Sex-specific post-transcriptional regulation of cardiovascular complications in diabetes

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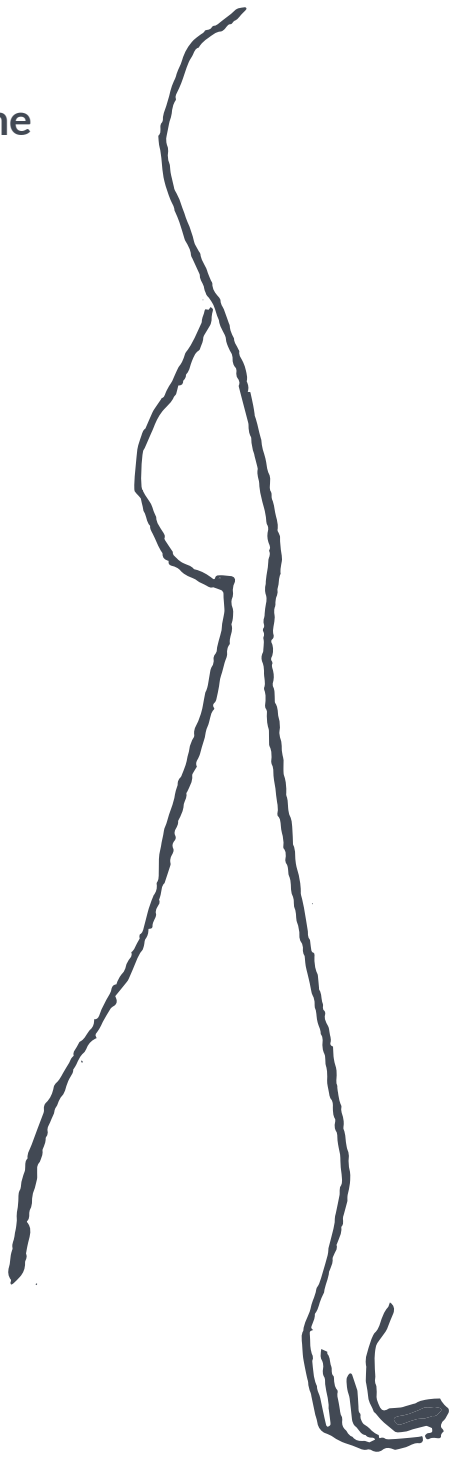
Chapter 1

General Introduction and outline



General introduction and outline

Women have a lower cardiovascular disease (CVD) prevalence compared to men albeit that this advantage is lost in the context of Diabetes Mellitus (DM) (1). In women with diabetes, energy metabolism disorders such as obesity and insulin resistance (2-4) have become well known destabilizers (5, 6) of the microvasculature (7). Given the observed increase in global diabetes prevalence and steep rise in obesity amongst women (8), this diabetic, female microvascular disadvantage will remain a highly prevalent clinical presentation (9). Unfortunately, our comprehension of how diabetic comorbidities induce microvascular injury in women is incompletely understood and therefore often misdiagnosed in daily clinical practice (10, 11). This pinpoints a potential profit for more sex-specific analysis in basic research which could improve our understanding of sex-specific cellular mechanisms in disease.

Physiology of energy metabolism

Glucose metabolism

In order to replenish glucose supply to the high energy demanding brain, glucose absorption and metabolism is tightly controlled in several different pathways. In the fasted state endogenous glucose is produced in the liver via glycogenolysis (glycogen breakdown to glucose) and gluconeogenesis (glucose formation from amino acids). However, upon feeding, glucose is absorbed as mono-sugars after breakdown of dietary carbohydrates in the gut. and absorption in peripheral tissues such as skeletal muscle and adipose tissue.

In physiological conditions, these aforementioned metabolic pathways are tightly regulated by two peptide hormones, insulin (secreted by pancreatic β-cells upon high postprandial plasma glucose levels) and glucagon (secreted by pancreatic α-cells upon fasting) (12). Of these, particularly insulin stimulates glucose uptake in skeletal muscle and adipose tissue and inhibits (hepatic) gluconeogenesis, glycogenolysis and adipocyte specific lipolysis. In contrast, glucagon secretion activates gluconeogenesis and glycogenolysis within the liver.

Insulin stimulates glucose uptake via glucose transporter receptor isoforms that are expressed in skeletal muscle and adipose tissue (Glut1 and Glut4) and

neurons (Glut3) (13). Particularly, the insulin stimulated translocation of Glut4 from intracellular sites to the cellular membrane of skeletal muscle and adipose tissue enhances glucose uptake. Glut4 translocation is dependent on insulin sensitivity of metabolic tissues which is tightly regulated via the insulin receptor (IR). This IR belongs to a class of tyrosine kinase receptors of which insulin receptor substrate (IRS)-1 and -2 are equally important in maintaining glucose homeostasis.

Fatty acid metabolism

Traditionally, white adipose tissue (WAT) is considered a fuel reservoir, in which excess energy is stored in the form of triglycerides during times of increased food intake or decreased energy expenditure (lipogenesis) but released in the form of free fatty acids (FFAs) and glycerol in conditions of increased energy demand (lipolysis). The majority of FFA taken up by metabolic organs is derived from triglyceride-rich lipoproteins, after liberation of fatty acids from triglycerides by LPL. WAT controls postprandial circulatory FFA fluxes because lipogenesis and lipolysis are tightly regulated by insulin (14, 15). Particularly postprandial insulin peaks inhibit lipolysis and FFA release within the circulation and stimulates FFA uptake in adipocytes.

Next to its particular function as repository in the process of fat storage, WAT is also a dynamic tissue type with an active role in metabolism. WAT exerts these metabolic effects via the production and release of several bioactive molecules known as adipokines of which leptin and adiponectin are particular examples (16). The primary role of leptin is suppression of appetite (leptin-deficient individuals gain massive body weight) - therefore, under physiological conditions leptin is a beneficial adipokine. In obese individuals however, leptin decreases the expression of insulin signalling genes thereby rendering WAT more insulin resistant and pro-inflammatory (17). In contrast, adiponectin is a beneficial adipokine with anti-inflammatory properties and insulin-sensitizing effects

In contrast to WAT, brown adipocytes constitute another class of adipocytes with the unique capacity to generate heat from stored FFAs and glucose. By inducing thermogenesis, active BAT improves whole-body energy metabolism (18), lowers fasting glucose levels (19) and restores peripheral insulin sensitivity (20). Brown adipocytes exert this function because they express UCP-1, a molecule that uncouples oxidative phosphorylation by allowing proton return (without

simultaneous ATP synthesis) across the inner mitochondrial membrane. Taken together, active BAT improves energy metabolism to reduce body weight, and as a consequence improves insulin sensitivity (21).

Pathophysiology of energy metabolism

Insulin resistance and adipocyte specific energy metabolism

DM is a cluster of metabolic pathologies characterized by an impaired glucose tolerance leading to a systemic hyperglycaemic state. In order to differentiate this cluster of pathologies, a distinction is made based on aetiology in which type 1 DM (T1DM) is characterized by the loss of insulin secretion and bioavailability due to autoimmune destruction of pancreatic beta cells. In contrast, individuals with prediabetic phenotypes such as obesity, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), have a high risk for developing type 2 DM (T2DM) in which hyperglycaemia results from an impaired pancreatic insulin secretion, increased hepatic glucose production and the loss of peripheral tissue (skeletal muscle and adipose tissue) insulin sensitivity (22).

The majority of IR-stimulated glucose uptake occurs in skeletal muscle while adipose tissue only captures a small fraction of plasma glucose (23). Nonetheless, skeletal muscle specific insulin receptor (IR) knockout mice have a normal systemic glucose tolerance (24), whereas an adipocyte specific IR knockout mice has impaired glucose tolerance due to insulin resistance in muscle and liver tissue (25). Consequently, skeletal muscle insulin stimulated glucose transport is severely impaired (26-28) and it is therefore now increasingly recognized that adipose tissue actively plays a directive role in the mechanisms leading to this pathophysiological phenotype (29).

Lastly, dysfunctional adipocytes in obesity and DM also start to express and secrete inflammatory chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) and tumour-necrosis factor-α (TNF-α) which activate an adipocyte specific pro-inflammatory state (30, 31). The resultant adipocyte specific inflammatory state negatively interferes with insulin signalling (32) and results in a higher rate of basal lipolysis, a mechanism meant to counteract the increase in adipocyte hypertrophy (31) but which increases plasma levels of FFA.

Consequently, a higher fat mass, leads to ectopic lipid deposition in the liver and skeletal muscle via lipid overflow, which renders these tissues insulin resistant.

Metabolic comorbidities, microvascular injury and clinical outcome in women

With insulin resistance, the microvasculature in diabetic women loses two important functions; insulin-induced vasodilation and insulin-induced microvascular recruitment (33). These insulin-regulated microvascular functions were first demonstrated in healthy subjects in whom insulin increased leg blood flow (34). Increased blood flow resulted from a decrease in skeletal muscle vascular resistance (35) a feature dependent on insulin concentration and insulin-stimulated glucose metabolism (36). Mechanistically, insulin induced these effects via nitric oxide (NO) release from endothelial cell (ECs) (37), a finding observed when inhibition of NO production with the NO synthase inhibitor L-N^G-monomethyl arginine (L-NAME) lowered the insulin mediated increase in blood flow (38).

Upon obesity, insulin resistance and DM, the presence of hypertrophic (visceral) adipose tissue associates with a reduction in NO-mediated (39), endothelium-dependent vasodilatation (40). As a result, the skeletal muscle vascular bed is characterized by a reduced capillary number (41) and extensive microvascular dysfunction (42). Moreover, expanding perivascular adipose tissue (PVAT) in obesity (43), secretes increasing amounts of TNF- α (44). This invokes reactive oxygen species (ROS) (45) that disrupt endothelial quiescence and impair microvascular tone (46). Interestingly, in women obesity increases PVAT size which impairs insulin-induced microvascular recruitment and lowers metabolic insulin sensitivity irrespective of PVAT inflammation (33).

Compared to diabetic men, diabetic women have more obesity-related comorbidities such as hyperlipidaemia (47) and decreased levels of high-density lipoprotein (HDL) (48), a metabolic profile that results in hypertension and loss of kidney function over time. This profile was invoked by dysfunctional adipose tissue which was found to secrete more leptin (49) that stimulated aldosterone release and synthesis (50) resulting in endothelial dysfunction and hypertension (51). Given that systemic blood pressure is more salt sensitive in aged women (52), it could be argued that these metabolic comorbidities also induce sex differences in the prevalence of hypertension which negatively affect kidney function in ageing women (53).

Taken together, the aforementioned studies suggest a female specific pathophysiological mechanism in which expanding (perivascular) adipose tissue in diabetic women lowers insulin sensitivity that promotes microvascular injury (Figure 1) (54). Following the resultant loss of endothelial quiescence in the skeletal muscle- (55) , renal- (56) and myocardial (57) microcirculation, women have an increased risk for diabetic cardiovascular comorbidities. In population studies, diabetic women have more coronary calcification (58), a higher prevalence of atrial fibrillation (59) and more diastolic dysfunction (60). Therefore, diabetic women need early detection to reduce this DM induced CVD risk which is why more sex-specific mechanistic studies are needed.

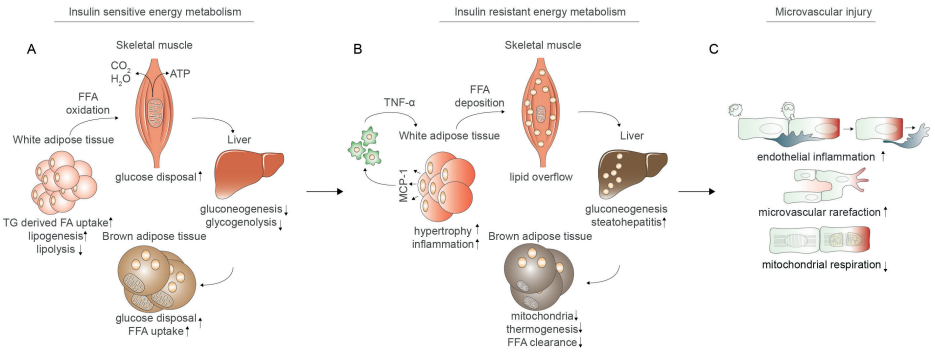


Figure 1. From insulin resistance to microvascular injury. (A) Normal physiology of insulin stimulated glucose disposal in skeletal muscle and brown adipose tissue (BAT) and lipid uptake in white adipose tissue (WAT). (B) Following ectopic lipid accumulation in skeletal muscle and in the liver as well as WAT specific insulin resistance and inflammation, glucose tolerance in skeletal muscle and BAT is lost. (C) Both insulin resistance and systemic inflammation in WAT impair endothelial eNOS secretion and insulin-induced microvascular recruitmen.

MicroRNAs

Traditionally the central dogma of molecular biology describes the transfer of genetic information from DNA into the intermediary template messenger RNA (mRNA) (transcription) of which the coding information is synthesized into proteins (translation). However, given that only a very minor amount of DNA (3%) encodes for proteins while a very large fraction of the genome is transcribed into RNA, transcription is not directly correlated with the expression of corresponding proteins (61). Over 95% of the genome is transcribed into so-called non-(protein)coding RNAs,

which cell-specific expression patterns, subcellular locations and intricate interplay coordinates the expression of multiple sets of functionally-related genes and proteins (62). As such, non-coding RNAs complement the physiological and adaptive cellular response to injury at the *post-transcriptional* level, thereby giving rise to biological complexity in development, health- and disease states.

A particular fraction of small regulatory non-coding RNA molecules comprises microRNAs (miRs). Following their biogenesis (Figure 2) these 20-25 nucleotide long-, single-stranded non-coding RNA molecules are key players in silencing or fine-tuning mRNA expression and protein levels through complementary base pairing at 3′-untranslated regions (3′-UTR) of target mRNAs. Currently, several thousand identified miRs bind multiple mRNA targets while a single mRNA molecule can be the target of multiple miRs. Because circulating miRs are potentially cell specific and have a remarkable plasma stability they can be used as plasma biomarker to reflect on-going disease processes.

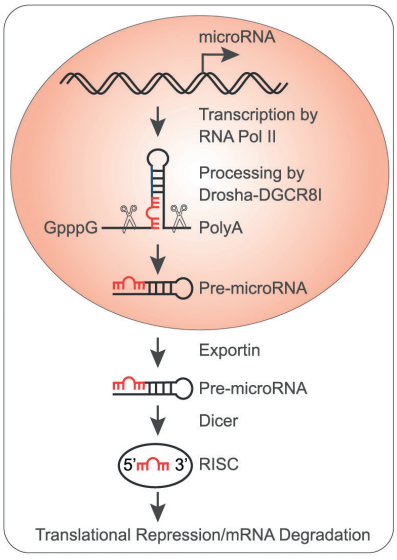


Figure 2. MiR biogenesis and function. MiRs are processed by the RNA polymerase II of independent genes or from introns of protein coding genes. The enzyme Drosha processes pri-miRs into a ~70 nucleotide precursor hairpin (pre-miR), which are exported by exportin to the cytoplasm where the enzyme Dicer cleaves pre-miRs to a single stranded miR which is incorporated into an RNA-induced silencing complex (RISC) that promotes mRNA degradation or inhibits protein translation.

Following their discovery, miRs have emerged as important candidates that have shown to improve disease diagnostics in CVD (63). A striking example of this potential of miRs as clinical biomarker has been the established association between plasma miRs and microvascular injury in type 2 diabetes (64). Moreover, next to its biomarker potential in disease, it is now known that miRs are distributed among plasma miRNA carriers, such as extracellular vesicles (EVs) (65), circulating high density lipoprotein (HDL) (66) or Argonaute-2 (Ago-2) (67). These plasma miRNA carriers enable the functional delivery of miRNAs at distal sites to pass on a miRNA mediated feedback loop in tissue inflammation, repair and regeneration.

Interestingly, sex differences in the expression of miRs could potentially improve diagnosis of female specific pathophysiological mechanisms. Sex differences in miR expression is the result of two main driving factors. First, many gene promoters contain oestrogen-responsive elements (EREs) (68) whereby estrogen binding drives miR expression. (69) Secondly, the X-chromosome encodes 118 miRs (70) and several of these X-linked miRs escape X-chromosome inactivation (71) resulting in higher expression levels of these miRs in particular cell types (72). Since plasma miRs originate from multiple molecular pathways, their identification could contribute to an increased understanding of the sex-specific cellular mechanisms in microvascular injury associated CVD.

Aims and outline of this thesis

This thesis studies sex-specific, post-transcriptional regulation in the microvascular injury associated cardiovascular complications of diabetes mellitus (DM) in women.

Chapter 2 discusses the aetiology and pathophysiology of cardiovascular disease (CVD) in women and provides a detailed focus on the post-transcriptional mechanisms of sex-specific miRs (miRs transcribed from the X-chromosome or regulated by estrogen). Furthermore, it provides an introduction to the sex-specific cellular mechanisms that regulate miR expression in endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and cardiomycotes (CMs) in response to metabolic and diabetogenic comorbidities. These sex-specific miRs either protect or predispose women to a systemic inflammatory state in which microvascular dysfunction may result in a diabetic CVD complication such as heart failure with a preserved ejection fraction (HFpEF).

In the setting of acute rejection of kidney transplants, a model of microvascular injury, **chapter 3** illustrates how plasma miRs can be used to monitor microvascular integrity in disease progression. As such, this study demonstrates that plasma miRs could serve in the identification of patients at risk of microvascular injury following a systemic inflammatory state. Moreover, the collective expression of these miRs expresses more insight into the related pathophysiology that activates a systemic inflammatory state.

The X-chromosome origin or estrogen regulation of miRs could provide a sex-specific biomarker particularly for female patients. Therefore **chapter 4** studies plasma miR patterns in (female) patients with idiopathic atrial fibrillation (iAF, a borderline AF phenotype). Given that iAF is more prevalent following T2DM, these patients provide a unique opportunity to study whether sex-specific plasma miR profiles provide a novel sex-specific strategy for the detection of microvascular injury in women.

Having focused on altered plasma miR profiles in the setting of a systemic inflammatory state, **chapter 5** illustrates that plasma miRs are actually carried in EVs or associate with HDL and the RNA-binding protein Ago-2. This chapter demonstrates that the distribution of these carrier-specific miRs is altered in patients with DM and diabetic nephropathy (DN) while some carrier-specific miRs display an improved biomarker potential compared to total plasma miR profiles as measured in the aforementioned studies (chapter 4 and 5). In addition, in *in vitro* studies carrier-specific miRs were found to rescue microvascular integrity suggesting that carrier specific miRs enable cell-to-cell communication at distal sites. As such, carrier selective miRs are more potent biomarkers for microvascular injury while their endocrine function rescues microvascular integrity.

Chapter 6 outlines how estrogen in male-to-female transgenders (transwomen) lowers plasma levels of two miRs derived from an X-chromosome miR cluster (miR-224 and miR-452). This X-linked miR cluster decrease associated with insulin resistance and its effects were further studied in mice in which silencing of both miRs actually activated a pre-diabetic phenotype in which brown adipocytes specific glucose uptake decreased while lipid uptake in white adipocytes was promoted. Next generation sequencing based differential gene expression analysis

identified a pre-diabetic genotype of genes involved in lipid uptake, insulin signaling and mitochondrial respiration which were further studied in in vitro studies.

In **chapter 7** we demonstrate that the adipocyte-derived miR-224 and miR-452 (and other miRs) as well as a well-known sex-specific miR, namely miR-34a can be used as sex-specific biomarker for microvascular injury in diabetic women with left ventricular diastolic dysfunction (LVDD) and heart failure with a preserved ejection fraction (HFpEF).

Lastly, **Chapter 8** presents a general discussion of the research presented in this thesis and studies the contribution of sex-specific post-transcriptional mechanisms in diabetic, microvascular injury associated CVD complications while **Chapter 9** offers a Dutch Summary of the research that is presented in this thesis.

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