAPK pathway, not the PI3K pathway, as demonstrated by experiments using specific inhibitors (wortmannin and PD98059) (Kim et al., 2006).

ET-1 also participates in heightened endothelial liberation of interleukin-6 (IL-6), increased vascular permeability, and VSMCs proliferation (Turco & Folli, 2012). NO is said to attenuate the generation and effect of ET-1, with studies reporting augmented NO-mediated insulin vasodilation following ET-1 receptor inhibition in animals and humans (Cardillo et al., 2002; Kelly et al., 2004; Potenza et al., 2005). Although the MAPK pathway participates in regulating gene expression, cell growth,

and differentiation, its activity in the endothelium is not as apparent compared to the PI3K/Akt pathway and does not contribute to insulin-mediated eNOS activation, especially in conditions of uninhibited NO production (Muniyappa & Quon, 2007; Turco & Folli, 2012).

Another insulin action via the MAPK pathway, but not PI3K pathway, is the stimulation of the expression of E-selectin, VCAM-1 and PAI-1, all of which regulate vascular function (Kim et al., 2006). Increased expression of these molecules in endothelial cells is linked to atherosclerosis in insulin-resistant conditions (Muniyappa & Quon, 2007). The suppression of the PI3K/Akt pathway enhances the MAPK activity, indicating that the MAPK pathway is involved in the pro-atherogenic effects linked to insulin signalling (Turco & Folli, 2012). The MAPK cascade is primarily involved in the initial phase of atherogenesis (Turco & Folli, 2012).

Alterations in insulin's vasoconstrictor and vasodilatory effects may contribute to vascular pathophysiology of insulin resistance (Turco & Folli, 2012). Hyperactivation of the MAPK pathway in insulin-resistant states can exacerbate inflammatory responses, promoting vascular injury and contributing to CVD (Kim et al., 2006). PI3K-dependent pathway is considered the primary insulin signalling route that governs metabolic and vascular functions, while Ras/MAPK pathway primarily facilitate vascular growth and mitogenic effects (Kim et al., 2006).

Under physiological conditions, insulin-induced ET-1 action on the expression of PAI-1 and cell adhesion molecules such as E-selectin, VCAM-1, and ICAM-1, are counteracted by NO production (Prieto et al., 2014). Insulin’s stimulation of the IRS1/2/PI3K/Akt pathway enhance eNOS expression, promoting vasodilation, mitigating atherosclerosis, and reducing oxidative stress and inflammation (Figure 2.3) (Fu et al., 2021). Conversely, insulin's activation of the Src/MAPK pathway

induces vasoconstriction via ET-1, trigger inflammation through PAI-1, and enhance VSMC proliferation and migration. Increased ET-1 expression and the consequent migration and proliferation of VSMC lead to vasoconstriction and ultimately trigger an atherogenic cascade (Fu et al., 2021)

2.4.3 Insulin resistance and endothelial dysfunction

The integrity of the functional endothelium is essential for vascular health. NO is recognized as the most effective endogenous vasodilator in the body, and decreased NO bioavailability is a hallmark of endothelial dysfunction (Ormazabal et al., 2018). Endothelial dysfunction represents a pathophysiological condition characterized by reduce NO synthesis and availability, leading to a functional and reversible impairment of the vasodilatory, anti-aggregating, and anticoagulant functions of endothelial cells (Anfossi et al., 2009; Prieto et al., 2014). It is regarded as the first step of atherogenesis, characterized by aberrant vascular reactivity and a pro-atherogenic interaction between the endothelium and circulating blood (Mather et al., 2004). Endothelial dysfunction also involves a blunted increase in blood flow response to endothelium-dependent vasodilators, including insulin, alongside elevated circulating levels of specific markers of endothelial activation, such as P-selectin, E-selectin, thrombomodulin, soluble VCAM-1, soluble ICAM-1 and von Willebrand factor (Anfossi et al., 2009).

Insulin resistance profoundly impacts the vascular endothelium. Endothelial dysfunction plays a significant role in CVD such as coronary artery disease, atherosclerosis, and hypertension, all of which are also associated with insulin resistance (Ormazabal et al., 2018). The impairment of vascular reactions correlates with the severity of insulin resistance, particularly in relation to vascular insulin action

(Anfossi et al., 2009). Furthermore, insulin resistance in endothelial cells results in elevated concentrations of pro-inflammatory markers, pro-thrombotic factors, and reactive oxygen species (ROS), which contribute to an elevated intracellular concentration of ICAM-1 and VCAM-1 (Ormazabal et al., 2018). The relationship between endothelial function and insulin metabolism is significant, as the correlation between insulin resistance and disturbances in endothelial signalling leads to inflammation and disrupts the equilibrium between vasodilatory and vasoconstrictive mechanisms, thereby increasing cardiovascular risk (Ormazabal et al., 2018).

In the context of vascular insulin resistance, the equilibrium between the vasodilatory effects of insulin mediated by the NO/cGMP/PKG pathway and the vasoconstrictive effects driven by ET-1 shifts in favor of the latter. This indicates a "selective" impairment of insulin signalling via the PI3K pathway, while the MAPK pathway remains intact (Anfossi et al., 2009). Insulin resistance and endothelial dysfunction are influenced by mechanisms such as lipotoxicity, inflammation and glucotoxicity. The molecular and pathophysiological pathways that establish reciprocal association between insulin resistance and endothelial dysfunction create a vicious cycle, further strengthening the connection between metabolic disorders and CVD (Kim et al., 2006). Therefore, insulin signalling in the vascular endothelium is fundamental to sustain vascular homeostasis. The PI3K/Akt pathway, through its role in NO production, plays a vital role in this process. Insulin resistance disrupts this pathway, leading to endothelial dysfunction and contributing to CVD. Therapeutic strategies targeting the restoration of insulin signalling pathways hold promise in preventing and managing vascular complications in metabolic disorders.

2.5 Obesity

2.5.1 Definition and epidemiology

The word ‘obese’, from which ‘obesity’ is derived, originates from the Latin word obesus, meaning ‘stout, fat or plump (Achike et al., 2011). Obesity is a chronic metabolic disease that is increasing in prevalence worldwide. It plays a major role in insulin resistance, metabolic syndrome and type 2 diabetes mellitus, all of which are cardiovascular risk factors (Prieto et al., 2014). Several organizations, including the World Health Organization (WHO), have classified obesity as a global pandemic requiring urgent national and international intervention (Prentice, 2006; World Health Organization, 2024).

Obesity occurs due to disequilibrium between energy intake and expenditure, leading to excessive or abnormal build-up of fat, which increases adipose tissue mass and ectopic fat accumulation (Alice et al., 2022). According to WHO, obesity is defined by a body mass index (BMI) greater than 30 kg ⁄m^2 (World Health Organization, 2024). It typically results in negative health impacts due to the excessive storage of fat, which results in greater number and/or size of adipocytes, thereby expanding adipose tissue (Martins et al., 2022).

Although genetic predisposition is involved in the onset of obesity, its causes are multifactorial. Complex interactions between epigenetics, lifestyle, cultural and environmental factors contribute to the onset of obesity (Martins et al., 2022). Most studies confirm that BMI continues to increase across almost all continents. This trend is driven by economic growth in developing countries, changes in nutrition patterns, and the availability of inexpensive, nutritionally unbalanced diets rich in carbohydrates and fats (Barton et al., 2012). Excessive food intake is further aggravated by unhealthy lifestyle, a lack of exercise, and the intake of high-calorie,

non-alcoholic and alcoholic drinks (Malik et al., 2010). This results in disequilibrium between energy consumption and expenditure, leading to fat storage in adipocytes as triglycerides (Alvarez et al., 2023). One alarming trend is the rise in childhood obesity, which is due to children's increased access to and little control over the intake of sweets and junk food (Cesare et al., 2019; Lee & Yoon, 2018).

In 2022, the WHO reported that 1 in 8 people globally were living with obesity, and 43% (2.5 billion) of adults were overweight, of whom 890 million were obese. Adult obesity has more than doubled, while juvenile obesity has quadrupled since 1990 (World Health Organization, 2024). In the same year of 2022, 37 million children under the age of 5 were classified as overweight, while over 390 million older children and adolescents were also categorized as overweight, including 160 million who were obese (World Health Organization, 2024). Overweight prevalence differs across regions: it is 67% in the WHO American Region, while in South-East Asia and the African Region, it is 31%. Obesity is now common in both developed and developing countries, affecting all ages and social classes (World Health Organization, 2024). If the current trends continue, by 2030, 57.8% of the global population will be overweight or obese placing a significant strain on individuals, societies and health care systems ((NCD-RisC), 2016).

Obesity and its consequent metabolic effects, such as diabetes and metabolic syndrome, are significant contributors to cardiovascular morbidity, posing a global threat to human health (Head, 2015). The WHO predicts that obesity and overweight will probably become the most significant causes of morbidity, besides infectious diseases and malnutrition (World Health Organization, 2024).

2.5.2 Pathophysiology: adipose tissue distribution and function in obesity

Accumulated body fat often results from consuming more nutrients than the body requires, with the extra nutrients stored as triglycerides in adipocytes (Ahmed et al., 2021). Inability of adipocytes to store excess triglycerides leads to generation of new adipocytes via the process of adipogenesis, therefore creating additional fat storage capacity. The increase in fat associated with obesity occurs via the multiplication (hyperplasia) and enlargement (hypertrophy) of adipocyte (Ahmed et al., 2021). Adult-onset obesity is characterized by adipocyte hypertrophy, which begins in adulthood, whereas juvenile obesity involves both adipocyte hyperplasia and hypertrophy starting in childhood (Achike et al., 2011).

Adipocytes are the major constituents of adipose tissue which accounts for 2–70% of human body weight and serves as both an energy store and a large secretory organ (Rigamonti et al., 2011). Adipose tissues are deposited in two main areas: subcutaneous depots such as subcutaneous abdominal, femoral and gluteal fat, and visceral depots including mesenteric, perirenal and omental fat (Kahn et al., 2019). Excess dietary consumption leads to fat accumulation in these depots, causing them to enlarge and become unhealthy (Ahmed et al., 2021).

In healthy state, fat storage is regulated by food consumption and hormones including insulin and leptin. Obesity results from prolonged disequilibrium between energy intake and expenditure leading to excess nutrients that affect the cellular activities of adipose tissue, the liver, muscles, the vascular and the immune system (Prieto, 2014). A small fraction of obese individuals remains metabolically healthy due to preserved insulin sensitivity, although further study suggests that they still have a high risk of cardiovascular events, debunking the concept of metabolically healthy obesity (Eckel et al., 2018). In contract, metabolically unhealthy obese individuals