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

Florijn, B.W.

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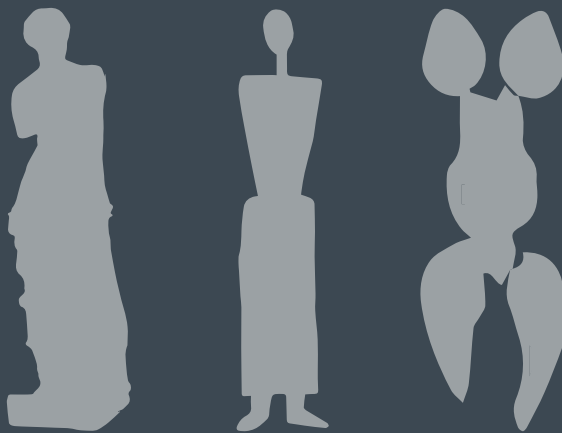
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Barend W. Florijn

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Barend Willem Florijn

Colofon

Sex-specific post-transcriptional regulation of cardiovascular complications in diabetes - Barend Willem Florijn

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Promotor

Prof. dr. A.J. van Zonneveld

Copromotor

Dr. R. Bijkerk

Leden promotiecommissie

Prof. dr. M.J.H. Wermer

Prof. dr. P.C.N. Rensen

Prof. dr. D.E. Atsma

Dr. E.P. van der Veer

Dr. B.B. van Rijn, Erasmus Medisch Centrum Rotterdam

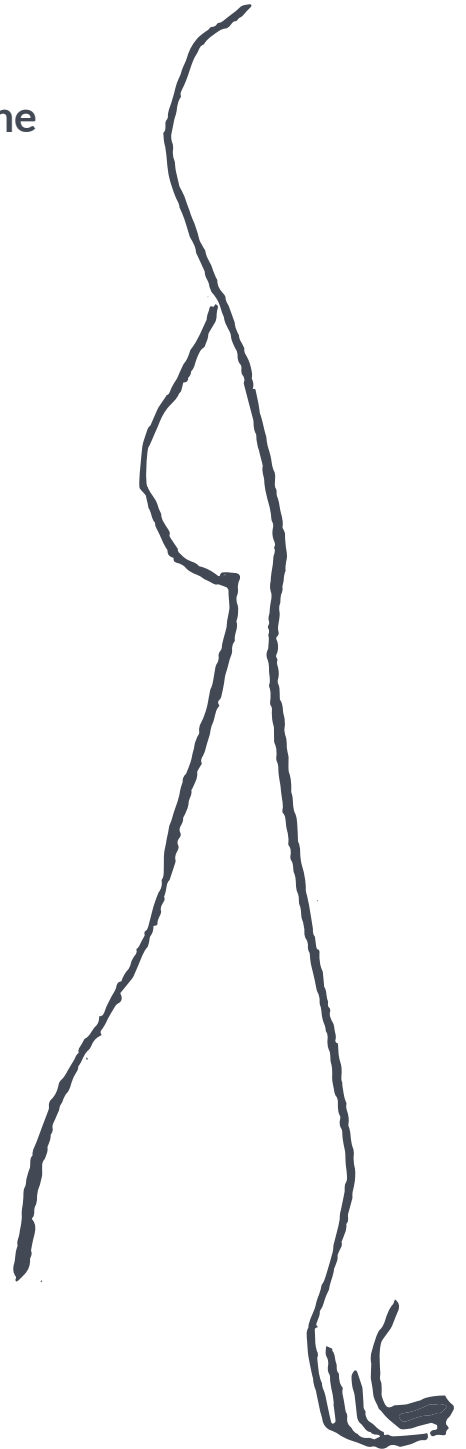
Dr. H.M. den Ruijter, Universitair Medisch Centrum Utrecht.

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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.

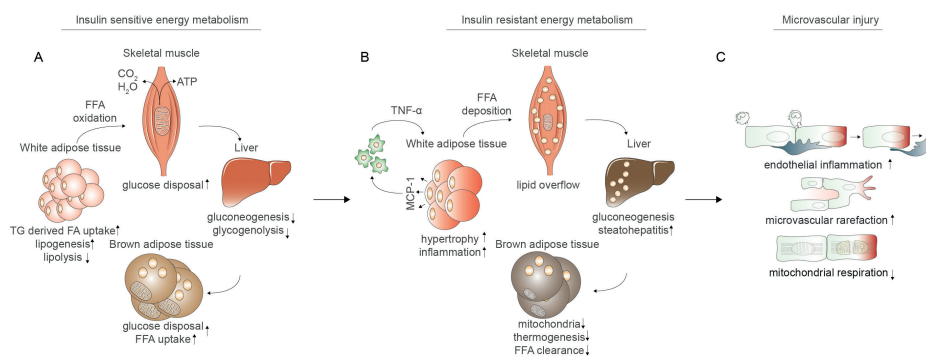


Figure 1. From insulin resistance to microvascular injury. (A) Normal physiology of insulin stimulated glucose disposal in skeletal muscle and brown adipose tissue (BAT) and lipid uptake in white adipose tissue (WAT). (B) Following ectopic lipid accumulation in skeletal muscle and in the liver as well 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 recruitment.

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.

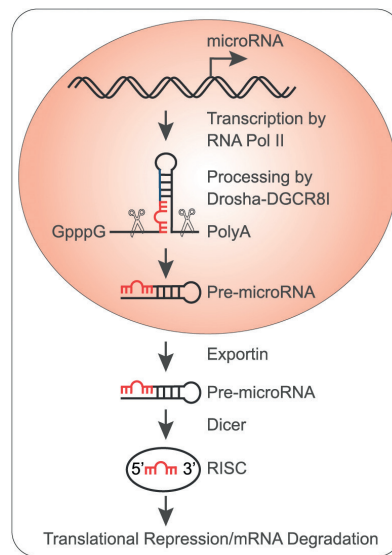


Figure 2. MiR biogenesis and function. MiRs are processed by the RNA polymerase II of independent genes or from introns of protein coding genes. The enzyme Drosha processes pri-miRs into a ~70 nucleotide precursor hairpin (pre-miR), which are exported by exportin to the cytoplasm where the enzyme Dicer 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 cardiomyocytes (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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Chapter 2

Gender and cardiovascular disease: are sex-biased microRNA networks a driving force behind heart failure with preserved ejection fraction in women?

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B.W.Florijn, R. Bijkerk, E.P. van der Veer,
A.J. van Zonneveld

Abstract

Cardiovascular disease (CVD) is the primary cause of death among men and women worldwide. Nevertheless, our comprehension of how CVD progresses in women and elicits clinical outcomes is lacking, leading CVD to be under-diagnosed and under-treated in women. A clear example of this differential presentation of CVD pathophysiologies in females is the strikingly higher prevalence of heart failure with preserved ejection fraction (HFpEF). Women with a history of pre-eclampsia or those who present with co-morbidities such as obesity, hypertension, and diabetes mellitus are at increased risk of developing HFpEF. Long understood to be a critical CVD-risk factor, our understanding of how gender differentially affects the development of CVD has been greatly expanded by extensive genomic and transcriptomic studies. These studies uncovered a pivotal role for differential microRNA (miRNA) expression in response to systemic inflammation, where their co-ordinated expression forms a post-transcriptional regulatory network that instigates microcirculation defects. Importantly, the potential sex-biased expression of the given miRNAs may explain sex-specific cardiovascular pathophysiologies in women, such as HFpEF. Sex-biased miRNAs are regulated by oestrogen (E2) in their transcription and processing or are expressed from loci on the X-chromosome due to incomplete X-chromosome inactivation. Interestingly, while E2-induced miRNAs predominantly appear to serve protective functions, it could be argued that many X-linked miRNAs have been found to challenge microvascular and myocardial integrity. Therefore, menopausal E2 deficiency, resulting in protective miRNA loss, and the augmentation of X-linked miRNA expression, may well contribute to the molecular mechanisms that underlie the female-specific cardiovascular aetiology in HFpEF.

Introduction

Cardiovascular disease (CVD) is the leading cause of death among women worldwide and more women than men die of CVD every year (1). Nonetheless, women remain underdiagnosed and undertreated because clinical standards are largely based on male pathophysiology and outcomes (2). A clear example of this differential presentation of CVD pathophysiology in females is the strikingly higher prevalence of heart failure with preserved ejection fraction (HFpEF) in women. While HFpEF is diagnosed in 40-70% of all heart failure (HF) cases in general (3), women outnumber men in HFpEF by a 2:1 ratio in population-based studies (4,5). Although this sex difference in HFpEF does not affect the prognosis of women compared to HFrEF patients (6), a recent study among 3385 HFpEF patients demonstrated that risk estimates for HF hospitalization, stroke, and (cardiovascular) death are higher in women who simultaneously present with atrial fibrillation (AF) (7). Therefore, a better understanding of the sex-specific mechanisms leading to HFpEF in women could reduce their risk for HFpEF and could improve female specific clinical care standards.

Various co-morbidities display strong associations with HFrEF and HFpEF, while also serving as HF phenotype determinants (8). While HFrEF is linked with ischemia and cardiomyocyte (CM) loss (9,10), HFpEF is associated with advanced age (11) obesity (12), hypertension (13), preeclampsia (14), diabetes mellitus (DM) (15) and renal dysfunction (16). A mechanistic understanding of HFpEF in cardiomyocytes (CM) has been restricted by limited access to human myocardial biopsies and the lack of animal models that sufficiently mimic human pathology. However, available histopathological studies of ventricular tissue have revealed that the aforementioned metabolic comorbidities induce extensive myocardial expression of endothelial adhesion molecules (17). An ensuing progressive reduction in capillary density (i.e. microvascular rarefaction) in HFpEF hearts (18) activates a transforming growth factor- β (TGF- β)-signalling cascade that is profibrotic and leads to myocardial collagen accumulation (19, 20). The subsequent loss of EC-derived nitric oxide (NO)-signalling limits myocardial cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signalling (21). Since cGMP-PKG signalling maintains CM elasticity via phosphorylation of titin, decreased cGMP-PKG levels trigger titin hypophosphorylation (22) yielding hypertrophic CMs with a higher resting tension (23-25). Interestingly, in women with HFpEF, CM remodelling

favours a sex-specific cardiac geometry whereby concentric remodelling is tightly coupled with diastolic dysfunction (26).

Collectively, this pathophysiological model has been put forth as the inflammatory microvascular paradigm (27). Clinical studies have demonstrated that this paradigm is also predictive for CVD morbidity and mortality due to the fact that subjects with HFpEF display reduced coronary bloodflow (28) while peripheral endothelial dysfunction independently correlates with future cardiovascular events (29).

Sex specific cardiomyocyte remodelling patterns upon vascular stress

To explain the above mentioned sex-differences in CM remodelling, the transverse aortic constriction (TAC) model is often used in animal research (30). This model enables the study of CM hypertrophy followed by a transition to heart failure and thus revealed that female mice develop a concentric form of hypertrophy and are more protected against fibrosis compared to male mice (31). Moreover, also female rats seem to be better protected against pressure overload because TAC induced LV hypertrophy resulted in a depressed contractile reserve in male hearts compared to female hearts (32). Similarly, also in humans, men and women display a different, left ventricular response to chronic load alteration during progression of aortic stenosis (AS) and this sex-specific remodelling of CMs, has also been attributed to the female predominant prevalence of HFpEF (33). While men tend to develop an eccentric left ventricular remodelling response of CMs upon stress, women have a more concentric remodelling response which manifests during aging (34), and with systolic hypertension (35) and aortic stenosis (36). Due to this concentric pattern of remodelling, women have a smaller LV cavity with a larger wall thickness, less cardiac collagen deposition (37) and a better preserved ejection fraction and contractility as compared to men (36). However, a complete understanding of the sex-specific cellular mechanisms within CMs is currently lacking.

Sex-biased miRNAs; potential mediators of the sex-specific cardiovascular pathophysiology in HFpEF

It is becoming increasingly apparent that the cellular “response to stress or injury” is predominantly regulated at the post-transcriptional level, involving an intricate interplay between noncoding RNAs, such as miRNAs and long-noncoding RNAs (lncRNA) (38), and RNA-binding proteins (39). Following this notion, many studies have addressed the coordination of gene expression in cardiovascular tissue by

miRNAs which play a pivotal role in silencing or fine-tuning mRNA expression and protein levels based on the complementarity of base-pairing at 3'-untranslated regions (3'-UTR) of target mRNAs (40). The expression of non-coding RNAs is sex-biased as a result of two main driving factors. First, many gene promoters contain estrogen-responsive elements (EREs) (41), whereby E2 binding drives miRNA expression (42). Second, the X-chromosome encodes 118 miRNAs (43) and several X-linked miRNAs are known to escape X-chromosome inactivation (44) resulting in higher expression levels of these miRNAs in particular cell types (45).

Striking examples of a resultant sex-specific myocardial miRNA expression were reported in a miRNA array study which demonstrated that myocardial miRNA expression from healthy, mature mice exhibited a sex-specific expression of a subset of miRNAs. Interestingly, the expression of the X-chromosome located miR-222 was to be significantly augmented in males compared to females resulting in a sex-specific myocardial eNOS expression (46). Furthermore, expression levels of other miRNAs, namely miR-1, miR-106b, miR-720, and miR-29b were augmented in females compared to males and their differential sex-specific myocardial expression, may result in a sex-specific response of CMs to injury or therapeutic inhibition.

A sex-specific therapeutic inhibition in myocardial tissue was demonstrated in a miRNA-sequencing study on RNA from mouse ventricular tissue which revealed a sex-specific myocardial miRNA expression within the healthy heart, but also in mouse models of dilated cardiomyopathy (DCM) and AF (47). Interestingly, this particular study demonstrated that the inhibition of a particular miRNA, namely miRNA-34a, in DCM female mice, resulted in less cardiac remodelling, lower expression of cardiac stress genes and less cardiac fibrosis compared to male mice (47). Given that the inhibition of this miRNA resulted in a more effective therapeutic inhibition in women, this suggests that sex-biased myocardial miRNA expression determines myocardial cellular fate in women with cardiac disease.

The above studies pinpoint sex-specific myocardial miRNA networks that may target multiple genes, thereby coordinating the control of distinct cellular pathways that together drive cellular functions, including those involved in the cellular response to injury in HFpEF (48). Consequently, by the simultaneous repression of multiple genes, miRNAs directly influence the output of functionally related biological pathways, the coordination of signalling networks and cellular fate

(49). The fact that the coordination of signalling networks within cells starts via X-chromosome-located miRNAs or miRNAs whose transcription is regulated by E2, increases the likelihood that this regulatory control will manifest differently between men and women. We believe that this hypothesis could provide additional mechanistic insight into HFpEF and the clinical heterogeneity associated with this syndrome, which could arise from different pathophysiological conditions being induced by different co-morbidities in men and women.

Therefore, this review postulates that sex-biased miRNAs may function as post-transcriptional mediators of the sex-specific cardiovascular pathophysiology driving HFpEF in women. Here, we will first discuss both the beneficial impact of E2 and the possibly injurious effects of X-chromosome gene dosage disequilibria on the cardiovascular system in women. Second, we provide insight into how the pathogenesis of HFpEF is linked with sex-biased miRNA expression and their regulation of functionally related genes in endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and CMs. To achieve this, we detail how E2-regulated miRNAs primarily seem to protect the microvasculature against inflammation and also limit CM remodelling and fibrosis. In contrast, as a result of incomplete X-chromosome inactivation, the augmentation of X-chromosome located miRNAs, could activate the cellular response to hyperglycaemia thereby driving microvascular inflammation and rarefaction (i.e. aberrant angiogenesis). These processes represent key pathophysiological features leading to HFpEF. In CMs for example, it could be argued that X-linked miRNAs act as accessory stimuli in the disruption of mitochondrial respiration and autophagy, leading to hypertrophic responses that are strongly associated with HFpEF.

Estrogen attenuates the cellular response to injury in HFpEF

Sex-steroid hormones markedly impact the pathophysiological differences in CVD development in men and women (50). Particularly the effects of 17 β -estradiol (E2), the major circulating estrogen, and the estrogen receptors (ER) have been well investigated (51). Two ERs, namely ER α and ER β are widely expressed in virtually all tissues with higher expression levels in women than in men (52). Upon E2 binding, nuclear translocation and subsequent attachment of the E2-ER complex to the DNA at EREs drives expression of genes that mostly have been described to have beneficial impact on the functional status of ECs, VSMCs and CMs (51).

In ECs, E2-ER signalling exerts anti-inflammatory effects by augmenting eNOS induced-NO signalling (53), attenuating the major cellular source of ROS, NADPH oxidase (54) and inhibiting the expression of endothelial leukocyte adhesion molecules such as vascular cell adhesion molecule 1 (VCAM1) and intercellular cell adhesion molecule (ICAM1) (55). VSMCs equally profit from protective E2 activity, as E2 decreases stress-induced proliferation and contractility of VSMCs (56, 57). In CMs, E2 stimulates the expression of the pro-survival kinase, Akt, which inhibits cellular apoptosis in women (58) and promotes cell survival (59). Moreover, E2 attenuates CM remodelling in response to pressure overload (60) and augments myocardial angiogenesis, thereby increasing capillary density (61).

Estrogen counteracts adverse hemodynamic and metabolic HFpEF comorbidities

The renin-angiotensin-aldosterone system (RAAS) controls water- and electrolyte homeostasis, and the classical activation of this pathway is initiated by binding of angiotensin II (ANG II) to the ANGII type 1 receptor (AT1R). Non-classical RAAS stimulation by E2 in premenopausal women opposes classical activation of this pathway, thereby triggering vascular vasodilation and enhanced blood flow (62, 63). However, menopausal E2 deficiency is associated with classical RAAS activation (64) with prospective population studies indicating that postmenopausal women present higher arterial pressures than aged-matched premenopausal women (65).

In lipoprotein metabolism, E2 decreases low density lipoprotein (LDL)-cholesterol (66) and prevents its vascular accumulation (67) and uptake by macrophages (68). Furthermore, E2 elevates high-density lipoprotein (HDL) levels (69) and amplifies its reversed cholesterol transport capacity (70). Similar protective effects of E2-ER signalling have been observed in glucose metabolism, as ER α knockout mice are hyperinsulinemic and have abnormal glucose levels (71), a phenomenon which is reversed upon ER α stimulation (72). Interestingly, this ER deficiency is also associated with less insulin secretion, less insulin sensitivity and more insulin resistance in postmenopausal women (73, 74).

These beneficial E2 effects in premenopausal women led to the hypothesis that hormone replacement therapy (HRT) might reduce CVD in post-menopausal women. However, unexpectedly several large prospective HRT studies failed to reduce CVD expression in women (75) and some trials even reported an adverse impact of HRT on the prevalence of myocardial infarctions (76). Therefore these

observations make way for the alternative hypothesis that elevated E2 levels, as reported in obese postmenopausal women may actually serve as a potential driver of elevated susceptibility for CVD in women with diabetes (77).

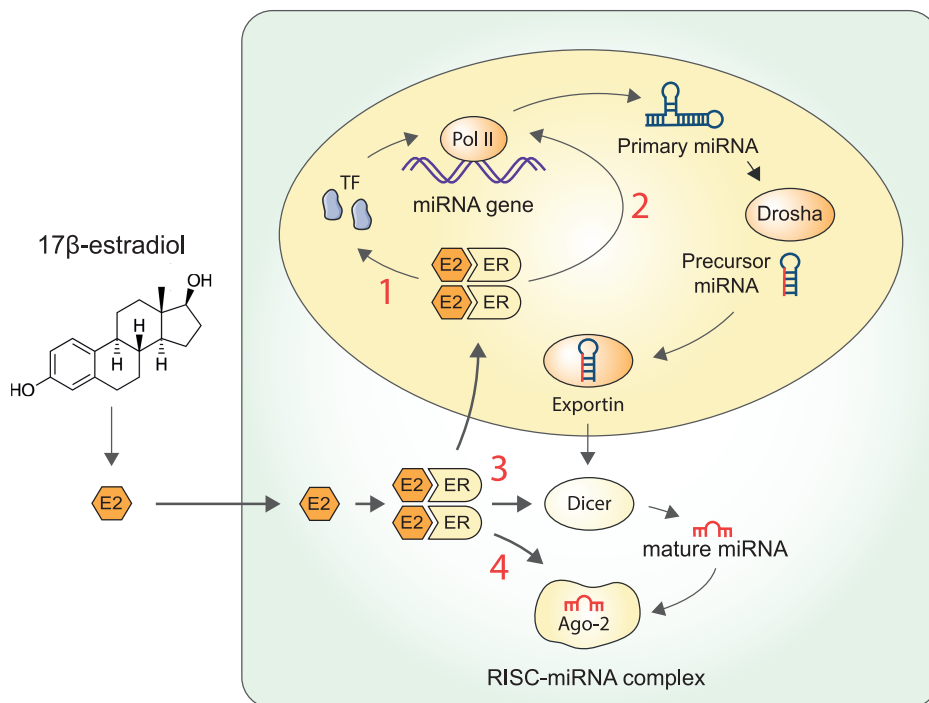


Figure 1. Levels of estrogen (E2) control of miRNA transcription and processing.

(1) E2 can impact on miRNA transcription via recruitment of transcription factors (TF). (2) E2 can affect RNA polymerase II (Pol II) activity. (3) E2 can augment the expression of the pre-miRNA processing protein Dicer. (4) E2 can increase the expression of the Argonaute-2 protein that is essential for miRNA target recognition and function.

Estrogen regulates miRNA transcription and processing

E2 can regulate miRNA expression and function at multiple levels (Figure 1). MiRNAs are expressed from intronic regions of protein-coding mRNA or located intergenically. Intergenic miRNAs have independent promoters, transcript sequences and terminators while intronic miRNAs share these regulatory motifs with the host gene (78). ER binding to such regulatory motifs in promoters drives miRNA transcription which can directly impact miRNA expression levels (42). This subsequently regulates multiple downstream targets (in both a direct and indirect fashion) (79). This was particularly demonstrated in ER-positive breast

cancer cell lines that have a reduced expression of miR-181a and miR-26a upon E2 stimulation. Selective overexpression of these miRNAs limited E2-dependent cell growth, suggesting that this E2 induced miRNA decrease was required for E2-dependent cell growth (80).

Alongside a role in driving miRNA transcription, E2 has recently been found to also impact expression levels of essential components of the miRNA processing machinery. Binding of the ER-E2 complex at an enhancer region upstream of the Dicer (known for cleaving pre-miRNAs) transcription start site has been shown to augment Dicer protein levels (81, 82). Furthermore, genome-scale studies in breast cancer cell lines found that the ER α binds the enhancer region upstream the DICER transcription start site, which suggests E2-ER signalling modulates miRNA processing (83). Further downstream in miRNA processing, ER α depletion has been shown to increase expression of the catalytic subunit of the RISC complex, the Argonaute-2 (Ago-2) protein in ER positive human breast cancer cell lines and tumours, which mediates the miRNA-dependent cleavage function (84).

Collectively, it is worth noting that the aforementioned studies, which have demonstrated that miRNA transcription is under E2-ER signalling control, are carried out in MCF-7 cell lines, which have a deregulated cell-cycle control and abnormal gene expression profiles. However, miRNA expression in healthy human umbilical vein endothelial cells (HUVECs), skeletal muscle and adipocytes has also been found to be regulated by E2-ER signalling.

In human umbilical vein endothelial cells (HUVECs), 24h of E2 treatment (10 nM) was found to increase both pri-miR-126 and mature miR-126-3p expression in an ER α -dependent manner (85). This suggests that E2 is involved in miRNA biogenesis in HUVECs. Furthermore, in skeletal muscle from female mice, pri-miR-22 expression was found to be more abundantly expressed than in male mice. Interestingly, upon mutating the ER α binding site in the pri-miRNA-22, the E2-induced repression of miR-22 processing was abolished. Therefore, this study concluded that the ER α represses the processing of the pri-miR-22 by binding to a conserved ER α binding element within the pri-miR-22 (86).

Similarly, miRNA profiles of skeletal muscle from monozygotic post-menopausal twin pairs discordant for estrogen-based hormone replacement therapy (HRT),

revealed that miRNA-182, miRNA-223 and miRNA-142-3p expression decreased as a result of HRT (87). However, no additional expression analysis of pri-miRNA sequences nor mutation analysis of ER binding sites in these particular miRNAs were performed in this study. Furthermore, in omental adipose tissue samples from women with gestational diabetes mellitus, miRNA-222 was upregulated compared to healthy pregnant controls and displayed a negative correlation with ER α and GLUT4 protein levels (88). Upon silencing of miRNA-222 in cultured adipocytes, GLUT4 translocation to the membrane improved, thereby resulting in more insulin stimulated glucose uptake compared to healthy controls (88). However, whether this effect was causally mediated via the ER α was not addressed, nor were any additional miR-222 overexpression experiments performed to determine direct miRNA-222 effects on ER α , independent of E2 signalling.

Taken together, these studies demonstrate that it is plausible to hypothesize that E2 affects miRNA transcription and processing in healthy human cells. Therefore, the observed enrichment of ERs within ECs could support the notion that miRNA transcription is regulated by E2 within the female vasculature (8).

Estrogen-regulated miRNAs attenuate the cellular response to microvascular inflammation

In healthy subjects, endothelial quiescence is established by laminar flow that marks a major physiological parameter affecting the transcriptome (90) via endothelial transcription factors KLF2 (91) and KLF4 (92). KLF2 and KLF4 activate NO signalling to prevent endothelial leukocyte adhesion and to reduce VSMC proliferation which enhances vascular vasodilation (93). In contrast, endothelial ROS and systemic pro-inflammatory signalling in HFpEF reduce NO signalling, leading to a reduction in endothelial migration and proliferation (94). Because the resulting decrease in capillary density further aggravates myocardial NO and oxygen (O₂) bio-availability in HFpEF patients (95), VSMCs can lose their vasodilator response and revert to a proliferative, migratory, and synthetic state to aid in vessel repair (96). In subadjacent CMs, reduced NO and O₂ levels perturb mitochondrial respiration (97) and contraction (98) which initiates a remodelling response (99) that correlates with left ventricular (LV) diastolic dysfunction (100).



Figure 2. Sex-biased miRNAs are potential mediators of the sex-specific cardiovascular pathophysiology in HfPEF.

The middle panel (blue panel) describes the cellular response to injury in the progression of HfPEF on which estrogen-regulated miRNAs (green panel) and X-linked miRNAs (red panel) were shown to have an impact. Green fonts depict miRNA/targets relations in HfPEF protective mechanisms, red fonts depict HfPEF progressive mechanisms.

In ECs, VSMCs and CMs, E2 regulates the aforementioned cellular processes. Whereas E2 induces EC proliferation and migration (51), it inhibits these processes in VSMCs *directly* (56) but also, *indirectly* via miRNA-induced changes in gene expression (Figure 2, E2 regulated miRNAs). In ECs, E2 was found to increase the expression of pri-miR-126 and mature miR-126-3p in an ER α dependent manner, which promoted endothelial proliferation and tube formation (85). Interestingly, in vitro both miRNA-126 and E2 acted synergistically to reduce the expression of VCAM-1, while in vivo experiments in female mice demonstrated that miRNA-126 and E2 were capable of reducing aorta sinus plaque size (85). Even though the anti-inflammatory effects in the in vitro experiment were very modest, the data strongly suggest the presence of a synergistic, anti-inflammatory effect of miRNA-126 and E2 in endothelial cells.

In mouse and human VSMCs, interaction of the ER-E2 complex at the promoter region of miRNA-203 in the 14q32 locus, along with indirect activation of transcription via transcription factors Zeb-1 and AP-1, was found to increase expression of miRNA-203 (101). Although no functional ERE was identified in the predicted miR-203 promoter region, this study demonstrated that ER α binding to the miR-203 promoter was mediated by AP-1 while Zeb-1 repressed E2 regulation of miR-203 expression through an interaction with the ER α at the miR-203 promoter. The resultant high miR-203 levels in ER α -overexpressing mouse VSMCs attenuated levels of the pro-proliferative proteins Abl1 and p63, resulting in less VSMC proliferation (101). Causal evidence that miR-203 contributed to the E2-induced inhibition of VSMC proliferation was demonstrated when miR-203 knockdown abolished E2-mediated inhibition of VSMC proliferation while miR-203 overexpression suppressed cellular proliferation (101).

Estrogen-regulated miRNAs attenuate myocardial remodelling and fibrosis

In CMs, E2 impacts cellular function *indirectly* by transcriptionally regulating miRNA expression. This was particularly striking in an animal model of cardiac fibrosis, where the levels of miRNA-24, 27a/b and miRNA-106a/b were increased within the hypertrophied hearts of wild type- and ER- β - deficient C57Bl/6J male mice, as compared to female mice (102). It was demonstrated that these miRNAs operate together targeting different negative regulators of the pro-fibrotic MAPK-ERK1/2 signalling pathway, namely Spry1, Rasa1 and Rasa2. In female mice, E2 repressed the expression of these miRNAs thereby limiting pressure overload

induced cardiac fibrosis (102), although no additional perturbation experiments with these E2 regulated miRNAs were performed. Nonetheless, this E2-miRNA mediated repression of fibrosis in the female sexes is in line with results from a genome-wide gene expression profiling study of human myocardial LV tissue samples, where transcriptome profiling revealed that fibrosis-related genes were induced to a higher degree in males as compared to females (103).

Importantly, E2 not only inhibits fibrosis, but is also protective against concentric remodelling of CMs. Decreased E2 levels in ovariectomized (OVX) rats were found to increase miRNA-23a expression, leading to gap junction defects in CMs as a result of reduced Connexin 43 (Cx43) protein levels, a phenomena which was reversed upon E2 suppletion (104). Interestingly, gap junction defects and Cx43 downregulation in cultured CMs were restored by miRNA-23a knockdown with antisense oligonucleotides (104). However, the mechanism through which E2 regulates miRNA-23a was not assessed while E2 also induced an upregulation of the miRNA-23a cluster members, miRNA-27a and miRNA-24-2. These miRNAs could have had a modulatory effect on Connexin-43 expression depending on their seed-sequence. Nonetheless, more detrimental effects of increased miRNA-23a levels were seen in response to E2 deficiency that induced structural damage to the mitochondrion in postmenopausal and OVX mice. This led to an impairment in respiratory function due to reduced production of peroxisome proliferator-activated receptor- γ co-activator 1- α (PGC-1 α), a key mitochondrial protein (105). Interestingly, both miRNA-23a overexpression and knockdown experiments altered mitochondrial respiration in cultured CMs because of PGC-1 α expression level changes (105).

Together, these studies clearly indicate a critical role for E2 regulated miRNAs and their subsequent regulation of gene expression in ECs, VSMCs and CMs. However, it should also be mentioned that phenol red in cell culture medium contains a higher concentration of E2 than the concentration of E2 in female serum (106). Therefore, unless miRNA expression experiments are carried out with phenol red free media or charcoal stripped FCS to exclude the estrogenic actions of phenol red, these experiments should be regarded to have been performed under chronic E2 stimulation. As such, one should be cautious in drawing strong conclusions on estrogen-induced mechanisms from these studies., Nonetheless, the aforementioned miRNAs pinpoint consequences of alterations in sex-hormone

levels in post-menopausal women which negatively impacts metabolism and cardiac function (107).

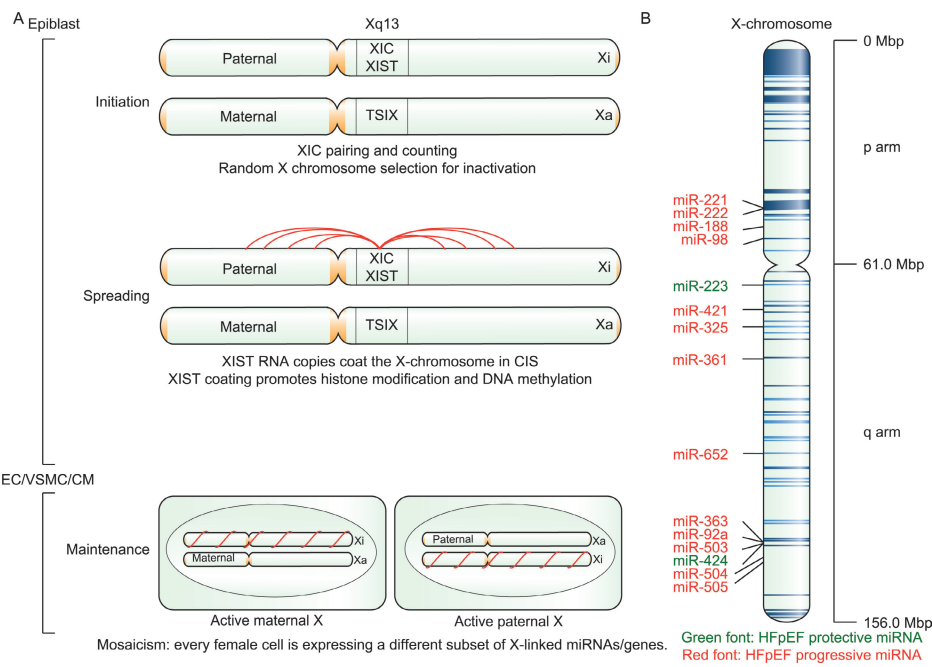


Figure 3. (A) Mechanism of X-chromosome inactivation (B) MiRNA map of the human X chromosome depicting X-linked miRNAs that regulate the cellular response to injury in HFpEF. (A) During embryonic development, XCI inactivation is carried out by the long-non-coding RNA XIST which is expressed from the X-inactivation center (XIC) on the X-chromosome. By spreading along the X-chromosome, XIST induces transcriptional silencing resulting in either an inactive maternal X- or paternal X-chromosome in women. This coating of the inactive X-chromosome by XIST should be maintained throughout adult live in ECs, VSMCs and CMs. Transcription of an antisense long-non-coding RNA to XIST, TSIX restricts XIST activity on the future active X-chromosome. (B) Chromosomal loci with a high or moderate probability of escaping X-chromosome inactivation are depicted in dark blue and light blue respectively. Data of chromosomal loci that escape X-chromosome inactivation was derived from reference (27). Italic and bold depicted X-linked miRNAs that are involved in the cellular response to injury in HFpEF are in close proximity to chromosomal loci that are known to escape X-chromosome inactivation.

X-chromosome mosaicism: a causal factor for sex-biased cardiovascular comorbidities?

Sex chromosome gene expression differences are the natural result of two X-chromosome copies versus one X and one Y chromosome copy in somatic cells of females and males, respectively. To avoid a female X-chromosome gene dosage disequilibrium, one of each of the X-chromosome copies is neutralized by random X-chromosome inactivation (XCI, Figure 3A) during embryonic development (108). XCI is coordinated by an X-inactivation center (Xic), located on the longer arm of the chromosome, which controls XCI by initiating X chromosome counting and random X chromosome choice (109). The Xic harbours the lncRNA 'X-inactive specific transcript' (XIST) which will coat the X-chromosome from which it is expressed (110). XIST coating subsequently directs chromatin and transcriptional changes by binding polycomb repressive complexes that induce methylation and subsequent silencing of the genes located on the X chromosome (110). These steps are completed in the peri-implantation embryo within the 10–20 cell epiblast lineage, and should be maintained in all somatic cells. However, 10% of X-chromosome located (X-linked) genes show variable expression patterns as a result of incomplete XCI, while 15% of X-linked genes permanently escape XCI, albeit that this proportion displays region-dependent variation (45). In female somatic cells, this results in mosaicism and heterogeneity in X-chromosome gene expression patterns.

This mosaicism of XX women has been demonstrated to lead to sex differences in the immune response to both self and foreign antigens. Surprisingly, the X-chromosome is enriched for immune response genes, such as Toll-like receptor 7 (TLR7) and CD40 ligand (CD40L) (111). Therefore, variations in these gene copies can result in distinct alleles with different regulatory and response capacities thereby conferring that X chromosome allelic diversity benefits to women. However, the increased dosage of immunity-related genes could also potentially induce a hyper-responsiveness to acute inflammatory stimuli thereby rendering women more susceptible to inflammatory- and autoimmune diseases than men (112). Furthermore, and relevant for understanding sex-specific aetiology in CVD, also in autoimmune disease patients, eNOS uncoupling plays a central role in the amplification of oxidative signalling pathways that chronically activate the microvascular endothelium (113, 114). Because far more women are autoimmune

disease patients, this phenomenon is also likely to predispose women towards more cardiovascular morbidity and mortality than men (115).

In line with these notions, the presence of two X chromosomes in female cells has also been associated with more cardiovascular comorbidities, based on research using the so-called four-core genotype (FCG) mouse model. In FCG mice, the testis-determining Sry gene is deleted from the Y chromosome (XY⁻), yielding a Y⁻ chromosome that is no longer testis determining. This creates XY⁻ mice that are gonadal females with ovaries. When a Sry transgene is inserted onto an autosome, mice are gonadal males (XY⁻Sry) with testes (116). Mating XY⁻Sry with XX females produces the FCGs which are XX females, XY⁻ females, XXSry males and XY⁻Sry mice (116).

In *gonadectomised* FCG mice it is thus possible to study sex chromosome induced cardiovascular effects, independently of gonadal hormone-mediated effects that are normally known to suppress the cellular response towards HFpEF inducing comorbidities. This has revealed that XX mice develop a higher mean arterial pressure after RAAS activation than XY mice (117) as well as a much larger myocardial infarct size following ischemia/reperfusion (I/R)- injury than XY mice (118). Moreover, XX mice on a high fat diet gain more obesity and develop more subcutaneous inguinal adipose tissue depots while they are also more insulin resistant than XY mice (119). Importantly, the development of these HFpEF inducing comorbidities (i.e. inflammation, obesity and hypertension) could solely be attributed to the number of X chromosomes rather than the absence of a Y chromosome.

X-chromosome located miRNAs escape X-chromosome inactivation

Both in humans and mice, the X-chromosome is relatively enriched for miRNAs with over two fold higher miRNA densities compared to the autosomes (43). In contrast, the male Y-chromosome possesses only two annotated miRNAs in the pseudo-autosomal region, which undergoes recombination with the X-chromosome (43). Currently, 118 miRNAs are annotated on the human X-chromosome (source: miRBase vs.21), of which the majority fall into six regional clusters (Figure 3B). These clusters comprise 78 X-linked miRNAs, of which 83% have been reported to escape XCI (44, 120) and modulate EC, VSMC and CM function and could thus

potentially be involved in the pathophysiology leading to HFpEF. (Figure 2, X-linked miRNAs).

X-linked miRNAs mediate the cellular response to hyperglycaemia

HFpEF is more prevalent in diabetic women than diabetic men (121) and obesity stands out as a significant risk factor for HFpEF in postmenopausal women (122). This suggests that hyperglycaemia and hyperinsulinemia in women are associated with HFpEF development. Interestingly, the endothelial response to hyperglycaemia is regulated by differential expression of several X-linked miRNAs. This was particularly evident when hyperglycaemia-induced EC dysfunction stimulated miRNA-221 expression in human umbilical vein endothelial cells (HUVECs) which triggered a reduced expression of c-kit, the receptor for stem cell factor that in healthy conditions activates EC migration. Subsequent miR-221 knockdown in HUVECs cultured under high glucose conditions, restored c-kit protein expression and abolished the inhibitory effect of high glucose exposure on HUVECs transmigration, although miR-221 knockdown itself had no significant effect on migration (123). More inhibition of EC migration was seen when high glucose levels decreased the expression of miRNA-98 in streptozotocin (STZ) treated rats fed a high fat diet resulting in increased expression of the cell-cycle protein cyclin D2 (124). Similar EC proliferation inhibition by glucose was seen when hyperglycaemia stimulated miRNA-503 expression in ECs (125). MiR-503 overexpression inhibited EC proliferation and migration while upon miR-503 knockout, ECs improved in proliferation and angiogenesis, suggesting an anti-angiogenic function for miRNA-503 in patients with DM (125). In ECs in vitro, these effects upon miR-503 knockdown associated with a restoration of cdc25A and CCNE1 protein levels, although no siRNA mediated experiments were carried out to confirm the cause-effect-relation between these observed post-transcriptional regulation of cell-cycle maintainer proteins and the aberrant endothelial angiogenic response. Interestingly, the microparticle-mediated release of miRNA-503 from diabetic ECs was efficiently taken up by neighbouring pericytes, where it was found to target the EFNB2 and VEGFA mRNAs resulting in impaired pericyte coverage of capillaries and increased permeability but also impaired post-ischaemic angiogenesis in limb muscles (126).

In contrast to the X-linked miRNA expression profiles observed in hyperglycaemic ECs, VSMCs isolated from internal mammary artery (IMA) segment of diabetic

patients display elevated levels of the miR-221/222 cluster and decreased p27^{Kip1} mRNA levels which resulted in more VSMC proliferation in vitro (127). Whether diabetes indeed promoted VSMC proliferation through higher levels of this particular miR-221/222 cluster could not be concluded from this study. However other studies have shown that the VSMC response to diabetes is mediated via other miRNA expression level changes. For instance increased miRNA-504 expression levels upon diabetes were responsible for skewing VSMCs to adopt an inflammatory phenotype with higher cellular expression of the pro-inflammatory genes Ccl2 and IL6. This was shown to enhance VSMC proliferation and migration, effects that were blocked by miR-504 inhibition in VSMCs (128).

These findings suggest that while EC-derived X-linked miRNAs negatively impact the proliferative and supportive capacities of ECs and pericytes respectively, they stimulate the proliferative capacity of VSMCs. This pinpoints towards an indirect manner by which hyperglycaemia could potentially be responsible for EC dysfunction, a decrease in pericyte coverage and vascular hyperactivity in the setting of HFpEF in women.

X-linked miRNAs mediate the cellular response to microvascular inflammation

The increased circulating levels of the pro-inflammatory factors C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in HFpEF patients (129), stimulate endothelial VCAM-1 and E-Selectin expression, thereby promoting leukocyte recruitment and adhesion to (coronary) ECs (19). The expression of these endothelial adhesion molecules is actively modulated by X-linked miRNAs either potentiating or attenuating the endothelial inflammatory response. Culturing ECs in such inflammatory conditions was found to significantly increase miRNA-92a expression levels, a miRNA that was also elevated in atherosusceptible regions of swine aorta (130, 131). Subsequent studies convincingly demonstrated that this endothelial miRNA-92a elevation was mediated via the oxidative stress induced sterol regulatory element-binding protein 2 (SREBP2), an activator of the endothelial inflammatory response. In endothelial cell-specific SREBP2 transgenic mice, miR-92a knockdown attenuated inflammasome activation, improved vascular vasodilation, and inhibited ANG II-induced or aging-related atherogenesis (132). This endothelial recovery was accomplished because the elevation of this particular miRNA during inflammation was responsible for decreased KLF2, KLF4 and eNOS levels while also leading to inflammasome activation (132). Similar effects on

endothelial inflammation are carried out by miRNA-223. Low baseline expression levels of endothelial miRNA-223 can be increased by the transport capacities of HDL which has been shown to be involved in the transfer of miRNA-223 to ECs (133). This way HDL augments endothelial miRNA-223 expression, thereby leading to a functional decrease in endothelial ICAM-1 expression levels (134). However, these beneficial anti-inflammatory properties of miRNA-223 expression are reduced in ECs upon continuous TNF- α treatment which enhances the expression of tissue factor (TF) by ECs (135). The in vitro transfection of ECs with a miRNA-223 mimic was subsequently found to decrease both TF mRNA and protein levels, suggesting a potential inhibitory role for miRNA-223 in the pro-thrombotic complications of endothelial inflammation (135).

Taken together, this shows that the endothelial response to pro-inflammatory cytokine signalling is either potentiated or attenuated by differential expression of X-linked miRNAs. Consequently, incomplete X-chromosome inactivation may result in a sex-biased expression of endothelial inflammatory genes in women with HFpEF that contributes to its pathophysiology of vascular endothelial dysfunction.

X-linked miRNAs mediate the angiogenic response to microvascular inflammation

ECs are essential for angiogenesis, a process that involves the formation of new capillaries from pre-existing vessels during development, and in response to O₂ deprivation and inflammation (136). A well-known promoter of this regenerative process is vascular endothelial growth factor (VEGF), a protein whose expression is initiated by exogenous ROS production (105) enabling the sprouting and migration of ECs from capillaries into surrounding tissues (137, 138). Following their generation, nascent vessels must be stabilized to ensure survival. The angiogenic growth-factor Angiopoietin-1 (Ang-1), produced by pericytes, plays a critical role in this process, as it provides structural support of newly formed capillaries, thereby enhancing EC-mediated barrier function (139).

Chronic exposure to hyperglycaemia (in DM) is known for critically diminishing Ang-1 levels and skewing the balance in favour of EC-derived Ang-2, an Ang-1 antagonist that promotes endothelial destabilization. Ang-2 is destabilising ECs by sensitizing them towards TNF- α and by stimulating the TNF- α -induced expression of endothelial adhesion molecules (140). This vascular destabilization results in a profound impairment in endogenous myocardial angiogenesis (141). The resultant

reduced capillary surface area relative to CM surface area in HFpEF patients, limits myocardial perfusion and NO signalling thereby inducing left ventricular diastolic dysfunction and cardiac reserve function impairment (18). This pathophysiologic process leading to HFpEF was recently demonstrated in HFpEF patients in whom cardiac positron emission tomography (PET) demonstrated a reduction in myocardial flow reserve (MFR, i.e. the ratio of maximal hyperemic to resting myocardial blood flow). Interestingly, the more MFR reduction was observed, the higher the severity of diastolic dysfunction in these HFpEF patients (28). Therefore, enough evidence thus far suggests that an aberrant myocardial, angiogenic response to a systemic inflammatory milieu can cause diastolic dysfunction and HFpEF (142).

Interestingly, miRNAs have come to light as fundamental guiders of angiogenic processes, as inhibition of miRNA processing via genetic knockdown of Dicer was shown to impair EC proliferation and angiogenesis (143). One mechanism by which VEGF controls angiogenesis is by stimulating the expression of X-linked miRNA-424 in ECs. While this miRNA is pro-angiogenic in hypoxic conditions (144), it inhibits angiogenesis in normoxic ECs by reducing the levels of VEGF receptor (145). Similarly, miR-223 levels were decreased by FGF and VEGF treatment, resulting in enhanced EC migration and proliferation, suggesting that the attenuation of miR-223 reprograms ECs towards a more angiogenic state (146). Similarly, like miR-223 is anti-angiogenic in ECs, so are increased endothelial expression levels of miR-505, which were found to reduce EC migration and angiogenesis. Gene expression analyses and a luciferase reporter assay in this study revealed that FGF18, a proangiogenic factor, is a directly regulated target of miRNA-505 in ECs. However, this study did not perform additional experiments into whether reduced angiogenesis of ECs was indeed regulated via the inhibition of FGF18 protein (147).

Further evidence that X-linked miRNAs critically impact angiogenesis can be derived from the identification that the miRNA-221-222 cluster was induced by stimulating ECs with IL-3 and bFGF. By activating ECs with these inflammatory stimuli, the miRNA-221/222 cluster negatively correlated with STAT5A expression, a transcription factor that mediates angiogenesis in ECs (148). More disruption of angiogenesis via this cluster was seen via three other ways: 1) inhibition of endothelial eNOS expression (149-151); 2) reduction of the pro-proliferative stem cell factor c-Kit (152,153); and 3) augmentation of the anti-angiogenic gene GAX by repressing ZEB2, a transcriptional inhibitor that targets GAX (154).

Evidence that a disturbed angiogenic response of ECs, via an increased expression of X-linked miRNAs, could have consequences for myocardial function was given in a larger preclinical model of pigs that underwent myocardial ischemia/reperfusion injury. With this model, this study found that the inhibition of the X-linked miRNA-92a was found to reduce infarct size as compared with control pigs, which resulted in an improved LV ejection fraction (155). Interestingly, this model as well as studies performed in mice, demonstrated that miRNA-92a knockdown was associated with higher capillary density, decreased inflammation and cell death. Although this particular study did not investigate the mechanisms by which miR-92a inhibitors affect inflammation, it provided evidence that the deletion of an X-linked miRNA preserves cardiac function after infarction. This suggests that increased expression of this particular miRNA in females or as a result of incomplete X-inactivation could render women more susceptible to a worse outcome after myocardial infarction compared to men.

Collectively, these studies demonstrate that several X-linked miRNAs impact microvascular stability and angiogenesis. Therefore, their involvement within the aberrant angiogenic response of (myocardial) ECs in HFpEF may further aggravate microvascular integrity in women which culminates in ventricular diastolic dysfunction. This way, X-linked miRNA regulation of angiogenesis may provide a novel molecular mechanism explaining the resultant distinct HF phenotypes in men and women.

X-linked miRNAs mediate mitochondrial fission-fusion events in cardiomyocytes

Mitochondria are highly abundant in CMs because CMs continuously employ oxidative phosphorylation to replenish their ATP supply for the maintenance of cellular, and therefore, myocardial contraction (156). Physiologic mitochondrial function is morphology dependent, and is determined by mitochondrial fission and fusion. Fission leads to fragmentation and the promotion of cell apoptosis, whereas fusion results in mitochondria elongation, a CM-protective process (157). However, microvascular rarefaction and therefore a reduced myocardial perfusion stimulates mitochondrial fission, leading to myocardial ROS production (158, 159). Consequently, this lowers protein kinase G activity in CMs, a phenomena which has been observed in CMs from both diabetic- (160) and HFpEF patients (21) which is known for promoting CM remodelling.

X-linked miRNAs have also been pinpointed to play a central role in regulating multiple sets of functionally related genes that modulate mitochondrial morphology. Particular examples of such genes are the Prohibitin family members, namely Prohibitin-1 (PHB-1) and PHB-2, which have been found to be involved in maintaining mitochondrial inner membrane macromolecular structure, a function that is vital for both the preservation of morphology, as well as energy production. Interestingly, mitochondrial fission in apoptotic CMs was found to be associated with decreased PHB-1 protein levels, an event triggered by miRNA-361-mediated targeting of the PHB-1 mRNA (161). Interestingly, antagomiR-mediated miRNA-361 knockdown prevented mitochondrial fission while overexpression of this miRNA in miR-361 transgenic mice exacerbated mitochondrial fission. Therefore both in vivo situations persuasively demonstrate that more miRNA-361 triggers CM apoptosis following myocardial ischemic injury thereby inducing an enlarged infarct zone as compared to WT mice (161). Further evidence that X-linked miRNAs critically impact mitochondrial morphology can be derived from the fact that they regulate PTEN-induced putative kinase 1 (PINK1), a mitochondrial protein kinase that is involved in ensuring mitochondrial quality control which identifies damaged mitochondria and targets them for degradation. In CMs exposed to high levels of oxidative stress, an inverse relationship was observed between miR-421 and Pink1 expression levels (162). Although miRNA-421 in this particular setting was regulated via the transcription factor E2F1 (which role in HFpEF development has not been studied yet but which interplay with NF- κ B coordinates the inflammatory response of CMs (163)), this study indicates that miRNA-421 balance is a determinant of myocardial infarct size by determining the degree of mitochondrial fragmentation in CMs in response to oxidative stress (162).

Taken together, the fact that overexpression of the X-linked miRNA-361 and -421, perturbs mitochondrial function could provide additional mechanistic insight into female-specific aspects of CM functioning and remodelling following microvascular rarefaction and inflammation. The double dosage of these miRNAs, resulting from incomplete X-chromosome inactivation could potentially provide an alternative explanation for the fact that mitochondria in CMs from female hearts display less ROS upon damage than male hearts (164).

X-linked miRNAs mediate autophagy signalling in cardiomyocytes

Autophagy is a process whereby damaged cellular components are sequestered into lysosomes and degraded (165). In CMs, the degradation of dysfunctional mitochondria is called macro-autophagy which is crucial for CM function, because CMs display increased oxidative stress upon the accumulation of dysfunctional mitochondria (11). However, macro-autophagy efficiency is diminished by hyperglycaemia (166) and with ageing (167) which are both associated with HFpEF development. Therefore, the tight regulation of genes controlling autophagy is crucial for the degradation of dysfunctional mitochondria to preserve CM function and prevent HFpEF.

ATG7 is a key-protein required for autophagy of damaged cells and cellular remnants, with expression of this protein being increased following I/R-induced myocardial injury. The X-linked miRNA-188 was recently found to decrease expression levels of ATG7 after I/R-injury, suggesting that this miRNA is capable of fine-tuning autophagy (168). Another autophagy-related gene that is impacted by X-linked miRNAs is the apoptosis repressor with caspase-recruit domain (ARC). This anti-autophagy protein is highly expressed in healthy CMs, while ARC expression is decreased as a consequence of ischemia/reperfusion (I/R)-injury. ARC expression was found to be suppressed as a result of induced miRNA-325 expression. Short hairpin-mediated knockdown of miR-325 restored ARC levels and normalized autophagy which showed that the repression of autophagy is protective against cell death induced by myocardial I/R injury (169). In the setting of the HFpEF inducing comorbidities, hyperglycaemia and inflammation, miR-221 is involved in a maladaptive autophagy response, as cardiac-specific overexpression of miRNA-221 was found to inhibit autophagy in CMs by modulating expression levels of the cyclin-dependent kinase (CDK) inhibitor p27. This was subsequently found to activate the mammalian target of rapamycin (mTOR), which as a consequence promotes inflammatory responses to injury thereby driving CM-mediated hypertrophy and fibrosis, leading to HF (170).

Collectively, chronically elevated expression of several X-linked miRNAs may impair normal autophagy and long term CM function. This is further underscored by the fact that disruption of autophagy by miR-221 in the setting of hyperglycaemia and inflammation activates a CM remodelling response resulting in HF. Therefore,

defective autophagy in CMs, induced by incomplete silencing of X-linked miRNAs may activate a HFpEF progressive mechanism in women.

X-linked miRNAs mediate the cardiomyocyte remodelling response to injury

Reduced endothelial NO levels in HFpEF, promote CM stiffening and interstitial fibrosis (18) thereby triggering left atrial (LA) enlargement (171) and concentric LV hypertrophy (172, 173). This is particularly evident in women with HFpEF who have a higher prevalence of LV concentric remodelling resulting in a more pronounced diastolic dysfunction compared to men (26).

The finding that a decreased cardiac NO bioavailability in HFpEF is linked to hypertrophy is highly relevant given that sex-dependent differences in cardiac eNOS expression were found to be mediated by changes in miRNA-222 expression (46). Here, a decrease in miRNA-222 expression in CMs from female mice was found to increase expression levels of the transcription factor V-ets erythroblastosis virus E26 oncogene homolog (ets-1), a miRNA-222 target gene, that subsequently enhanced expression of eNOS (46). Further ex vivo experiments with adult rat ventricular myocytes (ARVM) subjected with both a miR-222 mimic and a miR-222 inhibitor, demonstrated that eNOS expression inhibition was indeed regulated through miRNA-222 expression differences (46).

The central post-transcriptional role played by miRNAs in regulating CM function is further exemplified by the discovery that inhibition of the X-linked miRNA-652 in the setting of pressure overload-induced cardiac hypertrophy in mice attenuated LV thickening (174). The resultant improvement in heart function was a direct result of changes in Jagged1 protein levels, a direct target of miRNA-652, which is a Notch signalling ligand that drives excessive LV remodelling, a phenomena commonly observed in HFpEF. Interestingly the improvement in heart function in these mice was associated with well-preserved angiogenesis and therefore a reduction in cardiac fibrosis. However, miR-652 knockdown in this study was performed using systemic delivery of antisense oligonucleotides in mice while gene expression analysis was carried out 8 weeks after injection which probably resulted in no effects on CM proliferation, despite the other observed beneficial effects.

Alongside miRNA-652, hypertrophied CMs also express decreased levels of X-linked miRNA-223 and miRNA-221, which normally inhibit cardiac troponin

I-interacting kinase (TNNT3K) mRNA, and p27 respectively (175, 176). Similarly, also the X-linked miR-98 displayed protective effects in CMs because this particular miRNA has been found to inhibit CM hypertrophy, induced by Ang II-mediated cyclinD2 expression. This study demonstrated that cyclinD2 overexpression by Ang-II triggers the antihypertrophic actions of miRNA-98 via inhibition of cyclinD2 (177). Nonetheless, it should be noted that the inhibitory effects were partial, suggesting that the antihypertrophic actions of miR-98 could potentially be mediated by cyclin D2-independent mechanisms as well. This suggests that the expression of miRNA-223 (in rats), miRNA-98 (in mice) and miRNA-221 (in mice and humans) in CMs, is protective and serves to limit chronic remodelling of CMs.

X-linked miRNAs augment the diagnostic potential of currently known heart failure biomarkers

Diagnosing HFpEF requires imaging evidence of normal systolic LV function (LV ejection fraction, LVEF, $\geq 50\%$) in combination with increased LV wall thickness and diastolic stiffness (26, 94). However, given that Doppler imaging techniques fail to adequately reflect LV filling pressures (178) alternative approaches to improve the detection of HFpEF have been proposed, including the identification of novel HFpEF biomarkers (179). Importantly, currently employed diagnostic HF biomarkers, such as natriuretic peptides, are substantially lower in HFpEF than HFrEF, and therefore do not adequately provide a diagnosis of this primarily female-associated microvascular disease (180). Furthermore, recently discovered HFpEF biomarkers are primarily geared towards the detection of inflammatory bioactive molecules, while the levels of such inflammation-related biomarkers are traditionally lower in women than men (181). Evidence that existing approaches for detecting HFpEF are insufficient can be derived from the higher prevalence of unrecognized HFpEF in women with type 2 diabetes (>60 years of age) (28%) as compared to men (18.4%) (121).

Clinical studies have shown that miRNAs could be useful as a biomarker for a diagnosis of HFpEF, or may clinically differentiate between HFpEF and HFrEF. For instance, a miRNA profiling study by Wong et al, identified a subset of 6 significantly altered circulating miRNAs in HFpEF patients (miR-1233, -183-3p, -190a, -193b-3p, -193b-5p, and -545-5p) as compared to healthy controls (182). Interestingly, this study revealed that the most significant increase in expression for HFpEF was seen for the X-chromosome located miRNA-545-5p (3.02 ± 1.13 ; $p < 0.05$) which

exceeded the other identified miRNAs in mediating an improvement in NT-proBNPs potential to discriminate between HFpEF and healthy controls (182). In contrast, another miRNA profiling study by Watson et al. identified that miR-375, miR-146a, miR-30c, miR-328 and miR-221 differed in plasma from HFpEF compared to HFrEF patients, as well as in plasma from HF and no-HF patients (183). Surprisingly, while the X-chromosome located miRNA-221 differentiated HFpEF from HFrEF in this study, it distinguished HF from healthy controls in the study by Wong et al. These differences have been attributed to variances in methodology (184), but future studies could potentially test whether this X-chromosome located miRNA is indeed female-specific in the aforementioned disease cohorts. Furthermore, although miRNA profiling as described by Watson et al. was performed using pooled samples with a single array readout per cohort, their comparative expression analysis of the previously mentioned miRNAs in 225 HF patients is elegant. These studies uncovered a reduction of these miRNAs in HFpEF, suggesting their potential use as biomarkers for the early detection of HFpEF-related symptoms. Finally, another attempt to enable more early detection of HFpEF-related symptoms was provided with a miRNA profiling study in patients with diastolic dysfunction, a key feature of HFpEF. Interestingly, this study demonstrated a reduction in circulating levels of the X-chromosome located miRNA-500 in diastolic dysfunction (185). Although these expression values were based on a limited number of study samples and the results were not confirmed in a larger population, this study provides evidence that the X-linked miRNA-500 could be useful as a biomarker for diastolic dysfunction in female HFpEF cohorts.

Collectively, these studies have identified numerous miRNA biomarkers that are located on the X chromosome that could enhance the diagnostic potential of NT-proBNP for HFpEF. The combination of circulating miRNAs and NT-proBNP levels could augment the screening, diagnostic and prognostic potential of BNP. Given that BNP levels are generally lower in HFpEF patients (186), and that the prognostic value of BNP is different between men and women (187) the clinical addition of circulating miRNA-545-5p or miRNA-221 could improve the prognostic and diagnostic potential of BNP for HFpEF. Therefore, a stratification of circulating miRNA levels for women within the studies by Wong and Watson et al. could indicate whether testing for these miRNAs could improve HFpEF detection in women.

X-linked miRNAs have shown a (sex-biased) biomarker potential in CVD

The X-chromosome origin of circulating miRNAs may potentiate their diagnostic potential resulting in a sex-biased biomarker for female patients. However, despite the fact that these miRNAs have a sex-specific biomarker potential for a sex-specific CVD such as HFpEF, it is worth noting that several of these X-linked miRNAs have also shown a biomarker potential for other type of diseases. Nonetheless, striking examples of this sex-specific biomarker potential were recently found in female patients with metabolic syndrome, a clinical phenotype of abdominal adiposity and insulin resistance (188). As compared to males, elevated circulating miRNA-221 levels in these patients were positively correlated with metabolic syndrome-associated risk factors such as high blood pressure and low HDL (188). This female specific diagnostic potential of miRNA-221 was also seen in obese females during pregnancy (189) and with preeclampsia (190). Furthermore, women with pulmonary hypertension show a more pronounced decrease in circulatory miRNA-223 levels than men (191). While extensive studies have identified differential expression of other circulating miRNAs in association with numerous CVD and HFpEF co-morbidities, the potential sex-bias in these settings was not determined (192-198). Therefore, a meta-analysis of these datasets in which stratifications are introduced for females and males could indicate whether these miRNAs could accelerate the detection of HFpEF in women.

Concluding remarks

In conclusion, the cellular response of ECs, VSMCs and CMs to metabolic stress and vascular inflammation is preceded by marked changes within the cellular transcriptome. Upstream these transcript changes, it has become clear that in particular post-transcriptional networks drive the cellular response to the pathophysiology that leads to HFpEF. The comorbidities-induced vascular inflammatory state in HFpEF patients could potentially activate sex-biased miRNA networks which integrate the regulation of multiple sets of functionally related genes in ECs, VSMCs and CMs. As such these sex-biased miRNA networks could be responsible for the pathophysiological differences that are observed between women and men (Figure 4) with HFpEF. Even though gender-specific therapies for HFpEF have not been identified yet, a focus on the miRNA induced-post-transcriptional mechanisms provoking this female gender preponderance in HFpEF could offer an alternative understanding of this heterogeneous clinical phenotype. This demonstrates that while E2-induced miRNAs predominantly appear to serve

protective functions, many of the X-linked miRNAs that have been reviewed here, seem to associate with a deterioration in microvascular and myocardial integrity. Therefore, the relative overexpression of these X-linked miRNAs, due to incomplete allelic silencing, may well contribute to the molecular mechanisms that underlie the female-specific cardiovascular aetiology such as observed for HFpEF. This observation pinpoints the profit of sex-specific analysis in basic research, which incorporation could improve our understanding of sex-specific cellular mechanisms in disease (199). Given the recent progress in clinical RNA-therapeutic approaches that allow silencing or activation of these upstream regulators, this could in time allow the development of female-specific therapies to prevent HFpEF in women.

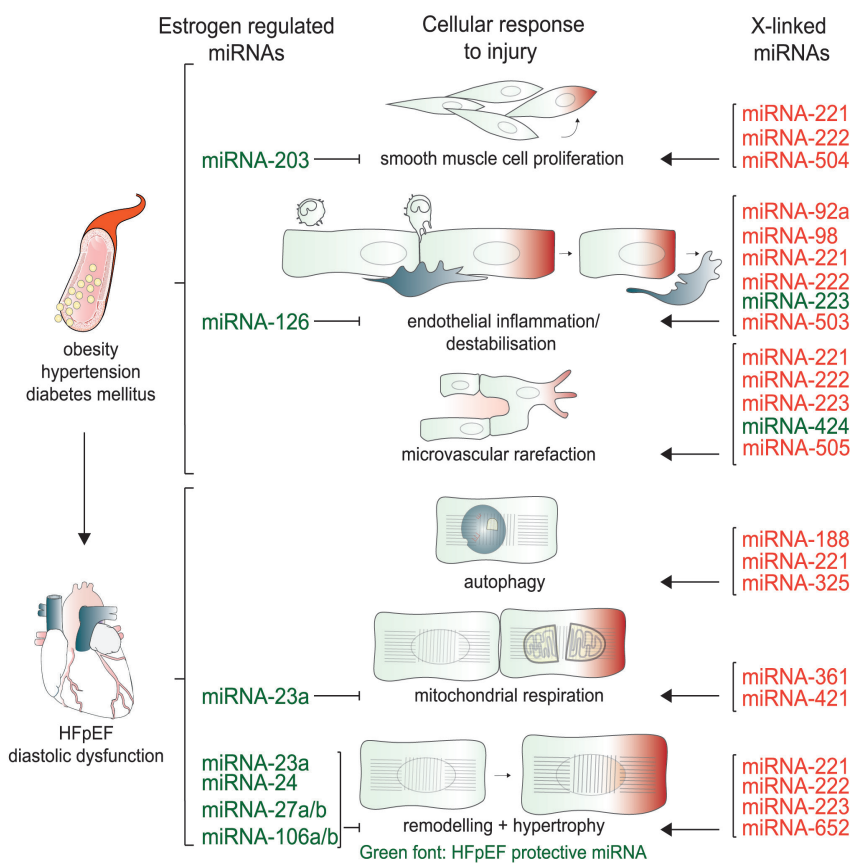


Figure 4. Overview of sex-biased miRNA involvement in the successive pathophysiological steps leading to HFpEF.

Figure 4. Continued.

Figure depicts the sex-biased miRNA with regulatory roles within the different steps of the HFpEF cascade from endothelial inflammation towards global cardiac remodeling and dysfunction. The middle panel depicts the cellular response to injury in the progression of HFpEF on which estrogen-regulated miRNAs (left side figure) and X-linked miRNAs (right side figure) were shown to have an impact.

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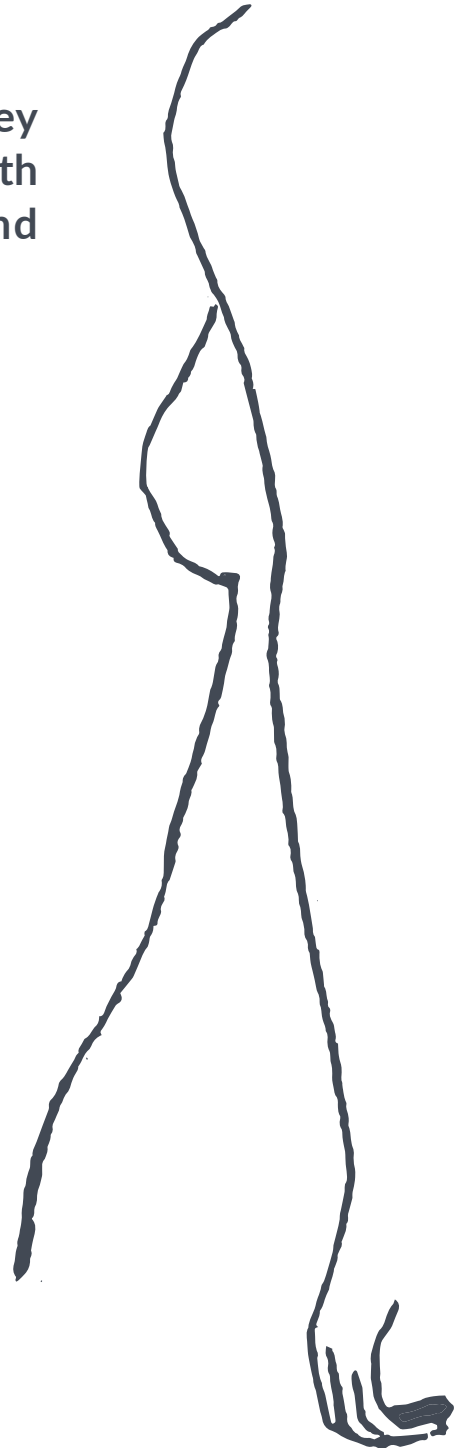
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Chapter 3

Acute Rejection After Kidney Transplantation Associates With Circulating MicroRNAs and Vascular Injury

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R. Bijkerk, B.W. Florijn, M. Khairoun,
J.M.G.J. Duijs, G. Ocak, A.P.J. de Vries,
A.F. Schaapherder, M.J.K. Mallat, J.W. de
Fijter, T.J. Rabelink, A.J. van Zonneveld,
M.E.J. Reinders



Abstract

Background. Acute rejection (AR) of kidney transplants is associated with the loss of endothelial integrity, microvascular rarefaction and, ultimately, graft dysfunction. Circulating angiogenic microRNAs (miRNAs) may serve as markers for microvascular injury. Here, we investigated short-and long-term effects of AR after kidney transplantation on systemic vascular injury and the associated circulating miRNA profile. **Methods.** Systemic vascular injury was determined by measuring capillary tortuosity and density within the oral mucosa as well as by assessing circulating levels of angiopoietin-2 (Ang-2)/angiopoietin-1 (Ang-1)-ratio, vascular endothelial growth factor (VEGF) and soluble thrombomodulin (sTM). Following a pilot study, we selected 48 miRNAs to assess the AR- and microvascular injury associated circulating miRNAs. **Results.** In stable transplant recipients (n=25) and patients with AR (n=13), that were also studied longitudinally (1, 6 and 12 months post-AR), we found an AR-associated increase in markers of systemic vascular injury, of which VEGF and sTM normalized within one year after AR. Out of 48 selected miRNAs, eight were either decreased (miR-135a, miR-199a-3p and miR-15a) or increased (miR-17, miR-140-3p, miR-130b, miR-122 and miR-192) in AR. Of these, miR-130b, miR-199a and miR-192 associated with markers of vascular injury while miR-140-3p, miR-130b, miR-122 and miR-192 normalized within 1 year after AR. **Conclusions.** AR after kidney transplantation is characterized by systemic microvascular injury and associates with specific circulating miRNA levels.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Website (www.transplantationdirect.com).

Introduction

In the context of transplantation, microvascular endothelial cells (ECs) are very susceptible to injury that can result from episodes of acute rejection (AR). Following the alloimmune response, cytokines and growth factors are produced that can lead to EC activation and microvascular destabilization (1-6). These rejection-associated events can result in perpetual EC damage and promotion of (aberrant) angiogenesis within the allograft (1, 3, 5). Together, these insults lead to the loss of the microvasculature, chronic ischemia and cell death (7, 8) and, ultimately, to the development of interstitial fibrosis/tubular atrophy (IFTA) and graft dysfunction (1, 2, 5). The rate-limiting role of microvascular integrity in AR associated graft loss was supported in a study demonstrating that when EC injury was inhibited (by limiting ongoing inflammation) and the microvasculature was maintained intact, physiological remodelling occurred and allograft tissue returned to normal (5, 8, 9).

We previously demonstrated that microRNAs (miRNAs) play a major role in maintaining microvascular integrity (10). Additionally, miRNA dysregulation is involved in the pathophysiology of diseases associated with microvascular injury (11, 12). Recently, we also demonstrated that circulating miRNAs could serve as markers of such microvascular injury after simultaneous pancreas kidney transplantation (SPK) (13). Furthermore, these circulating miRNAs might provide insight in and contribute to the underlying pathology (14, 15). Several microRNAs, both in the allograft tissue as well as circulating, have been described to associate with AR (16-24). However, their relation to vascular injury during and after the rejection episode is largely unexplored. Here, we investigated whether circulating miRNAs could serve as markers of microvascular injury in the context of AR. To that end, we first explored the relation of AR with systemic microvascular injury. Then, in a cross-sectional study of patients with AR, we measured circulating miRNA levels and associated them with markers of systemic vascular injury, including vascular endothelial growth factor (VEGF), soluble thrombomodulin (sTM) and angiopoietin-2 (Ang-2), which all play a central role in the angiogenic and inflammatory responses (1-3, 6, 25-27). Additionally, we employed sidestream darkfield (SDF) imaging, a non-invasive tool to visualize the human microcirculation (28) that we previously used to investigate the labial mucosal capillary tortuosity as marker for systemic microvascular disease in diabetes mellitus type I (DM) patients before and after SPK (29). Finally, in a longitudinal study, patients were followed

up for one year after the AR episode to evaluate longer term effects on vascular injury and circulating miRNAs. Taken together, we demonstrate that circulating miRNAs associate with microvascular injury in AR and could potentially be used to monitor microvascular integrity in disease progression.

Subjects and Methods

Transplantation

All procedures were approved by the Leiden University Medical Center's Medical Ethical Committee, all methods were performed in accordance with the relevant guidelines and regulations and all patients in the studies were enrolled after given informed consent on all aspects of the study. All patients underwent solitary kidney transplantation at the Leiden University Medical Center (LUMC) between 2003 and 2012. Kidney transplantation was performed as described previously (24). Average treatment regimen involved prednisone (tapered to a dose of 10 mg by 6 weeks and a dose of 7.5 mg by 3 months), cyclosporine (targeted to an area under the curve (AUC) 5400 ng.h/ml first 6 weeks then 3250 ng.h/ml) or tacrolimus (AUC 210 ng.h/ml first 6 weeks, then 125 ng.h/ml) and mycophenolate mofetil (MMF) (AUC 30-60 ng.h/ml). In case of side effects patients were converted to everolimus (AUC 120-150 ng.h/ml). All patients received induction treatment with basiliximab (40 mg at day 0 and 4) or daclizumab (100 mg/day on the day of transplantation and 10 days after transplantation).

Cross sectional and longitudinal acute rejection (AR) study population

In the cross sectional study, a total of 38 patients (24 males and 14 females) were enrolled. This cohort (marked as 'cohort A' for clarity purposes) is used in the studies found in Figures 1 to 5. All measurements were performed before patients received treatment for AR. None of the 38 patients had signs of infection at the time of the measurements. From those 38 patients, the control group (stable group) consisted of 25 kidney transplant recipients with of eGFR>30 ml/min and stable renal function after transplantation. Thirteen patients were included in the AR group because of a decrease in renal function and biopsy proven acute rejection. Biopsies were evaluated using the Banff criteria by two independent pathologists. Pathological characteristics including Banff grade are summarized in Table 1 and Supplementary Table 1 (<http://links.lww.com/TXD/A45>). All transplant recipients were negative for pre-transplant donor specific antibodies (DSA), as well as *de*

novo DSA after transplantation. Also, viral (BK, CMV) or urinary tract infections were absent. Patients with SPK, active infection, liver failure, active auto-immune disease, epilepsy or malignancy in the last 5 years (except patients treated for basal cell carcinoma who were in full remission) were excluded from the study. The patients in the AR group were also studied prospectively in a longitudinally study at 1 (M1, n=13), 6 (M6, n=13) and 12 months after the AR episode (M12, n=11, two patients discontinued their participation after a second rejection episode).

Stable transplantation study population

In this control cohort (marked as 'cohort B' for clarity purposes), a total of 48 patients were enrolled. This cohort is used in the studies found in Supplementary Figures 1 and 2 (<http://links.lww.com/TXD/A45>) and served to assess the effect of transplantation itself on the levels of markers of vascular injury and circulating miRNAs. The control group of this cohort (controls) consisted of 20 healthy controls. Twenty-eight chronic kidney disease (CKD) patients were included because they received a kidney transplant. The patients in the CKD group were studied in a longitudinally study at 1 (M1, n=28), 6 (M6, n=28) and 12 months after transplantation (M12, n=28). Patient characteristics are summarized in Supplementary Table 2 (<http://links.lww.com/TXD/A45>).

Microcirculatory imaging

The SDF microscan (MicroVision Medical Inc., Wallingford, PA, U.S.A) was performed as described previously (28, 29). Before analysis, the video files were anonymized so that the assessor was blinded to the subject's identity. Capillary loops were assessed by two individual assessors in a randomized, blind fashion. From the forty video files obtained of the microcirculation in each subject, four technically best files were selected from each lip quadrant for analysis. Capillary density (capillaries/mm²) was calculated by counting the number of vessels per screen shot. Subsequently, tortuosity of capillary loops was assessed using a validated scoring system described previously and the average of assessed capillary tortuosity was used to calculate mean tortuosity index per patient (28).

Laboratory and urinary assessments

All patients underwent routine venous blood sampling that was collected before the morning intake of immunosuppression to assess creatinine, urea, HbA1c, glucose and hemoglobin. Proteinuria was measured in collected 24-hours urine. The eGFR

was calculated with plasma creatinine concentration using the Modification of Diet in Renal Disease (MDRD) formula. For miRNA analysis, plasma was harvested by centrifugation of EDTA-anti-coagulated blood for 10 min at 3000 rpm and subsequently stored at -80°C. At the same time, blood was collected for analysis of serum Ang-1, Ang-2, VEGF (VEGF-A) and sTM concentrations. Both Ang-1 and Ang-2 were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer supplied protocol. Likewise, VEGF and sTM (Gen-Probe Diacolor Research, Besançon, France) levels were assessed. Since the Ang-2/Ang-1 rather than the absolute levels of either cytokine has been considered to determine the functional status of the microvasculature (26, 27, 30), this ratio was calculated for the different groups and time points.

RNA isolation

Total RNA was isolated from 200 µL plasma using 800 TRIzol µL reagent (Invitrogen, Breda, The Netherlands) using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands) with an adapted protocol. In short, 160 µL of chloroform was added to the plasma/TRIzol sample and centrifuged for 15 min at 12000 rpm. The aqueous phase was combined with 1.5 volumes of 100% ethanol and transferred to a MinElute Spin column and centrifuged for 15 sec at 13000 rpm. Subsequently the column was washed with 700 µL RWT buffer, twice with 500 µL RPE buffer and centrifuged for 15 sec at 13000 rpm after the first two washing steps and 2 min at 13000 rpm after the last washing step. Finally, RNA was eluted with 15 µL RNase-free water.

Profiling miRNAs

For miRNA cDNA synthesis, reverse transcription of total RNA was performed using the miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). cDNA was preamplified using Megaplex PreAmp primers pools A V2.1 (Applied Biosystems) according to the protocol of the manufacturer. For the pilot, for each cDNA sample, 384 miRNAs including 6 controls (RNU44, RNU48, 4*U6) were profiled using TaqMan® Array MicroRNA Human Card A V2.0 (Applied Biosystems). For the main study, custom designed Megaplex cards were generated to determine the expression of selected set of miRNAs. Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). miRNAs levels were assessed in patients for which sufficient amounts of plasma were available (i.e. stable group, n=13; AR D0, n=13; AR M1, n=8; AR M6, n=9; AR

m12, n=11). We used the difference in total signal of all miRNAs as a correction factor for normalization.

Statistical analyses

Continuous normally distributed data are presented as mean $\pm$ SEM, unless stated otherwise. Differences between two groups in the cross-sectional study were analysed using the unpaired two-sample T-test. When criteria for parametric testing were not met, median and interquartile range (IQR) are presented and tested with the Mann-Whitney test. For categorical variables cross-tables were used and analysed with the chi-square test. In addition, multivariable linear regression was used to adjust for possible confounders. Comparisons of mean differences between the different time points in the longitudinal study were performed using ANOVA analysis. Correlations between interval variables were calculated using the Spearman rank correlation coefficient. Differences were considered statistically significant with $p < 0.05$. Data analysis was performed using SPSS version 17.0 (SPSS Inc, Chicago, IL) and GraphPad Prism, version 5.0 (GraphPad Prism Software Inc, San Diego, CA).

Results

Characteristics of cross sectional and longitudinal acute rejection (AR) study population

To investigate the relation of circulating miRNAs and markers of systemic vascular injury with AR, we set up a cross sectional study ('cohort A') including AR patients and stable controls (stable allograft function after transplantation), allowing us to collect plasma for the assessment of vascular injury markers and circulating miRNAs. In addition, these AR patients were studied longitudinally at 1, 6 and 12 months after rejection to determine long-term effects. Baseline subject and transplantation characteristics are presented in Table 1 and Supplementary Table 1 (<http://links.lww.com/TXD/A45>) (Banff Scores and pathological characteristics). All rejections in this cohort involved T-cell-mediated rejections (TCMR). Renal function of the patients with stable allograft function (47.2 ± 13 ml/min/ 1.73 mm²) was better compared to AR patients (38.1 ± 13 ml/min/ 1.73 mm², $p < 0.05$). In the longitudinal study (Table 1) patients showed significant decreased renal function during all time points after AR compared to stable patients.

Table 1: Patients characteristics of acute rejection patients during (D0) and one (M1), six (M6) and twelve (M12) months after AR compared with stable renal transplant recipients.

Patients characteristics	Stable renal transplant (N=25)		D0 (N=13)		M1 (N=13)		M6 (N=13)		M12 (N=11)	
Age (years)	53.2	± 13	51.7	± 10	51.8	± 10	52.4	± 10	52.7	± 10
Sex, male N (%)	15	(60%)	9	(69%)						
Smoking, N (%)	3	(12%)	1	(8%)	1	(8%)	1	(8%)	0	
Median time since transplantation (months) (IQR)	5	(1-19)	12	(3-22)						
Primary kidney disease, N (%)										
Glomerulonephritis	8	(32%)	0		0		0		0	
Diabetes Mellitus	3	(12%)	1	(8%)	1	(8%)	1	(8%)	1	(8%)
Pyelonephritis or interstitial nephritis	1	(4%)	2	(15%)	2	(15%)	2	(15%)	2	(15%)
Focal segmental glomerulosclerosis	3	(12%)	0		0		0		0	
Urologic	1	(4%)	1	(8%)	1	(8%)	1	(8%)	1	(8%)
Polycystic kidney disease	4	(16%)	4	(31%)	4	(31%)	4	(31%)	4	(31%)
Hypertension	2	(8%)	2	(15%)	2	(15%)	2	(15%)	2	(15%)
Unknown	2	(8%)	2	(15%)	2	(15%)	2	(15%)	2	(15%)
Other	1	(4%)	1	(8%)	1	(8%)	1	(8%)	1	(8%)
BMI (kg/m ²)	25.8	± 4	24.3	± 3	24.3	± 3	23.8	± 4	25.2	± 4
Dialysis, N (%)	4	(16%)	0		0		0		0	
Systolic BP (mmHg)	137	± 20	142	± 19	132	± 10	132	± 16	127	± 19
Diastolic BP (mmHg)	82	± 9	83	± 13	77	± 9	79	± 12	72	± 10
eGFR (ml/min/1.73 m ²)	47.2	± 13	38.1	± 13 [#]	35.4	± 15 [#]	40.2	± 16 [#]	36.0	± 18 [#]
Median proteinuria (g/24hr) (IQR)	0.2	(0.2-0.4)	0.4	(0.3-1.0) [#]	0.3	(0.2-0.7)	0.2	(0.2-0.3)	0.3	(0.1-0.3)
Glucose (mmol/L)	6.0	± 2	5.5	± 1	5.7	± 2 [*]	6.3	± 3	5.9	± 1 [*]
Hemoglobin (mmol/L)	7.9	± 1	7.6	± 1	7.3	± 1	7.7	± 1	7.7	± 1
Hematocrit (L/L)	0.40	± 0.06	0.38	± 0.04	0.36	± 0.05	0.39	± 0.05	0.39	± 0.05

Table 1: Continued.

Patients characteristics	Stable renal transplant (N=25)		D0 (N=13)		M1 (N=13)		M6 (N=13)		M12 (N=11)	
Anti-hypertensives, N (%)										
ACE inhibitor	5	(20%)	2	(15%)	3	(23%)	3	(23%)	2	(18%)
Diuretics	6	(24%)	3	(23%)	4	(31%)	2	(15%)	1	(9%)
β-blockers	11	(44%)	6	(46%)	7	(54%)	7	(54%)	6	(55%)
Calcium antagonists	14	(56%)	8	(62%)	9	(69%)	6	(46%)	6	(55%)
Angiotensin-II antagonists	1	(4%)	3	(23%)	3	(23%)	2	(15%)	3	(27%)
Statins, N (%)										
	9	(36%)	5	(39%)	6	(46%)	7	(54%)	7	(64%)
Immunosuppressive, N (%)										
Cyclosporine	3	(12%)	3	(23%)	3	(23%)	2	(15%)	0	
Tacrolimus	19	(76%)	9	(69%)	9	(69%)	10	(77%)	9	(82%)
Prednisone	24	(96%)	13	(100%)	13	(100%)	13	(100%)	11	(100%)
Everolimus	2	(8%)	1	(8%)	0		0		1	(9%)
Mycophenolate Mofetil	21	(84%)	9	(69%)	9	(69%)	10	(77%)	7	(64%)
Anti-rejection, N (%)										
Methylprednisolone			11	(84%)						
ATG			1	(8%)						
Alemtuzumab			1	(8%)						
Donor characteristics										
Age (years)	52.3	± 9	46.4	± 11						
Sex, male N (%)	8	(32%)	4	(31%)						
Donortype, deceased donors, N (%)	5	(20%)	5	(39%)						

All data are mean ±SD, unless otherwise specified. *p<0.05 compared to stable patients. †P<0.05 compared to AR. BMI, body mass index; BP, blood pressure ACE, angiotensin converting enzyme; eGFR, estimated glomerular filtration rate; IQR, interquartile range.

Patients with AR have increased capillary tortuosity and elevated levels of Ang-2/Ang-1 ratios, VEGF and sTM

We investigated whether AR resulted in changes in capillary tortuosity, indicating systemic vascular injury. Indeed, patients with AR showed significantly increased capillary tortuosity compared to stable patients (1.70 ± 0.1 vs. 1.41 ± 0.06 , $P > 0.01$) (Fig 1AB), also after adjustment for age, sex, BMI, blood pressure, glucose levels and smoking. Capillary density showed no significant difference between the stable renal function group and patients with AR ($p = \text{NS}$, data not shown).

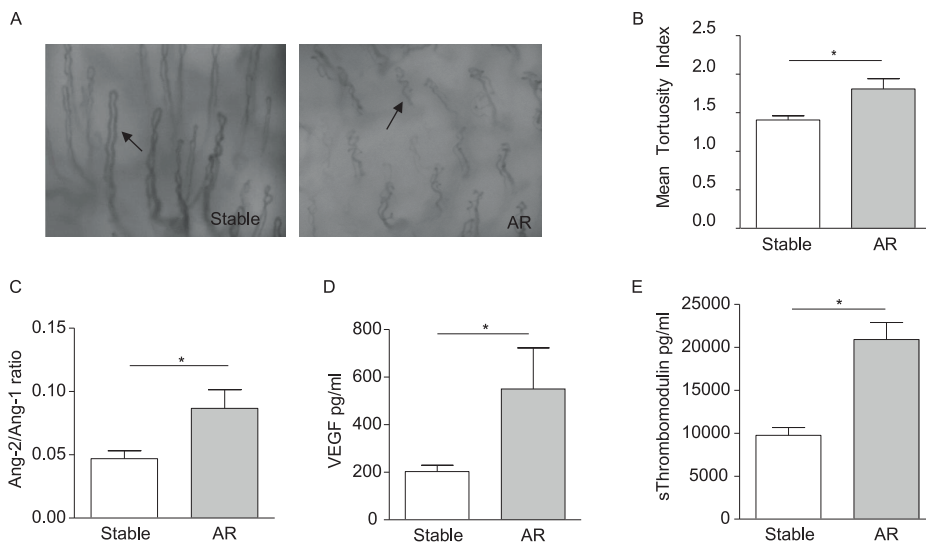


Figure 1. Increase in markers of vascular injury in acute rejection.

(A) Sidestream darkfield images of the oral mucosa visualizing the microvascular capillaries of a representative patient in stable and AR group. Black arrow: capillary loops. (B) Mean tortuosity index of microvascular capillaries in the stable (n=25) and AR (n=13) group. (C-E) Ang-2/Ang-1 ratio (C), VEGF-A (D) and soluble thrombomodulin (E) serum levels (pg/ml) in stable and AR group. Data shown are mean ± SEM. *P<0.05 compared to stable group.

Next, to evaluate the effects of AR on the expression of angiogenic factors, we calculated the Ang-2/Ang-1 ratio and measured serum levels of VEGF and sTM. The Ang-2/Ang-1 ratio was significantly increased in AR group (0.09 ± 0.02) compared with stable renal transplant recipients (0.05 ± 0.01 , $p=0.01$) (Fig 1C). In line with these observations, both VEGF (567 ± 188 pg/ml vs. 202 ± 27 pg/ml, $p=0.02$) and sTM (19667 ± 1809 pg/ml vs. 9667 ± 921 pg/ml, $p<0.0001$) levels were increased in AR patients compared to stable recipients (Fig 1DE). The differences remained significant after adjustment for age, sex, BMI, blood pressure, glucose levels and smoking ($p<0.05$). When adjustment for kidney function was performed, sTM, VEGF and Ang2/1 ratios remained significantly different between stable and AR, while the change in capillary tortuosity became borderline significant ($p=0.07$).

Acute rejection results in a shift in circulating miRNA levels

Next, we sought to determine circulating miRNA levels, since they could serve as markers of microvascular injury as they are central regulators of microvascular pathophysiology. To select candidate miRNAs that are associated with AR we

performed a pilot study assessing plasma profiles of 384 miRNAs from 4 stable controls and 6 patients with acute rejection (Supplementary Table 3A and 3B (<http://links.lww.com/TXD/A45>)). Based on (i) differential expression (based on p-value) between the AR and stable group, (ii) high range expression levels and (iii), a reported relation of a miRNA with rejection or processes related to microvascular destabilization, we selected 48 miRNAs (indicated in Supplementary Table 3A (<http://links.lww.com/TXD/A45>)) that associated with AR, for further evaluation in the main study.

The 48 selected miRNAs were subsequently assessed in the plasma of AR and stable patients. We found 8 of them to be significantly decreased (miR-135a, miR-199a-3p and miR-15a) or increased (miR-17, miR-140-3p, miR-130b, miR-122 and miR-192) in AR compared to the stable group, after adjustment for sex and age as possible confounders (Figure 2). Complete miRNA data (AR vs. stable) can be found in Supplementary Table 4 (<http://links.lww.com/TXD/A45>).

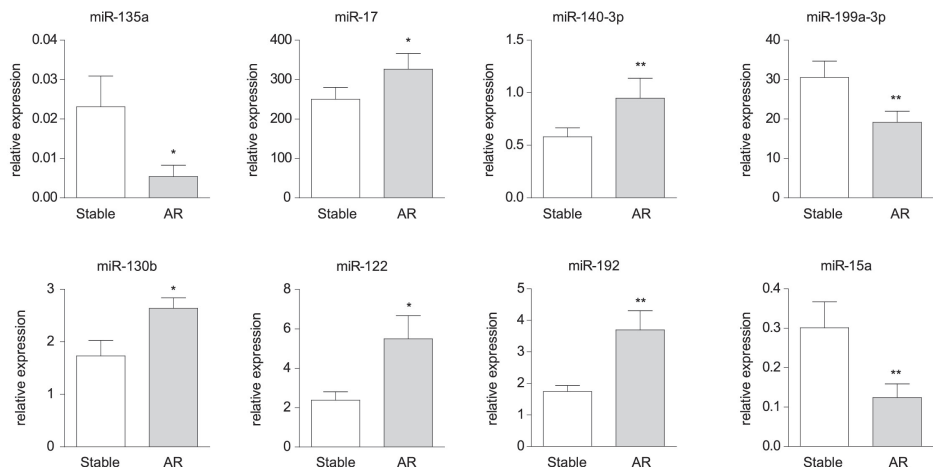


Figure 2. Differential miRNA levels in acute rejection.

MiRNAs that show statistically significant differences between stable and acute rejection (AR) group (* $P < 0.05$, ** $P < 0.001$). Means are relative expression levels and can also be compared amongst miRNAs. Stable group, $n = 13$; AR, $n = 13$. Data shown are mean $\pm$ SEM and adjusted for sex and age as possible confounders.

Selective effects on vascular injury markers and miRNAs in the first year after acute rejection

During the follow-up period after AR, capillary tortuosity remained significantly increased at 1 month (1.72 ± 0.1 , $p < 0.01$), 6 (1.71 ± 0.4 , $p < 0.05$) and 12 months (1.74 ± 0.1 , $p < 0.001$) compared to patients with a stable renal function (Fig 3AB). Also the Ang-2/Ang-1 ratio remained elevated at 1 month (0.07 ± 0.02 , $p = 0.35$), 6 months (0.06 ± 0.02 , $p = 0.44$) and 12 months (0.07 ± 0.02 , $p = 0.36$) after AR compared with stable renal transplant recipients (0.05 ± 0.01), although not statistically significant (Fig 3C). Furthermore, VEGF serum levels remained significantly increased up to 1 month after rejection (465 ± 109 pg/ml) compared with stable renal transplant recipients (202 ± 27 pg/ml, $p = 0.03$) but decreased at 6 (368 ± 150 pg/ml, $p = 0.23$) and 12 months (257 ± 95 pg/ml, $p = 0.42$) (Fig 3D). Mean sTM levels similarly decreased at 6 months (14927 ± 2330 pg/ml, $p = 0.02$) and 12 months (10875 ± 1549 pg/ml, $p < 0.05$) after rejection compared to stable patients (Figure 3E), while 12 months after rejection sTM was also significantly different from the AR group ($P < 0.05$). The differences remained significant after correction for age, sex, BMI, blood pressure, glucose levels and smoking.

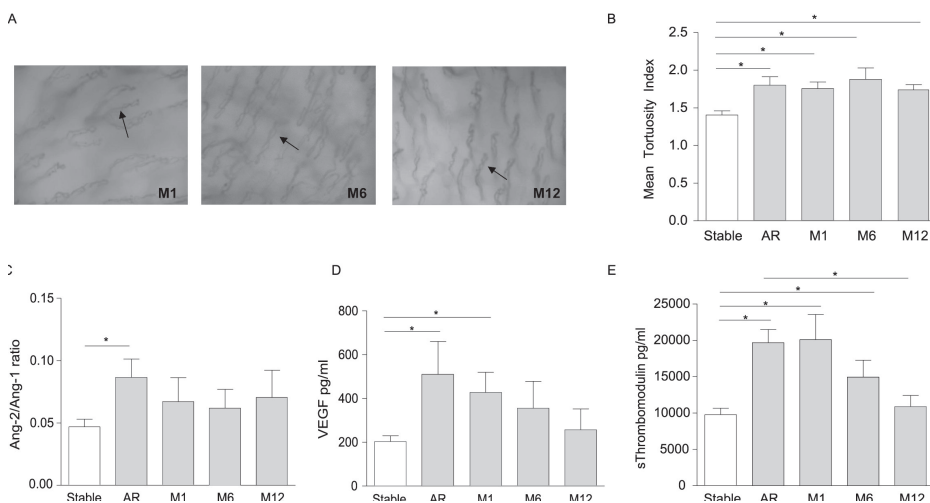


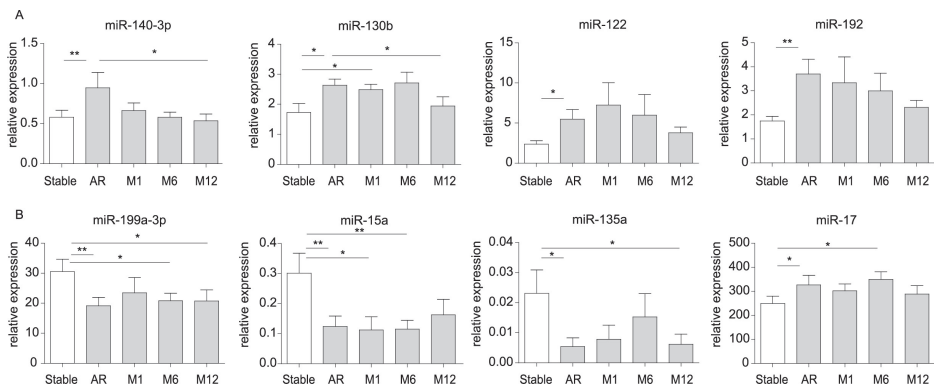
Figure 3. Normalization of VEGF and sTM, but not of Ang-2/Ang-1 ratio and microvascular tortuosity, within one year after acute rejection.

(A) Sidestream dark field images of the oral mucosa visualizing the microvascular capillaries of a representative stable kidney transplant patient, one (M1), six (M6) and twelve (M12) months after rejection in the longitudinal rejection study. Black arrows: capillary loops. (B) Longitudinal course of the mean tortuosity index of microvascular

Figure 3. Continued.

capillaries in the stable group (n=25), acute rejection (AR) group (n=13) and at one (M1, n=13), six (M6, n=13) and twelve (M12, n=11) months after rejection. (C-E) Longitudinal course of serum Ang-2/Ang-1 ratio (C), VEGF-A (D) and soluble thrombomodulin (E) levels at one, six and twelve months after rejection. Data are shown as mean $\pm$ SEM. *P<0.05.

We next assessed whether we could detect differences in the levels of the 48 selected miRNAs in the patients with AR after 1 year follow up. For the 8 miRNAs that differed statistically significant between stable and AR, we found 4 of them (miR-140-3p, miR-130b, miR-122 and miR-192) to show (a trend towards) normalization of their levels within 1 year after AR (Figure 4A). In contrast, miR-199a-3p, miR-15a, miR-135a and miR-17 remained either decreased or increased until 1 year after AR (Figure 4B). The longitudinal course of all 48 miRNAs can be found in Supplementary Table 5 (<http://links.lww.com/TXD/A45>).

**Figure 4. Circulating miRNA levels in patients with acute rejection, followed up for one year.**

(A-B) Longitudinal course of the circulating miRNA levels in the stable group, acute rejection (AR) group and at one (M1), six (M6) and twelve (M12) months after rejection. (A) miRNAs that show (a trend towards) normalization within 12 months after AR. (B) miRNAs that do not normalize within 12 months after AR. *P<0.05, statistically significant. Means are relative expression levels, and can also be compared amongst miRNAs. Stable group, n=13; AR D0, n=13; AR M1, n=8; AR M6, n=9; AR M12, n=11. Data shown are mean $\pm$ SEM.

Given that (time after) transplantation itself might contribute to changes in these vascular injury markers and miRNA levels, we included an additional control patient cohort ('cohort B') that includes kidney transplantation patients with stable renal function after transplantation, that were followed up to 12 months after transplantation. This allows distinguishing the effects of transplantation itself versus AR for these markers. Patient characteristics of this cohort are included

as Supplementary Table 2 (<http://links.lww.com/TXD/A45>). In this cohort we determined capillary tortuosity, Ang-2 (as we found Ang-2/Ang-1 ratio to be mainly determined by Ang-2 levels) and sTM. We found capillary tortuosity, Ang-2 and sTM levels to restore within 12 months after transplantation (Supplementary Figure 1 (<http://links.lww.com/TXD/A45>)). Furthermore, we measured the levels of the 8 miRNAs from Figure 2 in this additional control patient cohort (cohort B). These data are illustrated in Supplementary Figure 2 (<http://links.lww.com/TXD/A45>) demonstrating that the differences induced by transplantation itself are occurring within 1 month after transplantation, suggesting that the differences in the AR cohort cannot be the result of the transplantation or differences in time post-transplantation.

Specific miRNAs associate with markers of vascular injury

Given the relation between AR and vascular injury, we next sought to determine if we could establish correlations of vascular injury markers with specific circulating miRNAs (in cohort A). In addition, we determined correlation with renal function (creatinine levels, proteinuria and eGFR). As shown in Supplementary Table 6, which contains p-values and correlation coefficients for all comparisons, we found multiple significant correlations. Figure 5 illustrates the highlights of the significant correlations that we observed. Specifically, miR-130b, miR-199a-3p and miR-192, three miRNAs that were significantly different between the stable and AR group, showed significant correlations with sTM (miR-130b and miR-199a-3p) and capillary density (miR-192). In addition, we also found several correlations for miRNA with vascular injury markers, although their levels were not affected by AR, e.g. miR-29a, miR-93 and miR-361-5p significantly correlated with Ang-2-1 ratio, capillary tortuosity and VEGF levels, respectively (Supplementary Table 6 (<http://links.lww.com/TXD/A45>)).

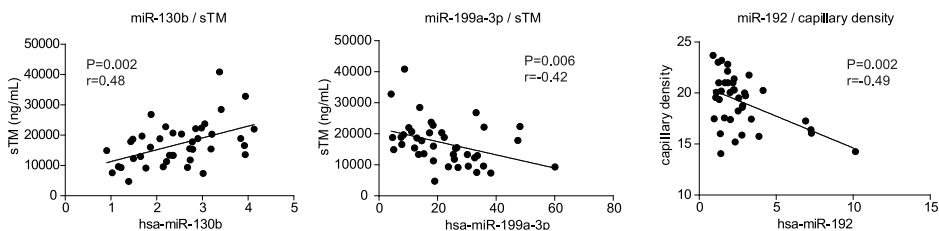


Figure 5. Correlation of circulating miRNAs with markers of (micro)vascular injury.

MiRNAs that were different between stable and AR group correlate with markers of systemic vascular injury.

Discussion

In the present study, we found that the systemic microvasculature is markedly disturbed in patients with AR compared with stable renal transplant recipients. In addition, we demonstrated that specific circulating miRNAs associate with AR and vascular injury.

Although it cannot be excluded that transplantation itself contributes to altered levels of vascular injury markers, our data regarding systemic vascular injury markers in the longitudinal AR study show that Ang-2 and capillary tortuosity maintain elevated after AR, but reverse within 1 year after transplantation, as determined in stable transplantation patients that were followed for a similar time (Figure 3 and Supplementary Figure 1 (<http://links.lww.com/TXD/A45>)). These data suggest AR-specific effects on capillary tortuosity and Ang-2. This prolonged Ang-2 induced destabilization promotes the dissociation of pericytes from ECs, resulting in the formation of abnormal capillary networks and abnormal blood flow (26). Hence, early induced endothelial injury appears to be sustained which might impact long-term graft function (31).

While initially it was thought that circulating miRNA populations were merely shed by-products of tissue damage, recent studies suggest that circulating miRNAs can be transferred to vascular target cells thereby playing an active role in vascular (dys)function (14). MiRNAs in the bloodstream are found packaged in exosomes (32) or associated with RNA-binding proteins (Argonaute2 [Ago2]) (33) or lipoprotein complexes (high-density lipoprotein [HDL]) (34). In particular exosomal miRNAs have been described to be transferred to and be functional in target cells in various tissues (35). The source of these miRNA-containing exosomes during AR may be the immune cells that drive the inflammatory response (36) or they possibly originate from the donor graft (37). As such, we believe that the circulating miRNAs we identified may play a role in the pathophysiology of AR and associated vascular injury. A promising candidate miRNA potentially relevant to AR pathophysiology that emerged from our study is miR-130b. In our previous study on circulating miRNAs in the setting of diabetic nephropathy (13), we found a strong correlation of miR-130b with sTM levels as well, similar to the findings in this study. Furthermore, miR-130b was previously found to be present and increased in (adipocyte-derived) exosomes in metabolic disease (38), while it was recently demonstrated that these

adipose-derived exosomal circulating miRNAs are transferred to target cells in various tissues exerting their function (35), providing proof of principle of (distant) cell-cell communication via exosomal miRNAs. As such, in our study it can be argued that exosomal-miR-130b (regardless of the source) can be transferred to endothelial cells where it may have pathogenic effects e.g. via targeting PPAR γ (39), which subsequently leads to endothelial cell activation (and thus increased sTM levels) and an aberrant angiogenic response (40, 41). Of note, increased levels of circulating miR-130b were also found to be associated with renal damage in lupus nephritis (42).

Similarly, we found high miR-192 levels to correlate with decreased capillary density, while miR-192 was previously found to be increased in exosomes in the setting of cardiovascular disease (43) and to play an essential role in (tumour) angiogenesis (44). Additionally, it has been demonstrated that exosomal miR-192 can be transferred to endothelial cells and subsequently abrogate the normal angiogenic program by targeting IL-8, ICAM and CXCL1 (45). Furthermore, miR-192 has been described to be involved in the development of renal fibrosis (46), whereas IF/TA is often observed in rejection and causing deterioration of renal allograft function (47). These data suggest a pathogenic link of these miRNAs to AR and vascular injury, although additional functional studies are required to further validate these associations and mechanisms. Circulating miRNAs thus represent promising candidate biomarkers for early monitoring of microvascular disease, since it is increasingly recognized that miRNAs play a major role in maintaining microvascular integrity (10), while these circulating miRNAs may also provide insight in the identification of disease specific deregulated molecular mechanisms (14, 15).

Furthermore, although most patients received a similar drug treatment regimen, we cannot exclude a role for (dose-dependent) immunosuppression-mediated effects on vascular injury and miRNA levels in these patients. Also, as levels of several miRNAs associate with kidney function, it can be argued that in patients with kidney dysfunction, a reduced clearance effect could cause increased levels of these circulating miRNAs. However, a recent study provided evidence against such an effect (48), suggesting kidney function independent effects as a cause for these differential miRNA levels. Indeed, statistical adjustment for kidney function (eGFR) showed limited influence on our results (in Figure 1).

Although speculative, selected circulating miRNAs could potentially serve as a biomarker for AR, but additional validation studies may be necessary to evaluate this potential. An additional application for the use of these noninvasive biomarkers might be found in the monitoring of the impact of novel therapeutics on vascular integrity. For instance, belatacept, which is a selective blocker of co-stimulation in acquired immunity designed to provide effective immunosuppression while avoiding the toxicities associated with calcineurin inhibitors (49), may be sparing the vasculature as well (50). Furthermore, assessment of vascular integrity might be valuable in currently performed therapies with mesenchymal stromal cells in renal transplant recipients (51, 52). Circulating miRNAs might serve as such vascular injury markers, and could potentially be ideally suited to monitor these effects.

Taken together, the current study demonstrates that circulating microRNAs associate with AR and (systemic) microvascular injury. Although additional (functional) studies are necessary to gain more insight, our data suggest that specific circulating miRNAs might participate and provide insight in the pathophysiology of AR, and may potentially serve as a readily assessable biomarker for monitoring the injury status of the microvasculature.

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Disclosures

The authors declare no conflicts of interest.

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Chapter 4

Vascular Calcification and not Arrhythmia in Idiopathic Atrial Fibrillation Associates with Sex Differences in Diabetic Microvascular Injury miRNA Profiles

MicroRNA
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B.W. Florijn[‡], E.M.G.J. Dudink[‡], B. Weijs,
J.M.G.J. Duijs, J. Luermans, E. Peeters, L.
Schurgers, J. Wildberger, U. Schotten, R.
Bijkerk, H.J. Crijns, A.J. van Zonneveld

[‡]Both authors contributed equally

Abstract

Background Atrial fibrillation (AF) in patients without concomitant cardiovascular pathophysiological disease, is called idiopathic atrial fibrillation (iAF). Nonetheless, iAF patients have oftentimes subclinical coronary (micro)vascular dysfunction and, particularly in women, a higher prevalence of subsequent cardiovascular comorbidities. Previously, we identified a plasma miRNA association with diabetes and microvascular injury in diabetic nephropathy (DN) patients. Therefore, in this study we assessed whether plasma levels of these diabetic, microvascular injury associated miRNAs reflect microvascular integrity in iAF patients, associate with the presence of paroxysmal arrhythmia or instead are determined by concealed coronary artery disease. **Methods** Circulating levels of a pre-selected set of diabetic, (micro) vascular injury associated miRNAs, were measured in 59 iAF patients compared to 176 sinus rhythm (SR) controls. Furthermore, the presence of coronary artery and aortic calcification in each patient was assessed using cardiac computed tomography angiography (CCTA). **Results** Paroxysmal arrhythmia in iAF patients did not result in significant miRNA expression profile differences in iAF patients compared to SR controls. Nonetheless, coronary artery calcification (CAC) was associated with higher levels of miRs-103, -125a-5p, -221 and -223 in men. In women, CAC associated with higher plasma levels of miR-27a and miR-126 and correlated with Agatston scores. Within the total population, ascending aortic calcification (AsAC) patients displayed increased plasma levels of miR-221, while women in particular demonstrated a descending aorta calcification (DAC) associated increase in miR-212 levels. **Conclusions** Diabetic microvascular injury associated miRNAs in iAF associate with subclinical coronary artery disease in a sex-specific way and confirm the notion that biological sex identifies iAF subgroups that may require dedicated clinical care.

Introduction

There is a strong association between atrial fibrillation (AF) and vascular disease, as this arrhythmia usually occurs in patients with cardiovascular comorbidities (1, 2) and, particularly in women upon diabetes mellitus (3). However, in a subset of patients with AF, meticulous phenotyping cannot identify any concomitant cardiovascular disease, a condition called idiopathic AF (iAF). Mechanistic studies in iAF patients have identified several underlying pathophysiological mechanisms that pinpoint myocardial, microvascular perfusion abnormalities in iAF (4), including oxidative stress (5), systemic inflammation (6) and subclinical coronary artery disease (7). Interestingly, in conjunction with female sex, these (micro)vascular comorbidities in iAF have been shown to augment the relative risk and event rates for stroke and transient ischemic attack (8). Therefore, early monitoring of (micro)vascular integrity in iAF patients could aid to better characterize iAF subgroups that might benefit from dedicated treatment strategies.

MicroRNAs (miRNAs) are short non-coding RNA molecules that fine-tune gene expression and thereby control functionally-related biological pathways (9). In human plasma, selected miRNAs circulate in levels that are different across (micro) vascular disease phenotypes (10) or biological sex (11). Following this notion, we recently demonstrated that a subset of circulating miRNAs associate with diabetic induced microvascular injury in diabetic nephropathy (DN) patients and correlate with conventional circulating markers of microvascular injury, such as angiotensin-2 (Ang-2), soluble thrombomodulin (sTM) and insulin-like growth factor (IGF) (12). Previous miRNA expression profiling studies in AF patients demonstrated that, miRNA-126 (13) and miRNA-152 (14) levels associated with prevalent arrhythmia, yet no relationship with vascular integrity or biological sex was established in these studies.

To determine whether circulating miRNAs could also serve as markers of (micro) vascular integrity in iAF, we assessed the association of plasma levels of 48 selected diabetic microvascular disease associated miRNAs which we previously found to be associated with (diabetic) conditions of (micro)vascular dysfunction (12, 15). Next, because iAF has also been associated with increased subclinical coronary artery disease (7), we determined whether selected circulating miRNA levels associated with coronary artery disease on cardiac computed tomography angiography (CCTA). Finally, because diabetes in women is a significant predictor of AF development (3) and women with iAF are at a higher risk of stroke or transient ischemic attack (8), we stratified circulating

miRNA levels according to biological sex, to evaluate whether biological sex is a determinant of the association between vascular calcification and circulating miRNAs.

Methods

Patient selection

This study was approved by the Institutional Review Board and complied with the ethical principles of the Declaration of Helsinki. All patients gave written informed consent. Of all consecutive patients who underwent CCTA in the Maastricht University Medical Center between January 2008 and March 2011, 115 iAF patients referred for work-up for pulmonary vein isolation (PVI) and 275 age, sex and PROCAM-score-matched (16) SR-controls were selected as previously described (7). For RNA isolation we selected the 235 patients (59 iAF, 176 SR) of whom EDTA-plasma was available.

Computed tomography

Non-contrast enhanced coronary calcium scans were performed in all patients as described previously (7). Scans were performed using an uniform scan protocol (240 to 400 mA (depending on body weight), at 80 to 120 kV) on a 64-slice CT platform (Brilliance, Philips Healthcare, Best, the Netherlands) and second generation dual source CT (Definition Flash, Siemens Healthcare, Forchheim, Germany). Cases and controls were equally distributed among both scanners. Two independent observers determined calcification of the coronary arteries (CAC), ascending (AsAC) and descending aorta (DAC) using dedicated vendor specific software. Presence of calcification was defined as Agatston > 0; the Agatston score was determined using a 3 mm CT slice thickness and a detection threshold of ≥ 130 HU involving $\geq 1\text{mm}^2$ area/lesion (3 pixels)(17). In case of ambiguity, final judgment was reached in a consensus conference. Calcium above the origin of the right coronary artery to the end of scan range, or up to the origin of the brachiocephalic artery, was considered to be in the ascending aorta. Calcium present distal from the origin of the left subclavian artery up to the diaphragm was considered to be localized in the descending aorta.

RNA isolation and microRNA profiling

RNA was isolated from 200 μL EDTA-plasma with 800 μL Trizol reagent (Invitrogen, Breda, the Netherlands) using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands) with an adapted protocol. Briefly, chloroform was added to the plasma/Trizol mixture

and centrifuged for 15 min at 15000g. The aqueous phase was combined with 1.5 volumes of 100% ethanol, conveyed to a MinElute Spin column (Qiagen) and centrifuged for 15 s at 18000 g. RNA was washed with 700 μ L RWT buffer, twice with 500 μ L RPE buffer and centrifuged for 15 s at 18000 g after the first two washing steps and 2 min at 18000 g after the last washing step. RNA was eluted with 15 μ L RNase-free water.

MiRNAs were selected based on their previously established association with (micro)vascular dysfunction in profiling studies (12, 15). (Supplementary table 1) For miRNA cDNA synthesis, reverse transcription of total RNA was performed using the miR reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was pre-amplified using Megaplex PreAmp primers pools A V2.1 (Applied Biosystems) according to the manufacturer's protocol. Custom designed Megaplex cards were generated to determine the expression of the selected microRNAs. Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Obtained miRNA cycle threshold (Ct)-values were normalized using the median Ct value of the expression of 48 measured miRNAs per patient (18).

Statistical analysis

Baseline characteristics are presented as mean $\pm$ SD and differences between groups were compared using a student T-test, or number (%) and compared using χ^2 -tests. MiRNA-values are presented as mean $\pm$ SD and the between groups differences were assessed using a Student's T-test and general linear modeling to correct for age, sex and vitamine K antagonist-use. In addition, miRNA-126 and miRNA-223 levels were corrected for aspirin use since aspirin inhibits platelet activation thereby decreasing the levels of both miRNAs. (19).

Results

Baseline characteristics of iAF patients compared to SR controls

Baseline characteristics of the iAF patients and the SR controls are listed in Table 1. Patients with iAF had a significantly larger left atrial diameter (LAd) and end systolic volume (ESV), while the left ventricular posterior wall diameter (LVPWd) was significantly smaller. Significantly different CAC, AsAC and DAC Agatston scores between groups are listed in Table 1. Patients with CAC were on average older, more often male and had larger LAd while patients with or without aortic calcification were comparable except for their age.

Table 1: Clinical characteristics of patients with or without idiopathic atrial fibrillation, calcification in the coronary arteries, ascending- or descending aorta.

	SR (n=176)	iAF (n=59)	p-value	CAC- (n=161)	CAC+ (n=71)	p-value	AsAC- (n=184)	AsAC+ (n=51)	p-value	DAC- (n=178)	DAC+ (n=57)	p-value
Age (years)	54.3 ± 9.3	54.4 ± 10.1	0.93	52.6 ± 9.5	57.8 ± 8.1	<0.001	52.8 ± 9.8	59.8 ± 5.3	<0.001	52.6 ± 9.6	59.9 ± 6.2	<0.001
Sex (female)	51 (29.0)	19 (32.2)	0.64	59 (36.6)	10 (14.1)	0.001	48 (26.1)	22 (43.1)	0.02	49 (27.5)	21 (36.8)	0.18
Atrial fibrillation				37 (23.0)	21 (29.6)	0.29	48 (26.1)	11 (21.6)	0.51	45 (25.3)	14 (24.6)	0.91
AF duration (months)		45.3 ± 49.7		35.5 ± 53.4	58.2 ± 64.6	0.18	42.3 ± 60.3	57.5 ± 58.0	0.45	37.9 ± 54.8	69.3 ± 70.3	0.16
Systolic blood pressure (mm Hg)	127 ± 9	127 ± 13	0.66	126 ± 9	127 ± 11	0.52	127 ± 10	127 ± 10	0.80	127 ± 10	126 ± 9	0.37
Diastolic blood pressure (mm Hg)	80 ± 10	78 ± 12	0.25	79 ± 10	82 ± 11	0.03	80 ± 11	79 ± 10	0.67	80 ± 11	79 ± 8	0.93
BMI (kg/m ²)	26.0 ± 3.5	26.1 ± 3.0	0.85	26.0 ± 3.5	26.3 ± 3.1	0.62	26.0 ± 3.4	26.2 ± 3.2	0.79	26.2 ± 3.4	25.8 ± 3.3	0.51
BSA (m ²)	1.9 ± 0.2	2.0 ± 0.2	0.13	2.0 ± 0.3	2.0 ± 0.2	0.53	2.0 ± 0.2	1.9 ± 0.2	0.18	2.0 ± 0.2	1.9 ± 0.2	0.45
Creatinine (umol/L)	84 ± 13	87 ± 13	0.08	83 ± 13	88 ± 12	0.01	85 ± 13	82 ± 13	0.14	85 ± 13	83 ± 13	0.23
eGFR (ml/min/1.73m ²)	97 ± 24	97 ± 22	0.96	99 ± 25	92 ± 19	0.07	99 ± 26	89 ± 18	0.02	99 ± 24	89 ± 21	0.01
Total cholesterol (mmol/L)	5.5 ± 1.0	5.5 ± 1.0	0.88	5.5 ± 0.9	5.5 ± 1.0	0.96	5.4 ± 0.9	5.6 ± 1.0	0.42	5.4 ± 1.0	5.7 ± 0.9	0.11
LDL-cholesterol (mmol/L)	3.4 ± 0.9	3.5 ± 0.8	0.19	3.4 ± 0.8	3.5 ± 1.0	0.46	3.4 ± 0.9	3.5 ± 0.8	0.30	3.4 ± 0.9	3.6 ± 0.8	0.06
HDL-cholesterol (mmol/L)	1.3 ± 0.4	1.3 ± 0.4	0.22	1.3 ± 0.4	1.3 ± 0.4	0.45	1.3 ± 0.4	1.4 ± 0.4	0.20	1.3 ± 0.4	1.4 ± 0.5	0.04
Triglycerides (mmol/L)	1.7 ± 0.9	1.5 ± 0.9	0.35	1.6 ± 0.9	1.7 ± 0.9	0.27	1.6 ± 0.9	1.7 ± 0.9	0.86	1.7 ± 0.9	1.5 ± 0.7	0.10
Glucose (mmol/L)	5.6 ± 0.7	5.3 ± 0.6	0.02	5.5 ± 0.7	5.4 ± 0.8	0.41	5.5 ± 0.8	5.5 ± 0.6	0.91	5.5 ± 0.8	5.6 ± 0.5	0.52
Echocardiography												
Left atrial diameter (mm)	37 ± 5	39 ± 5	0.003	37 ± 5	39 ± 5	0.02	37 ± 5	39 ± 5	0.11	37 ± 5	38 ± 5	0.47
IVSd (mm)	8.8 ± 1.1	8.5 ± 0.7	0.08	8.6 ± 1.0	8.8 ± 0.9	0.25	8.7 ± 1.0	8.6 ± 1.1	0.73	8.6 ± 1.0	8.8 ± 1.0	0.33

Table 1: Continued.

	SR (n=176)	iAF (n=59)	p-value	CAC- (n=161)	CAC+ (n=71)	p-value	AsAC- (n=184)	AsAC+ (n=51)	p-value	DAC- (n=178)	DAC+ (n=57)	p-value
LVPWd (mm)	8.7 ± 1.1	8.4 ± 0.6	0.02	8.5 ± 1.1	8.7 ± 0.8	0.21	8.6 ± 1.0	8.5 ± 1.0	0.74	8.5 ± 1.1	8.7 ± 0.8	0.33
EDV (ml)	112 ± 25	118 ± 25	0.23	115 ± 25	114 ± 27	0.96	115 ± 25	110 ± 25	0.29	116 ± 26	108 ± 23	0.27
ESV (ml)	35 ± 12	46 ± 13	<0.001	40 ± 13	38 ± 13	0.35	40 ± 14	40 ± 12	0.99	40 ± 14	38 ± 12	0.51
LVEF (%)	60 ± 5	62 ± 5	0.39	61 ± 5	61 ± 5	0.96	60 ± 5	62 ± 5	0.25	61 ± 5	61 ± 4	0.31
CT Agatston Scores												
Coronary artery	27.1 ± 86.7	69.8 ± 154.4	0.04		123.4 ± 168		27.7 ± 89.9	73.3 ± 159.3	0.06	28.8 ± 93.9	65.3 ± 143.4	0.08
Aortic Valve	6.4 ± 25.1	16.8 ± 56.6	0.17	5.6 ± 22.8	17.1 ± 54.9	0.09	3.2 ± 19.9	30.0 ± 63.2	0.004	2.6 ± 15.7	29.1 ± 63.7	0.003
Ascending aorta	5.4 ± 24.7	7.7 ± 35.9	0.58	2.9 ± 11.0	13.3 ± 47.3	0.07		27.7 ± 54.9		1.8 ± 9.2	19.1 ± 52.4	0.02
Descending aorta	28.2 ± 124.8	44.8 ± 192.2	0.44	18.0 ± 94.3	66.3 ± 218.4	0.08	25.4 ± 133.0	57.4 ± 178.8	0.16	133.4 ± 271.0		
Medication												
VKA	0 (0.0)	15 (25.4)	<0.001	6 (3.7)	9 (12.7)	0.01	12 (6.5)	3 (5.9)	0.87	9 (5.1)	6 (10.5)	0.14
Aspirin	21 (12.1)	33 (56.9)	<0.001	34 (21.4)	19 (27.5)	0.31	43 (23.8)	11 (22.0)	0.80	42 (24.0)	12 (21.4)	0.69
Beta blocker	28 (16.2)	23 (39.7)	<0.001	30 (18.9)	20 (29.0)	0.09	41 (22.7)	10 (20.0)	0.69	39 (22.3)	12 (21.4)	0.89
Digoxin	0 (0.0)	8 (13.8)	<0.001	6 (3.8)	2 (2.9)	0.74	5 (2.8)	3 (6.0)	0.27	5 (2.9)	3 (5.4)	0.37
Nondihydropyridine CCB	1 (0.6)	5 (8.6)	0.001	1 (0.6)	5 (7.2)	0.004	6 (3.3)	0 (0.0)	0.19	3 (1.7)	3 (5.4)	0.14
Statin	19 (10.8)	7 (11.9)	0.82	17 (10.6)	9 (12.7)	0.64	17 (9.2)	9 (17.6)	0.09	20 (11.2)	6 (10.5)	0.88

Data is expressed as mean ± SD or n (%). AF, Atrial Fibrillation; SR, Sinus Rhythm; CAC, Coronary Artery Calcification; AsAC, Ascending Aorta Calcification; DAC, Descending Aorta Calcification; BMI, Body Mass Index; BSA, Body Surface Area; CAD, Coronary Artery Disease; VKA, Vitamin K Antagonist; CCB, Calcium Channel Blocker; ACE, Angiotensin Converting Enzyme; ARB, Angiotensin Receptor Blocker; IVSd, Interventricular Septum diameter; LVPWd, Left Ventricular Posterior Wall diameter; EDV, End Diastolic Volume; ESV, End Systolic Volume; LVEF, Left Ventricular Ejection Fraction

Coronary artery calcification and not arrhythmia associates with sex-specific circulating miR profiles in iAF

To investigate whether the presence of paroxysmal arrhythmia in iAF patients associates with circulating levels of the selected panel of 48 miRNAs, we assessed their plasma levels in iAF patients compared to SR controls (Table 2). After correction for biological sex and age, no plasma miRNAs were found to be associated with a history of AF in the total iAF patient population. Next, to determine the contribution of subclinical-CV comorbidities on circulating miRNA expression, we assessed the association of CAC with plasma miRNAs. In the total population, CAC associated with significantly higher levels of miRs-103, 125a-5p, 221 and 223, of which the latter three were increased in men only (Table 3; Figure 1). In contrast, analysis of CAC associated miRNAs in women revealed a significantly increased expression of miR-27a and miR-126.

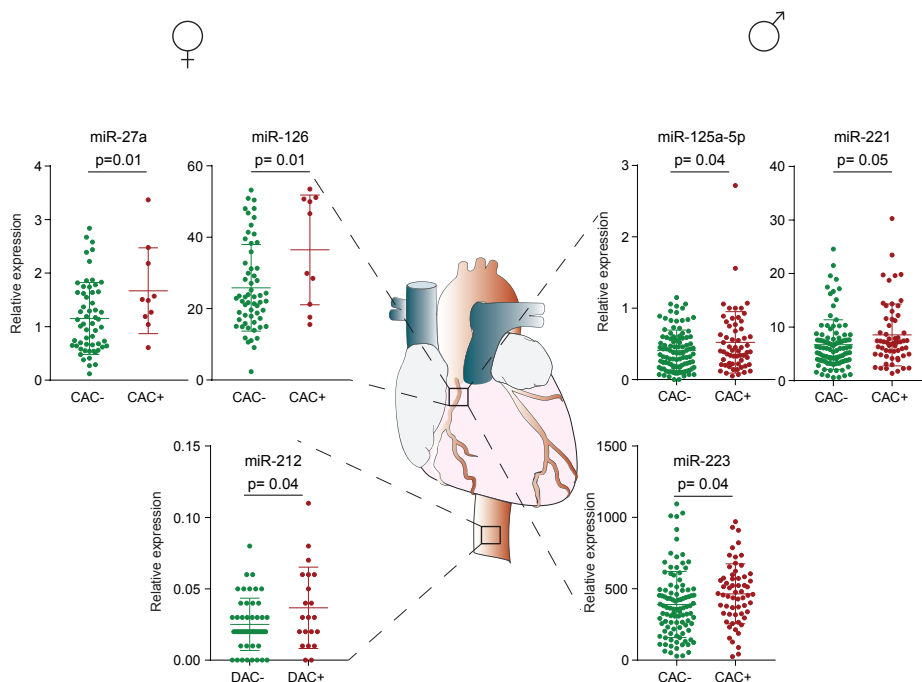


Figure 1: Differentially expressed circulating miRNAs in women and men either with or without coronary artery calcification (CAC), ascending aorta calcification (AsAC) and descending aorta calcification (DAC). Comparisons are adjusted for age, aspirin and Vitamin K antagonist use. Only significantly differently expressed miRNAs are shown.

Table 2: Differential expression of miRNAs in patients with or without calcification in the coronary arteries, ascending- or descending aorta.

	Total population			Female			Male		
	CAC- (n=160)	CAC+ (n=71)	p-value	CAC- (n=58)	CAC+ (n=10)	p-value	CAC- (n=100)	CAC+ (n=61)	p-value
miR-27a	1.54 ± 1.91	1.35 ± 0.75	0.44	1.17 ± 0.66	1.67 ± 0.81	0.013	1.76 ± 2.32	1.31 ± 0.74	0.457
miR-103	0.36 ± 0.32	0.50 ± 0.36	0.013	0.30 ± 0.28	0.49 ± 0.51	0.130	0.39 ± 0.33	0.50 ± 0.34	0.150
miR-125a-5p	0.41 ± 0.30	0.55 ± 0.52	0.027	0.37 ± 0.27	0.47 ± 0.37	0.371	0.43 ± 0.31	0.56 ± 0.54	0.042
miR-126#	26.78 ± 13.76	30.07 ± 13.70	0.05	25.83 ± 12.15	36.47 ± 15.38	0.012	27.33 ± 14.64	29.04 ± 13.14	0.341
miR-221	6.66 ± 6.54	9.03 ± 7.56	0.038	6.23 ± 8.68	7.69 ± 4.03	0.782	6.91 ± 4.98	9.25 ± 7.99	0.05
miR-223#	397.05 ± 229.29	472.14 ± 215.75	0.035	415.69 ± 227.16	515.57 ± 256.38	0.103	390.54 ± 231.53	465.02 ± 209.96	0.043
miR-221	AsAC- (n=184)	AsAC+ (n=51)	0.024	AsAC- (n=48)	AsAC+ (n=22)	0.124	AsAC- (n=136)	AsAC+ (n=29)	0.119
	7.76 ± 7.53	5.79 ± 3.48w		6.99 ± 9.61	4.93 ± 1.98		8.03 ± 6.67	6.42 ± 4.18	
miR-212	DAC- (n=178)	DAC+ (n=57)	0.62	DAC- (n=49)	DAC+ (n=21)	0.035	DAC- (n=129)	DAC+ (n=36)	0.346
	0.039 ± 0.039	0.034 ± 0.020		0.028 ± 0.016	0.041 ± 0.027		0.042 ± 0.045	0.034 ± 0.019	

Comparisons within the total population are adjusted for age, sex, and vitamin-K antagonists, while female and male group comparisons are adjusted for age and the use of vitamin K antagonists. MiRNA expression values marked with # are corrected for aspirin use. MiRNA relative expression data are presented as mean ± SD.

Circulating levels of miR-221 and miR-212 levels in women, associate with aortic calcification

Next, following the identification of coronary calcification associated miRNAs, we examined the relationship between specific miRNAs and aorta calcification. Within the total patient population, the presence of AsAC resulted in lower levels of miRNA-221. Interestingly, upon stratification for biological sex, the AsAC association with miRNA-221 still displayed a trend towards lower levels, although these differences were not significant for both men and women. However, the presence of DAC in women but not in men was associated with higher expression levels of miR-212 expression in women.

Discussion

This study shows that in iAF, instead of arrhythmia, subclinical CV comorbidities such as CAC, AsAC and DAC, associate with plasma levels of a selected set of miRNAs. Interestingly, when CAC, AsAC and DAC group comparisons were stratified for biological sex we observed a sex-specific, significant increase in miRNA-27a and miRNA-126 levels in women with a positive CAC score, while the levels of miRNA-125a-5p, miRNA-221 and miRNA-223 were higher in CAC positive men. Furthermore, circulating levels of miRNA-221 were elevated in patients with ascending aorta calcification while miRNA-212 expression associated with descending aorta calcification in women only.

Several of the differentially expressed miRNAs in this study have already shown promising potential as biomarker for clinical diagnostics and patients' response to treatment. As such, we previously demonstrated that plasma levels of miR-126 increase while miR-223 levels decrease in DN patients and normalize to healthy control levels, upon a simultaneous pancreas kidney (SPK) transplantation (12). Furthermore, in type 2 diabetes patients, both miR-126 and -223 display a positive response to drug treatment (20) while miR-126 was found to predict platelet activation and the response to aspirin therapy (21). Also miR-223 can be used in clinical care because its elevated levels were prognostic for cardiovascular mortality in patients with coronary artery disease (4). Interestingly, plasma levels of miR-27a were similarly found to provide additional aid in dedicated patient care because a reduction in its circulating levels associated with rehospitalization for heart failure patients (5). Therefore, the observed association of these miRs with vascular calcification in this study could potentially indicate a pathophysiology of subclinical coronary (micro)vascular dysfunction in iAF patients. Nonetheless, more miRNA expression profiling and functional studies in iAF patients are warranted to validate this relationship.

Studies on the relationship between iAF and circulating miRNAs are limited, although several studies reported changes in circulatory miRNAs in AF. In particular, miRNA-150 has consistently been associated with AF, and was repeatedly found to be decreased in (older) patients with AF (22-24). We could not confirm this association in the present study with iAF patients. It could be argued that the lowering of miRNA-150 levels in AF are mostly related to the contribution of CV comorbidities,

particularly because miRNA-150 is a marker of advanced atherosclerosis (25). Therefore, next to its association with arrhythmia in AF, this miRNA-150 change could also be related to the simultaneous prevalence of hypertension (22), heart failure (23) or diabetes (24) in these patient cohorts. Likewise, a similar relationship was identified between AF and plasma levels of miRNA-21 (24), a marker of cardiac fibrosis (26) and miRNA-152 (14) which is decreased in high glucose conditions (27). Again these miRNAs levels were also not altered in our iAF patients. A possible explanation for this discrepancy could be that our patients were not diabetic and relatively young whereas previous miRNA-21 expression changes were predominantly observed in older patients with a more severe AF phenotype, and an ensuing state of atrial ischemia and fibrosis. (26) Taken together, it could be argued that the previously identified circulatory miRNA profiles in AF are predominantly determined by an ensuing presence of arrhythmia in conjunction with severe CV comorbidities that are absent or less manifest in our iAF patient cohort.

The observed increase of the identified circulating miRs in this study could be explained by looking at their functional role within the vasculature system. For instance, in the setting of atherosclerosis, increased miR-103 expression in activated endothelial cells (ECs) represses endothelial KLF4 expression and thereby cellular quiescence (28) whereas an increased endothelial miR-212 expression is anti-angiogenic (29). As such, the higher levels of miR-103 and miR-212 in this study may suggest a pathophysiology of endothelial dysfunction and inflammation. Furthermore, although miR-126 normally favours vascular regeneration (30), the association of increased levels of miR-126 with vascular calcification in this study could indicate that, although speculative, more plasma miR-126 reflects a vascular regenerative feedback response in these iAF patients. Moreover, miRNAs are distributed among plasma miR carriers such as exosomes (31), circulating high density lipoprotein (HDL) (32) or Argonaute-2 (Ago2) (33). These plasma miR carriers enable the functional delivery of miRs at distal sites to pass on a miR mediated feedback loop in tissue inflammation, repair and regeneration. The observed increased expression of plasma miR-126, -27a, -125a-5p, -212 and -223 could have such carrier dependent impact. For instance, CD34+ peripheral blood mononuclear cells (PBMCs) derived exosomal miR-126 was found to be proangiogenic (34) whereas HDL-miR-223 transfer repressed endothelial activation and inflammation (35). Taken together, it could be argued that the observed increase of plasma miRs may reflect underlying pathophysiology or a regenerative response.

Diabetes mellitus is a highly significant predictor of AF in women (3). Furthermore, in iAF patients the relative risk of stroke, transient ischemic attack (TIA) and heart failure are at least doubled in woman compared to man (8) further emphasizing the action of sex-specific mechanisms in cardiovascular pathophysiology. These mechanism may also involve the action of sex-specific miRNAs. In woman, miRNA expression levels can differ from those in man due to oestrogen-dependent production or as a consequence of a gene-dose effect following incomplete X-chromosome inactivation (36). This could be possible because the X-chromosome in humans and mice is relatively enriched for miRs (118 annotated miRs on the human X-chromosome, source: miRBase vs. 22) with a twofold higher miR density compared to autosomes. These X-chromosome-located miRs (X-linked miRs) could potentially display an increased expression in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) from the cardiovascular system due to incomplete X-chromosome inactivation (XCI) (11). This could not explain that the X-linked miR-221 and miR-223 in this study were increased in male patients only. Therefore, another explanation for their sex biased miR expression could be the simultaneous expression with a sex-biased host gene or the sex specific expression of components of the miR processing machinery such as Dicer or Drosha (11). Therefore, another interesting mechanism for sexual dimorphism of miR expression could be the preferential binding of certain transcription factors (TFs) to the proximal genomic regions of particular sex-biased miR genes (sex-biased miRs have 15.7 TF binding sites in vicinity compared to 9.1 TF binding sites for non-biased miRs) (37). Because so far 33 female specific miR-regulating TFs have been identified compared to 56 male specific miR regulating TFs (37) it increases the likelihood that this regulatory control will develop differently between men and women.

In this study, women with coronary artery- and aortic calcification displayed higher circulating levels of miRNA-27a, miRNA-212 and miRNA-126 compared to men. Since women display higher levels of miRNA-126 during the ovulatory phase, and oestrogen was demonstrated to increase miRNA-126 expression to control the inflammatory response of ECs, a role for oestrogen in transcription of this vascular-protective miRNA in CAC positive women can be proposed (38). Furthermore, the observed female-related difference in miRNA-27a expression is consistent with observations that this miRNA is decreased in women with gestational diabetes (39) but increased in women with polycystic ovary syndrome (40), suggesting that miRNA-27a is responsive to a synchronous state of high

estrogen and metabolic dysfunction in women. Moreover, in contrast to the studied women in this cohort, CAC positive men displayed significantly higher levels of miRNA-221 and miRNA-223. Interestingly, miRNA-221, is known as a promoter of vascular calcification (41) and regulated by androgen hormone levels (42), while miRNA-223 is a known biomarker of CAC. (43) Therefore, although speculative, the male specific expression of these miRNAs in this cohort could be related to a male-specific cause or outcome of CV comorbidities in iAF patients. Taken together, it could be argued that the association of these sex-specific circulating miRNAs with vascular calcification in iAF patients confirms the notion that biological sex should be taken into account when deciding on vascular prophylactic therapy, including anticoagulation (8, 44).

This miRNA profiling study in iAF patients has several limitations. Measured miRNAs were selected based on their previous association with vascular injury. However, since numerous miRNAs are (mechanistically) involved in CV disease, we cannot exclude more miRNA changes in iAF than those observed in this study. Furthermore, this study was not followed by miRNA expression validation studies in independent iAF cohorts nor did we conduct intervention studies to evaluate whether the identified miRNAs are the cause or the outcome of (vascular calcification in) iAF. Therefore, future studies to validate these circulating levels of miRNAs in larger iAF patient cohorts are needed. However, since vascular calcification and age in iAF patients both augment coronary calcification (45) and because platelet activation hampers the biomarker potential of miRNA-126 and miRNA-223 (19), a strength of this study is the statistical adjustment of differentially regulated miRNAs for the use of aspirin and vitamin K antagonists.

Conclusion

Circulating miRNAs in iAF do not associate with the mere presence of paroxysmal arrhythmia but instead display a sex-specific association with subclinical vascular calcification. This sex specific miRNA profile association with vascular calcification in iAF patients, confirms the existence of sex specific etiologies of vascular disease, and suggests that sex specific strategies for the prevention of vascular complications should be employed.

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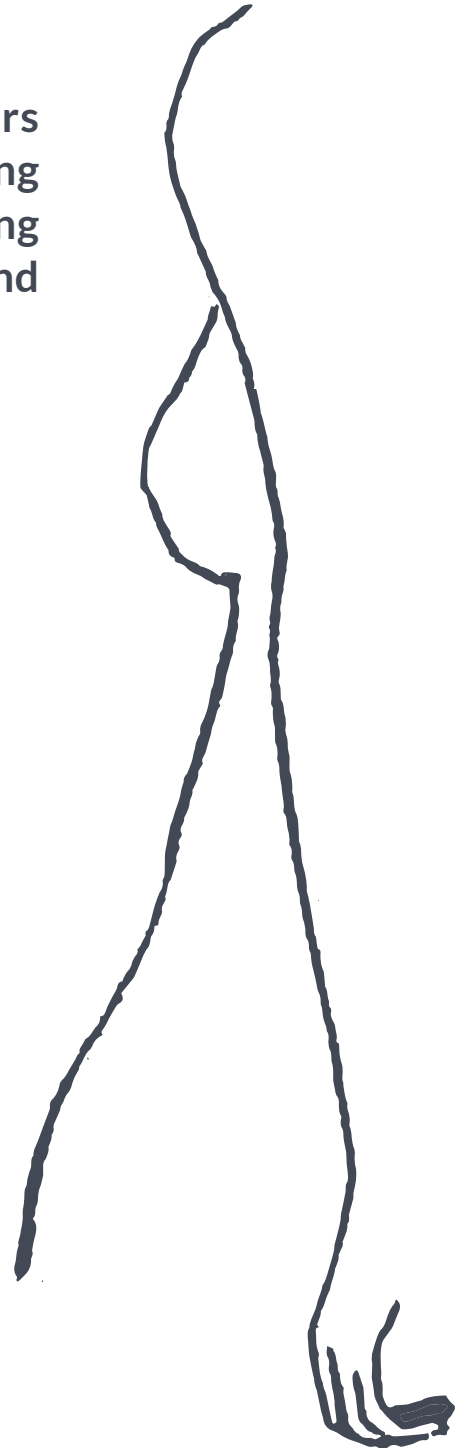
Chapter 5

Diabetic Nephropathy Alters the Distribution of Circulating Angiogenic MicroRNAs Among Extracellular Vesicles, HDL, and Ago-2

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B.W. Florijn, J.M.G.J. Duijs, J.H. Levels,
G.M. Dallinga-Thie, Y. Wang, A.N. Boing,
Y. Yuana, W. Stam, R.W.A.L. Limpens,
Y.W. Au, R. Nieuwland, T.J. Rabelink,
M.E.J. Reinders, A.J. van Zonneveld*,
R. Bijkerk*

*Both authors contributed equally



Abstract

Previously we identified plasma microRNA (miR) profiles that associate with markers of microvascular injury in diabetic nephropathy (DN) patients. However, miRs circulate in extracellular vesicles (EVs) or in association with high-density lipoprotein (HDL) or the RNA-binding protein Argonaute-2 (Ago-2). Given that the EV- and HDL-mediated miR transfer towards endothelial cells (ECs) regulates cellular quiescence and inflammation, we hypothesized that the distribution of miRs amongst carriers affects microvascular homeostasis in DN. Therefore, we determined the miR expression in EVs, HDL- and Ago-2 fractions isolated from EDTA-plasma of healthy controls, patients with diabetes mellitus +/- early DN (eGFR>30 ml/min/1.73 m²) and DN patients (eGFR<30 ml/min/1.73 m²). Consistent with our hypothesis, we observed alterations in miR-carrier distribution in plasma of DM and DN patients compared to healthy controls. Both miR-21 and miR-126 increased in EVs of DN patients, whereas miR-660 increased in the Ago-2 fraction and miR-132 decreased in the HDL fractions. Moreover, in vitro, differentially expressed miRs improved EC barrier formation (EV-miR-21) and rescued the angiogenic potential (HDL-miR-132) of ECs cultured in serum from DM and DN patients. In conclusion, miR measurement in EVs, HDL and Ago-2 may improve the biomarker sensitivity of these miRs for microvascular injury in DN while carrierspecific miRs can improve endothelial barrier formation (EV-miR-21/126) or exert a proangiogenic response (HDL-miR-132).

Introduction

Global diabetes mellitus (DM) prevalence has increased to over 400 million patients worldwide (1). As a consequence, microvascular complications such as diabetic nephropathy (DN) and heart failure with preserved ejection fraction (HFpEF) have become major causes of mortality and morbidity (2-4). Driven by the presence of metabolic comorbidities such as hyperinsulinaemia and hyperglycaemia, the loss of microvascular integrity in these complications is characterized by a chronic systemic inflammatory state that initiates endothelial cell (EC) dysfunction and pericyte detachment (5). Although these pathophysiological associations are well documented, the mechanisms that explain its onset and progression are incompletely understood.

The cellular response to (microvascular) injury is largely regulated at the post-transcriptional level involving non-coding RNAs (ncRNAs), such as microRNAs (miRs). MiRs are (negative) regulators of gene expression that predominantly interact with complementary sequences in the 3' untranslated regions (UTR) of their mRNA targets (6). In plasma, circulating miRs differ across disease phenotypes, which is why miRs have gained interest as biomarkers of disease (7). Previously we demonstrated microvascular injury in DM and DN patients (8) and subsequently showed that a selected set of plasma miRs, among others miR-21, -126, -132 and miR-223, strongly associate with DN and systemic microvascular injury and normalize after simultaneous pancreas kidney transplantation (SPK) (9). However, it is now recognized that plasma miRs circulate in plasma EVs (10), associate with the RNA-binding protein Ago-2 (11) or are carried by lipoproteins such as high density lipoprotein (HDL) (12). The clinical relevance of these plasma miR carriers resides in their ability to transfer miRs involved in vascular homeostasis and regeneration to vascular target cells (13). For instance, HDL transfers a functional miR-223 to ECs thereby exerting an anti-inflammatory response by lowering the expression of intercellular adhesion molecule 1 (ICAM-1) (14). Similarly, glucose tolerance improves following distant delivery of adipose tissue derived EV miRs (15) while the Ago-2 protein facilitates miR transfer to brain ECs of mice reduced tumour growth (16). Nevertheless, still little is known about the blood miR distribution over EVs, HDL and Ago-2 in DM and DN patients compared to healthy controls. Isolation of these carriers to study their (functional) miR content could indicate whether carrier specific miRs are a cause or consequence of microvascular injury in DN.

Here, we isolated plasma EVs, HDL and Ago-2 from type 1 DM +/- early DN (with eGFR>30 mL/min/1.73m²) and DN (eGFR<30 mL/min/1.73m²) patients and healthy controls to determine whether the distribution of miRs over these carriers is altered upon DM or DN. We subsequently explored whether a specific difference in distribution of miRs in these carriers is a better explanation for microvascular injury in DM and DN patients as compared to the overall absolute quantity of total plasma miRs. Furthermore, we analysed the impact of differentially expressed EV- and HDL-miR complexes on EC functions such as angiogenesis and endothelial barrier formation.

Research design and methods

Pilot and main study

This study was approved by the Institutional Review Board and complied with the ethical principles of the Declaration of Helsinki. Informed consent was obtained from all studied patients. Prior to the main study, an observational, cross-sectional pilot study was performed in which the expression of 48 selected, previously established microvascular-injury associated miRs (9; 17; 18) were assessed in the EV- and HDL- I fractions from 9 DM +/- early DN patients and 7 DN patients. The main study cohort was composed of a control group (control, n= 12) of healthy, age matched volunteers, a group of DM type 1 patients +/- early DN with an estimated GFR (eGFR) of ≥ 30 mL/min/1.73 m² (DM +/- early DN, n=14) and a Diabetes Page 4 of 45 5 group of DM1 patients with DN with an eGFR<30 mL/min/1.73 m² (DN, n=19). The DM +/- early DN and DN group are divided based upon their eGFR above or below 30 mL/min/1.73 m². As such, the DM +/- early DN group may contain patients with DN (although with higher eGFR) as their eGFR may fall in category 3 regarding CKD stage (KDOQI guidelines).

Table 1: Patient characteristics of the main study cohort

	Controls (N=12)	DM +/- early DN (N= 14)	DN (N= 19)
Sex, female, N (%)	6 (50%)	8 (57%)	4 (21%)
Age (years)	45 $\pm$ 11	55 $\pm$ 13	44 $\pm$ 6 ²
BMI (kg/m ²)	24.7 $\pm$ 3.9	23.6 $\pm$ 2.6	25.4 $\pm$ 3.4
Systolic blood pressure (mm Hg)	131 $\pm$ 12	131 $\pm$ 14	144 $\pm$ 18 ^{1,2}
Diastolic blood pressure (mm Hg)	84 $\pm$ 7	71 $\pm$ 8 ¹	86 $\pm$ 11

Table 1: Continued.

	Controls (N=12)	DM +/- early DN (N= 14)	DN (N= 19)
Hemoglobin (mmol/L)	8.8 ± 0.7	8.3 ± 1.3	7.7 ± 0.7 ¹
Hematocrit (L/L)	0.42 ± 0.03	0.40 ± 0.05	0.37 ± 0.04 ^{1,2}
HbA1c (%)		7.0 ± 0.7	9.2 ± 2.3 ²
Glucose (mmol/L)	5.5 ± 1.2	12.8 ± 4.7 ¹	14.5 ± 6.1 ¹
HDL (mmol/L)	1.54 ± 0.43	1.29 ± 0.35	1.73 ± 0.44
LDL (mmol/L)	3.3 ± 0.93	2.77 ± 1.16	2.21 ± 0.47 ¹
Triglycerides (mmol/L)	1.44 ± 1.04	1.75 ± 1.02	0.93 ± 0.35
eGFR* (mL/min/1.73m ²)	90 ± 13	72 ± 23 ¹	23 ± 19 ^{1,2}
Median proteinuria (g/24 hr) (IQR†)		0.29 (0.13-0.29)	0.68 (0.31-1.19)
Duration diabetes (years)		40 ± 10	37 ± 10
Smoking	0 (0%)	2 (14%)	0 (0%)
Acetylsalicylic acid, N (%)		2 (14.3%)	2 (11%)
Antihypertensive drugs, N (%)			
ACE‡ inhibitor		6 (43%)	13 (72%)
Angiotensin-II antagonist		3 (21%)	11 (58%) ²
β-blocker		0 (0%)	9 (47%) ²
Calcium antagonist		1 (7%)	8 (42%) ²
Diuretic		5 (36%)	9 (47%)
Statin, N (%)		7 (50%)	13 (72%)

*eGFR, estimated GFR; †IQR, interquartile range; ‡ACE, angiotensin converting enzyme. Parametric data are presented as mean ± SD. Non parametric data are presented as median and IQR. Differences between groups were analysed using the one-way analysis of variance (ANOVA) (Sidak or Tukey post-hoc test). Nonparametric data are shown as median and interquartile range (IQR) and were analysed using a Kruskal–Wallis test. Categorical data are presented as frequency and percentage.

¹p-value <0.05 versus controls

²p-value <0.05 versus DM ≥ 30 mL/min

Clinical chemistry and ELISA

Blood was drawn in EDTA-coated tubes. Plasma was obtained by centrifugation of EDTA blood for 10 min at 1000 g and subsequently stored at -80°C. Plasma creatinine, haemoglobin, HbA1c, glucose and urea were measured as well as proteinuria in 24 h urine. GFR was calculated with plasma creatinine concentration

using the MDRD formula. Angiopoietin-2 (Ang-2) levels in plasma were determined by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturers' protocols.

Plasma EV isolation

For EV (70 nm) isolation, 125 μ L human plasma was applied to a 3.64 mL Sepharose CL-2B size exclusion chromatography (SEC) column. 250 μ L fractions were eluted with PBS. A newly packed column was used for each sample. Different fractions were analysed for the presence of particles using nanoparticle tracking analysis (NTA, NS500; Nanosight, Amesbury, UK). Subsequently fractions were analysed for the presence of HDL with ELISA (Abcam, Cambridge UK, ab125961). Presence of EVs was confirmed by Western Blot and Transmission Electron Microscopy (TEM). After establishing in which fractions EVs elute, fractions were combined until a total of 2 mL eluate was collected. Eluate was applied to an Amicon Ultra-4 Centrifugal Filter unit for concentration and treated with proteinase-K (Thermo Fisher Scientific, The Netherlands). Trizol was added to isolate RNA.

Nanoparticle tracking analysis plasma EVs

EV quantification was done using the NTA system combined with an EMCCD camera and a 405 nm diode laser (19). With silica beads (100 nm diameter; Microspheres-Nanospheres, Cold Spring, NY) the NTA was configured and calibrated. Fractions were diluted 10-1,000-fold in PBS to reduce the number of particles in the field of view below 200/image. Of each fraction, 30 videos of 10-seconds duration, were captured with the camera shutter set at 1205 and the camera gain set at 437.

Transmission Electron Microscopy

EVs were absorbed for 1 min to freshly glow-discharged, carbon-coated pioloform grids. Next, samples were negatively stained with a 2% PTA solution for 30 seconds and examined with a Tecnai 12 Biotwin microscope (FEI Company, Eindhoven, The Netherlands) at 120 kV, equipped with a 4 $\times$ 4 k charged-coupled device camera (FEI Company).

HDL isolation and purification

HDL (density 1.063-1.21 g/mL) was isolated with a potassium bromide (KBr)-density gradient ultracentrifugation (DGUC) using a Beckman Optima Max bench-top ultracentrifuge with a fixed angle TLA 110 rotor (Beckman). 900 μ L plasma

aliquots were adjusted to the desired density with solid KBr and ultracentrifuged in 13x48 mm polycarbonate tubes at 100,000 rpm (435,680 = g) for 2 hours at 10°. Apo A-1 concentration was analysed using an immunoturbidimetric assay. The HDL fraction was isolated and dialyzed for 2 hours against PBS for KBr removal, applied to an Amicon Ultra-4 Centrifugal Filter unit for further concentration and applied to a Sepharose-CL-2B SEC column to deprive HDL from contamination with CD63 positive EVs (20).

Plasma Argonaute-2 immunoprecipitation

Ago-2 from plasma was isolated as described before (11). Briefly, goat anti-mouse IgG Magnetic Beads (Thermo Scientific) were incubated with 10 µg of mouse monoclonal anti-human Ago2 (Abcam, ab57113) or mouse normal IgG (Santa Cruz Biotechnology) antibodies for 2 hours at 4 °C. Preincubated Ago-2 beads were added to 400 µL of diluted plasma and incubated overnight at 4 °C. Beads were washed three times with 1% Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA) and split in half for RNA isolation and Western Blot.

Western blot

EV and Ago-2 protein lysates were generated using RIPA buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1mM ethylenediamine tetraacetic acid, 1% Triton X-100) supplemented with protease inhibitors cocktail (Complete, Roche). Using BCA Protein Assay Kit (Pierce) the total protein content was determined and 5–20 µg of total protein was applied on Any-kD Mini-PROTEAN TGX Precast SDS page gels (Biorad). Gels were blotted onto nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Biorad) and blocked with 5% non-fat milk powder in PBS with 0.01% of Tween (PBST). Primary antibodies against the following proteins were used: CD63 (Abcam, Cambridge, UK) and Ago-2 (Abcam). Membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary HRP-labeled antibodies for 1 hour at room temperature. Upon PBST washing, membranes were incubated with SuperSignal West Dura Chemiluminescent Substrate (Pierce) and exposed on BioMax XAR Film (Kodak) or Ultracruz autoradiography film (Santa Cruz).

RNA isolation and quantitative RT-PCR

RNA from EVs, HDL and Ago-2 fractions was isolated with 500µL Trizol reagent (Invitrogen, Breda, the Netherlands) using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Chloroform was added to the plasma/Trizol mixture and centrifuged for 15min at 15000g. Subsequently, the aqueous phase was combined with 1.5x volume of 100% ethanol, conveyed to a MinElute Spin column (Qiagen) and centrifuged for 15s at 18000g. EVs, HDL, and Ago-2 RNA was washed with 700 µL RWT buffer, twice with 500 µL RPE buffer and centrifuged for 15 s at 18000g after the first two washing steps and 2 min at 18000g after the last washing step. RNA was eluted with 15 µL RNase-free water. With a 5-minute 65° C incubation step, 500 ng RNA was reverse transcribed using specific Taqman prime pools and miR probes (below) or dinucleotide triphosphates (Invitrogen) and oligo(dT) (Invitrogen). Using a M-MLV First-Strand synthesis system (Invitrogen) cDNA was synthesized and validation of mRNA expression was carried out using SYBR Green Master Mix (Applied Biosystems). Target gene mRNA primer sequences are as follows: SPRED-1 (sense): CAGCCAGGCTTGGACATTCA; SPRED-1 (antisense): TGGGACTTTAGGCTTCCACAT; MEF2C (sense) CTGGGAAACCCCAACCTATT; MEF2C (antisense) GCTGCCTGGTGAATAAGAA; p120RASGAP (sense): TTATGATGGGAGGCCGCTATT; p120RASGAP (antisense): CTGCATTGGTACAGGTTCCCTT. mRNA expression was determined by normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

MiR profiling

Custom designed miR Megaplex cards were designed containing miRs that were selected based on their previously established association with (micro)vascular dysfunction in profiling studies (9; 17; 18). For miR cDNA synthesis of plasma EVs, HDL and Ago-2 RNA, reverse transcription of total RNA was performed using the miR reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was pre-amplified using Megaplex PreAmp primers pools A V2.1 (Applied Biosystems) according to the manufacturer's protocol. Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Obtained miR cycle threshold (Ct)-values in the pilot study were normalized using the median Ct value (21). In the main study, we performed an experimental validation of differentially expressed miRs in the different plasma carriers (HDL, EVs and Ago2) that we assessed in the pilot study (miR-1, miR-21, miR-29a, miR-126, miR-132, miR-145, miR-152, miR-212, miR-223, miR-574 and miR-660). This subset of

miRs was previously found to associate with microvascular injury in total plasma (9) and were therefore now assessed in the separate EV, HDL and Ago2 fractions (isolated as described above) using single TaqMan qRT-PCR assays as previously described (9). For normalization of carrier specific miR expression in the main study we tested several strategies such as normalization using the median of measured miRs and normalization to single endogenous controls such as miR-16. Because of its stable expression amongst carriers and strong correlation with the median of 11 measured miRs (data not shown) in EVs, HDL and Ago-2, miR-16 was selected for normalization.

In addition, we tested the correlation between the number of EVs using NTA and the level of EV-miR-16, as well as the correlation between Ago-2 levels determined with ELISA and the levels of AgomiR-16, and found again significant correlations ($r=0.75$, $p<0.001$ and $r=0.55$, $P<0.05$, respectively). Since HDL isolation efficiency was variable (in contrast to that of EVs and Ago2), HDL-miR expression was calculated per mg plasma ApoA1 protein.

Microvascular imaging and analysis

With side stream dark field imaging (SDF Microscan; MicroVision, Inc., Wallingford, PA) the average capillary tortuosity and the mean tortuosity index (MTI) per patient was assessed as previously described (22).

Endothelial cell culture with patient serum, EV-miRs and HDL-miRs

Human pooled serum derived EVs (System Biosciences) were transfected with a positive control siRNA TexRed, a scramble miR mimic or miR-21, -126 and -132 mimics using the Exo-Fect EV Transfection Reagent (System Biosciences). The EV transfection solution was incubated at 37°C in a shaker for 10 minutes placed on ice and centrifuged for 3 minutes at 20000 g. The transfected EV pellet was incubated with human umbilical vein endothelial cells (HUVECs) that were cultured in EV/exosome-depleted fetal calf serum (FCS, Thermofischer) for 48 hours at 37°C. Similarly, a complex of (fluorescently-random labeled), 80 µg HDL (ab204717, Abcam Cambridge UK) was incubated overnight at 4°C rotating with 2 µM cel-miR-39 or hsa-miR-132 mimic (Dharmacon). Unassociated miRs were removed by dialysis, while the HDL-miR complex was applied to HUVECs cultured in either 10% FCS or 10% serum from DM and DN patients.

Endothelial cell barrier measurements

Endothelial barrier was measured by Electric-cell impedance sensing. Confluent HUVEC cultures were treated with miR loaded EVs or HDL. After 48 hours, the cells were detached using trypsin-EDTA solution, counted using trypan blue and seeded in the electrode arrays (Ibidi, 96 wells, 20 electrodes per well, coated for 10 minutes with L-cysteine and subsequently with gelatin (1% in PBS)) at a density of 50.000 cells/well. Measurements of trans-endothelial electrical resistance were performed in real time by means of an Electric Cell-substrate Impedance Sensing (ECIS)-Z θ instrument (Applied Biophysics) at 37 °C, 5% CO₂. Cell spreading and monolayer formation were subsequently monitored by measuring the resistance at 4000 Hz. For the mathematical modelling to calculate resistance attributable to the functions cell-cell adhesion and cell-matrix interaction, the resistance and capacitance were measured at 11 different AC frequencies ranging from 62.5Hz up to 64000Hz

Vascular network formation

After EV- and HDL specific miR transfection, HUVECs (25.000 cells/well) were cultured in a 96 well plate coated with 100 μ l Corning Matrigel Basement Membrane Matrix (BD Biosciences). Tube length was quantified using the Angiogenesis Analyzer tool in Fiji Image J by measuring the cumulative tube length in three random microscopic fields using a digital imaging software (AxioVision Rel. 4.8, Carl Zeiss).

Statistical Analyses

Continuous normally distributed data are presented as mean $\pm$ SEM. Variable distribution was tested using the Kolmogorov-Smirnov test for normal distribution. Differences between groups in the main study were analysed using the one-way analysis of variance (ANOVA) (Sidak or Tukey post-hoc test). Nonparametric data are shown as median and interquartile range (IQR) and were analysed using a Kruskal-Wallis test. Categorical variables are presented as numbers and percentages, and differences between two groups were analysed with Fishers exact test. Correlations between variables were calculated using the Spearman rank correlation coefficient. In addition, multivariable linear regression was used to adjust for possible confounders. Results of the in vitro experiments are expressed as mean $\pm$ SEM while statistical analysis was performed using the student's T-test or a one-way ANOVA. Differences were considered statistically significant with

P less than 0.05. Data analysis was performed using SPSS version 17.0 (SPSS Inc, Chicago, IL) and GraphPad Prism, version 7.0 (GraphPadPrism Software Inc, San Diego, CA).

Results

Optimization carrier isolation methods

To investigate the distribution of miRs over different carriers (EVs, HDL and Ago-2), we first optimized their isolation methods (Figure 1). Plasma EVs were isolated using SEC (Sephacrose CL2B column). EVs were obtained from the first eight fractions because the majority of EVs elute in these fractions while HDL and other proteins elute in fraction 9-20. Subsequently, EV concentration and HDL presence was demonstrated by NTA analysis and HDL (ApoA1) ELISA of fractions respectively (Figure 1A). EV presence was demonstrated by CD63 expression on western blot of pooled fractions (Figure 1B) and TEM (Figure 1C). HDL from patient plasma was isolated with a density gradient ultracentrifugation (Figure 1D). Given that CD63 positive EVs have a similar density (20), as demonstrated by the presence of CD63 protein in the HDL fraction (Figure 1D), we further separated HDL from CD63 positive EVs and other particles using SEC (Figure 1E). Lastly, selective immunoprecipitation (Figure 1F) of Ago-2 fractions from plasma was validated by western blot (Figure 1G).

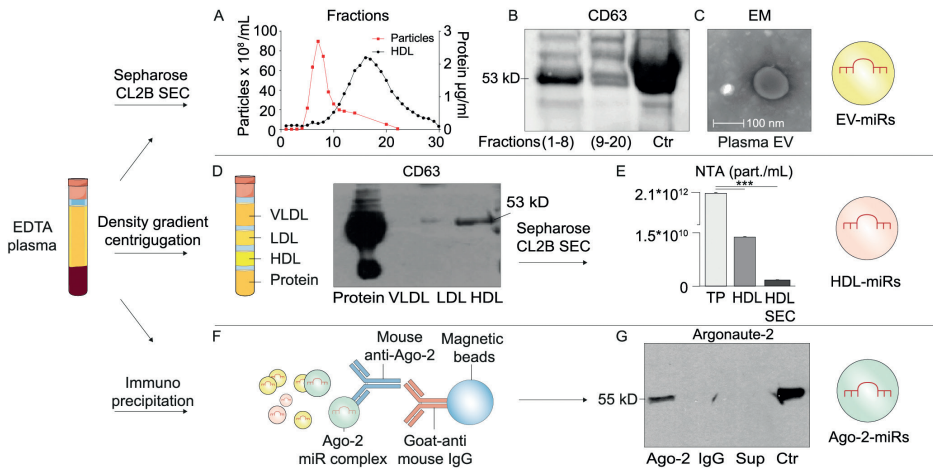


Figure 1. Carrier isolation procedures

Figure 1. Continued.

(A) Sepharose CL-2B size exclusion chromatography (SEC) was used to isolate CD63 positive extracellular vesicles (EVs) (Graph shows SEC separation of 125 μ L human plasma.) EV concentration was validated with Nanoparticle Tracking Analysis (NTA) in fractions 1-8, red line. Black line depicts HDL concentration as measured by Elisa. (B) CD63 Western Blot (WB) confirms EV presence in isolated fractions from human plasma. A commercial pure EV/exosome preparation (control) served as positive control. (C) EM picture of isolated EVs in fraction 1-8. (D) Schematic representation of KBr density gradient ultracentrifugation (DGUC) that was used to isolate lipoprotein fractions from human plasma (VLDL; $d < 1.006$ g/ml, LDL; $1.006 < d < 1.063$ g/ml, HDL $1.063 < d < 1.21$ g/ml and protein; $d > 1.21$ g/ml). (D) CD63 WB shows HDL is contaminated with EVs because of overlapping densities of EVs ($d = 1.13$ - 1.19 g/ml) and HDL ($d = 1.063$ - 1.21 g/ml). (E) NTA results (particles/mL) validating the isolation of EV free HDL. (F) Ago-2 immunoprecipitation protocol. (G) Successful immunoprecipitation (IP) for Argonaute-2 (Ago-2) as shown by Ago-2 WB. IP with IgG as negative control confirms Ago2 IP specificity. Supernatant (Sup) is plasma obtained after Ago-2 immunoprecipitation. HEK293T cell lysate was used as positive control. Significant correlations are depicted in bold. Data are represented as mean $\pm$ SEM. *** $p \leq 0.001$

Pilot identification of EV- and HDL-specific miRs

Because it appears that particularly EVs (23) and HDL (14) are relevant in cell-cell communication and to investigate the presence of EVs and HDL- selective miR profiles, we first performed a pilot study to identify candidate miRs in EVs and HDL from EDTA-plasma of 9 DM +/- early DN and 7 DN patients (Supplementary Table S1). Based on differential miR expression (p-value) analysis in this pilot study (Supplementary Table S2 and -S3) in combination with high range expression and/or a previously identified relation to microvascular injury (9; 17; 18), 11 miRs (miR-1, miR-21, miR-29a, miR-126, miR-132, miR-145, miR-152, miR-212, miR-223, miR-574 and miR-660) were selected for further analysis in the main study.

EV-miR-21 and -126 associate with diabetes and diabetic nephropathy

To validate whether the selected carrier-miRs significantly associate with DM (GFR ≥ 30 mL/min/1.73 m²), DN and (micro)vascular injury, we measured the eleven selected miRs that were identified in the pilot study in EVs (Supplementary table 4), HDL (Supplementary Table 5) and Ago-2 (Supplementary Table 6) using single TaqMan miR assays in the main patient cohort study (Table 1). To investigate whether miR profiling in EVs, HDL and Ago-2 resulted in a different expression pattern compared to total plasma, we also selected and re-analysed total plasma miR expression (Supplementary Table 7) of the same patients from which plasma miR expression results were previously published (9). Particularly the expression of miR-21 was significantly increased in plasma EVs of both DM and DN patients (Figure 2A). Although HDL-, Ago-2- and total plasma miR-21 displayed a similar

increasing trend (Figure 2A), these findings were not statistically significant. Also miR-126 was increased in plasma EVs from DN patients (Figure 2B). This miR was not altered in the HDL fraction, significantly increased in the plasma Ago-2 protein fraction of DM and DN patients and increased in total plasma of DM patients only (Figure 2B). When we investigated the association of EV-miRs with vascular injury markers we found that amongst miR-21 and miR-126 carriers, only EV-miR-21 ($R=0.34$, $p=0.03$) and EV-miR-126 ($R=0.30$, $p=0.05$) associated with capillary tortuosity (MTI) (Table 2, Supplementary Figure 1).

Table 2: Correlations between carrier specific miRs and markers of microvascular injury.

Angiopoietin-2 (pg/mL)				
	EVs	HDL	Ago	plasma
miR-21	$R=0.11$, $p=0.51$ FDR= 0.68	$R=0.05$, $p=0.77$ FDR= 0.87	$R=0.02$, $p=0.91$ FDR= 0.94	$R=0.12$, $p=0.95$ FDR= 0.95
miR-126	$R=0.13$, $p=0.41$ FDR= 0.60	$R=0.18$, $p=0.25$ FDR= 0.52	$R=0.13$, $p=0.40$ FDR= 0.60	$R=0.11$, $p=0.49$ FDR= 0.68
miR-132	$R=-0.15$, $p=0.36$, FDR= 0.60	$R=-0.34$, $p=0.03$ FDR=0.14	$R=0.19$, $p=0.26$ FDR= 0.52	$R=0.10$, $p=0.54$ FDR= 0.69
miR-660	$R=0.26$, $p=0.10$ FDR= 0.32	$R=0.17$, $p=0.30$ FDR= 0.56	$R=0.41$, $p=0.007$ FDR= 0.06	$R=0.47$, $p=0.001$ FDR= 0.02
Mean tortuosity index				
	EVs	HDL	Ago	plasma
miR-21	$R=0.34$, $p=0.03$ FDR= 0.14	$R=0.18$, $p=0.25$ FDR= 0.52	$R=0.34$, $p=0.15$ FDR= 0.44	$R=0.39$, $p=0.01$ FDR= 0.06
miR-126	$R=0.30$, $p=0.05$ FDR= 0.18	$R=0.08$, $p=0.63$ FDR= 0.75	$R=0.12$, $p=0.40$ FDR= 0.60	$R=0.32$, $p=0.04$ FDR= 0.16
miR-132	$R=0.30$, $p=0.85$ FDR= 0.91	$R=0.08$, $p=0.61$ FDR= 0.75	$R=0.04$, $p=0.79$ FDR= 0.87	$R=0.21$, $p=0.17$ FDR= 0.45
miR-660	$R=0.14$, $p=0.37$ FDR= 0.60	$R=0.20$, $p=0.19$ FDR= 0.47	$R=0.40$, $p=0.008$ FDR= 0.06	$R=0.46$, $p=0.001$ FDR= 0.02

EVs, extracellular vesicles; HDL, high density lipoprotein; Ago, Argonaute-2; R, correlation coefficient; p, p-value. Microvascular injury markers were adjusted for age, kidney function and diabetes parameters with multivariate analysis by partial correlation. Multiple testing was done through FDR with Benjamini Hochberg correction. Significant correlations are depicted in bold.

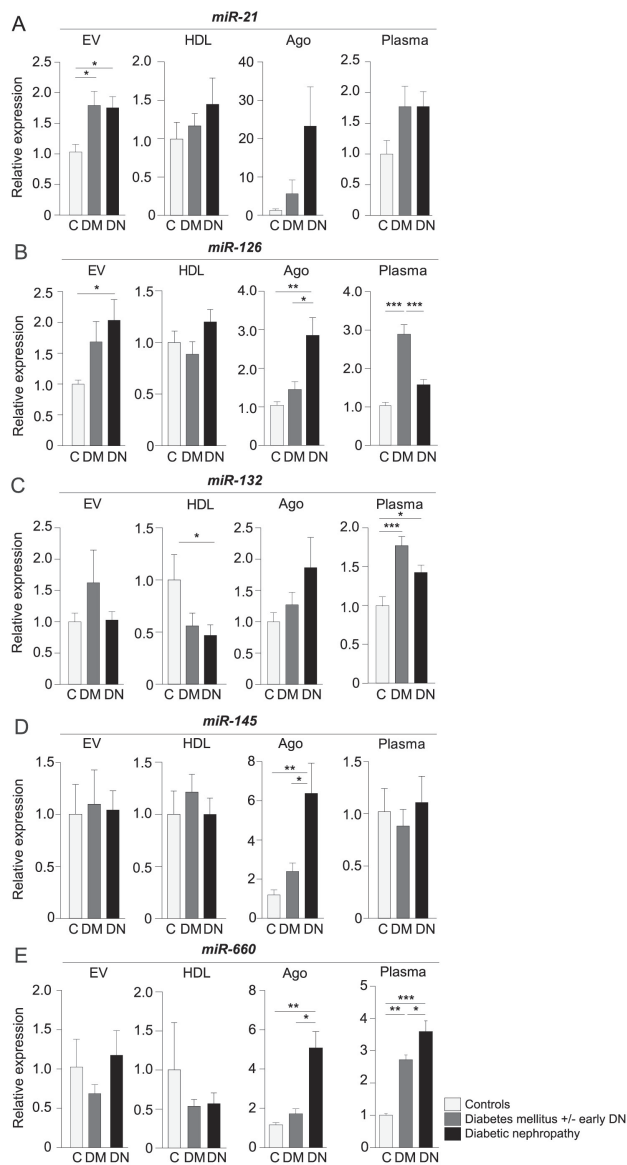


Figure 2. Differential expression of carrier specific plasma miRs.

(A) Increased expression of miR-21 in plasma EVs from DM +/- early DN and DN patients compared to healthy controls. (B) Increased expression of miR-126 in total plasma from DM +/- early DN patients and in plasma EVs and Ago-2 from DM +/- early DN and DN patients compared to healthy controls. (C) Decreased expression of miR-132 in the HDL fraction of DN patients and increased Diabetes Page 30 of 45 31 expression of miR-132 in total plasma of DM +/- early DN and DN patients compared to healthy controls. (D) Increased expression of miR-145 in AGO-2 from DM +/- early DN and DN patients compared to healthy controls. (E) Increased expression of miR-660 in total plasma and AGO-2 from DM +/- early DN and DN patients. Data are represented as mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Circulating HDL-miR-132 associates with diabetic nephropathy

Next, we screened for miRs to be differentially expressed within the HDL fraction. Compared to healthy controls and DM +/- early DN patients, miR-132 expression was decreased in the HDL fraction from DN patients, a finding which was not observed in EVs or Ago-2 (Figure 2C). Interestingly, while HDL-miR-132 expression displayed a significantly different decrease in DN patients compared to healthy controls, miR-132 in total plasma was increased in both DM +/- early DN patients (Figure 2C). Next, we determined whether HDL-miR-132 could improve the association with markers of (micro)vascular injury, compared to total plasma miRs. In contrast to total plasma miR-132 and EV- or Ago-miR-132, HDL-miR-132 displayed a novel correlation with Ang-2 ($R = -0.34$, $p = 0.03$, $FDR = 0.14$; Table 2, Supplementary Figure 1).

Ago-miR-660 associates with diabetes mellitus and diabetic nephropathy

Lastly, we sought to determine the contribution of plasma Ago-2-miR expression to the extracellular miR profile in DM +/- early DN patients. Interestingly, in plasma from DN patients, Ago-2 associated miR-126 (Figure 2B), miR-145 (Figure 2D), and miR-660 (Figure 2E) significantly increased compared to healthy controls. Furthermore, of these differentially expressed Ago-miRs, only Ago-miR-660 correlated with Ang-2 ($R = 0.41$, $p = 0.007$) and the MTI ($R = 0.40$, $p = 0.008$) (Table 2, Supplementary Figure 1).

Diabetes and diabetic nephropathy alter the carrier distribution of circulating miRs

To investigate how the presence of DM and DN influences the distribution of individual miRs amongst their carriers, we compared the relative expression for each miR in the EV-, HDL-, and Ago-2 protein fractions, and calculated its expression ratio amongst different carriers in healthy controls, DM +/- early DN patients (Figure 3). In contrast to Figure 2, which represents miR levels within either HDL, EV or Ago-2 in DM and DN patients, Figure 3 (a comparison of miRs amongst different carriers) points out the differential carrier distribution of these miRs in DM +/- early DN patients, DN and control subjects. The expression ratio calculation of these miR carriers demonstrated that under healthy conditions, miRs can be distributed throughout all carriers (miR-21, miR-126, miR-132, miR-145 and miR-574) or are predominantly expressed in one carrier (miR-1, miR-212, miR-29a in plasma EVs and miR-223, miR-21, miR-152 and miR-660 in Ago-2 protein). However,

the presence of either DM +/- early DN patients alters the distribution of these miRs among their carriers (Figure 3). For instance, the carrier-association of miR-223, -126, and -145 in plasma was shifted from Ago-2 to HDL in DN patients (Figure 3). Furthermore, the decrease in HDL and Ago-2 associated expression of miR-132 and the decrease in Ago-2 associated expression of miR-21 coincided with a shift in its association to plasma EVs or -HDL in DN patients (Figure 3).

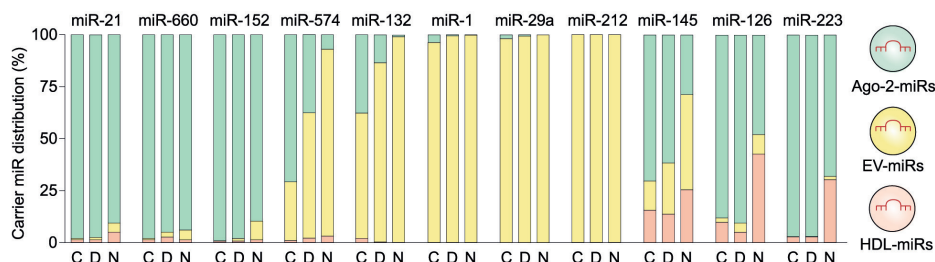


Figure 3. Distribution of carrier specific miRs in DM and DN.

'Absolute' values of selected carrier specific miRs (based on Ct values) were used to calculate the distribution of each miR in EVs, HDL and Ago-2. MiR Ct-value data are represented as mean per group and were normalized to plasma volume input. Green colour represents Ago2 carrier fraction while the yellow and red colour represent the plasma EV and HDL carrier fraction respectively.

EV-miR-21 and -126 regulate endothelial barrier formation in vitro

Since we hypothesized that the differentially expressed carrier-miRs in DN may be involved in specific cell-cell communication, and as such are involved the progression of DN and its cardiovascular complications, we next sought to investigate the functional impact of the differentially expressed carrier-miRs on ECs in vitro. To confirm that plasma EVs can indeed transfer their cargo into ECs, EVs from healthy controls were loaded with a Texas Red end-labelled siRNA and applied to HUVECs in culture. Upon the detection of fluorescent signal in ECs (Figure 4A-B), indicating successful transfer of EVs into HUVECs, we next loaded these EVs with miR-21 and miR-126 mimics to study whether endothelial expression of these miRs is increased upon EV transfer. Indeed, 48 hours after adding the EVs to the cells we observed increased endothelial expression of miR-21 (Figure 4C) and miR-126 (Figure 4E) with a concomitant decrease in the expression of their respective validated target genes MEF2C (Figure 4D) and SPRED1 (Figure 4F). Next, we investigated whether EVs loaded with these miRs stimulate endothelial angiogenesis or improve endothelial barrier formation by culturing

ECs in the presence of EVs loaded with miR-21 and miR-126. After 48 hours, ECs were seeded in an in vitro matrigel angiogenesis assay and in gelatin-coated culture chambers with electrodes present in the growth area to facilitate the measuring of electrical resistance by ECIS (24). As illustrated in Figures 4G-H, miR-21 and miR-126 in EVs did not improve endothelial tube formation, while HDL-miR-21 displayed a trend towards a decline in endothelial tube formation ($p=0.08$) (Figure 4I-J). However, after 24 hours the EV mediated transfer of miR-21 and miR-126 in ECs significantly improved the resistance of a monolayer of ECs, as compared to scramble miR control cells, indicating improved endothelial barrier function (Figure 4K-L) in contrast to HDL-miR-21 and HDL-miR-126 which did not affect endothelial barrier formation (Figure 4M-N)

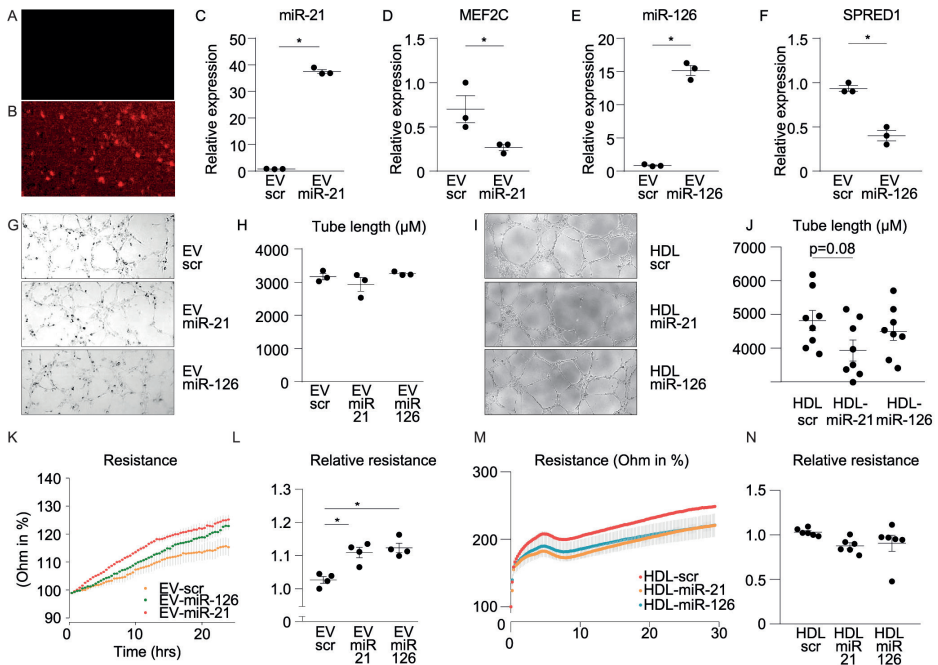


Figure 4. EV-miR function in endothelial cells.

Fluorescent signal detection in human umbilical vein endothelial cells (HUVECs) before (A) and after (B) transfer of EVs loaded with a positive control Texas Red-labeled siRNA. (C) Increased miR-21 expression after EV-miR-21 transfer in HUVECs followed by a decreased expression of its gene target MEF2C (D). Increased miR-126 expression after transfer of EV-miR-126 in HUVECs (E) followed by a decreased expression of its gene target SPRED1 (F). (G-H) EV-miRs did not affect the tube formation ability of HUVECs, as shown by a quantitative analysis of the total tube length. (I) HDL-miR-21 treated HUVECs displayed a trend towards less endothelial tube formation while HDL-miR-126 did not affect endothelial angiogenesis, as shown by quantitative analysis of total tube length (J).

Figure 4. Continued.

(K-) Transendothelial electrical resistance of EV-miR-21 and EV-miR-126 treated HUVECs seeded on ECIS electrodes. (L) Relative transendothelial electrical resistance of EV-miR-21 and EV-miR-126 treated HUVEC stable monolayer of 24 hr time frame in G. EV-miR-SCR set to 1. (M-N) In contrast to EV-miR-21 and -126, HDL-miR-21 and HDL-miR-126 did not significantly affect transendothelial electrical resistance. Data represented as mean $\pm$ SEM of n= 4-6.* $p \leq 0.05$

HDL-miR-132 regulates endothelial cell tube formation in vitro

Next, to investigate the functional impact of HDL-associated miRs on ECs, we combined fluorescently-labelled HDL with a *C. Elegans* (cel) miR-39 or a miR-132 mimic sequence and applied these complexes to cultured HUVECs. As shown in Figure 5A-B, fluorescent signal was detected within the cells indicating successful transfer of HDL. Next, using RT-qPCR we measured the expression of cel-miR-39 and miR-132 to investigate whether the transfer of HDL-miRs into HUVECs increased the expression of both miRs. Indeed, both the expression of cel-miR-39 (Figure 5C) and miR-132 (Figure 5D) in HUVECs was significantly increased. Moreover, the expression of p120RasGap, an established miR-132 target gene (25), was significantly lower compared to the HDL-scramble miR mimic control (Figure 5E) indicating a functional role of HDL associated miR-132 in HUVECs. We subsequently sought to investigate the function of HDL-miR-132 treated HUVECs in EC barrier formation and in an in vitro matrigel angiogenesis assay. As shown in Figure 5F, HDL-miR-132 treated HUVECs did not show altered endothelial barrier function. In contrast, HUVECs treated with HDL-miR-132 displayed a higher total tube length (Figure 5G-H) compared to HDL-scramble miR control treated HUVECs. Subsequently, we also included EV-miR-132 to check whether the HDL-miR-132 mediated effects were carrier specific. Interestingly, EV-miR-132 treated HUVECs did not have an improved barrier resistance (Figure 5I) nor angiogenic tube formation capacity (Figure 5J-K). Having identified that HDL-miR-132 improved tube formation ability, we tested whether HDL-miR-132 could improve the angiogenic function of ECs cultured in the presence of serum from DM and DN patients. In contrast to HUVECs cultured with healthy human serum (ctr-S), serum from DM- (DM-S) and DN patients (DN-S) decreased the angiogenic tube formation ability of ECs (Figure 5L). Nonetheless, HDL-miR-132 treated HUVECs that were cultured in the presence of DM (Figure 5L-M) or DN patient serum (Figure 5L-N) displayed a significantly increased tube formation capacity compared to HUVECs cultured in patient serum only.

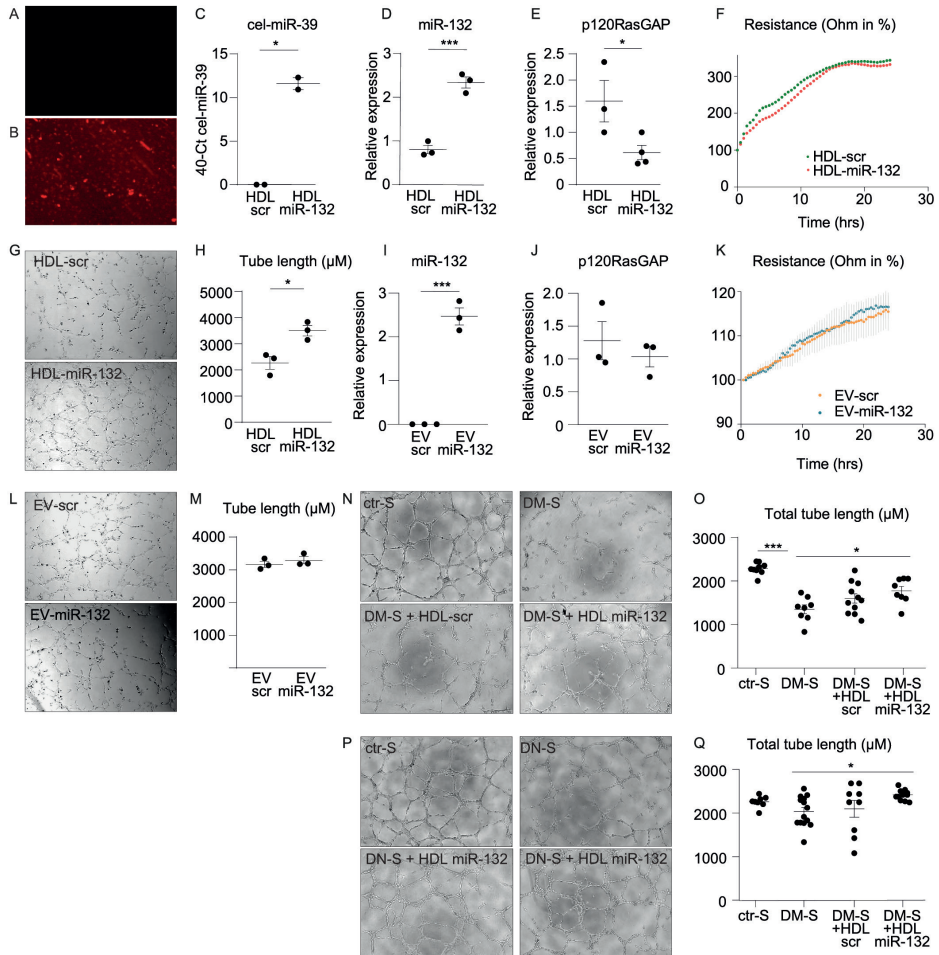


Figure 5. HDL-miR-132 function in endothelial cells.

Fluorescent signal detection in human umbilical vein endothelial cells (HUVECs) before (A) and after (B) transfer of fluorescently labelled HDL. (C) Increased cel-miR-39 expression after HDL-cel-miR-39 transfer in HUVECs. (D) Increased miR-132 expression after HDL-miR-132 transfer in HUVECs followed by a decreased expression of its gene target p120RasGAP (E). (F) No significant change in transendothelial electrical resistance of HDL-SCR and HDL-miR-132 treated HUVECs seeded on ECIS electrodes. (G-H) HDL-miR-132 increased the tube formation ability of HUVECs, as shown by a quantitative analysis of the total tube length (n=4). (I) Increased miR-132 expression upon EV-miR-132 transfer in HUVECs without differential expression of p120RasGAP (J). (K) No significant effect of EV-miR-132 on transendothelial electrical resistance and (L-M) angiogenic tube formation ability. (N) HUVECs cultured in 10% serum of diabetes mellitus- (DM-S) patients have a reduced tube formation capacity compared to healthy control serum, as shown by quantitative analysis of total tube length (n=4) in (O). (P-Q) HDL-miR-132 improves tube formation capacity of HUVECs cultured in serum of diabetic nephropathy patients (DN-S). Data are represented as mean $\pm$ SEM. * p $\leq$ 0.05, *** p $\leq$ 0.001

Discussion

This study demonstrates that the expression, distribution and function of selected plasma EV-, HDL-, and Ago-2 miRs is altered in DN compared to healthy control subjects. Specifically, we found decreased HDL-miR-132 and increased EV-miR-21, EV-miR-126, Ago-miR-126, Ago-miR-145 and Ago-miR-660 in plasma from DM and DN patients compared to healthy controls. Furthermore, we demonstrate that the selective uptake of the differentially expressed HDL-miR-132, EV-miR-21 and EV-miR-126 in ECs instigates carrier specific functions. While EV-miR-21 and EV-miR-126 enhanced endothelial barrier function, HDL-miR-132 significantly improved the angiogenic capacity of ECs. Thus, the changes in carrier specific miR distribution in DM and DN patients could reflect disease progression and provide a causal (HDL-miR-132) or compensatory (EV-miR-21 and EV-miR-126) mechanism in the advancement of DN and its cardiovascular complications.

Several mechanisms could be responsible for the altered distribution of miRs amongst carriers in DN. The cellular origin of circulating miR carriers could be altered as a result of metabolic comorbidities such as hyperglycaemia or insulin resistance. This was recently shown in a streptozotocin induced experimental rat model in which DN increased the plasma concentration of platelet-derived circulating microparticles with a causal role in glomerular endothelial injury (26). Because activated platelets are also a major source for circulating miRs in patients with type 2 diabetes (27) and platelets are an important source for plasma EVs (28), an altered miR-carrier distribution in DN could also be the expression of increased platelet-derived EVs in DN. Furthermore, a metabolic-comorbidity induced systemic inflammatory state could potentially also influence the cellular origin of plasma miR carriers. This has particularly been shown for HDL which macrophage-determined association with miR-223 was altered in inflammatory conditions (13). Likewise, white adipose tissue (WAT) inflammation upon DN (29) may result in more WAT derived EVs and exosomes, because it has been shown that predominantly WAT secretes the majority of plasma EVs and exosomes (15). Lastly, the loss of endothelial quiescence could potentially also influence carrier-miR content and distribution. Particularly in ECs it has been shown that EVs or exosome release is inhibited by endothelial nitric oxide synthase (eNOS) signalling (30) while in general, miR sorting into carriers is often a response to cellular activation (31).

This suggests than an altered distribution of plasma miRs amongst carriers could also be the result of a cellular phenotypic switch from quiescence to inflammation.

Circulating miRs have been increasingly recognized as biomarkers of cardiovascular disease (32). However, the lack of specificity and reproducibility of total plasma miRs could limit their application as clinically relevant biomarkers (33). We hypothesized that when the distribution of miRs in specific carriers instead of total plasma miRs is taken into account, a better association with DN and (micro)vascular injury could be observed. Indeed, the distribution of selected miRs in carriers changed upon disease (Figure 3) where the majority of plasma miRs is associated with Ago-2, while some particular miRs more efficiently associate with either EVs or HDL which is consistent with previous studies (11; 34-37). Nonetheless, EV-miR-21, EV-miR-126 and Ago-miR-660 altered upon DN but did not improve the correlation with vascular injury markers.

Interestingly, with regard to miR-126 we observed an increased expression of this miR in HDL (non-significant), EVs and Ago-2 (both significant) in DN patients, while this particular miR was reduced in total plasma. Since the term extracellular vesicle is an overall term used for all types of vesicles such as exosomes (30-100 nm) and microparticles (100-1000 nm) released from vascular cells (38) and circulating miRs can be identified to a lesser extent in low-density lipoprotein (LDL) as well (34), other particles or lipoproteins could have been missed from our isolation of EVs or HDL respectively. Also, studies have shown that next to Ago-2 protein, also other Ago proteins such as Ago-1, Ago-3 and Ago-4 might be associated with extracellular miRs (39). Although speculative, the shedding of miRs associated with these particular Ago proteins could have an effect on miR expression levels when those levels are measured in total plasma. In contrast to miR-126, HDL-miR-132 yielded a novel association with Ang-2 ($R = -0.34$, $p = 0.03$, $FDR = 0.14$). These data suggest that carrier-specific miRs could provide an improved association with markers of (microvascular) injury and as such could provide sensitive and -specific biomarkers of microvascular injury in DN patients. Since DN patients had a significantly lower eGFR than control and DM +/- early DN patients, it can be argued that a reduced eGFR could cause increased levels of carrier-specific miRs. However end-stage renal disease (ESRD)-patients with a low eGFR displayed a reduced concentration of plasma miRs without a concomitant increase of urine miRs (40), suggesting eGFR independent effects as a cause for differentially expressed plasma miRs in chronic

kidney disease (CKD). Also, because proteinuria was higher in DN patients, the observed changes in carrier-miRs could be due to proteinuria secondary to damage to the glomerular filtration barrier. However, we and others have previously found no relation between proteinuria and circulating miRNA levels (9; 17; 40) suggesting proteinuria is most likely not responsible for changes in miRNA levels in general. Nonetheless, glomerular filtration barrier damage might cause changes in specific carrier-miRs, e.g. glomerular miR-21 expression in DN patients associated with albuminuria (41), and thus proteinuria can not be excluded as a possible cause.. It should also be acknowledged that our DM +/- early DN group (with eGFR >30 mL/min/1.73 m²) contains a quite heterogeneous group of DM patients with variable eGFR that may also contain DN patients (although with higher eGFR than those in the DN group) as their eGFR may fall in category 3 regarding CKD stage based on KDOQI guidelines. As such, also given the relatively low DM +/- early DN and DN patient sample size, additional studies are warranted to further investigate the nature of the changes in levels of these carrier-specific miRs.

Because this study mainly focused on the function of EVs- and HDL- specific miRs, it cannot be excluded that Ago-2-miR complexes have specific functions in DN as well. Notably, we found most miRs to display a higher relative abundance of miRs in the Ago-2 fraction in DM +/- early DN patients, which suggests a disease specific cause. This could be explained by endothelial activation in DN patients (42) which may result in an increased plasma shedding of Ago-2 (and its associated miRs) compared to healthy controls. However, Ago-2-miR complexes were demonstrated to be secreted carried in microvesicles derived from platelets, possibly indicating that Ago-2-miR function is dependent on EV transfer. (43). Furthermore, we found EV-miR-21 and EV-miR-126 to improve endothelial barrier formation while HDL-miR-21 displayed a trend towards a decrease in endothelial angiogenesis and endothelial barrier formation (Figure 4). Consistently, circulating EV-miR-21-3p (44), platelet derived EV-miR-126 (45) and peripheral blood mononuclear cell (PBMC) derived EV-miR-126 (46) previously demonstrated to display similar proangiogenic and homeostatic responses within the vasculature while EV-miR-21 was previously found to associate with DN (47; 48). Interestingly, HDL-miR-132 decreased its target p120RasGAP and improved the angiogenic capacity of ECs, whereas EV-miR-132 did not, indicating a carrier-specific endothelial response exerted by this particular miR. Notably, a decrease in plasma miR-132 was previously demonstrated to cause a decrease in microvascular density in the heart via its target gene p120RasGap

(49). Given the decrease in HDL-miR-132 in DN patients, together with the notion that HDL-miR-132 correlates with levels of vascular injury marker Ang-2, and despite the observation that the HDL-miR-132 amount is only a minor part of total plasma miR-132 (Figure 3), this suggests that HDL-miR-132 exerts a pro-angiogenic response in these patients. More generally, as this provides proof of principle that carrier-miRs have carrier specific functions, the observed shift in miR distribution amongst carriers could have functional consequences.

Since miRs often target multiple functionally related genes (50), it is likely that in addition to the function of the miRs illustrated in this study, they also exert other functions. For instance, context- and cell type-specific functions for miR-21 and miR-132 have been observed previously. While increased tubulointerstitial miR-21 expression amplifies kidney fibrosis in acute kidney injury mouse models, silencing of miR-21 in mice reduced glomerular sclerosis in CKD (51). Similarly, increased expression of miR-132 in tubulointerstitial myofibroblasts promotes kidney fibrosis (52), while podocyte specific amplification of this miR improved actin polymerization thereby healing the injured glomerular capillary (53). Moreover, many other miRs, as well as other types of ncRNA, in EVs, HDL and Ago-2 will be involved in cell-cell communication and vascular injury upon metabolic comorbidities and inflammation. Finally, it cannot be excluded that the concentration and properties of HDL, EVs and Ago-2 themselves change in function upon diabetes such as has been described for HDL in CKD (54). Of note, although not significant, HDL-dependent rescue effects were seen in the matrigel angiogenesis assay which suggests that differences in HDL properties (as compared to HDL from DM and DN patients) may have contributed to the rescue of EC function in Figure 5L-N. Although this study provides proof of concept that HDL, EV and Ago-2 associated miRs can have carrier-specific functions, further *in vivo* studies are necessary to establish their relevance in DN induced microvascular injury.

Taken together, this study demonstrates that the carrier specific miR distribution is altered in plasma of patients with DN and that carrier-specific miRs could potentially improve the correlation with markers of microvascular injury compared to total plasma miRs. Furthermore, we demonstrate that carrier-specific miRs protect from (micro)vascular injury (EV-miR-21/126) or exert a compensatory pro-angiogenic response (HDL-miR-132) in diabetes.

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Author Contributions

BWF researched data and wrote the manuscript, JD, JL, GDT, AB, YY, YW and WS researched the data, RN, TR and MR contributed to the discussion and reviewed the manuscript. AJVZ and RB reviewed and edited the manuscript. AJVZ and RB are guarantors of this work and, as such, had full access to all the data in the study and responsibility for the integrity of the data and the accuracy of the data analyses.

Duality of Interest

The authors have no conflicts of interest.

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Chapter 6

Estrogen-driven control of metabolism associates with reduced circulating extracellular vesicle microRNA-224/452 in transwomen

Submitted

B. W. Florijn, J.M.G.J. Duijs[‡], M. Klaver[‡],
E. N. Kuipers, S. Kooijman, J. Prins, H.
Zhang, H.C.M. Sips, W. Stam, M.A.J.
Hanegraaf, R.W.A.L. Limpens, Rienk
Nieuwland, B. B. van Rijn, T.J. Rabelink,
P.C.N. Rensen, M. den Heijer, R. Bijkerk[‡]
and A.J. van Zonneveld[‡]

[‡]Both authors contributed equally

Abstract

Women with diabetes have a higher prevalence of cardiovascular complications than men, suggesting that sex-steroid hormones like estrogen may impact on female health in diabetes. Here we demonstrate that estrogen suppletion and insulin resistance in male-to-female transgenders coincides with lower plasma levels of miR-224 and miR-452 carried in extracellular vesicles. Systemic silencing of miR-224 and miR-452 in mice impaired energy metabolism with higher plasma insulin levels, increased white adipose lipogenesis and less glucose uptake and mitochondrial respiration in brown adipose tissue. Differentially expressed genes in adipocytes following RNA sequencing were involved in lipogenesis in white adipose tissue and mitochondrial respiration and glucose uptake in brown adipose tissue. In vitro studies confirmed the estrogen-dependent lowering of miR-224 (brown adipocytes) and miR-452 (white adipocytes) and their impact on adipocyte-specific metabolic processes. Collectively, we identified novel estrogen-driven, post-transcriptional networks that could drive the progression to insulin resistance in transwomen and provide potential anti-diabetic therapeutic targets in women.

Introduction

Women with diabetes mellitus (DM) have more fatal vascular events compared to men with diabetes (1). Both glucose control and lipid management in diabetic women is challenging (2, 3) and promotes striking sex differences in diabetes driven all-cause mortality (4, 5). These difficulties in diabetes management in women have been attributed to an association with endogenous sex steroids such as estrogen. However, it is debated whether estrogen itself is detrimental or beneficial for metabolic health in diabetes (6). While most studies consider estrogen to be beneficial, recent findings challenge this assumption because estrogen was found to interfere with glucose and lipid metabolism (7, 8) and to promote systemic inflammation (9). Therefore, a better understanding of the sex differences in the cardiovascular consequences of type 2 DM (10) could improve female-specific clinical care.

It has become increasingly clear that posttranscriptional networks through, an intricate interplay of non-coding RNAs such as microRNAs (miRs) and RNA binding proteins, can coordinate the expression of multiple sets of functionally related genes, that together shape the functional response of cells to either tissue injury or a change in metabolic demand (11). Also in human metabolic tissues such as adipose tissue, studies have addressed this miR-mediated coordination of gene expression via the complementarity of base pairing at 3'-untranslated regions (3'-UTR) of target mRNAs (12). Also, sex-specific expression of miRs in women may result via at least two mechanisms: (i) double dosage of X-chromosome located (X-linked) miRs due to incomplete silencing of the X-chromosome and (ii) estrogen regulation of miR transcription and processing (13). Given that increasing evidence is linking estrogen regulation in adipose tissue to whole body metabolism (14) and miR expression (15), sex-biased miRs in adipose tissue could potentially also alter lipid and glucose metabolism in women explaining sex-differences in the progression to type 2 DM and its cardiovascular complications.

Male to female transgender persons (transwomen) provide a unique insight into the aforementioned estrogen effects on metabolism. Regular estrogen supplementation in transwomen has been found to increase total body fat (16), fasting insulin and HOMA of insulin resistance (HOMA-IR) (17), thereby reducing peripheral insulin sensitivity (18). This estrogen driven, prediabetic phenotype in transwomen

increases their risk for type 2 diabetes and future cardiovascular disease (CVD) (19). Mechanistically, ectopic fat accumulation following increased total body fat is a determinant of insulin resistance via adipokine secretion and cytokine secretion from infiltrated macrophages (20). More recently, adipose tissue was also found to be a major source of circulating plasma miRs that are carried in extracellular vesicles (EVs), identifying a novel class of circulating ‘adipokines’ that can act as post-transcriptional regulators of metabolism at distal tissue sites (21). Given that estrogen also regulates the EV-miR cargo and displays strong associations with body composition and metabolic status in women (22), the mechanism of EV mediated miR delivery at distal sites could potentially also be involved in the development of a prediabetic state in women.

To understand the post-transcriptional mechanisms underlying the estrogen-driven worsening of glucose and lipid metabolism, we assessed miR levels in plasma and EVs from male to female transgenders (transwomen) in whom estrogen suppletion is known to induce insulin resistance (17). Following these studies, we identified two particular miRs, namely miR-224 and miR-452, to be decreased upon estrogen treatment. Subsequent systemic silencing of both miRs in mice led to an increase in plasma insulin levels, a reduction in glucose uptake in brown adipose tissue (BAT) and an increase in lipid uptake in white adipose tissue (WAT). Following RNA sequencing (RNA-seq) of BAT and WAT tissues, differentially expressed genes strongly associated with alterations in glucose and lipid metabolism. Taken together, our study demonstrates that an estrogen-driven post transcriptional network impacts on energy metabolism in adipocytes thereby providing a potential explanation for sex differences in prediabetes.

Methods

Patients

This study complied with the ethical principles of the Declaration of Helsinki and was conducted following approval by the Institutional Review Board of the Amsterdam University Medical center (Amsterdam UMC) and the Leiden University Medical Center (LUMC). All patients gave written informed consent. Transwomen received oral treatment with a daily dose of both 50 mg cyproteroneacetate (CPA) (Androcur®, Bayer) and 4 mg Estradiol Valerate (Progynova®, Bayer) or 100 µg/24h transdermal estradiol (System®, Janssen-Cilag) twice a week, as previously

described (77). Paired plasma samples were obtained at the start of treatment and after one year of daily estrogen suppletion. Patient characteristics of the transwomen cohort 1 and 2 are displayed in Tables 1 and 2, respectively.

Plasma RNA isolation

Plasma RNA from each patient sample was isolated from 200 μ L EDTA-plasma with 800 μ L Trizol reagent (Invitrogen, The Netherlands) by using the RNeasy Micro Kit (Qiagen, The Netherlands) and an adapted protocol. In short, 160 μ L chloroform was added to the Trizol sample and centrifuged for 15 min at 12,000 rpm. The aqueous phase volume was merged with 1.5x 100% ethanol, transferred to a MinElute Spin column (Qiagen) and centrifuged for 15s at 14,000 rpm. RNA was washed with 700 μ L RWT buffer and centrifuged for 15s at 14,000 rpm. This was followed by two washing steps with 500 μ L RPE buffer after which the column was centrifuged at 14,000 rpm. Total plasma RNA was eluted with 15 μ L RNase-free water.

Library preparation and Next Generation Sequencing of Plasma miRs

Plasma miR sequencing was performed by Exiqon according to protocol. Briefly, plasma RNA was converted into microRNA NGS libraries using NEBNext library generation kit (New England Biolabs Inc.) according to the manufacturer's instructions. Each individual RNA sample had adaptors ligated to its 3' and 5' ends and converted into cDNA. Then the cDNA was pre-amplified with specific primers containing sample specific indexes. After 18 cycle pre-PCR the libraries were purified on QiaQuick columns and the insert efficiency evaluated by Bioanalyzer 2100 instrument on high sensitivity DNA chip (Agilent Inc.). The miRNA cDNA libraries were size fractionated on a LabChip XT (Caliper Inc.) and a band representing adaptors and 15-40 bp insert excised using the manufacturer's instructions. Samples were then quantified using qPCR and concentration standards. Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar concentrations. The library pool(s) were finally quantified again with qPCR and optimal concentration of the library pool used to generate the clusters on the surface of a flow cell before sequencing using v3 sequencing methodology according to the manufacturer's instructions (Illumina Inc.). Samples were sequenced on the Illumina NextSeq 500 system. Experiments were conducted at Exiqon Services, Denmark.

Plasma EV isolation

Plasma EVs (70 nm) were isolated by applying 125 μ L human plasma to a 3.64 mL Sepharose CL-2B size exclusion chromatography (SEC) column (78). Fractions (250 μ L) were eluted with PBS and for each sample a newly packed column was used. Particle presence in different fractions was analysed using resistive pulse sensing (RPS, qNano, Izon Science, Christchurch, New Zealand) as described before (79) while protein presence in fractions 11-35 was confirmed using a BCA Protein Assay Kit (Pierce). EV fractions (fraction 1-10) were combined until a total volume of 2 mL eluate was collected and applied to an Amicon Ultra-4 Centrifugal Filter unit for concentration after which the presence of EVs was validated using nanoparticle tracking analysis (NTA, NS500; Nanosight, Amesbury, UK) and confirmed by Negative-Stain Transmission Electron Microscopy (TEM). EV fraction was treated with proteinase-K (Thermo Fisher Scientific, The Netherlands) after which EV and protein fractions were combined with Trizol to isolate RNA.

qPCR validation of plasma miRNA, EV miRNAs and mRNA expression

Selected miRNAs were validated with quantitative PCR, using individual samples that comprised the pooled samples that were used for plasma miR sequencing. Taqman primers (Cat. 4427975, Thermo Fisher Scientific, Waltham, MA) were used according to manufacturer's protocols. Briefly, total plasma RNA and EV-RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Cat. 4366596, Thermo Fisher Scientific). Obtained miRNA Ct-values were normalized to plasma levels of miR-16 and U6 small nuclear RNA and expression was quantified using the $\Delta\Delta$ Ct method. RT-qPCR validation of NGS sequencing based differential gene expression was performed after 500 ng RNA was reverse transcribed using dinucleotide triphosphates (Invitrogen) and oligo(dT) (Invitrogen). Using a M-MLV First-Strand synthesis system (Invitrogen) cDNA was synthesized and validation of mRNA expression was carried out using SYBR Green Master Mix (Applied Biosystems). Target gene mRNA primer sequences are listed in Supplementary Table 2.

Animal experiments

Male C57Bl/6J mice (n=10 per group, age=8 weeks, Charles River Nederland, Maastricht, the Netherlands) were randomized in three groups and received two subcutaneous injections of 25 mg/kg locked nucleic acid (LNA) modified antisense miR-224 (antimiR-224), miR-452 (antimiR-452) or scrambled miR sequence

(scramblemiR). Five days before injection, mice were individually housed in fully automated metabolic cages up and until the day of sacrifice and had access to *ad libitum* standard chow diet and drinking water *ad libitum*. Following anti-miR injection blood was drawn to measure plasma insulin levels. Indirect calorimetry was performed in fully automated metabolic cages (LabMaster System, TSE Systems) during the 48 hours of the *in vivo* experiment. O_2 consumption (VO_2), CO_2 production (VCO_2), and caloric intake were measured 48 hours and total EE was estimated from the VO_2 and resting energy requirement. Carbohydrate oxidation was calculated using the formula $((4.585 \times VCO_2) - (3.226 \times VO_2)) \times 4$. Similarly, fat oxidation was calculated using the formula $((1.695 \times VO_2) - (1.701 \times VCO_2)) \times 9$.

Plasma ELISA

Plasma insulin concentrations were measured by ELISA (Mercodia, 10-1247).

Clearance of radiolabeled glucose and lipoprotein-like particles

Glycerol tri[^3H]oleate-labeled lipoprotein-like triglyceride (TG)-rich emulsion particles (80 nm) were prepared as previously described (80) and [^{14}C]deoxyglucose ([^{14}C]DG) was added in a $^3H:^{14}C = 4:1$ ratio. After 6 hours fasting, mice were injected with 200 μL of emulsion particles (1 mg TG per mouse) via the tail vein. After 15 minutes, organs were harvested and dissolved overnight at 56°C in Solvable (Perkin Elmer). Glycerol tri[^3H]oleate- and [^{14}C]DG-derived activity was quantified and expressed per gram of wet tissue weight.

Tissue histology and immunohistochemistry

Formalin-fixed interscapular BAT (iBAT), subcutaneous WAT (sWAT), and gonadal WAT (gWAT) were dehydrated in 70% EtOH, embedded in paraffin, and cut into 5- μm sections. Sections were stained with hematoxylin and eosin (H&E) using standard protocols. All sections were digitalized with Philips Digital Pathology Solutions (PHILIPS Electronics) for morphological measurement. White adipocyte size was quantified using ImageJ software (Version 1.50)

RNA-seq library preparation

The NEB Next Ultra Directional RNA Library Prep Kit for Illumina was used to process samples according to the manufacturer's protocol. Briefly, mRNA was isolated from total RNA using the oligo-dT magnetic beads. After fragmentation

of the mRNA, a cDNA synthesis was performed for ligation of with the sequencing adapters and PCR amplification of the resulting product.

Mapping and analysis of RNA-seq data

Mus musculus reference version GRCm38.p4 was used for alignment of samples. The reads were mapped to the reference sequences using a short-read aligner based on the Burrows-Wheeler Transform. The default mismatch rate of 2% (2 mismatches in a read of 100 bases) was used. Based on the mapped locations in the alignment file, the frequency of reads mapping to a transcript was determined. Read counts served as input for downstream RNA-seq differential expression analysis. The read counts were loaded into the DESeq package v 1.10.1, a statistical package within the R platform v2.15.3. Additionally, RPKM/FPKM (reads/fragments per kilobase of exon per million reads mapped) values were calculated.

Pathway Analysis

Pathway analyses was carried out using Ingenuity Pathway Analysis (IPA) software. Bias corrected p-values were determined, and pathways were selected with a p-value lower than 0.01.

Cell culture of murine brown adipocytes and 3T3-L1 white adipocytes

Brown preadipocytes were isolated from interscapular BAT depots of 5-week-old male C57BL/6J mice as previously described (81). Cells were reversibly immortalized using doxycycline-controlled expression of simian virus large T antigen and were grown confluent in growth medium consisting of DMEM/F-12 GlutaMAX (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies Europe), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). 100 ng/mL doxycycline (Sigma-Aldrich) was added to the growth medium for expansion of cells. Upon confluence, cells were differentiated in growth medium supplemented with 5.6 nM bovine insulin (Sigma-Aldrich), 126 µM sodium ascorbate (Sigma-Aldrich) and 1 µM of the peroxisome proliferator-activated receptor γ agonist rosiglitazone (Sigma-Aldrich), 10 mM HEPES (adjusted to pH 7.4 with NaOH). Experiments were performed on day 12-14 of differentiation. 3T3-L1 preadipocytes (Zenbio) were grown confluent in growth medium consisting of DMEM, high glucose, 10% heat-inactivated FBS (Life Technologies Europe), penicillin and streptomycin. Two days after grown confluent, cells were differentiated in growth medium (DMEM / Ham's F-12 medium (1:1, v/v)

supplemented with HEPES pH 7.4, 10% heat-inactivated FBS (Life Technologies Europe), human insulin, dexamethasone, penicillin and streptomycin, 3-isobutyl-1-methylxanthine (IBMX) and PPAR γ agonist rosiglitazone.

Cell treatment

Differentiated 3T3-L1 adipocytes and differentiated brown adipocytes were incubated with 100 nM 17- β estradiol (E2758, Sigma Aldrich) for 48 hours. After 48 hours cells were washed with PBS for 3 times

Oxygen consumption and extracellular acidification of murine brown adipocytes

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF96 analyzer (Agilent Technologies). LNA-antimiR-224 and -452 transfected murine brown adipocytes were cultured and differentiated in Seahorse cell culture plates following the protocol described above. One hour prior to the oxygen consumption measurements medium was replaced by Seahorse XF Base Medium (Agilent) with 5 mM glucose (Sigma-Aldrich), 0.5 mM sodium pyruvate, 1x GlutaMAX and 10% FBS (all from Life Technologies Europe). Basal respiration was measured three times, followed by three measurements after addition of 1.5 μ M oligomycin (Cayman Chemical), 3 μ M FCCP and 1 μ M rotenone and antimycin A (all from Sigma-Aldrich). For extracellular acidification measurements medium was replaced one hour prior to the run by Seahorse XF Base Medium (Agilent) with 1x GlutaMAX and 10% FBS (both from Life Technologies Europe) with pH adjusted to 7.4. Basal respiration was measured three times, followed by six measurements after addition of 10 mM glucose (Sigma-Aldrich), 1.5 μ M oligomycin (Cayman Chemical) and 50 mM 2-deoxy-glucose (Sigma-Aldrich). OCR and ECAR values were adjusted for total protein using the BCA protein assay kit (Thermo Scientific Pierce) according to the manufacturer's instruction.

Mitotracker experiment

Immortalized murine brown adipocytes were transfected with LNA-antimiR-224 and -452 and incubated for 30 min with MitoTracker Green FM (125 nM; Thermo Fisher) and MitoTracker RedCMXRos (250 nM; Thermo Fisher) in DMEM/F12 (Sigma) without FBS. Hereafter, the medium was changed, and live cells were imaged using a confocal LSM (Leica TCS SP8, Leica Microsystems). Control cells

were monitored to correct for potential photobleaching, and total cell fluorescence per condition was quantified using ImageJ.

Glucose uptake experiments

Glucose uptake by immortalized murine brown adipocytes transfected with LNA-antimiR-224 and -452 was performed using a glucose uptake colorimetric assay kit (Sigma-Aldrich/Merck, The Netherlands) according to the manufacturer's protocol. Briefly, transfected cells were starved in serum-free medium overnight, washed and then glucose starved. Subsequently, cells were stimulated with insulin after which glucose was added. Following incubation, cells were lysed after which the lysate was combined with assay buffers and enzymes. Following the recycling amplification reaction absorbance was measured at 412 nm.

Statistics

Differential expression analysis of plasma microRNAs in the Next Generation Sequencing experiment was done using the EdgeR statistical software package (Bioconductor). For normalization the trimmed mean of M-values method was used based on log-fold and absolute gene wise changes in expression levels between samples (TMM normalization). All other data are expressed as mean $\pm$ SEM. Variable distribution was tested using the Kolmogorov-Smirnov test for normal distribution. In addition, multivariable linear regression was used to adjust for possible confounders. Statistical analysis was performed with GraphPad software (La Jolla, CA) using two-tailed paired or unpaired Student's t-test or ANOVA with Bonferroni's post hoc test.

Study approval

These studies were approved by the Institutional Review Boards of both the VU University Medical Center (Amsterdam, The Netherlands) and the Leiden University Medical Center (Leiden, The Netherlands) and complied with the ethical principles of the Declaration of Helsinki. All patients gave written informed consent. All animal experiments and protocols were approved by the animal welfare committee of the veterinary authorities of the Leiden University Medical Center (Leiden, The Netherlands).

Results

Identification of circulating estrogen-responsive miRs in transwomen

To identify candidate estrogen-responsive plasma miRs, we performed a pilot study assessing plasma miR profiles in transwomen who received one year of oral estrogen suppletion prior to surgical transition (23). Following next-generation sequencing of miRs from four pooled samples containing EDTA-plasma from five transwomen, before (n= 20) and after (n= 20) one year of estrogen treatment, we identified 667 miRs (Figure 1A) of which 33 were estrogen responsive and differentially expressed (Supplementary Data 1). Specifically, after one year of estrogen suppletion, miR-224, -122-5p, -539-5p, 23a-3p, -133b, -452-5p, 23b-3p, -3913-5p, -144-5p, -331-5p, -766-3p, -874-3p, 30b-5p and miR-490-3p levels displayed a significant decrease, while circulating levels of miR-3138, -215-5p, -483-5p, -let-7b-5p, -6787-3p, -184, -3679-5p, -370-5p, -615-3p, -let-7d-3p, -432-5p, -139-3p, -6747-3p, -433b-3p, -584-5p, -3198, -3940-3p, -625-3p and miR-6786-3p increased (Figure 1B). The following thirteen miRs were selected for validation using RT- qPCR: miR-224, -122, -23a, -452, -139, -133b, -215, -9, -874, -30b, -483, -539 and -652. These miRs were selected based on (i) a sufficient level of regulation across sample groups (> 2-fold up- or down- regulation, corresponding to +/- 1.0 logFoldChange) and (ii) differential expression (based on p-value) as a result of one-year estrogen suppletion.

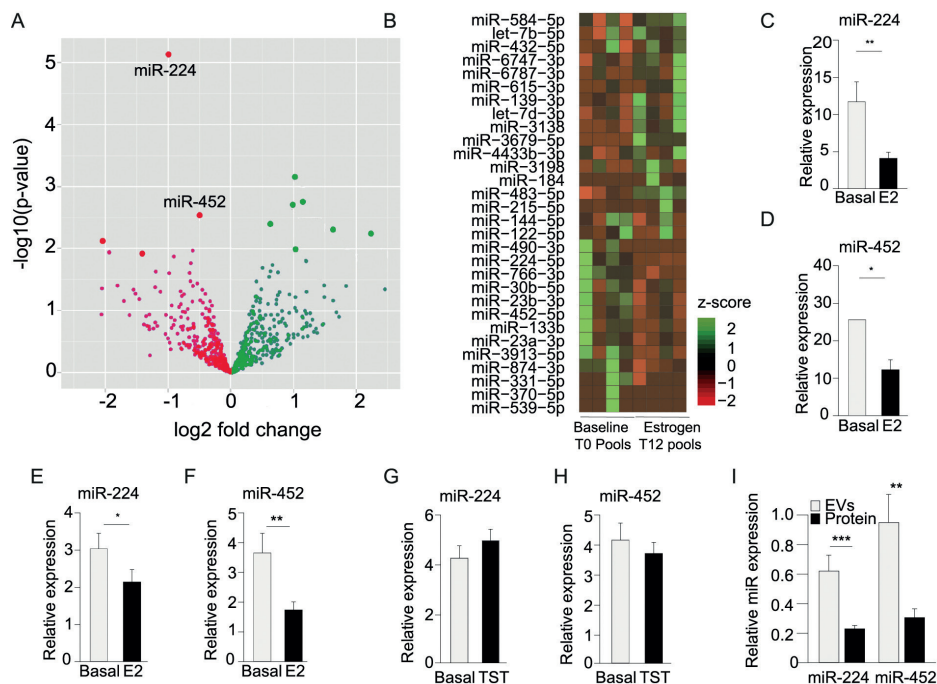


Figure 1. Identification of circulating miRNAs in human plasma of male-to-female transgender persons (transwomen).

(A) Volcano plot depicting differentially expressed miRNAs after estrogen treatment. (B) Heatmap depicting 33 differentially expressed circulating, estrogen responsive miRNAs. (C-D) RT-qPCR validation of the differentially expressed miR-224 and miR-452 in the same cohort of 20 transwomen. (E-F) RT-qPCR validation of miR-224 and miR-452 in a second, independent transwomen cohort (n=39). (G-H) Expression levels of circulating miR-224 and miR-452 in transmen after testosterone treatment (n=51). (I) Higher expression levels of miR-224 and miR-452 in extracellular vesicles (EVs) compared to total plasma protein both at baseline and after estrogen supplementation in transwomen (n=20). Basal, baseline state before hormone supplementation; E2, estradiol; TST, testosterone. Data are represented as means $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ according to the two-tailed paired and unpaired Student's t-test.

Validation of estrogen-responsive miRNAs in independent transgender cohorts

To validate whether the selected circulating miRNAs specifically associate with estrogen treatment in plasma of transwomen, we conducted a three-step validation process. First, we reassessed plasma levels of the miRNAs (Supplementary Figure 1) by RT-qPCR in the individual samples of the same transwomen cohort that was used in the pilot study, before (n=20) and after (n=20) estrogen supplementation (Table 1 for clinical characteristics). Out of the 13 selected miRNAs, we observed a significant decrease in circulating levels of miR-224 (Figure 1C), miR-452 (Figure 1D) and miR-

133b after a year of estrogen suppletion (Supplementary Figure 1). Subsequently, in a second independent transwomen cohort (Patient characteristics in Table 2) we again assessed plasma levels of miR-224, miR-452 and miR-133b. This study demonstrated that plasma miR-224 and miR-452 decreased (Figure 1E-F) while plasma miR-133b was not affected (Supplementary Figure 2). Because next to estrogen suppletion, transwomen also receive a daily dose of cyproterone acetate to block testosterone secretion, we sought to exclude that testosterone levels would be responsible for the observed differences in the circulating levels of the miRs. Therefore, we assessed plasma levels of miR-224, -452, and -133b in female-to-male transgender persons (transmen, n=50, clinical characteristics in Supplementary Table 1) who received one year of oral testosterone suppletion prior to surgical transition. In transmen, these miRs were not affected (Supplementary Figure 3). The supposedly coordinate regulation of miR-224 and miR-452 expression by estrogen could be consistent with the fact that both miRs are transcribed from a single transcript from the GABRE gene on the X-chromosome (Source: miRbase version 21). Following the above, and given that both miRs are predominantly expressed in adipose tissue (24) we subsequently focused our experiments on both miR-224 and miR-452. Therefore, we next assessed whether miR-224 and miR-452 associate with EVs or plasma proteins and used size-exclusion chromatography (SEC, Supplementary Figure 4) to isolate EVs from plasma of the transwomen pilot study cohort (n=20). Following qPCR measurement of miR-224 and miR-452 in the EV and protein fraction of these plasma samples, we found an approx. 3-fold higher expression of both miRs within the EV fraction compared to the plasma protein fraction after estrogen suppletion (Figure 1I). Subsequently, to assess the relation between plasma miR-224 and miR-452 levels and insulin resistance following estrogen suppletion we investigated the correlation of these miRs with the calculated homeostatic model assessment for insulin resistance (HOMA-IR) in transwomen. Interestingly, we observed that miR-224 negatively correlated with HOMA-IR ($R=-0.38$, $p=0.03$), while miR-452 and HOMA-IR were not significantly correlated (data not shown).

Table 1. Clinical characteristics of the pilot n=20 transwomen cohort

	Baseline	Estrogen	p-value
Age (years)	35 ± 13	36 ± 13	
BMI (kg/m ²)	25.2 ± 4.1	24.5 ± 6.8	0.623
Oestrogen (pmol/L)	96 ± 21	310 ± 246	0.001
Testosterone (nmol/L)	19.9 ± 8.7	0.8 ± 0.2	0.001
Systolic blood pressure (mm Hg)	133 ± 10	128 ± 12	0.051
Diastolic blood pressure (mm Hg)	86 ± 11	82 ± 9	0.167
Hemoglobin (mmol/L)	10.2 ± 0.5	9.0 ± 0.2	0.002
Hematocrit (L/L)	0.47 ± 0.02	0.43 ± 0.02	0.004
Glucose (mmol/L)	5.6 ± 0.5	5.5 ± 0.6	0.824
Insulin (pmol/L)	72 ± 44	105 ± 67	0.002
Creatinine (μmol/L)	79 ± 9	79 ± 13	0.913
Cholesterol (mmol/L)	4.59 ± 0.86	4.05 ± 0.71	0.002
Triglyceriden (mmol/L)	1.06 ± 0.42	0.97 ± 0.36	0.303
HDL-cholesterol (mmol/L)	1.38 ± 0.37	1.16 ± 0.30	0.001

BMI, body mass index; HDL, high density lipoprotein

Table 2. Clinical characteristics of the validation n=30 transwomen cohort

	Baseline	Estrogen	p-value
Age (years)	34 ± 12	35 ± 13	
BMI (kg/m ²)	23.5 ± 6.1	24.9 ± 4.3	0.074
Oestrogen (pmol/L)	86 ± 22	275 ± 231	0.001
Testosterone (nmol/L)	20.4 ± 6.3	0.8 ± 0.4	0.001
Systolic blood pressure (mm Hg)	127 ± 10	122 ± 9	0.003
Diastolic blood pressure (mm Hg)	80 ± 9	75 ± 8	0.003
Hemoglobin (mmol/L)	9.8 ± 0.5	8.8 ± 0.5	0.001
Hematocrit (L/L)	0.45 ± 0.03	0.42 ± 0.02	0.005
Glucose (mmol/L)	5.4 ± 0.7	5.2 ± 0.7	0.226
Insulin (pmol/L)	50.1 ± 30.9	71.8 ± 49.4	0.036
Creatinine (μmol/L)	78.2 ± 8.8	73.1 ± 9.0	0.001
Cholesterol (mmol/L)	4.7 ± 1.1	4 ± 0.8	0.001
Triglyceriden (mmol/L)	1.1 ± 0.5	0.9 ± 0.3	0.033
HDL-cholesterol (mmol/L)	1.4 ± 0.3	1.1 ± 0.3	0.001

BMI, body mass index; HDL, high density lipoprotein

Systemic silencing of miR-224 and miR-452 in mice affects adipocyte specific nutrient uptake

Next, we studied the function of miR-224 and miR-452 in lipid and glucose metabolism. First, male C57BL/6J mice (n=10) were housed in metabolic cages and injected with locked nucleic acid (LNA) antisense (anti)-miR-224, LNA anti-miR-452 or control scramble-miR (Figure 2A). Within 48 hours, anti-miR-224 and anti-miR-452 injection in mice led to a higher energy expenditure (Figure 2B) while respiratory exchange ratio, carbohydrate oxidation and fat oxidation were not affected (data not shown). Then we studied whether silencing of both miRs affected plasma insulin levels and found that in anti-miR-452 treated mice, insulin levels were significantly increased after 48 hours (Figure 2C). Therefore, to study the effect of both miRs on nutrient uptake, we next assessed the impact of anti-miR-224 and -452 treatment on glucose and triglyceride (TG)-derived fatty acid (FA) uptake in metabolic tissues such as skeletal muscle and adipose tissue. To that end we determined tissue-specific uptake of glucose, using [^{14}C]deoxyglucose (DG) and TG-derived FA, using glycerol tri[^3H]oleate packaged in TG-rich lipoprotein-like particles. Interestingly, anti-miR-224 treatment in mice resulted in a reduction of [^{14}C]DG uptake in subscapular brown adipose tissue (sBAT, Figure 2D), while anti-miR-452 treatment in mice reduced [^{14}C]DG in both interscapular brown adipose tissue (iBAT) and sBAT. In anti-miR-224 treated mice [^3H]oleate uptake was significantly increased in skeletal muscle and gonadal white adipose tissue (gWAT) and a trend towards increased [^3H]oleate uptake was observed in subcutaneous white adipose tissue (sWAT, $p=0.08$), while anti-miR-452 treatment of mice increased [^3H]oleate uptake in subcutaneous white adipose tissue (Figure 2E). Lastly, to study whether the observed increase in [^3H]oleate uptake led to an increase in white adipocyte size, we performed HE staining of sWAT tissue, which demonstrated a marked increase in white adipocyte size in the anti-miR-224 treated mice (Figure 2F-G).

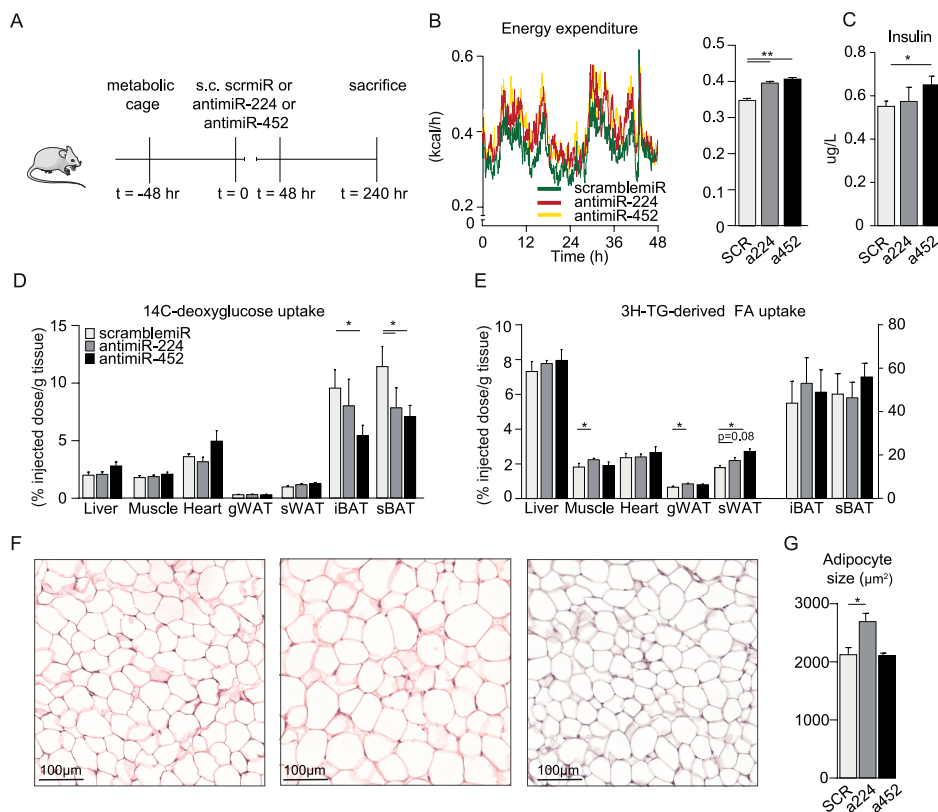


Figure 2. Systemic silencing of miR-224 and miR-452 affects adipocyte specific nutrient uptake.

(A) Experimental set up, mice were housed in calorimetric cages 48 hours before i.p. injection of scramble miR (n=10), anti-miR-224 (n=10) and anti-miR-452 (n=10). 240 hours after injection mice were sacrificed. (B) Energy expenditure (EE) was assessed and quantified over 48 hours. (C) MiR-452 silencing increased plasma insulin levels. (D) MiR-224 silencing reduced deoxyglucose uptake in subscapular BAT (sBAT) while miR-452 silencing decreased deoxyglucose uptake in interscapular BAT (iBAT) and subscapular (sBAT). (E) MiR-224 silencing increased triglyceride-derived fatty acid uptake in skeletal muscle, gonadal WAT (gWAT) and subcutaneous WAT (sWAT) while miR-452 silencing increased triglyceride-derived fatty acid uptake in subcutaneous WAT (sWAT). (F-G) Hematoxylin and eosin (H&E) staining of white adipocyte size in anti-miR-224 and anti-miR-452 treated mice compared to scramble control mice. SCR, scramble miR; a224, anti-miR-224; a452, anti-miR-452. Data represents means $\pm$ SEM. *p $\leq$ 0.05 according to a one-way ANOVA, Bonferroni's post-hoc test.

MiR-224 and miR-452 share a strong bias for target genes in mitochondrial energy metabolism, insulin signaling, glucose metabolism and lipogenesis

Given that miR-224 and miR-452 silencing in mice stimulates fatty acid uptake in skeletal muscle and WAT and decreases glucose uptake in BAT, we next performed RNA sequencing (RNA-seq) of WAT and BAT from anti-miR-224 and anti-miR-452 treated mice to identify alterations in the expression of genes related to the metabolic changes observed. Using *in silico* analysis of RNA-seq data with the Ingenuity Pathway Analysis (IPA) tool, in BAT we identified mitochondrial dysfunction and insulin receptor signaling as significant top canonical pathways affected after anti-miR-224 and anti-miR-452 treatment, respectively (Supplementary Figure 5). In contrast, in anti-miR-224 treated WAT tissue, adenosine monophosphate-activated protein kinase (AMPK) signaling (regulates adipocyte metabolism), and signal transducer and activator of transcription 3 (STAT3) (controls lipogenesis and adipocyte hypertrophy) were affected, while anti-miR-452 treatment affected the HIPPO-pathway (adipocyte differentiation) and insulin receptor signaling. Following the major metabolic impact induced by miR-224 and miR-452 silencing, we subsequently used IPA to identify all potential target genes for these miRs that could serve as upstream regulators of differential top canonical pathways. Using IPA, we found that both miR-224 and miR-452 have a strong bias towards target genes that associate with metabolism in general suggesting a very complex coordination of multiple metabolic pathways by these miRs (Figure 3A). Indeed, when we plotted the differential expression of potential miR-224 and miR-452 target genes in heatmaps, many of them were upregulated in BAT and WAT (Supplementary Figure 6, metabolic genes marked with an asterisk) and some key metabolism-related target genes could be validated by qPCR (Figure 3B-C). Specifically, we identified an increased expression of the miR-224 targets *Ndufa11*, *Cox17*, *Cox16b1* and *Id3* in BAT (Figure 3B), the increases of which associate with metabolic syndrome and obesity (25, 26). *Nr4a1* expression, an effector of BAT thermogenesis (27) was decreased with miR-224 silencing. Interestingly, in WAT (Figure 3C), anti-miR-224 treatment coordinately upregulated genes that regulate lipid metabolism such as *Arf6* (28, 29) and *Adam10* (30) or exacerbate insulin sensitivity like *Sema3C* (31) and *Sdc4* (32). Similarly, anti-miR-452 treated WAT tissue displayed higher expression of lipogenesis associated genes, such as *Pnrc2* (33) and *Stard4* (34) as well as genes that promote insulin resistance like *Cntf* (35) or associate with weight gain and adiposity (*Tgif-1*) (36, 37).

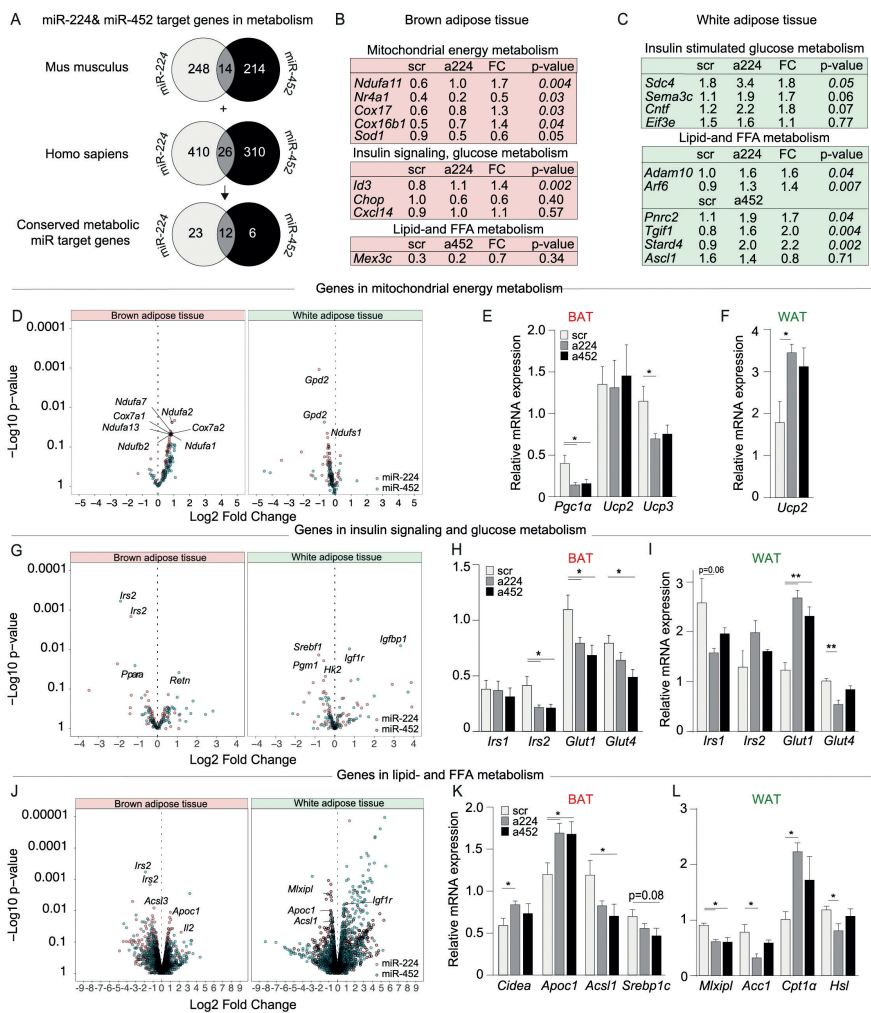


Figure 3. Next-generation sequencing (NGS)-derived differential expression and qPCR validation of (miR target) genes involved in mitochondrial energy, glucose and lipid metabolism. (A) Both miR-224 and miR-452 have a strong bias towards target genes that associate with metabolism in general. (B) Differentially expressed miR-224- and miR-452 target genes involved in mitochondrial energy metabolism, insulin signaling, glucose metabolism and lipid metabolism in BAT and (C) WAT tissue. (D) Volcano plot of NGS-derived differential expression of genes (n=4 per group) involved in mitochondrial energy metabolism. (E) RT-qPCR validation (n=8-10 per group) of NGS derived, differential expression of genes involved in mitochondrial energy metabolism in BAT and (F) WAT. (G) Volcano plot of NGS-derived differential expression of genes involved in insulin signaling and glucose metabolism followed by RT-qPCR validation of several key genes in (H) BAT and (I) WAT. (J) Volcano plot of NGS-derived differential expression of genes involved in lipid metabolism followed by RT-qPCR validation in (K) BAT and (L) WAT. Data are represented as means $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$ according to a one-way ANOVA, Bonferroni's post-hoc test.

Adipose tissue specific differential expression of genes involved in metabolism following miR-224 and miR-452 silencing

Next we sought to obtain evidence for the differential expression of genes downstream of miR-224 and miR-452 silencing that function in the top altered canonical metabolic pathways. To that end, we first generated gene-distribution volcano plots confirming marked differential expression of genes in mitochondrial energy metabolism (Figure 3D), insulin signaling and glucose metabolism (Figure 3G) and lipid metabolism (Figure 3J) (RNA-seq data can be found in Supplementary Data 2, while a selected subset of differentially expressed genes involved in these pathways is displayed in Table 3). Next we validated the differential expression of a selected set of genes whose functions are known to have a major impact on energy metabolism by RT-qPCR. With regard to mitochondrial energy metabolism in BAT, qPCR validation confirmed a reduced expression of *Ucp3* and *Pgc1 α* (Figure 3EF), which regulates mitochondrial biogenesis and which loss predisposes to insulin resistance (38). Given the *in vivo* decrease in BAT specific glucose uptake, we also validated the loss of BAT-specific glucose uptake genes namely *Glut4* and *Irs2* (39, 40) (Figure 3H). Similar decreases in key insulin signaling genes like *Irs1* and *Glut4* were observed in anti-miR-224 treated WAT (Figure 3I). Finally, in lipid metabolism in BAT we validated a lower expression of *Cidea* (Figure 3K), which associates with lipogenesis (41), a decreased expression of *Acs1*, a promoter of FA oxidation (42) and increased *Apoc1* expression. In WAT-specific lipid metabolism, the expression of *Mlxipl*, *Acc1* and *Hsl* was decreased (Figure 3L), which collective decrease is associated with more FA uptake (43-45).

Table 3. Next generation sequencing derived differential gene expression (FPKM, $p < 0.05$, $n = 4$) of a selected set of key-metabolism genes involved in glucose and lipid metabolism in brown and white adipose tissue after systemic silencing of miR-224 and miR-452 in mice.

	Glucose Metabolism	Lipid metabolism	Mitochondrial Energy Metabolism
AntimiR-224 - BAT	Irs1↓, Irs2↓, Eif4ebp1↑, Frs2↓, Jun↓, Fbp1↑, Pik3ca↑	Cidea↑, Fabp4↑, Fabp5↑, Slc27a3↓, Acaa2↑, Apoc1↑, Pdhab↑, Pdk4↓, Idh3b↑, Suclg1↑, Acaa1a↑, Acca2a↑, Acat1↑, Acadvl↑, Acsl3↓	Ppargc1a↓, Cox5b↑, Cox6b1↑, Cox6c↑, Cox7a2↑, Cox7a2l↑, Cox7b↑, Cyc1↑, Ndufa1↑, Ndufa11↑, Ndufa2↑, Ndufa3↑, Ndufa4↑, Ndufa6↑, Ndufa7↑, Ndufa8↑, Ppa2↑, Sdhb↑, Slc25a21↓, Ucp3↓,
AntimiR-452 - BAT	Insr↓, Irs2↓, Pfkfb3↓, Sorbs1↓, Pdpk1↓, Pir3cb↓, Prkc1↓, Srebf1↓, Sorbs1↓, Frs2↓	Scd2↓, Srebp1c↓, Acsl1↓	Ppargc1a↓, Atp6vao2↓, Cox6c↑, Lhpp↑, Slc25a15↓, Ucp2↑
AntimiR-224 - WAT	Igf1r↑, Grb2 ↑, Ptpn1↑, Ptprf↑, Adra1d↓, Srebf1↓, Glut4↓, PI3K↑, mTOR↓	Gpr120↓, Mlxipl↓, Acc1↓, Acc2↓, Srebp1c↓, Hsl↓, Acsl1↓, Apoc1↓	Acads↓, Acsl5↑, Cpt1a↑, Crat↓, Hadha↓, Lipe↓, Hmgcs2↑
AntimiR-452 - WAT	Gys1↓, Fbp2↑, Igf1r↑, Igfbp1↑, Ptprf↑, Araf↑	Gpr120↓, Mlxipl↓, Acc1↓, Apoc1↓	Slc27a2↑, Prkacb↑ Slc25a15↑, Slc25a21↑

In vitro validation of alterations in lipogenesis in WAT and mitochondrial function in BAT following miR-224/452 silencing

To further validate the hypothesis that estrogen alters miR-224 and miR-452 to modulate adipose tissue function, we first cultured immortalized murine white adipocytes (3T3-L1) and brown adipocytes (T37i) and exposed these cells to estrogen to confirm the downregulation of both miRs by estrogen. Addition of estrogen to 3T3-L1 adipocytes selectively decreased miR-452 (Figure 4A), while estrogen significantly reduced miR-224 expression in brown adipocytes (Figure 4B). Subsequently, we assessed whether silencing of both miRs in cultured brown and white adipocytes alters their cellular metabolism. Therefore, we assessed LNA-antimiR uptake in cultured 3T3-L1- and T37i-cells by transfecting them with Texas Red end-labelled siRNAs. Successful LNA transfer into both adipocyte cell lines was

demonstrated by the detection of a marked fluorescent signal demonstrating the usefulness of this approach to silence miR-224 and miR-452 in these cells (Figure 4C). Given the observed increase in lipid uptake in WAT upon silencing of miR-224 in the mice, we first tested whether miR-224 silencing in 3T3-L1 cells also affect basal lipolysis. Glycerol release into the culture medium was markedly lowered upon the silencing of miR-224 transfection (Figure 4D).

Next, since multiple genes involved in mitochondrial function were affected in BAT tissue from antimiR-224 and antimiR-452 treated mice, including *Pgc1- α* , we measured mitochondrial respiration with the oxygen consumption rate (OCR) using Seahorse respirometry in the cultured brown adipocytes (Figure 4E). Although maximal respiration was not significantly affected, we observed a decrease in basal respiration in antimiR-224 cells and a decrease in proton leak in antimiR-224 and -452 treated cells (Figure 4F). Next, we stained the mitochondria in antimiR-224 and -452 treated murine brown adipocyte cell cultures using Mitotracker green (MTG; total) and Mitotracker red (MTR; active) (Figure 4H), which stain mitochondria independent of- and dependent on membrane potential respectively. After quantification of relative fluorescence intensity (MTR/MTG), which measures mitochondrial depolarization, a decrease in mitochondrial depolarization was seen in antimiR-224 and -452 treated cells (Figure 4G).

Finally, given that deoxyglucose uptake is much lower both in BAT from our mouse study as well as in BAT from prediabetic individuals (46) we measured deoxyglucose uptake in the cultured brown adipocytes following miR-224 and miR-452 silencing, and found less glucose uptake following knock-down of both miRs (Figure 4I). Moreover, because brown adipocytes rely on glucose consumption and glycolytic flux in order to maintain mitochondrial respiration (47) we measured proton flux in culture media of antimiR-224 and antimiR-452 treated brown adipocytes with Seahorse and calculated the extracellular acidification rate (ECAR) as a measure of glycolysis. These studies identified a decrease in glycolysis, glycolytic capacity and glycolytic reserve in these cells (Figure 4J).

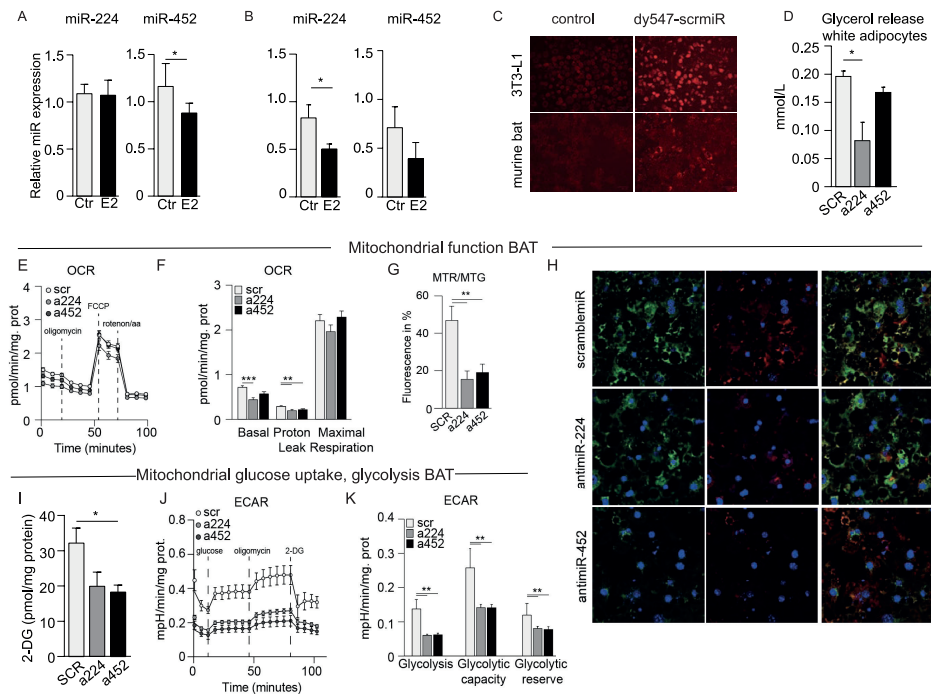


Figure 4. Silencing of miR-224 and miR-452 in cultured 3T3-L1 white adipocytes and immortalized brown adipocytes.

(A) 3T3-L1 exposed to estrogen displayed a decreased expression of miR-452 while miR-224 in estrogen-exposed brown adipocytes was reduced (B). (C) Transfer of Texas Red end-labelled siRNAs into 3T3-L1 white adipocytes and immortalized murine brown adipocytes. (D) Decreased glycerol release in culture media of anti-miR-224 treated white adipocytes (n=6) only. (E) MiR-224 and miR-452 were silenced in immortalized brown adipocytes (n=10) followed by measurement of oxygen consumption rate (OCR) in a Seahorse XF24 analyser and its quantification in (F). (G-H) Quantification using ImageJ of representative images of anti-miR-224 and anti-miR-452 treated, immortalized brown adipocytes (n=4-6) stained with MitoTracker Green FM (125 nM) and MitoTracker Red CMXRos (250 nM). Fluorescence of MitoTracker stained cells was imaged using a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems). (I) Glucose uptake was measured in anti-miR-224 and anti-miR-452 treated, immortalized brown adipocytes (n=4-6). (J) Extracellular acidification rate (ECAR) of anti-miR-224 and anti-miR-452 treated immortalized brown adipocytes (n=10) followed by its quantification in (K). Data are represented as means $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$ according to a one-way ANOVA, Bonferroni's post-hoc test.

Discussion

This study demonstrates that estrogen supplementation in transwomen lowers the X-linked miR-224 and -452 circulating in extracellular vesicles. Following the subsequent systemic silencing of miR-224 and miR-452 with LNAs in mice, lipid uptake was increased in skeletal muscle and WAT while glucose uptake was decreased in BAT. In silico analysis and RT-qPCR validation of RNA-seq based and adipocyte specific, differentially expressed genes identified mitochondrial energy metabolism, insulin-stimulated glucose uptake and lipogenesis as differential top canonical pathways. These metabolic pathways were also affected in cultured brown and white adipocytes after silencing of both miRs because we observed a decrease in mitochondrial respiration and glycolysis in brown adipocytes and less lipolysis in white adipocytes. Taken together, this study provides new evidence that the estrogen driven reduced expression of miR-224 and miR-452 activates a prediabetic phenotype in adipocyte and skeletal muscle energy metabolism. As such these results pinpoint a post-transcriptional mechanism that could explain prediabetes following estrogen supplementation in transwomen.

Estrogen and its two receptors, estrogen receptor (ER)- α and ER β , are predominantly protective against obesity, type 2 diabetes and CVD (48). However, in favor of the alternative hypothesis that elevated estrogen levels actually serve as a potential driver of diabetic cardiovascular complications, it has been shown that high endogenous estrogen levels in men predict the progression of carotid artery intima-media thickness (49) while exogenous estrogen supplementation in transwomen increases body fat (16) and reduces insulin sensitivity (18). These estrogen induced metabolic interruptions have also been observed in community studies, in which impaired fasting glucose is more often seen in men while a decline in glucose tolerance is predominantly present in women (50-53). Particularly during the female menstrual cycle, this prediabetic phenotype is present following rising levels of estrogen (54-55) while a systemic increase in estrogen levels after the use of oral contraceptives containing estrogen (56) or in gestational diabetes (GDM) (57, 58) promotes a peripheral insulin resistance that lowers glucose tolerance. Moreover, although exogenous estrogen supplementation in postmenopausal women was initially found to improve fasting glucose and insulin levels, a reduced glucose clearance over time upon a glucose challenge accounted for a decrease in peripheral insulin sensitivity, suggesting that estrogen does not improve hyperglycemia

(59-62). Nevertheless, a mechanistic understanding of the sex-specific cellular mechanisms that predispose women to prediabetes with rising estrogen levels was currently lacking.

With RNA-seq and subsequent RT-qPCR validation of plasma miRs in transwomen, we validated a decreased expression of circulating miR-224 and miR-452 (predominantly carried in EVs) in response to estrogen. This estrogen-mediated repression of the miR-224/452 cluster was demonstrated before in ER positive breast cancer patients (63) and is consistent with the idea that miR clusters tend to be regulated in a similar way (64). In addition, testosterone suppletion in transmen did not affect the expression of miR-224 and miR-452. Furthermore, because we previously did not identify an association between both miRs and plasma insulin levels in diabetic nephropathy (DN) patients (65) it can be argued that the increased levels of insulin in transwomen due to estrogen suppletion, do not affect the plasma levels of both miRs. Lastly, the *in vivo* relation between systemic silencing of both miRs with lipid and glucose metabolism in this study is in line with a recent functional enrichment analysis of X-linked miRs which demonstrated that miRs derived from the X-chromosome particularly regulate metabolism (66). Moreover, both miR-224 and miR-452 are predominantly expressed in adipose tissue (24) while plasma miRs are predominantly carried in EVs derived from adipocytes (21), a finding confirmed in this study for both miRs. However, we did not study whether the isolated EVs from plasma were derived from adipocytes nor did we study whether EVs secretion in plasma was increased following estrogen suppletion in transwomen. Nonetheless, adipocytes increase the secretion of EVs during obesity and DM, both in mice (67) and men (68). Estrogen in contrast, could interfere with this endocrine signal by making the lower expression of miR-224 and miR-452 in adipocyte specific EVs able to alter metabolism at distal sites thereby favoring prediabetes with systemic insulin resistance and white adipose hypertrophy (Figure 5).

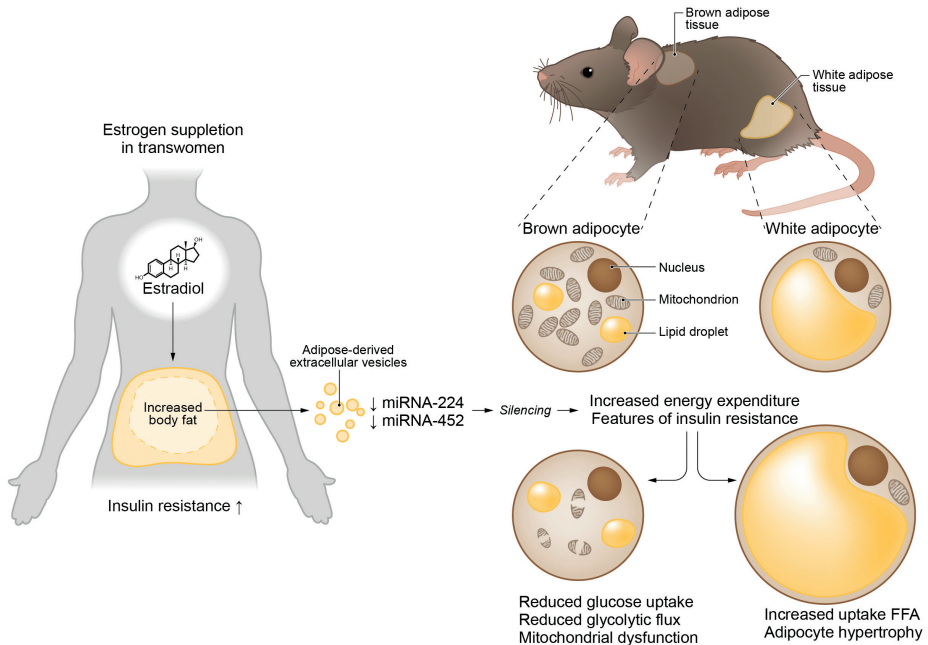


Figure 5. Proposed mechanism by which miR-224 and miR-452 mediate prediabetes in transwomen.

Increased total body fat following estrogen suppletion results in lower miR-224 and miR-452 in plasma extracellular vesicles. MiR-224 and -452 silencing in mice lowers mitochondrial respiration and glucose uptake in brown adipose and results in more triglyceride-derived fatty acid uptake in white adipose.

In adipocytes, post-transcriptional regulation of metabolism is well described for miR-224 and to a lesser extent for miR-452. The fact that miR-224 controls FA metabolism of 3T3-L1 adipocytes (69), prevents 3T3-L1 apoptosis upon inflammation (70) and controls low-density lipoprotein (LDL) metabolism (via its target PCSK9) (71) is consistent with the notion that miRs coordinately regulate cohorts of functionally related genes that participate in similar processes (72). Because a miR-452 expression change was also seen in human umbilical vein endothelial cells (HUVECs) from infants of gestational diabetic women it could be argued that both miRs are indeed involved in prediabetic pathways (73). The *in vitro* experiments demonstrate that a 100 nM estrogen dose lowers miR-224 in brown adipocytes and miR-452 in white adipocytes only. This suggests that both miRs are not simultaneously repressed by estrogen, which could question a simultaneous role in metabolism. Nonetheless, a striking example in which

the miR-224/452 cluster simultaneously controls cellular metabolism was found in malignant melanomas, in which both miRs targeted thioredoxin interacting protein (TXNIP), a key transcription factor involved in redox regulation and tumor suppression (74). We could not demonstrate a similar coordinate regulation of metabolic processes *in vivo* by this miR cluster in this study. Therefore, more studies involving this miR cluster in estrogen associated diabetic phenotypes are needed to investigate overlapping differentially expressed target genes of both miRs involved in adipocyte specific metabolism. Lastly, in both *in vitro* (miR-224) and *in vivo* (miR-224 and miR-452) experimental settings, mitochondrial respiration and glucose uptake was diminished. Interestingly, IPA analyses of the differential proteome of omental adipose tissue from women with GMD compared to adipose tissue from control subjects (75) revealed that mitochondrial dysfunction topped the list of differentially expressed pathways (data not shown) further supporting the relation between high levels of estrogen and a prediabetic state. Furthermore, particularly female rats were previously found to become obese and diabetic upon losing mitochondrial respiration in BAT (76). This could potentially explain the higher plasma levels of insulin in the transwomen cohorts in this study albeit that more (clinical) studies are needed to investigate whether estrogen lowers adipocyte specific EV secretion and its miR content.

Taken together, this study identified an estrogen-driven molecular mechanism mediated by silencing of miR-224 and miR-452 in adipose tissues. This could explain prediabetes following estrogen suppletion in transwomen. In mice, silencing of miR-224 and miR-452 elicits white adipocyte hypertrophy and impaired glucose uptake and mitochondrial dysfunction in BAT tissue. While these effects are most likely involving numerous genes and metabolic pathways, our results could aid to the development of novel approaches to counteract diseases associated with insulin resistance in general and in particular in women with known high endogenous levels of estrogen, such as pregnant women who develop gestational diabetes.

Author Contributions

BWF conducted experiments, acquired and analyzed data and wrote the manuscript. MK, ENK, RWALL and SK acquired and analyzed data. JD, JL, GDT, AB, YY and WS researched the data. RN, TJR, PCNR, AJVZ and RB contributed to the discussion and reviewed and edited the manuscript. BWF, AJVZ and RB are

guarantors of this work and, as such, had full access to all the data in the study and responsibility for the integrity of the data and the accuracy of the data analyses.

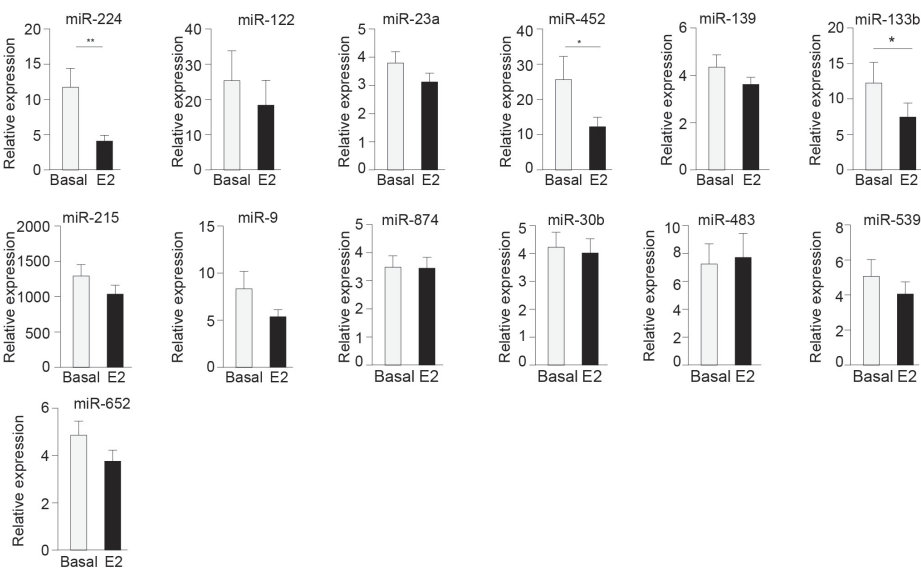
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Duality of Interest

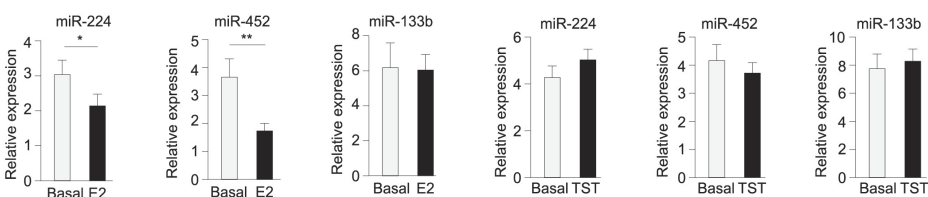
The authors have no conflicts of interest.

Supplements



Supplementary Figure 1.

RT-PCR validation of 13 estrogen responsive circulating miRNAs in human plasma from transwomen (n= 20). Upon RT-qPCR validation of selected miRNAs, miR-224, -452 and -133b display a significant decrease after one year of estrogen treatment. E2, estrogen.*p< 0.05, **p< 0.01.

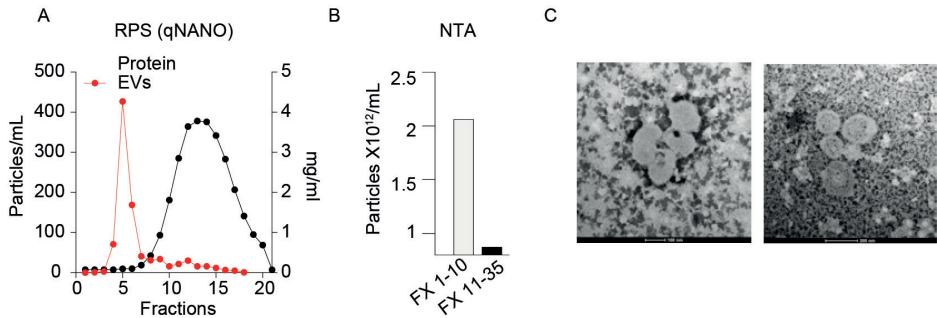


Supplementary Figure 2.

RT-PCR validation of plasma miR-224, miR-452 and miR-133b in male-to-female transgender persons (transwomen) n= 30. *p< 0.05, **p< 0.01 according to a paired student T-test.

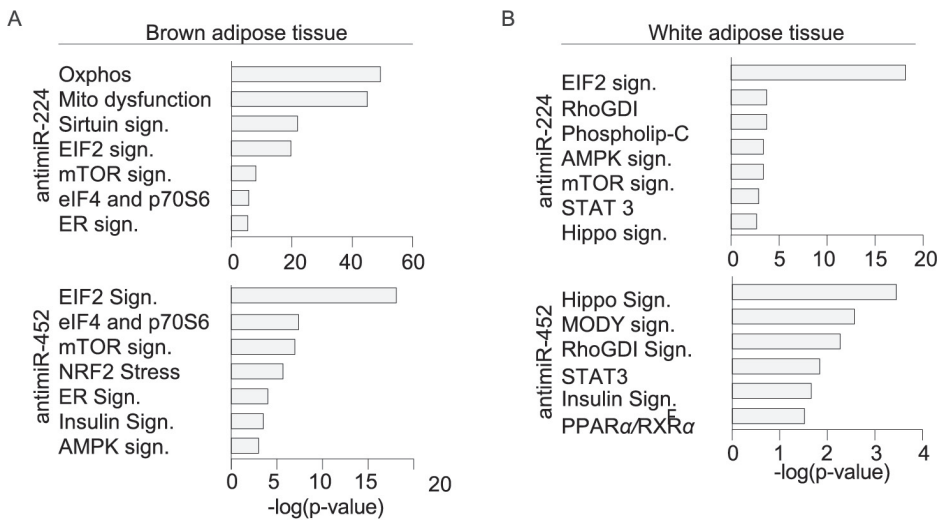
Supplementary Figure 3.

RT-PCR validation of miR-224, miR-452 and miR-133b in female-to-male transgender persons (transmen) n= 50. *p< 0.05, **p< 0.01 according to a paired student T-test.



Supplementary Figure 4.

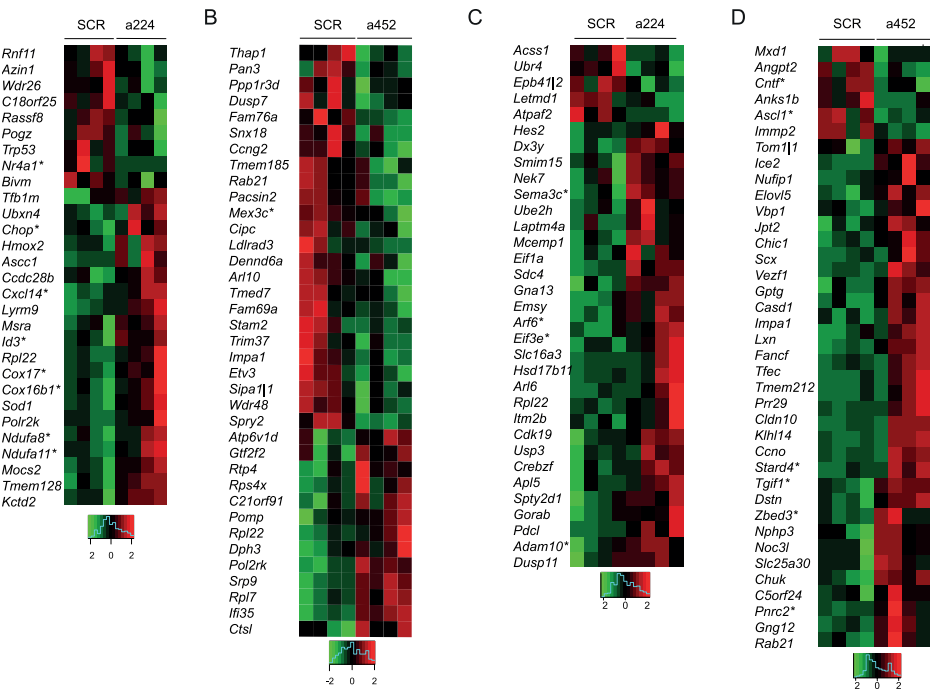
Plasma EV isolation. (A) EV concentration in different fraction was analysed using resistive pulse sensing (RPS) while protein presence in each fraction was assessed with a BCA protein assay. (B) After EV fractions were combined to a total volume of 2 mL, EV presence was validated using nanoparticle tracking analysis (NTA). (C) EV presence was validated with transmission electron microscopy.



Supplementary Figure 5. Ingenuity pathway analysis (IPA) derived top canonical pathways

next-generation sequencing (NGS)-based differential gene expression (FPKM, $p < 0.05$, $n = 4$).

(A) In BAT mitochondrial dysfunction and insulin receptor signalling were significant top canonical pathways in antimiR-224 and antimiR-452 treated BAT respectively. (B) In antimiR-224 treated WAT tissue, AMPK signalling (regulates adipocyte metabolism) and STAT3 (controls lipogenesis and adipocyte hypertrophy) were affected, while antimiR-452 treatment affected the HIPPO-pathway (adipocyte differentiation) and insulin receptor signalling.



Supplementary Figure 6. Heat maps depicting NGS-based differential expression (FPKM, $p < 0.05$, $n = 4$) of miR-224 and miR-452 target genes in brown adipose tissue (BAT) and white adipose tissue (WAT).

(A) Differentially expressed miR-224 target genes and (B) miR-452 target genes in BAT. (C) Differentially expressed miR-224 target genes and (D) miR-452 target genes in WAT tissue. Those target genes involved in lipid metabolism or insulin signalling are marked with an asterisk.

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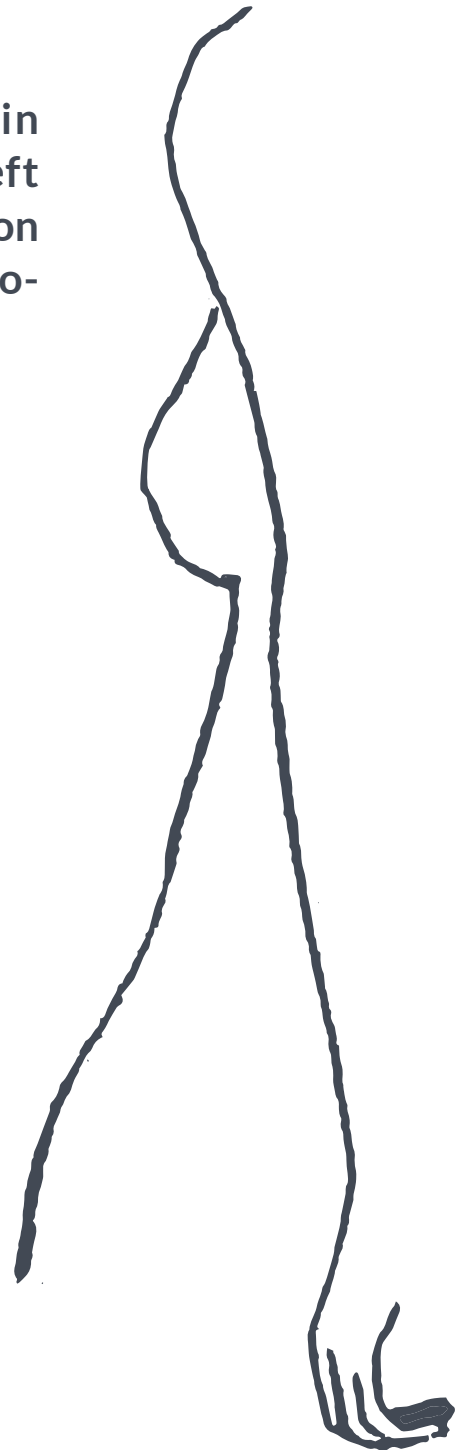
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Chapter 7

Sex-specific microRNAs in women with diabetes and left ventricular diastolic dysfunction or HFpEF associate with micro-vascular injury

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B.W. Florijn, G.B. Valstar, J.M.G.J. Duijs,
R. Menken, M.J. Cramer, A.J. Teske, C.
Ghossein-Doha, F.H. Rutten, M.E.A.
Spaanderman, H.M. den Ruijter, R.
Bijkerk, A.J. van Zonneveld



Abstract

Left ventricular diastolic dysfunction (LVDD) and heart failure with preserved ejection fraction (HFpEF) are microcirculation defects following diabetes mellitus (DM). Unrecognized HFpEF is more prevalent in women with diabetes compared to men with diabetes and therefore sex-specific diagnostic strategies are needed. Previously, we demonstrated altered plasma miRs in DM patients with microvascular injury (defined by elevated plasma Angiopoietin-2 (Ang-2) levels). This study hypothesized the presence of sex-differences in plasma miRs and Ang-2 in diabetic (female) patients with LVDD or HFpEF. After a pilot study, we assessed 16 plasma miRs in patients with LVDD (n= 122), controls (n= 244) and female diabetic patients (n= 10). Subsequently, among these miRs we selected and measured plasma miR-34a, -224 and -452 in diabetic HFpEF patients (n= 53) and controls (n= 52). In LVDD patients, miR-34a associated with Ang-2 levels (R^2 0.04, $R = 0.21$, $p = 0.001$, 95% CI 0.103-0.312), with plasma levels being diminished in patients with DM, while women with an eGFR < 60 ml/min and LVDD had lower levels of miR-34a, -224 and -452 compared to women without an eGFR < 60 ml/min without LVDD. In diabetic HFpEF women (n=28), plasma Ang-2 levels and the X-chromosome located miR-224/452 cluster increased compared to men. We conclude that plasma miR-34a, -224 and -452 display an association with the microvascular injury marker Ang-2 and are particularly targeted to women with LVDD or HFpEF.

Introduction

Heart failure with preserved ejection fraction (HFpEF), and its precursor left ventricular diastolic dysfunction (LVDD), are manifestations of microvascular injury in diabetes patients (1). The pathophysiology of microvascular injury in this patient population is associated with more hospitalization and cardiovascular death (2). Especially women with type 2 diabetes mellitus (DM) have a higher prevalence of HFpEF compared to men (28% vs. 18.4%), which often remains undiagnosed until the later stage of the disease (3-7). Therefore, the need to develop appropriate diagnostic strategies specific for women is critical, whereby the assessment of plasma microRNA (miR) levels could improve the detection of HFpEF and its precursor LVDD following type 2 DM.

Previously, we demonstrated that a select subset of circulating angiogenic miRs (among others miR-126, miR-130b, miR-223 and miR-660) are increased in plasma derived from diabetic nephropathy patients (both women and men) and associate with microvascular injury, as defined by elevated plasma levels of Ang-2 levels (8). In addition, we have demonstrated in a review of the literature that the differential expression of (plasma) miRs between women and men involves at least two potential mechanisms: (i) double dosage of X-chromosome located (X-linked) miRs due to incomplete X-chromosome inactivation and (ii) estrogen regulation of miR transcription and processing (9). The augmented expression of endothelial X-linked miRs has been established to instigate microcirculation defects (9) while higher plasma levels of these miRs have previously been shown to identify microvascular injury in women with other cardiovascular disease phenotypes such as idiopathic atrial fibrillation (iAF) (10).

The present study hypothesizes that sex-specific plasma miRs are differentially expressed in (female) patients with HFpEF and its precursor LVDD (in particular in patients with diabetes) and associate with microvascular injury (defined by elevated plasma Ang-2 levels). To test this, we assessed plasma Ang-2 (a tyrosine-protein kinase (TIE-2) receptor ligand that promotes endothelial activation and destabilization) and 16 candidate X-chromosome located (X-linked) miRs in (diabetic) LVDD and (diabetic) HFpEF patients. In addition, we performed sex-specific stratification of plasma- Ang-2 and miR level results and logistic regression

analysis to assess which clinical characteristics are associated with significant differentially expressed plasma miRs.

Materials and methods

Ethics and approval

This study complied with the ethical principles of the Declaration of Helsinki and was conducted following approval by the Institutional Review Board of the Maastricht University Medical Center (MUMC), Utrecht University Medical Center (UMCU) and the Leiden University Medical Center (LUMC). All patients provided written informed consent.

Study designs

Maastricht study

Women with a history of preeclampsia (PE) were evaluated routinely for cardiovascular function at approximately 1 year postpartum. For this study, those women who were at least 4 years postpartum were invited by mail to participate in a second follow-up cardiovascular assessment. PE was diagnosed according to the criteria set by the International Society of Hypertension in Pregnancy: new-onset hypertension, systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg, after 20 weeks' gestation and proteinuria exceeding 0.3 g/day (11).

HELPFul study

Study design and procedures of the "Discovery of biomarkers for the presence and progression of left ventricular diastolic dysfunction and HEart faiLure with Preserved ejection Fraction in patients at risk for cardiovascular disease" (HELPFul) study have been published in more detail (12). Briefly, HELPFul is a Dutch case-cohort in which patients from the UMCU participated who were referred by their GP for a diagnostic cardiac assessment. Patients who had a previous cardiac intervention, or who were known with congenital cardiac disease were excluded from participation. Patients that had a ratio of the peak early (E) diastolic filling velocity and early diastolic mitral annular velocity (e') (average of septal and lateral) (E/e') ≥ 8 with tissue Doppler echocardiography were considered to have a higher probability of having LVDD. 'Cohort' patients were randomly sampled from all patients aged 45 years or older, striving to include 25% of eligible participants.

Information on co-morbidities, medical history, and medication use was collected. The diagnostic work-up further consisted of physical examination, blood testing of standard cardiovascular biomarkers, electrocardiogram (ECG), bicycle exercise-ECG, and transthoracic echocardiogram. A structured case record form was used to assess symptoms suggestive of cardiac pathology. Hypertension was determined by (i) self-reporting, (ii) use of blood pressure lowering medication, or (iii) a mean (of at least two measurements) systolic blood pressure > 140mmHg at the outpatient center. Type 2 diabetes was determined by self-reporting or use of blood glucose lowering medication. Hypercholesterolemia was determined by self-reporting or use of lipid-lowering medication. Atrial fibrillation was determined by self-reporting or atrial fibrillation on ECG at the outpatient center. Body mass index (BMI) was calculated from dividing weight (kg) by squared height in meters (m²). Waist to hip ratio was calculated from dividing waist circumference (cm) by hip circumference (cm). The estimated glomerular filtration rate (eGFR) was calculated from both creatinine and cystatin-c with the validated CKD-EPI formula (13).

UHFO-DM study

UHFO-DM is a prospective diagnostic efficiency study from the UMCU (14). Patients aged 60 years and over with diabetes type 2, enlisted with the diabetes service of the Center for Diagnostic Support in Primary Care (SHL), Etten-Leur were eligible. This was a representative sample of all patients with diabetes type 2 registered with a general practitioner. The standard care of the diabetes service consists of periodically serum glucose and HbA1c assessment and yearly monitoring of other laboratory parameters and fundoscopy, to help the general practitioners with the management of patients with diabetes. Furthermore, the SHL provides a supporting service to the general practitioners of diabetic nurses, who work according to the current diabetes guidelines. In total 561 general practitioners in the region make use of the services of the Diabetic service of the SHL, with 48,175 patients with type 2 diabetes enlisted in the SHL service. In total, 100 nurse practitioners from the SHL support more than 200 general practitioners (GPs) in their work for diabetic patients. A random sample of approximately 1200 patients were enlisted within the SHL diabetic service database and when living within 60 kilometers of the cardiology outpatient department of the Oosterschelde hospital in Goes they were asked to participate in the study. To prevent duplicate investigations, patients known with a cardiologist-confirmed diagnosis of heart failure, were only asked to fill out the questionnaires. They also were asked for

permission to scrutinize their medical files for co-morbidities and date of diagnosis of heart failure.

Echocardiography

Echocardiography measurements were carried out as previously described (15) and performed in accordance with the recommendation of the American Society of Echocardiography (ASE) (16). Echocardiographic measurements were made using a phased-array echocardiographic Doppler system (Vivid 7, GE Vingmed Ultrasound, Horten, Norway). Left ventricular end-diastolic (LVEDd), end-systolic (LVESd) diameters (mm), end-diastolic interventricular septum thickness (IVST; mm) and the posterior (inferolateral) wall thickness (PWT; mm) were measured using the M-mode in the parasternal long-axis view. Left ventricular mass (LVM; g) was determined using the formula $0.8 \times (1.04((LVEDd + PWT + IVST)^3 - (LVEDd)^3)) + 0.6$, indexed for body surface area (17). Relative wall thickness (RWT) was computed using the formula $(2 \times PWT)/LVEDd$ (17). Left ventricular end-diastolic (LVEDV, mL) and end-systolic (LVESV, mL) volumes were determined using the Teichholz formula (18). Heart rate (HR, bpm) was calculated by multiplying by 60 the reciprocal of the mean of five consecutive RR intervals on the electrocardiogram while $((LVEDV - LVESV)/(LVEDV)) \times 100$ was used to calculate left ventricular ejection fraction (LVEF, %). Stroke volume (SV, mL) was computed by taking the product of VTI and mid-systolic cross-sectional area (cm²) at the level of the left ventricular outflow tract in the parasternal long-axis view. Mean aortic velocity time integral (VTI, cm) was calculated by averaging the outer edge tracings of five consecutive continuous wave Doppler registrations of the left ventricular outflow tract velocity. Cardiac output (CO, L/min) was calculated by multiplying SV by HR. The E/A ratio is the ratio of the early (E) to late (A) ventricular filling velocities while E/E' is the ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (E'). Using tissue doppler (TD), early (e') diastolic velocities were assessed at the septal and lateral insertion of the annulus of mitral valve. Assessments were executed offline using EchoPAC PC SW (GE Vingmed Ultrasound) version 6.1.2.

Adjudication of diagnosis of asymptomatic LVDD (Maastricht study), LVDD (HELPFul study) and HFpEF (UHFO-DM study)

Maastricht study

Asymptomatic LVDD (heart failure stage B) was diagnosed according to the guidelines of the American Heart Association (19). Asymptomatic LVDD was

defined as the presence of previous myocardial infarction, LV hypertrophy (left ventricular mass index (LVMI) $> 95 \text{ g/m}^2$), concentric remodeling ($\text{RWT} > 0.42$ and $\text{LVMI} \leq 95 \text{ g/m}^2$), mildly impaired LVEF ($> 40\%$ and $< 55\%$) or asymptomatic valvular disease (17). We defined asymptomatic valvular disease as mild aortic valve insufficiency or central aortic valve insufficiency. HF with preserved ejection fraction (HFpEF) in this subclinical stage was defined as $\text{LVEF} \geq 55\%$ but with the occurrence of one of the other criteria for asymptomatic LVDD (17).

HELPFul study

We applied consensus diagnosis for LVDD with an expert panel consisting of cardiologists (RM, MJC, AT) and a general practitioner specialized in heart failure (FR). This method is comparable to previous studies (20) (21). The expert panel used all available diagnostic information, including patient reported symptoms, risk factors, electrocardiography, echocardiography, results from the exercise test, (cardiovascular) medication use and plasma B-type natriuretic (BNP) levels. The panel based the diagnosis of LVDD on available diastolic function criteria and recommended cut-points of recent international guidelines (22-24). The panel categorized patients into four groups; no LVDD, possible LVDD, probable LVDD, and definite LVDD. For the purpose of this study, we combined probable LVDD with definite LVDD into 'LVDD', and no LVDD and possible LVDD into 'no LVDD'.

UHFO-DM study

Presence or absence of HF was determined by an outcome panel consisting of two cardiologists and one GP (14). The panel used all available information from the diagnostic work-up, including echocardiography, but except the NT-proBNP results (to prevent incorporation bias pertaining to this particular test). In case of no consensus the majority decision was used. For HFpEF patients had to have echocardiographic diastolic abnormalities in combination with indicative symptoms and signs (that is, peripheral or pulmonary fluid retention or raised jugular venous pressure) of heart failure or indicative symptoms and echocardiographic left ventricular hypertrophy, atrial fibrillation, or anginal complaints.

RNA isolation

Plasma RNA was isolated from 200 μL human plasma with 800 μL Trizol reagent (Invitrogen, Breda, the Netherlands) using the RNeasy Micro Kit (Qiagen, 1082 Venlo, the Netherlands) as described previously (25). Briefly, chloroform was added

to the plasma/Trizol mixture and centrifuged for 15 min at 15 000g. The aqueous phase was combined with 1.5x the volume of 100% Ethanol, conveyed to a MinElute Spin column (Qiagen) and centrifuged for 15s at 18.000g. Subsequently the RNA was washed with 700 μ L RWT buffer, twice with 500 μ L RPE buffer and centrifuged for 15 s at 18 000g after the first two washing steps and 2 min at 18 000g after the last washing step. Finally, RNA was eluted with 15 μ L RNase-free water.

MicroRNA profiling

For miR cDNA synthesis, reverse transcription of total RNA was performed using the miR reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was preamplified using Megaplex PreAmp primers pools according to the protocol of the manufacturer. For the pilot study custom designed megaplex cards were generated to determine the expression of 118 X-chromosome located miRs, 2 Y-chromosome located miRs, a selected set of 48 microvascular injury associated miRNAs (8,26) and U6 and miR-16 as controls. Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). In the main patient studies, expression of individual miRNAs was detected by dedicated TaqMan qRT-PCR assays as previously described (8). After comparison of miR-16 en U6 Ct-values and their respective standard deviation (SD) in asymptomatic LVDD patients, LVDD patients and HFpEF patients, we choose to normalize miR expression results using expression levels of miR-16 (15).

ELISA

In the Maastricht cohort, plasma soluble Flt-1 (sFlt-1), thrombomodulin (sTM) and Angiopoietin (Ang-2) concentrations were determined by ELISA (R&D Systems, Minneapolis, MN and Diaclone Research, Besancon, France) according to the manufacturers' supplied protocols. In the HELPFul and UHFO-DM cohort plasma Ang-2 concentration was determined by ELISA (R&D Systems, Minneapolis, MN and Diaclone Research, Besancon, France) according to the manufacturers' supplied protocols.

Data analyses

Baseline characteristics are presented as mean $\pm$ standard deviation (SD), while non-normally distributed data is presented as median with interquartile range (IQR) and categorical data is presented as frequency with percentage. Differences between groups were compared using a Pearson Chi-square test or a student T-test,

depending on the distribution of and type of variable. MiRNA-expression values are presented as mean $\pm$ standard error of the mean (SEM) and group differences were assessed using a student's T-test. To account for the effect of age we also assessed differences of miRNA expression values between groups with one-way analysis of covariance (ANCOVA), adjusting for age. Correlations between variables were calculated using the Spearman rank correlation while p-values were adjusted with multiple testing through FDR with Benjamini Hochberg correction. In the linear regression model, a simultaneous method of entry was used in which plasma miRs were selected as dependent variables while age, sex, eGFR, hypertension, diabetes mellitus and obesity as independent variables. A p-value < 0.05 was considered to be statistically significant. Data analysis was performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism, version 5.0 (GraphPad Prism Software, Inc., San Diego, CA).

Results

Patient characteristics

Asymptomatic LVDD patients (Maastricht study)

Supplemental Table 1 displays the clinical characteristics of patients with a previous history of preeclampsia and asymptomatic LVDD (n= 13) compared to healthy controls (n= 14). Women with asymptomatic LVDD had a higher ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (E') (E/E' ratio) and a higher left atrial volume index (LAVI) compared to healthy parous controls.

LVDD patients (HELPFul study)

Table 1 displays the clinical characteristics of diabetic patients with LVDD (n= 12), derived from the HELPFul cohort, consisting of patients with LVDD (Supplemental Table 2). The HELPFul cohort is an ongoing case-cohort study at a Dutch cardiology outpatient clinic enrolling patients aged 45 years and older with no prior history of cardiovascular disease, who were referred by the general practitioner for cardiac evaluation(12). Women with LVDD (n= 10) had a higher systolic- and diastolic blood pressure compared to women without LVDD (Table 1).

HFpEF patients (UHFO-DM study)

Table 2 displays the clinical characteristics of DM patients with HFpEF (n= 53). HFpEF women (n= 28) had a higher E/e' ratio than HFpEF men (n=27), while HFpEF

men had a higher LAVI and higher plasma levels of circulating brain natriuretic peptides (BNP).

Table 1. (Sex-stratified) clinical characteristics of diabetes mellitus (DM) patients with or without LVDD.

	Total population		Women		Men	
	DM Without LVDD (N= 17)	DM with LVDD (N= 12)	DM Without LVDD (N=7)	DM With LVDD (N=10)	DM Without LVDD (N= 10)	DM With LVDD (N=2)
Sex, male, n (%)	10 (59%)	2 (17%) ¹	0 (0%)	0 (0%)		
Age (years)	63.1 ± 8.7	66.2 ± 8.5	63.1 ± 6.2	66.2 (8.9)	61.5 ± 10	68.4 ± 9.9
BMI (kg/m ²)	27.8 ± 3.3	32.3 ± 7.0 ¹	28.9 ± 3.7	33.6 ± 6.9	26.9 ± 3.9	27.1 ± 3.4
Systolic BP (mm Hg)	149.1 ± 15.9	156.7 ± 16.3	153.3 ± 16.3	156.5 ± 14.5	147.4 ± 19.6	158.1 ± 16.4
Diastolic BP (mm Hg)	85.3 ± 12.8	87.9 ± 9.2	85.8 ± 5.8	88.0 ± 9.5	88.1 ± 10.9	93.0 ± 10.7
Current or former smoker, n (%)	11 (65%)	8 (67%)	4 (57%)	6 (60%)	55 (65%)	27 (73%)
Hypertension, n (%)	13 (76%)	11 (92%)	6 (86%)	9 (90%)	48 (58%)	23 (64%)
Diabetes mellitus, n (%)	17 (100%)	12 (100%)	7 (100%)	10 (100%)	10 (100%)	2 (100%)
eGFR <60 ml/min	2 (12%)	3 (25%)	1 (14%)	2 (20%)	1 (10%)	1 (50%)
Creatinine (umol/L)	68.8 (63.2-79.4)	67.6 (60.8-76)	68.1 (64.8-79.8)	63.8 (59.4-69.2)	83.2 (68.3-91.9)	95.1 (93.8-95.1)
Total cholesterol (mmol/L)	4.5 ± 0.9	4 ± 1.1	4.7 ± 0.7	4.0 ± 1.2	4.4 ± 1.1	3.8 ± 0.6
HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.2 ± 0.2	1.5 ± 0.4	1.2 ± 0.3	1.1 ± 0.1	1.1 ± 0.2
Triglycerides (mmol/L)	2.2 ± 1.0	1.7 ± 1.1	2.6 ± 1.2	1.8 ± 1.2	1.8 ± 0.8	1.3 ± 0.07
Echocardiography						
Ejection fraction	66.8 (62.9-76)	68.4 (63.5-73.7)	66.8 (56.7-77.6)	68.4 (63.8-75.9)	66 (63.4-74.2)	66 (61.5-66.6)
EA ratio	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1 ¹	0.8 ± 0.3	0.7 ± 0.1
E/E' ratio	8.9 (8.3-10.9)	9.4 (8.3-11.7)	9.6 (8.2-11.5)	10 (8.9-12.2)	8.8 (7.9-10.9)	8.2 (7.6-8.2)
LAVI (mL/m ²)	23.7 (16.7-25.4)	25.6 (16-33.1)	24.3 (13.5-27.0)	324.3 (15.8-35.5)	22.9 (16.8-24.3)	28 (26.6-28) ¹
LVMI (g/m ²)	70.0 ± 15.6	82 ± 22.0	67 ± 9	78.3 ± 20.5	72 ± 19	100.5 ± 26.8

Table 1. Continued.

	Total population		Women		Men	
	DM Without LVDD (N= 17)	DM with LVDD (N= 12)	DM Without LVDD (N=7)	DM With LVDD (N=10)	DM Without LVDD (N= 10)	DM With LVDD (N=2)
RWT	0.38 (0.31-0.47)	0.46 (0.41-0.48)	0.37 (0.31-0.39)	0.46 (0.42-0.47) ¹	0.45 (0.30-0.52)	0.46 (0.41-0.46)
BNP (pg/mL)	16.9 (5-23.4)	11.5 (2.5-35.9)	24.8 (0-47.9)	11.4 (0-34.2)	13.4 (7.5-21.2)	42.5 (10.2-42.5)
e' septal velocity (cm/sec)	7 (5-8.5)	6 (5-7)	8 (6-9)	6.5 (5-7.3)	6.5 (5-8.3)	5 (4-5)
e' lateral velocity (cm/sec)	9 (7-10)	8 (6.3-9)	10 (9-10)	8 (5.8-8.3) ¹	8 (6.8-9.5)	9.5 (9-9.5)

EA ratio, ratio of early (e) to late (a) ventricular filling; E/E' ratio, ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (E'); LAVI, left atrial volume index; LVMI, left ventricular mass index; RWT, relative wall thickness; BNP, brain natriuretic peptide; TPV, tricuspid valve regurgitation velocity. Parametric data is presented as mean $\pm$ SD or median $\pm$ SD. Non-parametric data is presented as median and IQR. Categorical data is presented as frequency and percentage. ¹p<0.05 versus no LVDD as determined by Independent samples t-test for all normally distributed continuous variables, Pearson chi-square test for binary variables and Independent Samples Mann Whitney U test for non-normally distributed variables.

Table 2. (Sex-stratified) clinical characteristics of diabetes patients with (N=53) or without HFpEF (N=52).

	Total population		Women		Men	
	Controls (N= 52)	HFpEF (N= 53)	Controls (N= 26)	HFpEF (N=28)	Controls (N=24)	HFpEF (N=27)
Age (years)	69.8 $\pm$ 6.9	75.4 $\pm$ 6.8 ¹	69.2 $\pm$ 7.5	75.2 $\pm$ 5.9 ¹	70.4 $\pm$ 6.3	75.6 $\pm$ 7.7 ¹
BMI (kg/m ²)	27.6 $\pm$ 5.1	30.1 $\pm$ 4.1 ¹	28.3 $\pm$ 6.0	31.5 $\pm$ 4.4 ¹	26.8 $\pm$ 4	28.7 $\pm$ 3.3
Systolic BP (mm Hg)	159.7 $\pm$ 21.7	162.3 $\pm$ 22.0	165.6 $\pm$ 24.8	162.7 $\pm$ 21.7	153.8 $\pm$ 16.5	162.7 $\pm$ 22.8
Diastolic BP (mm Hg)	89 $\pm$ 11	89.9 $\pm$ 11.1	92 $\pm$ 12.3	91.3 $\pm$ 11.6	86 $\pm$ 8.6	88.4 $\pm$ 10.5
Current or former smoker	5 (8%)	9 (15%)	3 (10%)	2 (7%)	2 (7%)	7 (23%)
Hypertension	38 (63%)	44 (73%)	23 (77%)	26 (87%)	15 (50%)	18 (60%)
eGFR<60 ml/min	7 (13%)	12 (22%)	5 (17%)	5 (19%)	2 (8%)	7 (25%)
Creatinine (umol/L)	77 (65-87)	77 (65-91)	70 (60.5-81)	67.5 (60.5-76.5)	82 (73.8-92.3)	89 (76-102)
Hypercholesterolemia	45 (75%)	38 (63%)	22 (73%)	20 (67%)	23 (77%)	18 (60%)

Table 2. Continued.

	Total population		Women		Men	
	Controls (N= 52)	HFpEF (N= 53)	Controls (N= 26)	HFpEF (N=28)	Controls (N=24)	HFpEF (N=27)
Echocardiography						
Ejection fraction	60.6 (54.5-65.9)	60.8 (55.0-65.6)	61.4 (55.8-66.6)	61.0 (56.9-68.2)	60.4 (54.2-63.0)	59.3 (53.4-64.9)
EA ratio	0.80 ± 0.19	0.83 ± 0.62	0.80 ± 0.19	0.80 ± 0.38	0.79 ± 0.19	0.87 ± 0.81
E/E' ratio	8.6 (7.2-10.2)	10.6 (9.2-12.7) ²	9.8 (8.0-10.8)	11.5 (10.3-14.7) ²	8.3 (6.3-9.0)	9.9 (7.8-11) ²
LAVI (mL/m ²)	24.2 (18-4-29.9)	29.3 (25-34.1) ²	24.4 (18-31.3)	27.7 (24.5-32.2)	24.0 (19.6-29.6)	32.4 (25.5-37.4) ²
RWT	0.31 (0.28-0.34)	0.31 (0.26-0.38)	0.32 (0.28-0.34)	0.33 (0.30-0.41)	0.31 (0.27-0.24)	0.29 (0.24-0.36)
BNP (pg/mL)	8 (5-12)	13.5 (8-28.3) ²	10 (5-16.5)	13 (8-22.5)	7 (4.8-9.3)	15.5 (8-37.3) ²

EA ratio, ratio of early (e) to late (a) ventricular filling; E/E' ratio, ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (E'); LAVI, left atrial volume index; RWT, relative wall thickness; BNP, brain natriuretic peptide. Parametric data is presented as mean ± SD or median ± SD. Non-parametric data is presented as median and IQR. Categorical data is presented as frequency and percentage. ¹p<0.05 (significant difference between groups) as determined by Independent samples T-test for normally distributed continuous variables. ²p<0.05 (significant difference between groups) as determined by Independent Samples Mann-Whitney u Test

Pilot study identifies candidate miRs that associate with microvascular injury

We performed a pilot study to select candidate plasma miRs for plasma profiling in LVDD and HFpEF patients. In this study we assessed plasma levels of (i) 118 X-chromosome located miRs in healthy women and men (n= 6) (Supplemental Table 3). Out of differentially expressed miRs, we selected the X-linked miR-660 for further analysis.

Furthermore, based on our previously published studies into the association of plasma miRs with microvascular injury in diabetic nephropathy (8), idiopathic atrial fibrillation(10) and kidney transplant rejection(26) as well as a review of the literature of miRs associated with angiogenesis, or their X-linked origin, we selected the following miRs for analysis in the second pilot study: miR-20a, miR-21, miR-26b, miR-34a, miR-92a, miR-125a, miR-126, miR-143, miR-193a, miR-221, miR-223, miR-224, miR-345, miR-452 (References can be found in Supplemental Table 4).

In the second pilot study, the aforementioned miRs were measured in plasma from women with a previous history of pre-eclampsia and asymptomatic LVDD (n= 13, Clinical characteristics in Supplemental table 1) compared to healthy controls (n= 14) to determine a potential association with diastolic dysfunction. We observed that of the measured miRs, only plasma levels of miR-224 (FC 1.5, $p = 0.03$, 95% CI 0.095-0.598) were increased in women with asymptomatic LVDD (Supplemental table 5 and Supplemental Figure 1A) compared to healthy parous controls.

Next, given that women with a history of preeclampsia have an increased risk for microvascular injury and heart failure (15), we assessed whether women with preeclampsia and asymptomatic LVDD indeed display an association with microvascular injury. To that extent we measured markers of microvascular injury in plasma from women with a previous history of pre-eclampsia and asymptomatic LVDD (n= 13, Supplemental table 1) As compared to healthy controls (n= 14), our assessment of Ang-2, soluble trombosmodulin (sTM) and soluble fms-like tyrosine kinase-1 (sFlt-1) revealed that only plasma Ang-2 levels were higher in women with a history of preeclampsia and asymptomatic LVDD (fold change (FC) 1.4, $p = 0.02$, 95% CI 230.5-1084) (Supplemental Table 5 and Supplemental Figure 1B).

Finally, we assessed the association between markers of microvascular injury (Ang-2, sTM, sFlt-1) and plasma miRs and observed moderate positive associations for Ang-2 and miR-34a ($R = 0.36$, FDR adjusted $p = 0.02$), -224 ($R = 0.32$, FDR adjusted $p = 0.03$), and miR-452 ($R = 0.39$, FDR adjusted $p = 0.01$). Similar associations were found for sTM and miR-125a ($R = -0.38$, FDR adjusted $p = 0.01$), -126 ($R = -0.30$, FDR adjusted $p = 0.04$) and miR-143 ($R = -0.39$, FDR adjusted $p = 0.01$) (Supplemental Figure 1C).

Plasma miR-34a in patients with diabetes and LVDD

Having identified a differential expression of plasma miR-224 and Ang-2 in women with asymptomatic LVDD, we next aimed to investigate whether patients with LVDD (Clinical characteristics in Supplemental Table 2) also have increased plasma miRs and Ang-2 levels. However, we observed that plasma miRs and Ang-2 were not significantly different in patients with LVDD (n= 122) compared to healthy controls (n= 244) (data not shown). Next, we assessed a potential association between plasma miRs and Ang-2 in patients with LVDD. Interestingly, in the entire cohort of patients with LVDD and controls we found that miR-34a was associated with plasma levels of Ang-2 (R^2 0.04, $R = 0.21$, $p = 0.001$, 95% CI 0.103-0.312, Figure 2A).

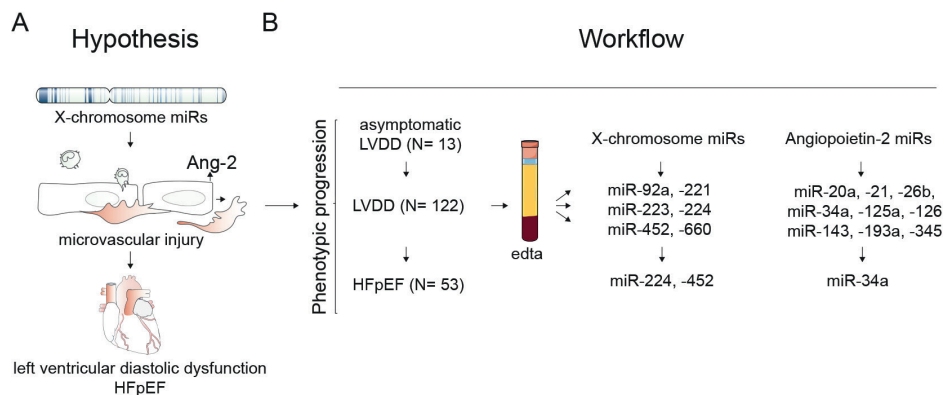


Figure 1. Hypothesis and workflow of the study.

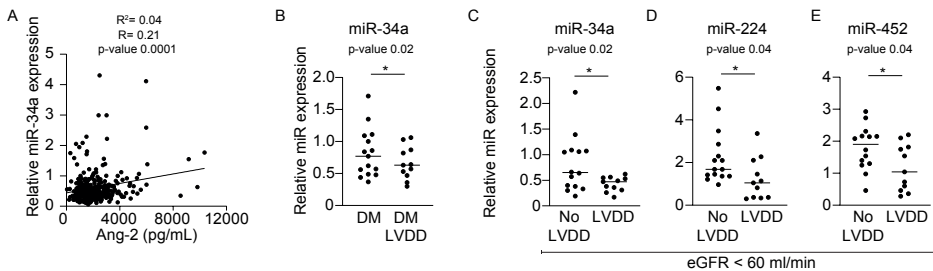
(A) This study hypothesized that sex-specific plasma miRs (derived from the X-chromosome) are differentially expressed in (female) patients with LVDD and HFpEF and associate with microvascular injury (defined by increased plasma Ang-2 levels). (B) In the workflow of the study we measured plasma Angiopoietin-2, X-chromosome located miRs and miRs that were previously found to associate with Ang-2 (ref. 8 and 13) in plasma from female patients with asymptomatic LVDD (n= 13). Following this discovery phase, a selected subset of 16 miRs and controls and plasma Angiopoietin-2 were measured in patients with LVDD (n= 122) compared to their controls (n= 244). Subsequently, miR-34a, -224 and -452 were selected and measured in patients with HFpEF (n= 52) compared to their respective controls (n= 53).

Subsequently, we conducted a linear regression analysis to assess which clinical characteristics determine plasma miR-34a in patients with LVDD. This analysis (Table 3) identified that particularly sex ($\beta = -0.160$, p-value= 0.002) and the presence of DM ($\beta = 0.121$, p-value= 0.02) were statistically significant determinants of this relationship while an eGFR < 60 ml/min displayed a trend towards an association, although non-significant ($\beta = -0.101$, p-value= 0.09). Given that the presence of DM was a significant determinant of plasma miR-34a, we assessed plasma levels of miR-34a in patients with diabetes, with or without LVDD (Table 1) and found significant lower plasma levels of miR-34a in diabetic patients with LVDD compared to diabetic patients without LVDD (FC 1.23, p = 0.02, 95% CI -0.428-0.117, Figure 2B).

Table 3. Multiple linear regression analysis of the association between clinical characteristics and plasma miRs.

	miR-34a		miR-224		miR-452	
	β	p-value	β	p-value	β	p-value
Age	0.106	0.097	0.004	0.955	0.002	0.970
Sex	-0.160	0.002	0.145	0.007	0.165	0.002
eGFR	-0.101	0.099	-0.035	0.579	-0.101	0.107
Hypertension	-0.098	0.065	-0.045	0.412	-0.046	0.402
Diabetes Mellitus	0.121	0.022	0.033	0.537	0.020	0.711
Obesity	0.113	0.035	0.010	0.851	-0.074	0.173

In the linear regression model, miRs are dependent variables while age, sex, eGFR, hypertension, diabetes mellitus and obesity are independent variables. A simultaneous method of entry was used in the regression model. " β " means standardized regression coefficients; "p-value" indicates significance.

**Figure 2.** Plasma Ang-2 levels and circulating miR-34a, -224 and -452 in (female) DM patients with LVDD compared controls.

(A) Particularly miR-34a displayed a significant correlation with Ang-2 in the total patient population of patients with LVDD (N=366, $R^2=0.04$, $R=0.21$, $p=0.001$, 95% CI 0.103-0.312.). (B) Lower levels of miR-34a in diabetic patients with LVDD compared to diabetic patients without LVDD (Fold change 1.2, $p=0.02$, 95% CI -0.428-0.117). (C) Lower levels of miR-34a, in women with an eGFR < 60 ml/min and LVDD compared to women without LVDD (Fold change 1.9, $p=0.03$, CI -0.737 to -0.030). (D) Decreased plasma miR-224 in women with an eGFR < 60 ml/min and LVDD compared to women without LVDD (Fold change 1.8, $p=0.04$, CI -1.998 to -0.059) and (E) Decreased miR-452 in women with an eGFR < 60 ml/min and LVDD compared to women without LVDD (Fold change 1.5, $p=0.04$, CI -1.213 to -0.039). Relative miR-expression values are normalized to plasma miR-16 levels, presented as mean $\pm$ standard error of the mean (SEM) and group differences are depicted as * $p \leq 0.05$. Correlations between variables were calculated using the Spearman rank correlation.

Sex-specific plasma miR-34a, -224 and -452 levels in women with an eGFR < 60ml/min and LVDD

In our pilot study in women with asymptomatic LVDD we observed significant correlations between plasma Ang-2 and miR-224 and -452. Therefore, we used linear regression analysis (Table 3) to investigate if sex could determine plasma miR-34a, -224 and -452 levels in patients with LVDD. We found that indeed sex was a significant determinant of this relationship for miR-34a ($\beta = -0.160$, $p = 0.002$), miR-224 ($\beta = 0.145$, $p = 0.002$) and miR-452 ($\beta = 0.165$, $p = 0.007$). Interestingly, linear regression analysis also demonstrated that an eGFR < 60 ml/min displayed a (non-significant) trend as determinant of plasma miR-34a ($\beta = -0.101$, $p = 0.09$), while an eGFR < 60 ml/min could not determine plasma miR-224 ($p = 0.58$) or miR-452 ($p = 0.11$).

Subsequently, we assessed differential expression of these miRs in patients with an eGFR < 60 ml/min with or without LVDD (clinical characteristics in Supplemental Table 6). Results from this analysis demonstrated that in particular female patients with LVDD with an eGFR < 60 ml/min displayed lower levels of miR-34a (FC 1.9, $p = 0.03$, CI -0.737 to -0.030, Figure 2C), miR-224 (FC 1.8, $p = 0.04$, CI -1.998 to -0.059, Figure 2D) and miR-452 (FC 1.5, $p = 0.04$, CI -1.213 to -0.039, Figure 2E) compared to female patients with an eGFR < 60 ml/min without LVDD.

Plasma Ang-2 and miR-34a, -224 and -452 levels in women with diabetes and HFpEF

Next we hypothesized that the phenotypic progression from LVDD to HFpEF in the setting of DM could be associated with pronounced microvascular injury, and increased Ang-2 levels. For this, we measured plasma Ang-2 levels in DM patients with HFpEF (Table 2, $n = 53$). Here, we observed increased levels of plasma Ang-2 (FC 1.3, $p = 0.02$, 95% CI 110.5-980.9, Figure 2A) in diabetic women with HFpEF ($n = 28$) as compared to diabetic women without HFpEF ($n = 27$).

Next, we selected miR-34a, -224 and -452 based on pilot study results and measured their plasma levels in DM patients with HFpEF compared to DM patients without HFpEF. In diabetic patients (both women and men) with HFpEF ($n = 53$) compared to diabetic patients without HFpEF ($n = 52$), we observed no significant differences in miR-224 and -452 levels (data not shown). However, plasma levels of miR-34a displayed a trend towards an increase in diabetic women with HFpEF ($n = 28$) compared to diabetic women without HFpEF ($n = 27$, FC 1.5, $p = 0.08$, 95%

CI -0.009-0.173, Figure 2B). In addition, when women with HFpEF (n= 28) were compared to men with HFpEF (n= 27), the plasma levels of miR-224 (FC 0.57, $p=0.04$, 95% CI -0.452 to -0.010, Figure 2C) and miR-452 (FC 0.58, $p=0.05$, -0.539 to -0.002, Figure 2D) were significantly higher in women versus men with HFpEF.

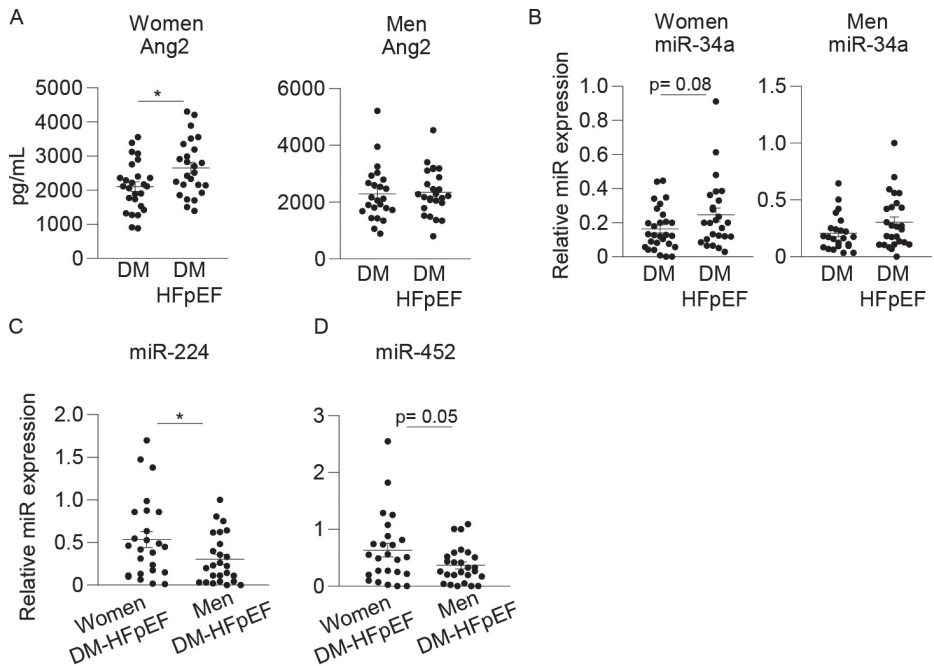


Figure 3. Plasma Ang-2 levels and plasma miR-34a, -224 and -452 in (female) DM patients with HFpEF.

(A) Increased plasma Ang-2 (Fold change 1.3, $p=0.02$, 95% CI 110.5-980.9) levels in diabetic women with HFpEF compared to diabetic women without HFpEF (but not in diabetic HFpEF men compared to diabetic men without HFpEF) (B) miR-34a displayed a trend towards an increase in diabetic women with HFpEF compared to diabetic women without HFpEF only (Fold change 1.5, $p=0.08$, 95% CI -0.009-0.173). (C) Increased plasma levels of miR-224 (Fold change 0.57, $p=0.04$, 95% CI -0.452 to -0.010) and (D) miR-452 (Fold change 0.58, $p=0.05$, -0.539 to -0.002,) in diabetic women with HFpEF compared to diabetic men with HFpEF. Relative miR expression values are normalized to plasma miR-16 levels, presented as mean $\pm$ standard error of the mean (SEM) and group differences are depicted as * $p \leq 0.05$ according to a student T-test.

Discussion

In this study, our main findings were (i) decreased levels of miR-34a, -224 and -452 in diabetes patients with LVDD and in female diabetes patients with an eGFR < 60 ml/min but increased plasma miR-224 and miR-452 in diabetic women with HFpEF versus diabetic men with HFpEF; (ii) Regression analysis demonstrated that sex, DM and renal dysfunction are associated with plasma miR-34a levels in patients with LVDD; and (iii) Increased levels of plasma Ang-2 in diabetic women with HFpEF but not in diabetic men with HFpEF

MiRs are extensively involved in manifestations of microvascular injury following type 2 DM (27). In the setting of HFpEF, the X-linked miR-545-5p differentiated HFpEF patients from healthy controls (28) while X-linked miR-221 distinguished HFpEF patients from patients with heart failure with a reduced ejection fraction (HFrEF) (29). Nonetheless, statistical analysis of these miR expression results were not stratified for women and men, and the identified miRs were not related to molecular pathways involved in microvascular injury (30). In this study, plasma miR-34a levels were decreased in diabetic patients with LVDD, while miR-34a, -224 and -452 were decreased in diabetic women with LVDD and an eGFR < 60 ml/min. In contrast, miR-34a was increased in diabetic women with HFpEF (although non-significant) while miR-224 and -452 were increased in diabetic HFpEF women versus men. This dichotomous outcome in plasma miR-34a levels could indicate different pathophysiological mechanisms of plasma miR release in different phenotypes of diastolic dysfunction. This was previously demonstrated with plasma miRs following different stages of cardiomyocyte remodeling in patients with aortic stenosis (31). It could also indicate that different stages of diastolic dysfunction change the distribution of circulating miRs among plasma carriers like high density lipoprotein (HDL), extracellular vesicles and plasma Argonaute-2 protein, like we previously demonstrated following a phenotypic progression from DM to diabetic nephropathy (25). Therefore, instead of the overall absolute quantity of total plasma miRs, a specific difference in the distribution of miRs among these carriers could potentially provide a better explanation for this dichotomous trend and its relationship to microvascular injury in diastolic dysfunction. Still, regression analysis in this study identified that sex was found to be a statistically significant determinant of plasma miR-34a in LVDD patients. This could potentially explain the observed increase in women as compared to men with HFpEF and suggests a similar

sex specific plasma expression pattern as was previously shown for miR-34a (32). Nonetheless, it could also very well be that the small sample size of diabetic women with LVDD (n= 10) has led to his differential expression of plasma miRs. Therefore, additional well powered studies are needed to investigate sex differences in plasma miR trends in different stages of diastolic dysfunction.

In this study we observed that women with asymptomatic LVDD as well as diabetic women with HFpEF had higher plasma Ang-2 levels, a finding not present in diabetic women with LVDD. Again, this could be related to the relatively small cohort size of diabetic women with LVDD (n= 10). Still, higher levels of plasma Ang-2 in the total cohort of LVDD patients and controls (n= 366) were modestly associated with miR-34a which could indicate a possible relevance of this marker for microvascular injury in these patients with LVDD. Higher plasma Ang-2 levels, indicating an association with microvascular injury in HFpEF women, is consistent with studies that demonstrated that coronary microvascular dysfunction is a female specific pathophysiology in HFpEF (33,34). In addition, significantly higher plasma levels of miR-224 and -452 were seen in HFpEF women compared to HFpEF men. It could very well be that the X-chromosome origin of both miRs is associated with a differential expression in women only. Previously we reviewed that particularly X-chromosome located miRs instigate microvascular injury phenotypes which could explain why both miRs were higher in HFpEF women together with higher levels of the microvascular injury marker Ang-2 (9). However additional validation studies are necessary to evaluate this potential.

Of the identified plasma miRs in this study, miR-34a is predominantly secreted by adipocytes and promotes a systemic inflammatory state (35). Interestingly, this miR has a well-established myocardial function in women because a targeted myocardial therapy with miR-34a in murine models of myocardial hypertrophy was previously found to reduce cardiac fibrosis and improved cardiac contractility in female mice (36). More evidence for sex-specific downstream effects of this particular miR comes from the fact that it regulates the expression of the long-noncoding RNA X-inactive-specific transcript (XIST), which regulates X-chromosome inactivation in female cells (37). In down regulating the expression of XIST (which normally mediates X-chromosome inactivation), miR-34a could increase the expression of both miR-224 and -452 which are located on a chromosomal cluster on the X-chromosome (source miRBase version 22). Of the X-linked miR-224/452

cluster, which is also predominantly expressed in adipose tissue (38), miR-224 was increased in our pilot study in women with asymptomatic LVDD (Supplemental Figure 1A) and in diabetic women with HFpEF versus HFpEF diabetic men, while both miRs were decreased in women with an eGFR < 60 ml/min and the presence of LVDD (Figure 2D-E). Regarding miR-224, this miR is known for regulating adipocyte differentiation (39), inflammation (40), and (micro)vascular quiescence or activation (41). Also miR-452 has a role in metabolism and inflammation because hyperglycemia regulates miR-452 expression (42) and its adipocyte-specific decrease activates a TNF- α induced inflammatory response (43). However, more studies are needed to investigate whether these miRs pinpoint adipocyte dysfunction as a co-contributor to microvascular injury in diabetic women with LVDD or HFpEF.

In this study we measured Ang-2 as marker of microvascular injury in patients with HFpEF and LVDD. However, Ang-2 is a single protein biomarker which may not be able to capture (microvascular) disease variability in patient populations. Therefore, additional studies are needed to assess additional parameters of endothelial and microvascular function to confirm the presence of microvascular injury over the phenotypic progression from LVDD to HFpEF. Alternatively, a comparative analysis of circulating non-coding RNAs and other protein biomarkers could potentially better capture the different molecular characteristics at the endothelial and cardiomyocyte level upon a systemic inflammation induced microvascular injury phenotype. An interesting approach for such a comparative analysis would be to measure the miR-224/452 cluster and Pentraxin-3, a miR-224 target, involved in the innate immune- and inflammatory response (44), produced in the coronary microcirculation and altered upon left ventricular (LV) diastolic dysfunction in patients with HFpEF (45). Such an approach might be further strengthened by adding the measurement of miR-34a, that regulates cardiac contractile function (46), and its gene target Bcl-2 which is pro-fibrotic and known to be involved in the myocardial structural abnormalities of HFpEF as well (47). This could clarify whether the identified plasma miRs in this study indeed pinpoint to adipocyte dysfunction as a pathophysiological substrate for endothelial dysfunction and microvascular injury in (female) patients HFpEF and LVDD.

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Author Contributions

BWF contributed to the conception and design of the work; the acquisition, analysis, and interpretation of data, and wrote the manuscript. GBV contributed to the analysis, and interpretation of data. JMGJD contributed to the acquisition and interpretation of data. RM, MJC, AJT, CGD, FHR, MEAS, and HMdR contributed to the discussion and reviewed the manuscript. AJVZ and RB reviewed and edited the manuscript and provided final approval of the version to be published. AJVZ and RB are guarantors of this work, had full access to all the data in the study and bear responsibility for the integrity of the data and the accuracy of the data analyses.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The datasets generated and analyzed during the current study are available in the tables as provided in this manuscript. Any additional information on the data can be requested from the corresponding author.

Supplemental table 1. Clinical characteristics of women with history of preeclampsia and asymptomatic LVDD compared to controls.

	Controls (n= 14)	Preclinical heart failure (n=13)
Age (years)	46.8 (6.2)	50.9 (8.6)
Months post-partum	217.2 (75)	260.6 (86.5)
Body weight (kg)	76.9 (12.9)	72.4 (13.4)
Body surface area (BSA) (m ²)	1.9 (0.1)	1.8 (0.1)
BMI (kg/m ²)	27 (5.6)	25.6 (5.3)
Systolic blood pressure (mm Hg)	120 (16.7)	114 (10.8)
Diastolic blood pressure (mm Hg)	71.1 (6.2)	69 (7.3)
Total cholesterol	5.4 (1)	5.4 (1)
HDL	2 (0.6)	1.5 (0.5)
LDL	3.0 (0.9)	3.4 (0.9)
Triglycerides	1 (0.4)	1.1 (0.5)
Echocardiography		
Left ventricular mass	112.1 (25.6)	131 (28.1)
End diastolic volume	110 (22.9)	105.3 (19.2)
End systolic volume	42 (10.9)	38.6 (8.5)
Ejection fraction (%)	61.9 (3.9)	63.4 (3.6)
Stroke volume	76.6 (14.4)	88.4 (23.7)
Cardiac output	4.9 (1.1)	5.0 (1.4)
EA ratio	1.32 (0.3)	1.24 (0.3)
SD ratio	1.17 (0.2)	1.38 (0.4)
E/E'	6.5 (1.6)	9.2 (2.0) ¹
Left atrial volume index (mL/m ²)	29.9 (5.6)	38.5 (9.5) ¹
Left ventricular mass index (g/m ²)	59.8 (12)	72.3 (15.5)
Relative wall thickness (RWT)	0.30 (0.04)	0.36 (0.06)

Parametric data are presented as mean $\pm$ SD. ¹p < 0.05 versus control according to an independent samples T-test. Controls were age, BMI and blood pressure matched.

Supplemental Table 2. (Sex-stratified) clinical characteristics of healthy controls and patients with LVDD.

	Total population		Women		Men	
	No LVDD (N= 244)	LVDD (N= 122)	No LVDD (N=160)	LVDD (N=85)	No LVDD (N= 84)	LVDD (N=37)
Sex, male, n (%)	84 (34%)	37 (30%)				
Age (years)	61.7 ± 9.1	67.4 ± 9.4 ¹	61.8 ± 8.6	67.1 (9.2) ¹	61.5 ± 10	68.4 ± 9.9 ¹
BMI (kg/m ²)	27.1 ± 4.3	27.4 ± 4.8	27.1 ± 4.6	27.6 ± 5.3	26.9 ± 3.9	27.1 ± 3.4
Systolic BP (mm Hg)	144.4 ± 18.8	155.5 ± 19 ¹	142.8 ± 18.2	154.5 ± 20 ¹	147.4 ± 19.6	158.1 ± 16.4 ¹
Diastolic BP (mm Hg)	86.1 ± 10.5	90 ± 10.9 ¹	85 ± 10.1	88.8 ± 10.7 ¹	88.1 ± 10.9	93.0 ± 10.7 ¹
Current or former smoker, n (%)	163 (67%)	74 (60%)	108 (67%)	47 (55%)	55 (65%)	27 (73%)
Hypertension, n (%)	143 (59%)	84 (70%) ¹	95 (60%)	61 (73%) ¹	48 (58%)	23 (64%)
Diabetes mellitus, n (%)	17 (7%)	12 (10%)	7 (4%)	10 (12%) ¹	10 (12%)	2 (6%)
eGFR<60 ml/min	19 (8%)	18 (15%) ¹	15 (9%)	11 (13%) ¹	4 (5%)	7 (19%)
Creatinine (umol/L)	68.8 (63.2-79.4)	67.6 (60.8-76)	65.3 (59.7-72.1)	64.9 (59.6-70.6)	81.1 (72.3-88.1)	78.3 (69-89.5)
Total cholesterol (mmol/L)	5.5 ± 1.1	5.3 ± 1.2	5.6 ± 1.1	5.5 ± 1.2	5.3 ± 1.1	5 ± 1.2
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	1.2 ± 0.3	1.2 ± 0.2
Triglycerides (mmol/L)	1.9 ± 1.3	1.9 ± 1.2	1.7 ± 0.8	1.8 ± 1.0	2.2 ± 2	2 ± 1.6
Echocardiography						
Ejection fraction	67.2 (63-73.4)	66.9 (63-72.8)	66.9 (62.5-73.4)	66.9 (63.3-72.8)	67.8 (63.9-73.6)	65.3 (62-72.8)
EA ratio	1.0 ± 0.3	0.9 ± 0.4 ¹	1.0 ± 0.3	0.8 ± 0.3 ¹	1.0 ± 0.3	1.0 ± 0.5
E/E' ratio	8.6 (7.9-9.7)	10.3 (9.1-12.8) ¹	8.9 (7.9-10.0)	10.5 (9.4-13) ¹	8.5 (7.1-9.6)	9.9 (8.3-11.7) ¹
LAVI (mL/m ²)	22.8 (18.8-27.4)	30.9 (23.6-39) ¹	22.1 (18.2-27.6)	30.4 (21.1-38.3) ¹	23.7 (19.7-26.6)	32.2 (26.1-41.2) ¹
LVMI (g/m ²)	71.0 ± 15.5	86.4 ± 22.8 ¹	68.9 ± 15.3	81.9 ± 20.1 ¹	75.1 ± 15.2	97 ± 25.2
RWT	0.40 (0.36-0.46)	0.45 (0.40-0.54) ¹	0.39 (0.36-0.44)	0.47 (0.39-0.55) ¹	0.41 (0.36-0.48)	0.44 (0.41-0.51) ¹
BNP (pg/mL)	17.9 (10-30.9)	32.4 (15.3-66.6) ¹	19.6 (10.5-32.9)	28.1 (14.1-50.2) ¹	16.4 (10-27.9)	48.3 (16.4-86.6) ¹
e' septal velocity (cm/sec)	7 (6-9)	6 (5-7) ¹	7 (6-9)	6 (5-7) ¹	8 (6-9)	6 (5-7) ¹

Supplemental Table 2. Continued.

	Total population		Women		Men	
	No LVDD (N= 244)	LVDD (N= 122)	No LVDD (N=160)	LVDD (N=85)	No LVDD (N= 84)	LVDD (N=37)
e' lateral velocity (cm/sec)	9 (8-11)	7 (6-9) ¹	9 (7-11)	7 (6-9) ¹	9 (8-11)	8 (6-10) ¹

EA ratio, ratio of early (e) to late (a) ventricular filling; E/E' ratio, ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (E'); LAVI, left atrial volume index; LVMI, left ventricular mass index; RWT, relative wall thickness; BNP, brain natriuretic peptide; TPV, tricuspid valve regurgitation velocity. Parametric data is presented as mean $\pm$ SD or median $\pm$ SD. Non-parametric data is presented as median and IQR. Categorical data is presented as frequency and percentage. ¹p<0.05 versus no LVDD as determined by Independent samples t-test for all normally distributed continuous variables, Pearson chi-square test for binary variables and Independent Samples Mann Whitney U test for non-normally distributed variables.

Supplemental table 3. Plasma profiling of X-chromosome located microRNAs in healthy women (n=3) and men (n=3).

Geometric mean of intensities in women (n=3)	Geometric mean of intensities in men (n=3)	Fold-change	MicroRNA ID	Parametric p-value	FDR
287,35	1176,27	0,24	hsa-miR-664b-5p	0,0044956	0,616
388,02	776,05	0,5	hsa-miR-363	0,027357	0,957
16383,99	31288,25	0,52	hsa-miR-660	0,0345057	0,957
131071,94	116771,88	1,12	hsa-miR-130a	0,0667662	0,957
37,62	1,41	26,6	hsa-miR-888	0,0777908	0,957
39420,71	23712,06	1,66	hsa-miR-27b	0,0937884	0,957
741455,2	1001223,8	0,74	hsa-miR-15b	0,0994014	0,957
630,35	1231,9	0,51	hsa-miR-503	0,1051315	0,957
3,25	31,27	0,1	hsa-miR-18b	0,1136259	0,957
488,88	1448,15	0,34	hsa-miR-31	0,1213119	0,957
194130,37	64039,15	3,03	hsa-miR-376c	0,1241101	0,957
111498,63	154081,44	0,72	hsa-miR-106b	0,142438	0,957
1,32	34,3	0,038	hsa-miR-708	0,1506042	0,957
4194304	5663777,2	0,74	miR-20a	0,1713483	0,957
2097151,1	3567956,1	0,59	hsa-miR-451	0,180914	0,957
691,38	1149,4	0,6	hsa-miR-214	0,1810847	0,957
228209,49	353986,23	0,64	hsa-miR-103	0,1933647	0,957
1048576	1352002,9	0,78	hsa-miR-491-5p	0,2018449	0,957

Supplemental table 3. Continued.

Geometric mean of intensities in women (n=3)	Geometric mean of intensities in men (n=3)	Fold-change	MicroRNA ID	Parametric p-value	FDR
99,27	1588,37	0,062	hsa-miR-374c	0,2042913	0,957
741455,2	1048576,5	0,71	hsa-miR-26a	0,2170785	0,957
38,5	261,98	0,15	hsa-miR-93	0,2309528	0,957
108,89	1072,43	0,1	hsa-miR-362-3p	0,2357035	0,957
2642244,8	3329021,3	0,79	hsa-miR-374b	0,2415183	0,957
14937,66	19710,36	0,76	hsa-miR-378	0,250814	0,957
122,22	13	9,4	hsa-miR-513-5p	0,2596716	0,957
1588,37	116,7	13,61	hsa-miR-138	0,2660159	0,957
17158,86	26615,89	0,64	hsa-let-7f	0,2666379	0,957
11863278	26632170	0,45	hsa-miR-19b	0,2841158	0,957
1702,38	140,39	12,13	hsa-miR-32	0,2912515	0,957
1,32	8	0,16	hsa-miR-504	0,297337	0,957
111566783	143850974	0,78	hsa-miR-223	0,2987522	0,957
13,61	222,86	0,061	hsa-miR-501-3p	0,304061	0,957
3565,77	11062,07	0,32	hsa-miR-34a	0,3057593	0,957
1,32	11,31	0,12	hsa-miR-6857-5p	0,3083428	0,957
88496,52	111498,63	0,79	hsa-miR-28-5p	0,3096702	0,957
3913423	5045851,8	0,78	hsa-miR-20a	0,3199371	0,957
322736,95	467087,51	0,69	hsa-miR-29a	0,32946	0,957
32767,99	45282,53	0,72	hsa-miR-532	0,3304597	0,957
2144,86	198,55	10,8	hsa-miR-545	0,3459876	0,957
630731,48	851708,44	0,74	hsa-miR-374	0,3539606	0,957
35940,75	55749,33	0,64	hsa-miR-98	0,3559323	0,957
2095,87	2702,35	0,78	hsa-miR-502-3p	0,3629847	0,957
2640,63	1097,5	2,41	hsa-miR-193a-5p	0,3902062	0,957
397335,93	287526,19	1,38	hsa-miR-421	0,4154777	0,957
35119,87	43237,63	0,81	hsa-miR-133b	0,4232167	0,957
4,29	14,59	0,29	hsa-miR-450b-5p	0,4275385	0,957
588493,12	912838,35	0,64	hsa-miR-125a-5p	0,4403853	0,957
198667,97	233543,75	0,85	hsa-miR-26b	0,4498537	0,957
362259,98	445994,51	0,81	hsa-miR-19a	0,4503709	0,957
26007,97	13619	1,91	hsa-miR-143	0,4517177	0,957
978355,74	1204497,6	0,81	hsa-miR-20b	0,4574599	0,957
31307384	40366832	0,78	hsa-miR-188-5p	0,4594748	0,957

Supplemental table 3. Continued.

Geometric mean of intensities in women (n=3)	Geometric mean of intensities in men (n=3)	Fold-change	MicroRNA ID	Parametric p-value	FDR
776521,47	1176986,8	0,66	hsa-miR-142-3p	0,4671931	0,957
456419,17	676001,14	0,68	hsa-miR-221	0,4950471	0,957
11856,03	17158,87	0,69	hsa-miR-193b	0,5027335	0,957
154081,37	108952,03	1,41	hsa-miR-133a	0,5085637	0,957
134,05	37,62	3,56	hsa-miR-326	0,5114109	0,957
222,86	1072,43	0,21	hsa-miR-500b	0,5200279	0,957
84,45	268,11	0,31	hsa-miR-548c-5p	0,5244142	0,957
9855,18	8579,43	1,15	hsa-miR-100	0,5316799	0,957
26615,88	29875,32	0,89	hsa-miR-128	0,5568818	0,957
1448,15	2896,31	0,5	hsa-miR-501-5p	0,5581623	0,957
62576,47	73561,67	0,85	hsa-miR-130b	0,5652022	0,957
1482	1702,38	0,87	hsa-miR-200a	0,5978843	0,957
50827,91	53231,77	0,95	hsa-miR-195	0,6086524	0,957
1912020	2353973,5	0,81	hsa-miR-150	0,6123738	0,957
12706,97	15286,81	0,83	hsa-miR-10a	0,6135451	0,957
15653692	17570683	0,89	hsa-miR-106a	0,6163931	0,957
86475,29	106463,56	0,81	hsa-miR-6086	0,6324049	0,957
58385,94	68635,45	0,85	hsa-miR-340	0,6416747	0,957
978355,74	934175,01	1,05	hsa-miR-331-3p	0,6433317	0,957
19710,36	24266,3	0,81	hsa-miR-142-5p	0,6457907	0,957
274541,8	250305,95	1,1	hsa-miR-21	0,652994	0,957
27874,66	25413,96	1,1	hsa-miR-210	0,6529946	0,957
111,43	353,77	0,31	hsa-miR-766-5p	0,6584996	0,957
22655109	25429511	0,89	hsa-miR-126	0,6612236	0,957
37,62	261,98	0,14	hsa-miR-384	0,6636462	0,957
256156,6	308162,88	0,83	hsa-miR-145	0,6645626	0,957
345900,99	308162,75	1,12	hsa-miR-590-5p	0,6692197	0,957
75281,07	82570,19	0,91	hsa-miR-25	0,6701786	0,957
3106085,9	3406833,8	0,91	mmu-miR-374-5p	0,6701786	0,957
2352,53	2896,31	0,81	hsa-miR-125b	0,6756459	0,957
2767207,9	3651353,4	0,76	hsa-miR-892b	0,6772483	0,957
54476	77040,7	0,71	hsa-miR-4767	0,6846429	0,957
3484,33	2765,52	1,26	hsa-miR-365	0,6847668	0,957
1956711,5	1703416,9	1,15	hsa-miR-146b-5p	0,6873322	0,957

Supplemental table 3. Continued.

Geometric mean of intensities in women (n=3)	Geometric mean of intensities in men (n=3)	Fold-change	MicroRNA ID	Parametric p-value	FDR
6658039,7	5534418,3	1,2	hsa-miR-320	0,6880374	0,957
11585,23	7643,4	1,52	hsa-miR-224	0,6883326	0,957
5042,77	6353,49	0,79	hsa-miR-212	0,6901877	0,957
2702,35	3326,99	0,81	hsa-miR-509-3p	0,6941416	0,957
13,61	6,81	2	hsa-miR-361-3p	0,6975489	0,957
1703416,9	1449041,2	1,18	hsa-miR-197	0,7085934	0,957
21137958	18401668	1,15	hsa-miR-146a	0,7125738	0,957
27874,66	33533,92	0,83	hsa-miR-132	0,7177405	0,957
978355,74	871615,88	1,12	hsa-miR-28-3p	0,7224054	0,957
1,32	1,55	0,85	hsa-miR-676	0,7235155	0,957
1783977,2	1415944,9	1,26	hsa-miR-574-3p	0,7237274	0,957
15653692	17169366	0,91	hsa-miR-17	0,7280707	0,957
353986,07	406623,26	0,87	hsa-miR-328	0,7384845	0,957
370727,44	445994,51	0,83	hsa-miR-155	0,7412804	0,957
4002,45	3649,12	1,1	hsa-miR-449a	0,7470899	0,957
103,97	238,86	0,44	hsa-miR-500	0,7542302	0,957
61147,23	53231,78	1,15	hsa-miR-320d	0,7598355	0,957
25,99	48,5	0,54	hsa-miR-424	0,7646508	0,957
9195,21	6809,5	1,35	hsa-miR-452	0,7681045	0,957
1589343,7	1415944,9	1,12	hsa-miR-197	0,768971	0,957
233543,65	217904,07	1,07	hsa-miR-27a	0,7700844	0,957
831,75	3649,12	0,23	hsa-miR-1298	0,7707723	0,957
287525,94	330280,74	0,87	hsa-miR-486-3p	0,7718609	0,957
61147,23	70239,75	0,87	hsa-miR-628-5p	0,7747372	0,957
15,63	10,56	1,48	hsa-miR-1468	0,7909246	0,957
477,71	116,7	4,09	hsa-miR-450a	0,7943075	0,957
23712,06	21125,04	1,12	hsa-miR-718	0,8094881	0,957
12133,15	13307,94	0,91	hsa-miR-422a	0,8138351	0,957
17559,93	18820,28	0,93	hsa-miR-181a	0,816752	0,957
6968,66	6353,49	1,1	hsa-miR-99a	0,8221977	0,957
18390,41	19710,36	0,93	hsa-miR-885-5p	0,823092	0,957
42250,08	46340,96	0,91	hsa-miR-423-5p	0,8246432	0,957
108951,98	101655,86	1,07	hsa-miR-652	0,8468222	0,969
2353972,5	2196334,4	1,07	hsa-miR-342-3p	0,8489077	0,969

Supplemental table 3. Continued.

Geometric mean of intensities in women (n=3)	Geometric mean of intensities in men (n=3)	Fold-change	MicroRNA ID	Parametric p-value	FDR
11068832	10327587	1,07	hsa-miR-222	0,8576284	0,971
294246,56	274541,92	1,07	hsa-miR-92a	0,8823522	0,978
57052,37	58385,94	0,98	hsa-miR-152	0,8899137	0,978
10321,27	9630,09	1,07	hsa-miR-671-3p	0,890179	0,978
24,25	40,32	0,6	hsa-miR-513C	0,9169698	0,978
24266,3	24833,5	0,98	hsa-miR-345	0,9188487	0,978
10,08	7,64	1,32	hsa-miR-508	0,9246038	0,978
891988,62	912838,35	0,98	hsa-miR-140-5p	0,9301869	0,978
9,4	12,41	0,76	hsa-miR-23c	0,9306357	0,978
1626492,3	1553042,9	1,05	hsa-miR-513B	0,9328287	0,978
3486463,5	3406833,8	1,02	hsa-miR-92	0,9413313	0,978
4002,45	4191,74	0,95	hsa-miR-1	0,9419048	0,978
416127,66	406623,26	1,02	hsa-miR-139-5p	0,9621393	0,991
3,4	3,56	0,95	hsa-miR-6858-5p	0,9786424	1

Supplemental Table 4. Selection of plasma miRs involved in angiogenesis, X-chromosome origin, diabetes or cardiovascular disease.

miR ID	Angiogenesis	X-linked	DM	DN	CVD
miR-20a	(1, 2)				(3)
miR-21			(11)		
miR-26b	(2)				(4)
miR-34a		(5)			
miR-92a		(6)			
miR-125a					(7)
miR-126	(8, 9)		(8)	(8, 9)	
miR-143	(10)				
miR-193a	(11)				
miR-221					(7)
miR-223			(8)	(8)	
miR-224		(12)			
miR-345			(8)	(8)	
miR-452		(13)			
miR-660			(8)	(8)	

References can be found below this table. X-linked, X-chromosome located; DM, Diabetes Mellitus; DN, Diabetic Nephropathy; CVD, cardiovascular disease

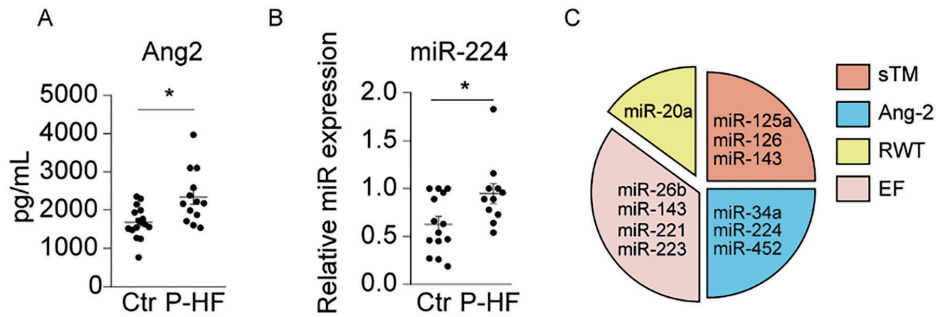
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Supplemental Table 5. Plasma miR levels in women with history of preeclampsia and asymptomatic LVDD compared to controls.

	Controls (N= 14)	Preclinical heart failure (N= 13)
miR-26a	40.3 ± 23.1	34.8 ± 22.7
miR-345	0.33 ± 0.13	0.37 ± 0.11
miR-193a	0.51 ± 0.29	0.95 ± 0.82
miR-223	592.3 ± 205.5	614.0 ± 299
miR-92a	34.9 ± 18.5	38.3 ± 12.3
miR-224	0.63 ± 0.30	0.95 ± 0.34 ¹
miR-143	0.15 ± 0.05	0.15 ± 0.10
miR-221	4.03 ± 3.2	3.05 ± 1.97
miR-660	0.60 ± 0.38	0.55 ± 0.26
miR-34a	0.06 ± 0.05	0.15 ± 0.21
miR-26b	3.33 ± 1.56	3.56 ± 1.90
miR-125a	0.76 ± 0.27	0.69 ± 0.38
miR-452	0.05 ± 0.03	0.09 ± 0.05
miR-126	51.32 ± 21.96	49.28 ± 21.92
miR-21	2.86 ± 1.23	2.65 ± 1.20
miR-20a	40.51 ± 18.88	35.44 ± 17.85
Ang-2 pg/mL	1685 ± 409.6	2342 ± 699.3
sFLT-1 pg/mL	89.28 ± 25.60	103.3 ± 28.20
sTrom pg/mL	3589 ± 669.4	3648 ± 1014

MiR expression values are presented as mean ± SD. ¹p < 0.05 versus control according to an independent samples T-test. Controls were age-, BMI- and blood pressure- matched. Ang-2, Angiopoietin-2; sFlt-2, soluble fms-like tyrosine kinase-1; sTrom, soluble Trombomodulin



Supplemental Figure 1 Ang-2 and miR expression profiling in plasma from women with a history of preeclampsia and preclinical heart failure (P-HF, n=13) compared to controls (n=14). (A) Plasma Ang-2 levels increased in women with a history of preeclampsia and P-HF (fold change (FC) 1.4, $p = 0.02$). (B) Plasma miR-224 increased in women with a history of preeclampsia and P-HF (FC 1.5, $p = 0.03$). MiR-expression values are presented as mean $\pm$ standard error of the mean (SEM) and group differences are depicted as $*p \leq 0.05$ an independent samples T-test. (C) Overview of miR association with echocardiography parameters (RWT and EF) and markers of microvascular injury (sTM and Ang-2). Correlations between variables were calculated using the Spearman rank correlation while p-values were adjusted with multiple testing through FDR with Benjamini Hochberg correction.

Supplemental Table 6. (Sex-stratified) clinical characteristics of patients with or without an eGFR < 60 ml/min with or without LVDD.

	Total population		Women		Men	
	eGFR< 60 ml/min Without LVDD (N= 19)	eGFR< 60 ml/min with LVDD (N= 18)	eGFR< 60 ml/min Without LVDD (N=15)	eGFR< 60 ml/min With LVDD (N=11)	eGFR< 60 ml/min Without LVDD (N= 4)	eGFR< 60 ml/min With LVDD (N=7)
Sex, male, n (%)	4 (21%)	7 (39%)	0 (0%)	0 (0%)	4 (100%)	7 (100%)
Age (years)	71.5 ± 9.1	76.9 ± 8.3	70.6 ± 9.2	76.3 (9.6)	75 ± 8.8	77.9 ± 6.3
BMI (kg/m ²)	27.4 ± 5.0	26.8 ± 6.8	27.4 ± 5.2	28.3 ± 8.2	27.5 ± 4.8	24.4 ± 2.3
Systolic BP (mm Hg)	150.8 ± 23.2	158.9 ± 14.7	149.6 ± 24.3	159.1 ± 13.9	155 ± 21.2	158.6 ± 17
Diastolic BP (mm Hg)	85.8 ± 12.7	88.9 ± 11.4	85 ± 13.4	88.6 ± 11.2	88.8 ± 11.1	89.3 ± 12.7
Current or former smoker, n (%)	9 (47%)	11 (61%)	5 (33%)	6 (55%)	4 (100%)	5 (71%)
Hypertension, n (%)	11 (61%)	14 (82%)	10 (71%)	11 (100%)	1 (25%)	3 (50%)
Diabetes mellitus, n (%)	2 (11%)	3 (19%)	1 (7%)	2 (20%)	1 (25%)	1 (17%)
eGFR<60 ml/min	19 (100%)	18 (100%)	1 (14%)	2 (20%)	1 (10%)	1 (50%)
Creatinine (umol/L)	97.3 (78.2-104.6)	93.9 (81.3-8-104)	87.6 (75.5-103.6)	84 (73.9-100)	107.9 (94-115.6)	98.6 (93.8-122.1)
Total cholesterol (mmol/L)	5.6 ± 1.0	5.2 ± 1.1	5.7 ± 1.0	5.4 ± 1.2	5.05 ± 0.8	4.8 ± 1.0
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.4 ± 0.3	1.5 ± 0.4	1.4 ± 0.4	1.1 ± 0.1	1.3 ± 0.2
Triglycerides (mmol/L)	2.0 ± 0.7	1.9 ± 1.3	2.0 ± 0.7	2.2 ± 1.5	2.1 ± 0.1	1.3 ± 0.3 [†]
Echocardiography						
Ejection fraction	66.5 (62.0-70.7)	64 (57.3-74.5)	68.3 (62.4-72.3)	69.2 (63.5-81.7)	63.7 (49.3-66.2)	61.5 (36-64)
EA ratio	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.3	0.6 ± 0.1
E/E' ratio	9.5 (8.4-11.6)	12.5 (9.0-14.4)	10.2 (8.9-14.5)	12.8 (8.5-14.3)	7 (6.2-8.1)	12.2 (9.7-14.8) [†]

Supplemental Table 6. Continued.

	Total population		Women		Men	
	eGFR < 60 ml/min Without LVDD (N = 19)	eGFR < 60 ml/min with LVDD (N = 18)	eGFR < 60 ml/min Without LVDD (N = 15)	eGFR < 60 ml/min With LVDD (N = 11)	eGFR < 60 ml/min Without LVDD (N = 4)	eGFR < 60 ml/min With LVDD (N = 7)
LAVI (mL/m ²)	26.2 (20.3-30)	28.9 (18.1-51)	24.4 (20.1-27.2)	28.9 (17-49.2)	29.4 (21.6-33.5)	30.1 (19.3-58.1)
LVMI (g/m ²)	73.1 ± 12.1	97.6 ± 28.7 [†]	73.4 ± 12	90 ± 30	72 ± 14	109.6 ± 23.8 [†]
RWT	0.41 (0.37-0.48)	0.45 (0.41-0.58) [†]	0.41 (0.36-0.49)	0.49 (0.42-0.61) [†]	0.40 (0.37-0.46)	0.43 (0.41-0.52)
BNP (pg/mL)	40.4 (11.9-45)	87.5 (33.2-213) [†]	27 (11.4-45)	83.6 (22.7-134.1) [†]	40.3 (25.5-49.2)	211.1 (74.8-265.3)
e' septal velocity (cm/sec)	6 (5-9)	5 (4-7)	6 (4-7)	5 (4-8)	9.5 (6.5-11.8)	5.5 (4-6.3) [†]
e' lateral velocity (cm/sec)	7 (5.5-10.5)	7 (6-10)	7 (5-8.5)	7 (7-10)	11 (7-11)	7.5 (5.3-9.8)

EA ratio, ratio of early (e) to late (a) ventricular filling; E/E' ratio, ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (E'); LAVI, left atrial volume index; LVMI, left ventricular mass index; RWT, relative wall thickness; BNP, brain natriuretic peptide; TPV, tricuspid valve regurgitation velocity. Parametric data is presented as mean ± SD or median ± SD. Non-parametric data is presented as median and IQR. Categorical data is presented as frequency and percentage. [†]p<0.05 versus no LVDD as determined by Independent samples t-test for all normally distributed continuous variables, Pearson chi-square test for binary variables and Independent Samples Mann Whitney U test for non-normally distributed variables.

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Chapter 8

Discussion and future directions

Discussion and future perspectives

Cardiovascular complications of diabetes in women remain underdiagnosed and undertreated because clinical standards are largely based on male pathophysiology and outcome (1). These female specific cardiovascular complications of diabetes often result from microvascular injury of which early detection is needed in order to enable female-specific diabetes care (2). To improve early microvascular injury detection in women, and in search for innovation in diagnostics and therapeutics, this thesis has its focus on sex-biased non-coding RNA (microRNA signatures in human plasma, adipose tissue and endothelial cells (ECs).

Previously, microRNAs (miRs) have been shown to provide a missing mechanistic link in metabolism, both as key-regulators of insulin secretion (3) and adipose tissue metabolism (4). Also, circulating in plasma from patients with diabetes mellitus (DM), miR concentrations are altered with adipocyte dysfunction (5), diabetic disease progression (6) and following therapy (7). These plasma miRs are distributed between plasma carriers such as extracellular vesicles (EVs) (8), high density lipoprotein (HDL) (9) and the Argonaute-2 (Ago-2) protein (10) which allow miRs to adopt an extracellular function as novel paracrine mediators of intercellular communication at distal sites (11). It has been argued, however, that these extracellular RNA concentrations are too low to induce a biological or pathological response in target tissues in health and disease (12). However, by working in a dose-response fashion with their mRNA binding sites, also low miR concentrations dictates target gene expression changes (13). Furthermore, miRs mostly function synergistically (regulating the same gene) and through feedback- and feed-forward loops that amplifies their effect (4). Given that different miRs can target multiple genes in a complementary fashion within the same molecular pathway (14), supposing a 1:1 stoichiometry between miRs and their mRNA targets is an oversimplification that needs to be avoided (11). As such, the relevance of (extracellular) miRs in the progression of DM and its (cardio)vascular complications following adipocyte dysfunction and microvascular injury has now become undisputed (15).

In women, sex-biased miR signatures in adipose tissue and plasma may result from a higher expression of X-chromosome located miRs or due to their estrogen driven transcription and processing. We describe in **chapter 2** how overexpression

of many X-linked-miRs, as a result of incomplete X-chromosome inactivation (XIC), may associate with a deterioration in microvascular integrity. Recently it was shown that many sex-biased miRs are not only expressed from sex-chromosomes like the X-chromosome, but are also particularly transcribed from autosomes and show a non-random distribution across chromosomes (16). This study investigated this by employing a computational framework analysis to identify sex-biased miR expression in 8 miR datasets comprising miR expression results from peripheral blood, 'brain'- and colorectal mucosa tissue. Although these tissues provide a small and selected subset of cells, the miR expression results derived from these expression datasets revealed that for 19 out of 23 chromosomes (except the X-chromosome) male specific miRs have a more enriched expression compared to female specific miRs on the same chromosome (7). Nonetheless, female-specific miRs were involved in a higher disease spectrum width, particularly in metabolic diseases (7). Therefore, further studies assessing whether metabolism associated X-linked miRs can be used to monitor microvascular injury in diabetes are needed.

In contrast to sex-chromosome associated miRs, **chapter 2** also shows that the systemic loss of estrogen-regulated miRs activates a deterioration in microvascular integrity. In vitro studies indicate this may be caused by an estrogen effect on the expression of key miR processing genes such as Dicer1 (17) and Ago-2 (18) of which the expression is increased and decreased in vascular cells respectively. It is worth mentioning that the identified mechanisms by which estrogen controls miR transcription and processing were identified in MCF-7 cell lines, which are malignant cells with a deregulated cell cycle control and abnormal gene expression profiles (19). Nonetheless, in human cultured endothelial cells (ECs) (20) and cardiomyocytes (21) it has been shown that estrogen influences miR transcription and downstream (target) mRNA expression as well, although further mechanistic studies were not performed. This could be of interest for female-specific microvascular integrity given the decline in estrogen levels in postmenopausal women and potential increase in CVD risk. With regard to premenopausal women, a comparison of the follicular and luteal state of the female menstrual cycle has identified no significant changes in plasma miRs (22). Still, given that estrogen regulates miR expression in an age- and estrogen dependent manner (23), more mechanistic studies focusing on the estrogen mediated miR transcription in adipocytes and vascular cells such as ECs, vascular smooth muscle cells (VSMCs) and cardiomyocytes (CM) are needed.

X-chromosome mosaicism in women has been found to lead to sex differences in the immune response to both self and foreign antigens (24). This is because the X-chromosome is enriched for immune response genes and their double dosage (due to variation in copy number) predispose women to a hyper-responsiveness against acute inflammatory stimuli. Post-transcriptional control of the resultant immune response is regulated by X-linked miRs which were found to be overexpressed in T-cells from women with systemic lupus erythematosus (SLE), leading to an exacerbation of disease activity (25). Estrogen-regulated miRs also contribute to the etiopathogenesis of SLE by controlling the immune response in splenocytes (26). This overactivation of the immune response in women due to a sex-biased miR expression renders women more susceptible to an inflammation induced microvascular EC injury (27). Still, it is unknown whether sex-biased (i.e. X-linked miRs or estrogen regulated miRs) miRs are involved in- or regulate the progression of microvascular injury in the setting of an overactive inflammatory response.

In **chapter 3** we investigate whether plasma miRs could be used to monitor microvascular integrity in the context of acute kidney transplant rejection, due to an alloreactive immune response against the donor kidney. This study shows that a more pronounced activation of the immune response markedly injures the microvasculature in these patients as shown by an increased mean tortuosity index of microvascular capillaries, an increased Ang-2/Ang-1 ratio and higher VEGF-A- and sTM serum levels. Following kidney transplant rejection, several plasma miRs associated with rejection (among others miR-130b, miR-135a and miR-199a-3p) and microvascular injury markers (miR-130b, miR-199a-3p and miR-192). It can be argued that a reduced glomerular filtration rate (GFR) may have caused increased levels of these circulating miRs in these patients but a study on plasma miRs in patients with different stages of chronic kidney disease (CKD) provided evidence against this (28). This study demonstrated a reduced concentration of plasma miRs in patients with a lower eGFR (stage 4 CKD patients and end-stage renal disease (ESRD) compared to healthy controls) without a concomitant increase of miRs in the urine of these patients (28). This suggests eGFR independent effects as a cause for differential miR levels induced by kidney transplant rejection. With regard to the sex-specificity of these miRs, none of them were located on the X-chromosome., but, interestingly, the estrogen regulated miR-130b, (which associated with acute rejection and microvascular injury) can be used to monitor diabetic disease progression (29) while its sex-specific expression was recently reported in animal

studies (30). Nonetheless, we did not perform further validation studies nor sex-specific stratifications of the identified circulating miRs for women and men. This would reduce the sample size of the studied patient cohorts thereby influencing the differential expression of these miRs. This could have been of interest as well, given that the differentially expressed miR-135a and miR-199a-3p in this study were previously also found to be regulated by estrogen in their transcription and processing (31, 32).

Having identified that plasma miRs can be used to monitor microvascular integrity following an ensuing alloreactive immune response, an additional hypothesis generating study in **chapter 4** identified that the X-chromosome origin or estrogen regulation of miRs in plasma provides a sex-biased expression which could indeed be used as a sex-specific biomarker for diabetic cardiovascular comorbidities. This hypothesis study was conducted in patients with idiopathic atrial fibrillation (AF), a microvascular injury associated *borderline* AF phenotype (33) with a high prevalence in women with type 2 diabetes mellitus (DM2) (34). Strikingly, miR expression results in women indicated that coronary calcification particularly increased circulating levels of miR-126, an endothelial specific miR regulated by estrogen (20). These findings, in which coronary calcification associates with a diabetic, microvascular miR profile, could suggest that differentially expressed miRs pinpoint endothelial dysfunction as pathophysiological substrate in women with iAF. Previously, this aetiology could not be demonstrated with conventional methods to assess endothelial function in the setting of coronary calcification (35). Whether microvascular injury indeed induced differential expression of miR-126 remains to be studied, because we did not assess levels of microvascular injury markers in these patients. Nevertheless, a differential expression of miR-126 in women is consistent with studies which have shown that miR-126 in women can also be used to monitor microvascular injury disease progression in hypertension and preeclampsia (36, 37). Importantly, we performed in **chapter 4** an extensive statistical adjustment of circulating miR levels for the use of aspirin and vitamin K antagonists. Previously it was demonstrated that the administration of platelet inhibitors such as aspirin reduced circulating levels of miRs (38). Therefore, adjusting miR expression levels in patients who receive a daily dose of aspirin is necessary because platelet activation in type 2 diabetes (DM2) promotes the release of miRs into the circulation which could potentially overestimate their expression levels.

Several limitations characterize the study in **chapter 4** as well. First, we could not confirm associations of previously identified miRs with arrhythmia in AF patients in plasma from patients with *idiopathic* atrial fibrillation. This is consistent with an often observed poor reproducibility of miR biomarker studies which is related to differences in normalizations methods, differences in assay technologies and differences in pre-analytical sample handling (39). Therefore, in order for plasma miRs to become a clinically relevant biomarker, more biomarker research in larger cohorts is needed in which plasma miRs are tested in several phases, including initial proof of concept studies as well as prospective validation studies in independent populations (11). This way, plasma miRs could potentially (and in conjunction with established biomarkers) improve disease stratification and prognostication, which could eventually allow for a better patient-specific intervention (11). Second, it could be argued that this study demonstrates that plasma miRs that are identified in robust disease phenotypes (atrial fibrillation) oftentimes cannot be used to differentiate borderline disease phenotypes (idiopathic atrial fibrillation) from healthy controls. This could be related to more rigorous pharmacological treatments in patients with a strong disease phenotype. Third, in this study with iAF patients we only measured the expression of a selected subset of 48 miRs while numerous miRs are known to be mechanistically involved in diabetic cardiovascular comorbidities. Although this particular subset of miRs was previously found to associate with markers of microvascular injury (6), it cannot be excluded that the association of more involved miRs was missed in our approach. Finally, the assessment of total plasma miRs in chapter 4 obviates the fact that plasma miRs are distributed among EVs and circulating proteins like HDL and the Ago-2 protein. These plasma carriers allow miRs to become extracellular thereby allowing a novel paracrine mechanism of non-coding RNAs for intercellular communication while a change in the distribution of these miR carriers could reflect disease progression in diabetes patients.

In diabetes, miRs carried in EVs participate in cell-cell crosstalk between liver- and adipose tissue, a mechanism that controls glucose uptake in skeletal muscle (40). This extracellular RNA mediated endocrine signal was confirmed in another study that demonstrated the adipokine-like character of these EV-miRs which were secreted from adipocytes in order to regulate peripheral insulin sensitivity (41). By functioning as biomarker, EV-miRs and not Ago-2 miRs can be used to monitor diabetic disease progression (42), which is consistent with results

that demonstrated that only EV-miRs can be used to monitor diabetic disease progression to microvascular injury (43). Furthermore, in ischaemic conditions particularly EV-miRs promote angiogenesis and stimulate the formation of new functional vessels (44). Interestingly, this functional role for extracellular RNA in angiogenesis is also particularly seen for miRs associated with HDL, a lipoprotein that promotes miR biogenesis (45) while particular HDL-miRs such as HDL-miR-223 suppresses the induction of angiogenesis in diabetes (46). Taken together, this suggests that extracellular RNA species (carried in EVs or associated with HDL) can act as functional regulators of metabolism and angiogenesis in target tissue by changing mRNA expression and translation (11). Assessing (sex-biased) extracellular miRs in diabetes could potentially provide a better mechanistic explanation for a diabetes induced microvascular injury compared to differences in the overall absolute quantity of total plasma miRs.

As such, in **chapter 5** we hypothesized whether DM and DN results in a shift in the plasma miR distribution between circulating EVs, HDL- and Ago-2 protein that contributes to microvascular injury. To study carrier-miR expression we first adopted novel methods to isolate HDL, EVs and Ago-2 from the same plasma samples of individual patients. First, HDL was purified from plasma using density gradient ultracentrifugation (DGU), a method that yielded an HDL recovery rate of more than 50% (data not shown) which is consistent with previously published methods (47). The obtained HDL fraction was further purified with size exclusion chromatography (SEC) in order to deprive microparticles with a similar density from the HDL fraction (48). Because EV- and HDL-fractions displayed a different extracellular RNA profile, it could be argued that this isolation method is accurate enough to isolate a relatively purified HDL fraction. However, still other lipoproteins such as low-density lipoprotein (LDL) or microparticles could have contaminated our HDL fraction. Additional contamination could be caused by the fact that HDL is highly heterogeneous and contains subfractions with a different density, size, and protein composition (49). With the EV isolation however, we experienced less difficulties due to the more distinct physico-chemical properties of EVs. We again used SEC to isolate EVs and we provide extensive validation of their presence in the isolate with quantitative (nanoparticle tracking analysis, NTA) and qualitative proof (CD63 Western Blot and electron microscopy visualization). This in-depth method for EV isolation is in compliance with methodological recommendations for EV isolation and characterization that were published recently (50, 51). Collectively,

these robust methods for HDL- and EV isolation allowed us to assume RNA was isolated from a purified HDL and a non-contaminated EV fraction.

Having established a novel method to isolate plasma-miR carriers, we assessed their miR expression levels in a pilot- and main study. Upon these studies described in **chapter 5**, we found in the HDL fraction that absolute levels of HDL-miR-132 decreased in plasma from DM and DN patients, while HDL-miR-132 correlated with levels of vascular injury marker Ang-2. In addition, we found HDL-miR-132 to rescue the decreased angiogenic capacity of DN patient serum in an in vitro angiogenesis assay. This demonstrates that (i) an HDL-miR-132 decrease is not only indicative of microvascular injury but (ii) also suggests that HDL-miR-132 plays an active important role in maintaining vascular integrity. The diagnostic potential for microvascular injury of miR-132 is consistent with similar data from a previous study which demonstrated that miR-132 is an angiomiR while its decrease associates with a reduction in microvascular density and microangiopathy (52). Moreover, our in vitro experiments particularly provide new evidence that this HDL-miR-132 decrease is causally related to microvascular injury in these diabetic patients because it restored microvascular injury in a matrigel angiogenesis assay in a carrier-dependent manner. Regeneration of microvascular endothelium was so far only shown for miR-132 carried in EVs derived from mesenchymal stem cells (MSCs) (53) and neurons (54) which enhanced angiogenesis and maintained brain vascular integrity respectively. However, not only miR-132 is involved in these angiogenic processes because other miRs (miR-223) that also associate with HDL were previously found to be elevated in the HDL fraction of diabetic mice and able to restore endothelial integrity (46).

With regard to miRs carried in EVs, it has been shown that the cellular source from which they originate determines whether their induced gene expression differences are beneficial or detrimental in diabetes patients (55). Beneficial EV-miRs are the proangiogenic EV-miR-21 (56) and -126 (57) derived from diabetic ECs, which expression acts as a protective stimulus to recipient vascular cells. In contrast, when EV-miR-21(58) and EV-miR-126 (59) are derived from diabetic, pancreatic beta cells, their increased shedding with a higher miR expression cargo results in more cellular apoptosis of recipient cells. In **chapter 5** we did not study the cellular source from which the identified plasma EV-miRs originated. So far known, the majority of plasma EV-miRs originate from platelets (60) or are derived from

adipocytes (40) while HDL receives its miR content via inflammatory cells such as monocytes or macrophages (9). Others suggest that extracellular RNA profiles reflect a secretory pattern of multiple nonhematopoietic and hematopoietic cells (61). Although speculative, it could very well be that the EV- and HDL-miRs in these DM and DN patients were derived from platelets since platelets are activated in diabetes patients (38). Another limitation of our study in **chapter 5** is that we did not perform stratifications of differentially expressed carrier miRs for women and men. This could have been of interest given that sex-steroids are known to regulate the differentially expressed miRs in this study because testosterone augments miR-132 expression which promotes angiogenesis (62) while the estrogen regulation of miR-21 stimulates inflammation (63). Therefore, determining carrier specific miR expression followed by a subsequent stratification of miR expression results for women and men is an interesting follow up approach for future studies.

Following the identification of differentially expressed HDL-miR-132, EV-miR-21 and -126 in plasma from diabetic patients in the study in **chapter 5**, we also performed additional in vitro studies with these carrier miRs to assess their functional role in endothelial cell biology and angiogenesis. HDL-miR-132 (and not EV-miR-132) restored microvascular injury induced by serum from DM and DN patients in a matrigel angiogenesis assay. Although carrier specific diagnostic potential (EV-miRs but not Ago-miRs) for microvascular injury has been demonstrated recently (42), our study in **chapter 5** is the first to demonstrate these carrier specific functions for extracellular RNA. Still, in these experiments we also observed a better angiogenic performance of ECs with recombinant HDL only, although not statistically significant. This suggests that the function of HDL changes with disease states as well, a finding consistent with studies which have demonstrated a change in the molecular characteristics and function of HDL in hyperglycaemic settings (64).

With the aforementioned proof that carrier-specific miRs induce post-transcriptional regulation of genes involved in metabolism at distal sites, we studied in **chapter 6** whether a sex-specific expression of carrier-specific miRs could explain a DM induced microvascular injury phenotype in women. With miR-sequencing of plasma RNA in transwomen (who receive a daily dose of estrogen prior to surgical transition) we identified two X-linked miRs in EVs, miR-224 and miR-452 to be decreased after one year of estrogen suppletion. Interestingly, the

X-chromosome contains a high amount of estrogen-responsive-elements (EREs) and the differential expression of the miR-224/452 cluster could potentially be explained by the presence of these EREs (65). We found that systemic silencing of this miRNA cluster promoted a prediabetic phenotype in mice due to insulin resistance and an aberrant metabolism in brown- and white adipose tissue. Previously, studies have shown that urinary miR-224 secretion increased following disease progression in type 1 DM patients (66) while EV-miR-224 was found to regulate skeletal muscle insulin sensitivity in gestational diabetes mellitus (GDM) (67). Interestingly, regarding miR-452 it was demonstrated that its expression in human umbilical vein endothelial cells (HUVECs) was promoted upon maternal gestational diabetes (68). The fact that the expression of both miRs is altered in the setting of GDM (with high levels of estrogen and insulin) is consistent with their differential expression in transwomen in our study. Like in GDM patients, also transwomen display both high estrogen and insulin levels following one year of estrogen suppletion. Therefore, future studies should investigate whether both miRs regulate maternal and fetal metabolism in GDM as well.

In **chapter 6** we found that both plasma miR-224 and -452 were predominantly carried in EVs. However, we did not investigate whether estrogen suppletion increases adipose tissue EV secretion or instead the EV miR copy number of miR-224 and -452. In addition, the way adipocytes induce packaging of both miRs and the mechanism of their selection and secretion was also not studied. Whether these are estrogen induced effects is very relevant for plasma miR concentrations and the resultant clinical phenotype (possibly instigated by EV mediated extracellular communication via miRs at distal sites). Furthermore, because transwomen get elevated plasma levels of insulin after estrogen suppletion, it could very well be that insulin itself suppresses both miRs. However our group has previously shown that fluctuations in plasma insulin levels in DM and DN patients (adjusted by a simultaneous pancreas kidney transplantation, SPK) do not significantly affect plasma levels of miR-224 and -452 (6).

Interestingly, systemic silencing of both miRs in mice in **chapter 6** increased their plasma insulin levels and lowered glucose uptake in brown adipocytes. Although plasma insulin levels in mice were possibly induced by less glucose uptake in brown adipose it could also be the result of more insulin secretion by pancreatic beta cells after a direct silencing effect of both miRs in these cells. We did not

investigate this in more detail which would require additional glucose clamp studies but since they were not fed a high fat diet the mice in our experimental setup could not have answered these particular questions. Such an experimental setup could have elucidated whether systemic silencing of both miRs is indeed a causal mechanism that induces insulin resistance via a peripheral effect on glucose and lipid metabolism. Furthermore, after anti-miR-224 and anti-miR-452 treatment of cultured murine white adipocytes, we observed less glycerol release in culture media from anti-miR-224 treated cells only. These results are consistent with *in vivo* data in **chapter 6** in which lipid uptake predominantly increased in anti-miR-224 treated mice. This suggests that miR-224 is predominantly involved in white adipocyte metabolism, an observation consistent with recent studies in which miR-224 particularly regulates white adipocyte differentiation (69) and fatty acid metabolism (70).

To investigate whether differential (target) mRNA expression was involved in glucose and lipid metabolism after miR-224 and miR-452 silencing in mice, we performed RNA sequencing (RNA-seq) of brown and white adipose tissue. The RNA-seq data of differentially expressed (target) genes in **chapter 6** shows that the lower concentration of both miRs upon estrogen secretion modulates only a subset of (target) genes. However, amongst differentially expressed genes we identified key regulators of mitochondrial metabolism (Pgc1a), insulin stimulated glucose uptake (Irs-1, Irs-2) and lipogenesis (Gpr120, Apoc1, Acs1l1). Furthermore, in additional mechanistic studies *in vitro* in **chapter 6**, we observed an estrogen-induced decrease of miR-224 in brown adipocytes and miR-452 in white adipocytes. However, it is likely that these effects are also partially time dependent since estrogen supplementation of cultured adipocytes was performed at a concentration of 100 nM and lasted for 48 hours. That such effects are indeed time- but also dose dependent is particularly seen in transwomen in whom estrogen supplementation lasts for over year. This is a time frame in which a daily estrogen supplementation promotes fat mass and lowers insulin sensitivity, suggesting that time and dosage should be taken into account in *in vitro* settings (71).

Interestingly, in cultured brown adipocytes, only anti-miR-224 led to a decrease in mitochondrial basal respiration. Nonetheless, glycolysis and glucose uptake in brown adipocytes were lower in anti-miR-224 and -452 treated cells, indicating that both miRs decrease glucose uptake in these cells as was seen in mice as well. This

reduction in glucose uptake was followed by a decrease in glycolysis in these cells, and a reduction in mitochondrial respiration which are crucial metabolic pathways that facilitate cellular thermogenesis (72). Moreover, adipocyte specific dysfunction of metabolism is a pathophysiological pathway causally related to microvascular injury and myocardial fibrosis (73). Therefore, we next studied whether both miRs are also involved in the aetiology of left ventricular diastolic dysfunction (LVDD) and the onset of heart failure with preserved ejection fraction (HFpEF) (left ventricular (LV) ejection fraction (EF) $\geq 50\%$) in women.

Currently, women and men are equally affected by the presence of LVDD (74) while LVDD in women oftentimes progresses to HFpEF (75). However, particularly obese and diabetic women outnumber men (76) in the prevalence of HFpEF (77-81). This female preponderance for HFpEF in the setting of diabetes is consistent with studies in which machine learning with model-based clustering defined a common HFpEF phenotype which mainly included women with DM, obesity and renal dysfunction leading to a systemic inflammatory state that promotes microvascular injury and subsequent myocardial remodelling (82-84). With regard to the involvement of miRs in this pathophysiology, several studies have addressed the differential expression of miRs involved in- and leading to LVDD and HFpEF. In these studies, particularly the involvement of miR-146 (a regulator of diabetes-induced myocardial cytokine production through NF- κ B) (85) and miR-155 (a macrophage-derived pro-inflammatory miR) (86) demonstrate that metabolic comorbidities and inflammation are driven by a differential expression of miRs leading to HFpEF. Nonetheless, in their analysis these studies did not investigate whether these miRs displayed a sex-specific differential expression through a stratification of their expression levels for women and men. Therefore, we assessed in **chapter 7** whether X-linked- or estrogen-regulated miRs (identified in the previous chapters and conducted pilot studies), display a sex-specific diagnostic potential for microvascular injury in patients with LVDD and in women with HFpEF.

In **chapter 7** we present plasma miR profiling results of three patient cohorts: (i) (formerly preeclamptic) women with LVDD and concentric remodeling, (ii) diabetic patients with LVDD and (iii) patients with DM and HFpEF. In formerly preeclamptic women, Ang-2 levels increased in women with LVDD while particularly miR-224 increased and associated with Ang-2. Furthermore, in diabetic women with LVDD (87) we identified a reduction of miR-34a as well as miR-224 and -452 following an

eGFR < 60 ml/min. Interestingly, particularly miR-34a is derived from adipocytes (88) and induces a sex-specific response to treatment following myocardial hypertrophy (89). In chapter 7 we have detailed the role for miR-224 and miR-452 in regulating adipocyte metabolism. Moreover, miR-224 regulates adipocyte differentiation (69) and inflammation (90), as well as (micro)vascular quiescence and its activation (91). Given that the silencing of these miRs in **chapter 7** associated with a prediabetic phenotype, their differential expression in women with LVDD could suggest that its development is not driven by hypertension. Instead it could be argued that these structural myocardial abnormalities are driven by obesity, although body weight and body mass index (BMI) were not significantly different in these women. However, an obesity driven progression to LVDD is consistent with recent studies that highlights differences in obesity- and hypertension-induced cardiac remodelling in obese rats, because obesity increased the E/e' ratio whereas hypertension did not (92).

Conclusion and future perspectives

This thesis demonstrates that sex-specific miR expression in women with DM pinpoint adipocyte metabolism as pathophysiologic substrate for microvascular injury. In addition, it clearly shows that in diabetes, carrier miRs, such as HDL and EVs, enable miRs a carrier-specific, extracellular function which controls adipocyte metabolism at the post-transcriptional level. In patients with prediabetic phenotypes such as insulin resistance and following the progression towards type 2 DM, particular miRs circulate in plasma and induce adipocyte dysfunction at distal sites which could affect endothelial quiescence over time.

In women, not only adipocyte dysfunction following macrophage infiltration and subsequent inflammation could serve as pathophysiological substrate for myocardial microvascular injury (73) in the setting of iAF, LVDD and HFpEF. Also a reduced skeletal muscle mitochondrial content (93), uncoupling of glycolysis from glucose oxidation (94), increased triglyceride lipolysis (95) and beiging of brown adipose tissue (96) are involved in its pathophysiology. Collectively, this suggests that diabetic cardiovascular complications are due to an altered systemic cellular metabolism that strongly affects the heart-adipose tissue connection.

To investigate a female-specific, heart-adipose tissue connection in diabetes in more detail, the miRNA-mRNA interactome could potentially give additional information about the loss of cellular quiescence and cellular phenotypic changes in response to DM. A combination of simultaneous single-cell profiling data of mRNAs and miRs in skeletal muscle, white- and brown adipocytes as well as cardiomyocytes could identify how the interaction of their cell-specific transcriptomes is involved in the development of a diabetic heart-adipose tissue connection. Single-cell sequencing results have currently been performed in white- and brown adipocytes (97, 98), cardiomyocytes (99) and endothelial cells (100) and a similar approach in sex-specific diabetic pathophysiology could increase our understanding of cellular phenotypic changes upon diabetes in women.

In conclusion, with the identification of sex-specific post-transcriptional regulatory networks, this thesis has identified molecular pathways that could potentially lead to novel therapeutic targets and approaches to improve female specific diabetes care that could prevent the onset and progression of female specific microvascular injuries.

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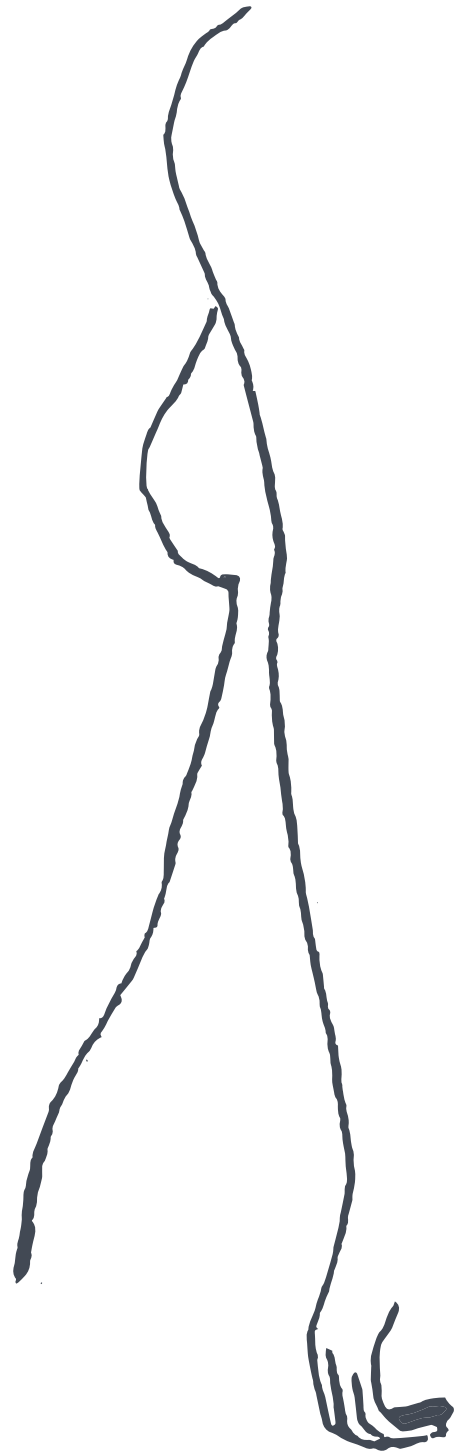
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Chapter 9

English and Dutch summary



Chapter 1 starts with a short introduction on the development of microvascular injury in women and continues with a mechanistic explanation of microRNA (miR) biogenesis and function.

In **chapter 2** this thesis provides a more in-depth introduction on the aetiology and pathophysiology of cardiovascular disease (CVD) in women. Attention is given into the mechanisms in which estrogen regulates microvascular function and homeostasis in ECs, or exacerbates cellular quiescence of adipocytes. This chapter further highlights the post-transcriptional mechanisms of sex-specific miRs (miRs transcribed from the X-chromosome or regulated by estrogen) in endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and cardiomyocytes (CMs).

To illustrate how plasma miRs can be used to monitor microvascular integrity, **chapter 3** is dedicated to a plasma miR profiling study in kidney transplant patients in whom an ensuing allogenic immune response coincides with microvascular injury. Using this unique patient cohort we could demonstrate that a selected subset of plasma miRs associated with inflammation, rejection and microvascular injury.

Chapter 4 of this thesis studies whether sex-specific circulating miRs in plasma can provide a female-specific biomaker for diabetic cardiovascular complications. In patients with idiopathic atrial fibrillation (AF), a microvascular injury associated *borderline* AF phenotype with a high prevalence in women with type 2 diabetes mellitus (DM2), sex-specific miR levels were measured and their association with microvascular injury was studied. Differentially expressed sex-biased miRs pinpoint endothelial dysfunction as pathophysiological substrate in women with iAF, an aetiology which could not be demonstrated with conventional methods previously.

Given that circulating plasma miRs are distributed among plasma carriers like extracellular vesicles (EVs), high density lipoprotein (HDL) and the Argonaute-2 (Ago-2) protein, we studied in **chapter 5** whether these carriers have a different miR distribution and function following DM and diabetic nephropathy (DN). With a novel method to isolate these carriers, this study identified an altered miR expression distribution following microvascular injury while different carrier specific miRs displayed different functions in ECs.

In **chapter 6** we report that male-to-female transgenders (transwomen) have a different plasma miRs profile following estrogen suppletion. In particular two miRs, namely miR-224 and -452 (derived from the same cluster on the X-chromosome and carried in EVs in plasma) associated with systemic insulin resistance. Upon silencing of both miRs in mice, we observed the onset of a prediabetic phenotype while RNA-sequencing of different adipose tissues led to the identification of a prediabetic, post-transcriptional gene network.

Lastly in **chapter 7** we assessed whether sex-specific miRs could function as biomarker for microvascular injury in (diabetic) women with left ventricular diastolic dysfunction or heart failure with preserved ejection fraction (HFpEF). Interestingly, particular those sex-specific miRs that were derived from adipocytes (miR-224, -452 and -34a) displayed a biomarker potential for microvascular injury in these female patient cohorts.

Finally **chapter 8** provides a general discussion of this thesis.

Hoofdstuk 1 van dit proefschrift bevat een korte inleiding op het ontstaan van microvasculair vaatlijden in vrouwen en geeft een beknopte moleculaire uitleg van de synthese en het werkingsmechanisme van microRNAs (miRs).

In **hoofdstuk 2** volgt een diepgaande analyse van de oorzaken en de pathofysiologie van vasculair lijden in vrouwen. Specifieke aandacht wordt gegeven aan het mechanisme waarmee het vrouwelijk hormoon oestrogeen de homeostase en functie van endotheelcellen, gladde spiercellen en cardiomyocyten reguleert. Daarnaast beschrijft dit hoofdstuk de rol van post-transcriptionele regulatie door geslacht-specifieke expressie van miRs (expressie van X-chromosoom miRs of miRs gereguleerd door oestrogeen) in de hierboven genoemde cellen.

Een illustratie hoe miRs kunnen worden gebruikt voor het monitoren van microvasculair lijden wordt gegeven in **hoofdstuk 3**. Specifiek is hoofdstuk 3 een studie in niertransplantatie patiënten in wie een voortgaande allogene immuunrespons leidt tot microvasculair lijden en resectie van de transplantatie nier. Met dit unieke patiënt cohort demonstreert deze studie de betrokken rol van miRs in de pathofysiologie van microvasculair lijden.

Hoofdstuk 4 bestudeert of geslacht-specifieke expressie van miRs in plasma bruikbaar is als vrouwspecifieke biomarker voor microvasculair lijden bij diabetes mellitus. In patiënten met een idiopathische vorm van atrium fibrilleren (een borderline fenotype met een hoge prevalentie in vrouwen met type 2-diabetes mellitus) werd geslacht-specifieke expressie van plasma miRs gevonden en hun associatie met plasma markers van microvasculair lijden. De differentieel tot expressie gevonden miRs wijzen op endotheel dysfunctie, een pathofysiologie die met conventionele methoden in deze patiënten niet kon worden aangetoond.

Plasma miRs zijn in plasma gebonden aan extracellular vesicles (EVs), high density lipoprotein (HDL) en het Argonaute-2 (Ago-2) eiwit. In **hoofdstuk 5** wordt een nieuw ontwikkelde methode beschreven om deze dragers in plasma te scheiden. Daarnaast toont deze studie aan dat diabetes mellitus de distributie en functie van miRs gebonden aan deze plasma dragers verandert.

Hoofdstuk 6 beschrijft miR expressie in transvrouwen door oestrogeen toediening. Deze studie identificeert twee miRs, namelijk miR-224 en miR-452

waarvan de remming in muizen leidt tot een prediabetes fenotype met insuline resistentie en een verstoord metabolisme in vet-cellen. Deze miRs verstoren mitochondriële functie (bruin vet) en de opname van vrije vetzuren (wit vet), een mechanisme wat in vrouwen associeert met microvasculair lijden.

Dat miR-224 en miR-452 inderdaad betrokken zijn bij microvasculair lijden en hartfalen wordt aangetoond in **hoofdstuk 7**. Dit hoofdstuk beschrijft dat deze miRs en een andere geslacht-specifieke miR (miR-34a) biomarker potentie hebben voor diastolisch hartfalen bij vrouwen met diabetes.

Ten slotte bevat **hoofdstuk 8** een algemene discussie van de resultaten beschreven in dit proefschrift.

Chapter 10

Appendices

Curriculum vitae

Barend W. Florijn was born on October 18th 1987 in Rotterdam, the Netherlands and completed VWO secondary school in Rotterdam in 2006. Following secondary school he studied Law (Leiden University) and Medicine in 2008 (Leiden University Medical Center, LUMC) and graduated in 2014. During his studies he worked as a teaching assistant at the Department of Surgery and started a research project at the Medical Psychology Department under supervision of prof. dr. A.A. Kaptein and at the Department of Internal Medicine (Nephrology) under supervision of dr. D. Soonawala. In addition, he was part of the Examination Appeals Board (College van Beroep voor de Examens, CBE) of the Leiden University. The doctoral research described in this thesis was conducted from 2014 through 2018 at the Department of Internal Medicine (Nephrology) and the Eindhoven Laboratory for Vascular and Regenerative Medicine of the LUMC under supervision of prof. dr. A.J. van Zonneveld. The research of this thesis was presented at the ASN Congress in Chicago in 2016, the ISEV congressed in Washington in 2014 and Rotterdam in 2016 and the Non-Coding RNA congress in Keystone Colorado in 2018. Moreover, based on his research he obtained a grant from the Diabetesfonds in 2018 (Doorbraakbeurs) entitled 'Estrogen driven posttranscriptional control of metabolism: the missing link in gestational diabetes'. During these years of research he was engaged in the development, coordination and organization of the half minor Heart and Bloodvessels (2014-2018), which was nominated as best half minor in 2018. In addition, he obtained a university teaching certificate (BKO) in 2018 and was a member of the team that won the Kolf Battle of the Universities (organized by the the Dutch Kidney Foundation). In 2019, he worked as a resident in Internal Medicine at the Haga Hospital, The Hague and as a resident in Neurology at the Reinier de Graaf Hospital (dr. L. Aerden). July 2020, he started as a neurologist in training at the LUMC (dr. C.S.M. Straathof).

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