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

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

ESC European Society of Cardiology

Cardiovascular Research (2018) **114**, 210–225
doi:10.1093/cvr/cvx223

REVIEW

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

Barend W. Florijn[1,2], Roel Bijkerk[1,2], Eric P. van der Veer[1,2], and Anton Jan van Zonneveld[1,2]*

[1]Einthoven Laboratory for Vascular and Regenerative Medicine, Leiden University Medical Center, Albinusdreef 2, 2300 RC Leiden, The Netherlands; and [2]Department of Internal Medicine (Nephrology), Leiden University Medical Center, Albinusdreef 2, 2300 RC Leiden, The Netherlands

Received 19 May 2017; revised 15 August 2017; editorial decision 3 October 2017; accepted 23 November 2017; online publish-ahead-of-print 24 November 2017

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.

Keywords Women • Sex-biased miRNAs • HFpEF • Microvascular injury

1. 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 under-diagnosed and under-treated 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 with HF with reduced ejection fraction (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 ischaemia and cardiomyocyte (CM) loss,[9,10] HFpEF is associated with advanced age,[11] obesity,[12] hypertension,[13] pre-eclampsia,[14] diabetes mellitus (DM)[15] and renal dysfunction.[16] Unfortunately, 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

* Corresponding author. Tel: +31 71 526 5195; fax: +31 71 526 6868, E-mail: a.j.van_zonneveld@lumc.nl

aforementioned metabolic co-morbidities 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 endothelial cell (EC)-derived nitric oxide (NO) signalling limits myocardial cyclic guanosine monophosphate (cGMP)–protein kinase G (PKG) signalling.[21] Because 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] whereas peripheral endothelial dysfunction independently correlates with future cardiovascular events.[29]

2. 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 HF and thus revealed that female mice develop a concentric form of hypertrophy and are more protected against fibrosis compared with male mice.[31] Moreover, female rats seem to be better protected against pressure overload because TAC-induced left ventricular (LV) hypertrophy resulted in a depressed contractile reserve in male hearts compared with the female hearts.[32] Similarly, in humans, men and women display a different, LV response to chronic load alteration during the progression of aortic stenosis (AS), and this sex-specific remodelling of CMs has also been attributed to the female predominant prevalence of HFpEF.[33] Men tend to develop an eccentric LV remodelling response of CMs upon stress, whereas women have a more concentric remodelling response that develops during ageing,[34] and with systolic hypertension[35] and AS.[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 EF and contractility when compared to men.[36] However, a complete understanding of the sex-specific cellular mechanisms within CMs is currently lacking.

3. 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 non-coding RNAs, such as miRNAs and long-non-coding RNAs (lncRNA),[38] and RNA-binding proteins.[39] Following this notion, many studies have addressed the co-ordination of gene expression in cardiovascular tissue by miRNAs, which play a pivotal role in silencing or fine-tuning messenger RNA (mRNA) expression and protein levels based on the complementarity of base pairing at 3′-untranslated regions (3′-UTR) of the 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 oestrogen-responsive elements (EREs)[41] whereby E2 binding drives miRNA expression.[42] Secondly, 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 that 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 with females resulting in a sex-specific myocardial endothelial nitric oxide synthase (eNOS) expression.[46] Furthermore, expression levels of other miRNAs, namely miR-1, miR-106b, miR-720, and miR-29b, were augmented in females compared with 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 mycoardial miRNA expression within the healthy heart but also in mouse models of dilated cardiomyopathy (DCM) and AF.[47] Interestingly, this 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 with 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-mentioned studies pinpoint sex-specific myocardial miRNA networks that may target multiple genes, thereby co-ordinating 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 co-ordination of signalling networks, and cellular fate.[49] The fact that the co-ordination 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 develop 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 possibe injurious effects of X-chromosome gene dosage disequilibria on the cardiovascular system in women. Secondly, we provide insight into how the pathogenesis of HFpEF is linked with sex-biased miRNA expression and their regulation of functionally related genes in 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.

4. Oestrogen attenuates the cellular response to injury in HFpEF

Sex steroid hormones markedly affect the pathophysiological differences in CVD development in men and women.[50] Particularly, the effects of 17β-estradiol (E2), the major circulating oestrogen, and the oestrogen receptors (ER) have been well investigated.[51] Two ERs, namely ERα and ERβ, 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 reactive oxygen species (ROS), NADPH oxidase[54] and inhibiting the expression of endothelial leucocyte 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]

5. Oestrogen counteracts adverse haemodynamic and metabolic HFpEF co-morbidities

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 pre-menopausal 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 post-menopausal women present higher arterial pressures than aged-matched pre-menopausal women.[65]

In lipoprotein metabolism, E2 decreases LDL-cholesterol[66] and prevents its vascular accumulation[67] and uptake by macrophages.[68] Furthermore, E2 elevates 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 ERα knockout mice are hyperinsulinaemic and have abnormal glucose levels,[71] a phenomenon that is reversed upon ERα stimulation.[72] Interestingly, this ER deficiency is also associated with less insulin secretion, less insulin sensitivity, and more insulin resistance in post-menopausal women.[73,74]

These beneficial E2 effects in pre-menopausal women have 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 post-menopausal women may actually serve as a potential driver of elevated susceptibility for CVD in women with diabetes.[77]

6. Oestrogen 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, whereas 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 affect miRNA expression levels.[42]. This subsequently regulates multiple downstream targets (in both a direct and an 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 affect expression levels of essential components of the miRNA processing machinery. Binding of the ER-E2 complex at an

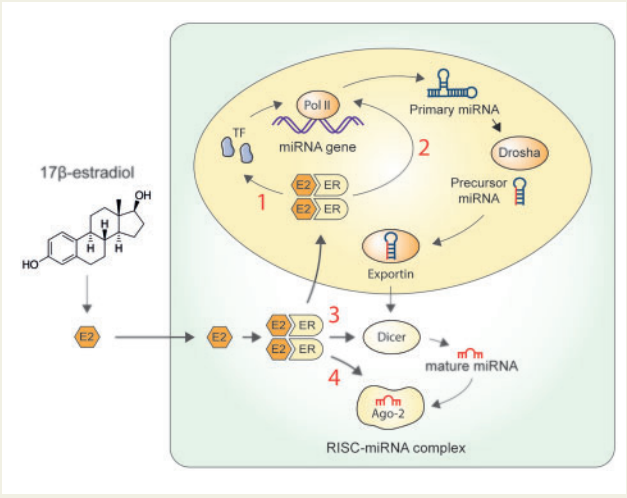

Figure 1 Levels of oestrogen (E2) control of miRNA transcription and processing. (1) E2 can impact on miRNA transcription via recruitment of transcription factors (TF). (2) E2 can affect RNA polymerase II (Pol II) activity. (3) E2 can augment the expression of the pre-miRNA processing protein Dicer.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

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, genomescale studies in breast cancer cell lines found that the ERα binds the enhancer region upstream the DICER transcription start site, which suggests E2–ER signalling modulates miRNA processing.[83] Further downstream in miRNA processing, ERα 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 HUVECs, 24 h of E2 treatment (10 nM) was found to increase both pri-miR-126 and mature miR-126-3p expression in an ERα-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 ERα-binding site in the pri-miRNA-22, the E2-induced repression of miR-22 processing was abolished. Therefore, this study concluded that the ERα represses the processing of the pri-miR-22 by binding to a conserved ERα binding element within the pri-miR-22.[86]

Similarly, miRNA profiles of skeletal muscle from monozygotic postmenopausal twin pairs discordant for oestrogen-based 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 or mutation analysis of ER-binding sites in these particular miRNAs was performed in this study. Furthermore, in omental adipose tissue samples from women with gestational diabetes mellitus, miRNA-222 was up-regulated compared with the healthy pregnant controls and displayed a negative correlation with ERα 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 with the healthy controls.[88] However, neither this effect was causally mediated via the ERα nor were any additional miR-222 overexpression experiments performed to determine direct miRNA-222 effects on ERα, 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.[89]

7. Oestrogen-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 leucocyte adhesion and to reduce VSMC proliferation that 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 (O_2) bioavailability 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 subjacent CMs, reduced NO and O_2 levels perturb mitochondrial respiration[97] and contraction,[98] which initiates a remodelling response[99] that correlates with LV diastolic dysfunction.[100]

In ECs, VSMCs and CMs, E2 regulates the aforementioned cellular processes. Although 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 ERα-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 ant-inflammatory effects in the *in vitro* experiment were very modest, the data strongly suggest that 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 the expression of miRNA-203.[101] Although no functional ERE was identified in the predicted miR-203 promoter region, this study demonstrated that ERα 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 ERα at the miR-203 promoter. The resultant high miR-203 levels in ERα-overexpressing mouse VSMCs attenuated the 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]

8. Oestrogen-regulated miRNAs attenuate myocardial remodelling and fibrosis

In CMs, E2 affects 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-ß- deficient C57Bl/6J male mice, when compared with 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 gender is in line with the results from a genomewide gene expression profiling study

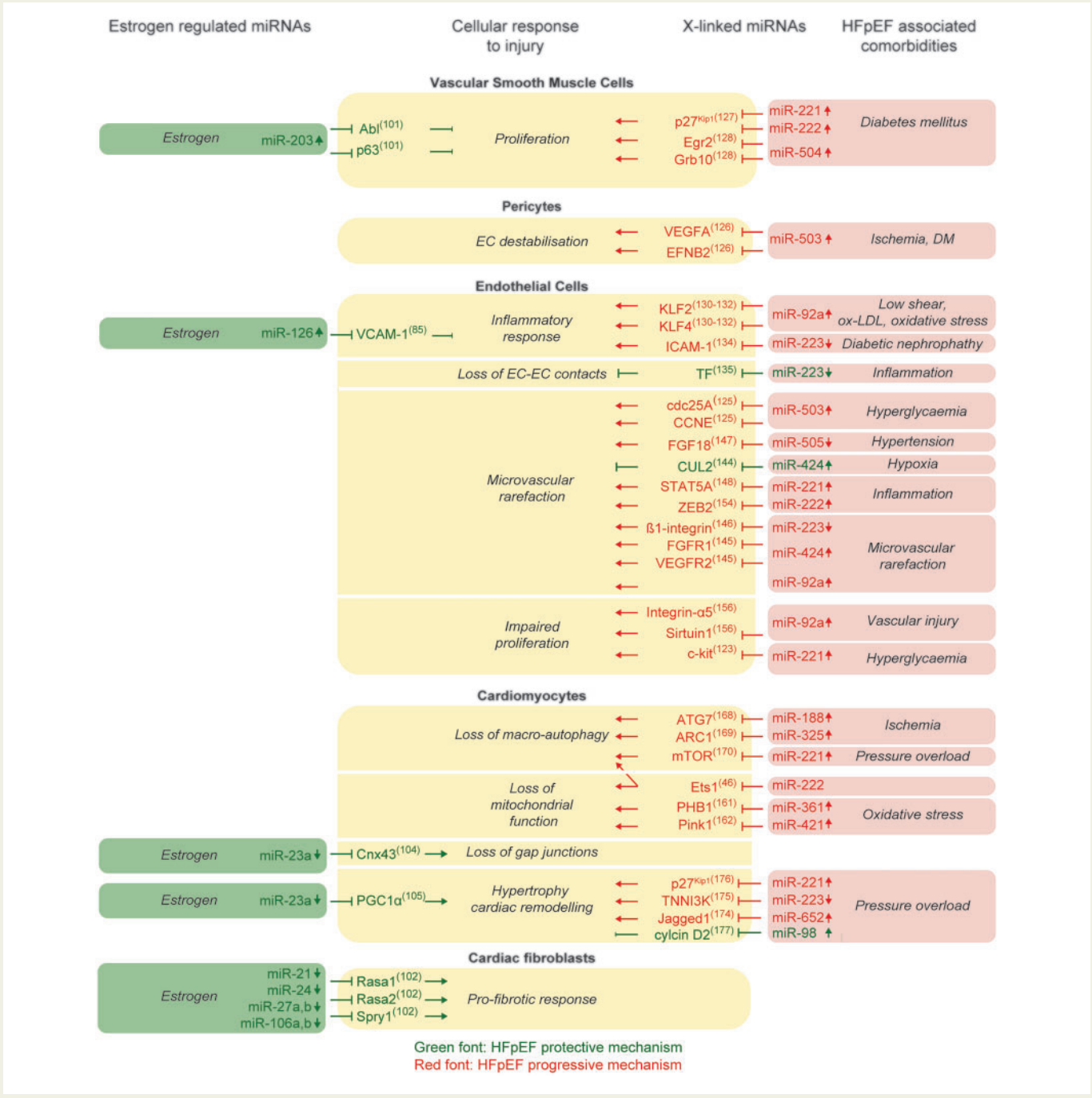

Figure 2 Sex-biased miRNAs are potential mediators of the sex-specific cardiovascular pathophysiology in HFpEF. The middle panel (yellow panel) describes the cellular response to injury in the progression of HFpEF on which oestrogen-regulated miRNAs (green panel) and X-linked miRNAs (red panel) were shown to have an impact. Green fonts depict miRNA/targets relations in HFpEF protective mechansms, red fonts depict HFpEF progressive mechanisms.

of human myocardial LV tissue samples, where transcriptome profiling revealed that fibrosis-related genes were induced to a higher degree in males when compared with 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 that was reversed upon E2 suppletion.[104] Interestingly, gap junction defects and Cx43 down-regulation 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 up-regulation 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 post-menopausal 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-1α), a key mitochondrial protein.[105] Interestingly, both miRNA-23a overexpression and knockdown experiments altered mitochondrial respiration in cultured CMs because of PGC-1α 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 fetal calf serum (FCS) to exclude the oestrogenic 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 oestrogen-induced mechanisms from these studies. Nonetheless, the aforementioned miRNAs pinpoint consequences of alterations in sex hormone levels in post-menopausal women, which negatively affects metabolism and cardiac function.[107]

9. 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 vs. 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 co-ordinated 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, whereas 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 *gonadectomized* FCG mice, it is thus possible to study sex chromosome-induced cardiovascular effects, independently of gonadal hormone-mediated effects that are normally known to supress the cellular response towards HFpEF-inducing co-morbidities. 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 ischaemia/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 co-morbidities (i.e. inflammation, obesity, and hypertension) could solely be attributed to the number of X chromosomes rather than the absence of a Y chromosome.

10. 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 with the autosomes.[43] In contrast, the male Y-chromosome possesses only two annotated miRNAs in the pseudoautosomal 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).

11. 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 post-menopausal 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

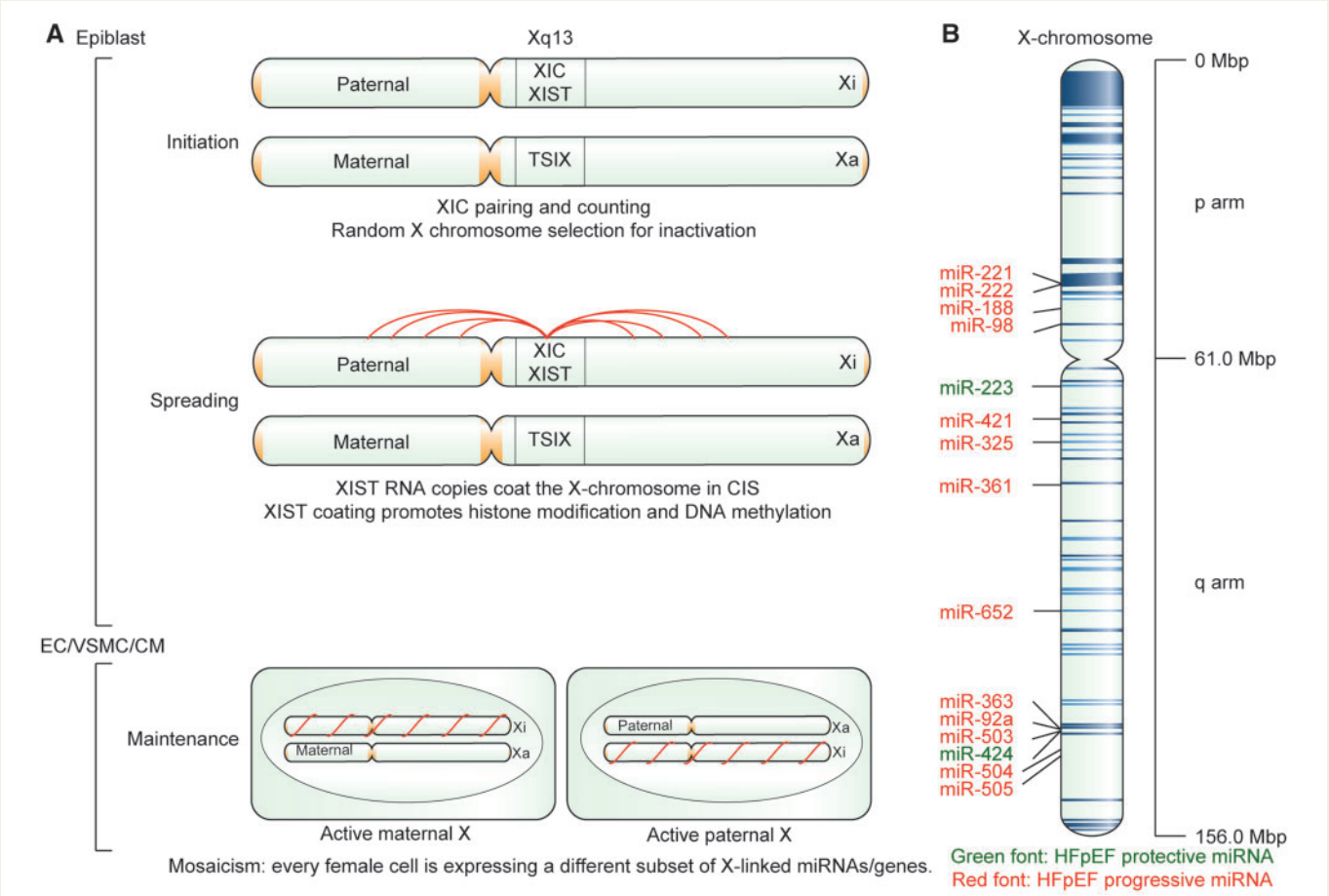

Figure 3 (*A*) Mechanism of X-chromomsome inactivation (*B*) MiRNA map of the human X chromosome depicting X-linked miRNAs that regulate the cellular response to injury in HFpEF. (*A*) During embryonic development, XCI inactivation is carried out by the long-non-coding RNA XIST which is expressed from 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 Carrel and Willard.[45] Italic and bold emphases 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.

expression in 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 $p27^{Kip1}$mRNAlevels, 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 affect the proliferative and supportive capacities of ECs and pericytes respectively, they stimulate the proliferative capacity of VSMCs. These pinpoint 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.

12. 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 tumour necrosis factor-α (TNF-α) in HFpEF patients[129] stimulate endothelial VCAM-1 and E-Selectin expression, thereby promoting leucocyte 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 ageing-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 an miRNA-223 mimic was subsequently found to decrease both TF mRNA and protein levels, suggesting a potential inhibitory role for miRNA-223 in the prothrombotic 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.

13. 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 O_2 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 the 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 destabilizes 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 LV diastolic dysfunction and cardiac reserve function impairment.[18] This pathophysiological process leading to HFpEF was recently demonstrated in HFpEF patients in whom cardiac positron emission tomography demonstrated a reduction in myocardial flow reserve (MFR, i.e. the ratio of maximal hyperaemic 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, sufficient 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 reprogrammes 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 pro-angiogenic 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 affect 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: (i) inhibition of endothelial eNOS expression[149–151]; (ii) reduction of the pro-proliferative stem cell factor

c-Kit[152,153]; and (iii) 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 I/R injury. With this model, this study found that the inhibition of the X-linked miRNA-92a was found to reduce infarct size when compared with control pigs, which resulted in an improved LV EF.[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 affect 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.

14. 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 that 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 and 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 ischaemic injury, thereby inducing an enlarged infarct zone as compared with wild-type mice.[161] Further evidence that X-linked miRNAs critically affect 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-kB 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]

15. X-linked miRNAs mediate autophagy signalling in cardiomyocytes

Autophagy is a process whereby damaged cellular components are sequestered into lysozymes 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 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 co-morbidities, 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 that 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.

16. 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 of 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, whereas 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 (TNNI3K) 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.

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

Diagnosing HFpEF requires imaging evidence of normal systolic LV function (LVEF ≥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, whereas 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%) in comparison with 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.*[182] identified a subset of 6 significantly altered circulating miRNAs in HFpEF patients (miR-1233, -183-3p, -190a, -193b-3p, -193b-5p, and -545-5p) when compared with the healthy controls. Interestingly, this study revealed that the most significant increase in the 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.*[183] identified that miR-375, miR-146a, miR-30c, miR-328, and miR-221 differed in plasma from HFpEF compared with HFrEF patients, as well as in plasma from HF and no-HF patients. Surprisingly, while the X-chromosome-located miRNA-221 differentiated HFpEF from HFrEF in this study, it distinguished HF from the 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 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 an 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

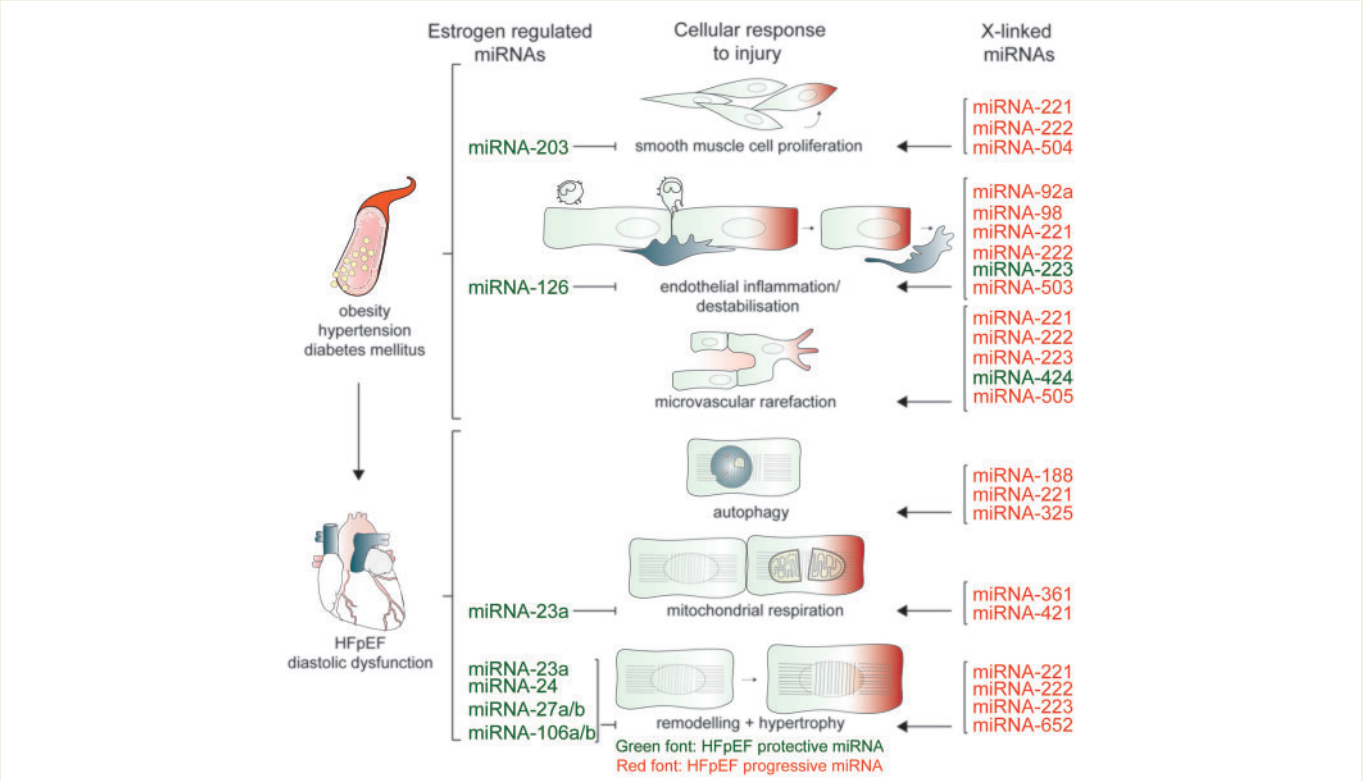

Figure 4 Overview of sex-biased miRNA involvement in the succesive pathophysiological steps leading to HFpEF. Figure depicts the sex-biased miRNA with regulatore roles within the different steps of the HFpEF cascade from endothelial inflammation towards global cardiac remodelling and dysfunction. The middle panel depicts the cellular response to injury in the progression of HFpEF on which oestrogen-regulated miRNAs (left-side figure) and X-linked miRNAs (right-side figure) were shown to have an impact.

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 *et al.* and Watson *et al.* could indicate whether testing for these miRNAs could improve HFpEF detection in women.

18. 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] When compared with 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 pre-eclampsia.[190] Furthermore, women with pulmonary hypertension show a more pronounced decrease in circulatory miRNA-223 levels than men.[191] Although 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 data sets in which stratifications are introduced for females and males could indicate whether these miRNAs could accelerate the detection of HFpEF in women.

19. 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 co-morbidities-induced vascular inflammatory state in HFpEF patients could potentially activate sex-biased miRNA networks that 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 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.

Funding

This work was part supported by grants from the Dutch Heart Foundation, Queen of Hearts: Improving diagnosis of CVD in women, [2013T084 to A.J.V.Z.], The Dutch Kidney Foundation innovation grant [14OIP13 to A.J.V.Z. and R.B.], and The European Fund for the Study of Diabetes [EFSD/BI to A.J.V.Z. and R.B.].

Conflict of interest: none declared.

References

1. Garcia M, Mulvagh SL, Bairey Merz CN, Buring JE, Manson JE. Cardiovascular disease in women: clinical perspectives. *Circ Res* 2016;**118**:1273–1293.
2. Appelman Y, van Rijn BB, Ten Haaf ME, Boersma E, Peters SA. Sex differences in cardiovascular risk factors and disease prevention. *Atherosclerosis* 2015;**241**:211–218.
3. Steinberg BA, Zhao X, Heidenreich PA, Peterson ED, Bhatt DL, Cannon CP, Hernandez AF, Fonarow GC. Trends in patients hospitalized with heart failure and preserved left ventricular ejection fraction: prevalence, therapies, and outcomes. *Circulation* 2012;**126**:65–75.
4. Bhatia RS, Tu JV, Lee DS, Austin PC, Fang J, Haouzi A, Gong Y, Liu PP. Outcome of heart failure with preserved ejection fraction in a population-based study. *N Engl J Med* 2006;**355**:260–269.
5. Devereux RB, Roman MJ, Liu JE, Welty TK, Lee ET, Rodeheffer R, Fabsitz RR, Howard BV. Congestive heart failure despite normal left ventricular systolic function in a population-based sample: the Strong Heart Study. *Am J Cardiol* 2000;**86**:1090–1096.
6. Abebe TB, Gebreyohannes EA, Tefera YG, Abegaz TM. Patients with HFpEF and HFrEF have different clinical characteristics but similar prognosis: a retrospective cohort study. *BMC Cardiovasc Disord* 2016;**16**:232.
7. O'Neal WT, Sandesara P, Hammadah M, Venkatesh S, Samman-Tahhan A, Kelli HM, Soliman EZ. Gender differences in the risk of adverse outcomes in patients with atrial fibrillation and heart failure with preserved ejection fraction. *Am J Cardiol* 2017;**119**:1785–1790.
8. Edelmann F, Stahrenberg R, Gelbrich G, Durstewitz K, Angermann CE, Dungen HD, Scheffold T, Zugck C, Maisch B, Regitz-Zagrosek V, Hasenfuss G, Pieske BM, Wachter R. Contribution of comorbidities to functional impairment is higher in heart failure with preserved than with reduced ejection fraction. *Clin Res Cardiol* 2011;**100**:755–764.
9. Deswal A, Bozkurt B. Comparison of morbidity in women versus men with heart failure and preserved ejection fraction. *Am J Cardiol* 2006;**97**:1228–1231.
10. Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, Kajstura J, Anversa P. Myocyte death in the failing human heart is gender dependent. *Circ Res* 1999;**85**:856–866.
11. Loffredo FS, Nikolova AP, Pancoast JR, Lee RT. Heart failure with preserved ejection fraction: molecular pathways of the aging myocardium. *Circ Res* 2014;**115**:97–107.
12. De Simone G, Devereux RB, Chinali M, Roman MJ, Barac A, Panza JA, Lee ET, Howard BV. Sex differences in obesity-related changes in left ventricular morphology: the Strong Heart Study. *J Hypertens* 2011;**29**:1431–1438.
13. Meyer S, van der Meer P, Massie BM, O'Connor CM, Metra M, Ponikowski P, Teerlink JR, Cotter G, Davison BA, Cleland JG, Givertz MM, Bloomfield DM, Fiuzat M, Dittrich HC, Hillege HL, Voors AA. Sex-specific acute heart failure phenotypes and outcomes from PROTECT. *Eur J Heart Fail* 2013;**15**:1374–1381.
14. Ghossein-Doha C, van Neer J, Wissink B, Breetveld NM, de Windt LJ, van Dijk AP, van der Vlugt MJ, Janssen MC, Heidema WM, Scholten RR, Spaanderman ME. Pre-eclampsia: an important risk factor for asymptomatic heart failure. *Ultrasound Obstet Gynecol* 2017;**49**:143–149.
15. Dhingra A, Garg A, Kaur S, Chopra S, Batra JS, Pandey A, Chaanine AH, Agarwal SK. Epidemiology of heart failure with preserved ejection fraction. *Curr Heart Fail Rep* 2014;**11**:354–365.
16. Gori M, Senni M, Gupta DK, Charytan DM, Kraigher-Krainer E, Pieske B, Claggett B, Shah AM, Santos AB, Zile MR, Voors AA, McMurray JJ, Packer M, Bransford T, Lefkowitz M, Solomon SD. Association between renal function and cardiovascular structure and function in heart failure with preserved ejection fraction. *Eur Heart J* 2014;**35**:3442–3451.
17. Franssen C, Chen S, Unger A, Korkmaz HI, De Keulenaer GW, Tschope C, Leite-Moreira AF, Musters R, Niessen HW, Linke WA, Paulus WJ, Hamdani N. Myocardial microvascular inflammatory endothelial activation in heart failure with preserved ejection fraction. *JACC Heart Fail* 2016;**4**:312–324.
18. Mohammed SF, Hussain S, Mirzoyev SA, Edwards WD, Maleszewski JJ, Redfield MM. Coronary microvascular rarefaction and myocardial fibrosis in heart failure with preserved ejection fraction. *Circulation* 2015;**131**:550–559.
19. Westermann D, Lindner D, Kasner M, Zietsch C, Savvatis K, Escher F, von Schlippenbach J, Skurk C, Steendijk P, Riad A, Poller W, Schultheiss HP, Tschope C. Cardiac inflammation contributes to changes in the extracellular matrix in patients with heart failure and normal ejection fraction. *Circ Heart Fail* 2011;**4**:44–52.
20. Kasner M, Westermann D, Lopez B, Gaub R, Escher F, Kuhl U, Schultheiss HP, Tschope C. Diastolic tissue Doppler indexes correlate with the degree of collagen expression and cross-linking in heart failure and normal ejection fraction. *J Am Coll Cardiol* 2011;**57**:977–985.
21. van Heerebeek L, Hamdani N, Falcao-Pires I, Leite-Moreira AF, Begieneman MP, Bronzwaer JG, van der Velden J, Stienen GJ, Laarman GJ, Somsen A, Verheugt FW, Niessen HW, Paulus WJ. Low myocardial protein kinase G activity in heart failure with preserved ejection fraction. *Circulation* 2012;**126**:830–839.
22. Borbely A, Falcao-Pires I, van Heerebeek L, Hamdani N, Edes I, Gavina C, Leite-Moreira AF, Bronzwaer JG, Papp Z, van der Velden J, Stienen GJ, Paulus WJ. Hypophosphorylation of the Stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. *Circ Res* 2009;**104**:780–786.
23. Borbely A, van der Velden J, Papp Z, Bronzwaer JG, Edes I, Stienen GJ, Paulus WJ. Cardiomyocyte stiffness in diastolic heart failure. *Circulation* 2005;**111**:774–781.
24. van Heerebeek L, Borbely A, Niessen HW, Bronzwaer JG, van der Velden J, Stienen GJ, Linke WA, Laarman GJ, Paulus WJ. Myocardial structure and function differ in systolic and diastolic heart failure. *Circulation* 2006;**113**:1966–1973.
25. van Heerebeek L, Hamdani N, Handoko ML, Falcao-Pires I, Musters RJ, Kupreishvili K, Ijsselmuiden AJ, Schalkwijk CG, Bronzwaer JG, Diamant M, Borbely A, van der Velden J, Stienen GJ, Laarman GJ, Niessen HW, Paulus WJ. Diastolic stiffness of the failing diabetic heart: importance of fibrosis, advanced glycation end products, and myocyte resting tension. *Circulation* 2008;**117**:43–51.
26. Gori M, Lam CS, Gupta DK, Santos AB, Cheng S, Shah AM, Claggett B, Zile MR, Kraigher-Krainer E, Pieske B, Voors AA, Packer M, Bransford T, Lefkowitz M, McMurray JJ, Solomon SD. Sex-specific cardiovascular structure and function in heart failure with preserved ejection fraction. *Eur J Heart Fail* 2014;**16**:535–542.
27. Paulus WJ, Tschope C. A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *J Am Coll Cardiol* 2013;**62**:263–271.
28. Srivaratharajah K, Coutinho T, deKemp R, Liu P, Haddad H, Stadnick E, Davies RA, Chih S, Dwivedi G, Guo A, Wells GA, Bernick J, Beanlands R, Mielniczuk LM. Reduced myocardial flow in heart failure patients with preserved ejection fraction. *Circ Heart Fail* 2016;**9**:pii: e002562.
29. Akiyama E, Sugiyama S, Matsuzawa Y, Konishi M, Suzuki H, Nozaki T, Ohba K, Matsubara J, Maeda H, Horibata Y, Sakamoto K, Sugamura K, Yamamuro M, Sumida H, Kaikita K, Iwashita S, Matsui K, Kimura K, Umemura S, Ogawa H. Incremental prognostic significance of peripheral endothelial dysfunction in patients with heart failure with normal left ventricular ejection fraction. *J Am Coll Cardiol* 2012;**60**:1778–1786.
30. Regitz-Zagrosek V, Kararigas G. Mechanistic pathways of sex differences in cardiovascular disease. *Physiol Rev* 2017;**97**:1–37.
31. Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Kararigas G, Dworatzek E, Staub E, Martus P, Ruiz Noppinger P, Kintscher U, Gustafsson J-A, Regitz-Zagrosek V. Female sex and estrogen receptor-beta attenuate cardiac remodeling and apoptosis in pressure overload. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R1597–R1606.
32. Weinberg EO, Thienelt CD, Katz SE, Bartunek J, Tajima M, Rohrbach S, Douglas PS, Lorell BH. Gender differences in molecular remodeling in pressure overload hypertrophy. *J Am Coll Cardiol* 1999;**34**:264–273.
33. Scantlebury DC, Borlaug BA. Why are women more likely than men to develop heart failure with preserved ejection fraction? *Curr Opin Cardiol* 2011;**26**:562–568.
34. Hayward CS, Kelly RP. Gender-related differences in the central arterial pressure waveform. *J Am Coll Cardiol* 1997;**30**:1863–1871.

35. Krumholz HM, Larson M, Levy D. Sex differences in cardiac adaptation to isolated systolic hypertension. *Am J Cardiol* 1993;**72**:310–313.
36. Carroll JD, Carroll EP, Feldman T, Ward DM, Lang RM, McGaughey D, Karp RB. Sex-associated differences in left ventricular function in aortic stenosis of the elderly. *Circulation* 1992;**86**:1099–1107.
37. Villar AV, Llano M, Cobo M, Expósito V, Merino R, Martín-Durán R, Hurlé MA, Nistal JF. Gender differences of echocardiographic and gene expression patterns in human pressure overload left ventricular hypertrophy. *J Mol Cell Cardiol* 2009;**46**: 526–535.
38. Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet* 2011;**12**:861–874.
39. de Bruin RG, Rabelink TJ, van Zonneveld AJ, van der Veer EP. Emerging roles for RNA-binding proteins as effectors and regulators of cardiovascular disease. *Eur Heart J* 2017;**38**:1380–1388.
40. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004;**5**:522–531.
41. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* 2006;**38**:1289–1297.
42. Klinge CM. miRNAs regulated by estrogens, tamoxifen, and endocrine disruptors and their downstream gene targets. *Mol Cell Endocrinol* 2015;**418(pt. 3)**:273–297.
43. Guo X, Su B, Zhou Z, Sha J. Rapid evolution of mammalian X-linked testis microRNAs. *BMC Genomics* 2009;**10**:97.
44. Song R, Ro S, Michaels JD, Park C, McCarrey JR, Yan W. Many X-linked microRNAs escape meiotic sex chromosome inactivation. *Nat Genet* 2009;**41**: 488–493.
45. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* 2005;**434**:400–404.
46. Evangelista AM, Deschamps AM, Liu D, Raghavachari N, Murphy E. miR-222 contributes to sex-dimorphic cardiac eNOS expression via ets-1. *Physiol Genomics* 2013; **45**:493–498.
47. Bernardo BC, Ooi JY, Matsumoto A, Tham YK, Singla S, Kiriazis H, Patterson NL, Sadoshima J, Obad S, Lin RC, McMullen JR. Sex differences in response to miRNA-34a therapy in mouse models of cardiac disease: identification of sex-, disease- and treatment-regulated miRNAs. *J Physiol* 2016;**594**:5959–5974.
48. van Zonneveld AJ, Rabelink TJ, Bijkerk R. miRNA-coordinated networks as promising therapeutic targets for acute kidney injury. *Am J Pathol* 2017;**187**:20–24.
49. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;**136**: 215–233.
50. Vitale C, Fini M, Speziale G, Chierchia S. Gender differences in the cardiovascular effects of sex hormones. *Fundam Clin Pharmacol* 2010;**24**:675–685.
51. Murphy E. Estrogen signaling and cardiovascular disease. *Circ Res* 2011;**109**:687–696.
52. Mendelsohn ME, Karas RH. Molecular and cellular basis of cardiovascular gender differences. *Science* 2005;**308**:1583–1587.
53. Mendelsohn ME. Protective effects of estrogen on the cardiovascular system. *Am J Cardiol* 2002;**89**:12E–17E; discussion 17E–18E.
54. Wagner AH, Schroeter MR, Hecker M. 17beta-estradiol inhibition of NADPH oxidase expression in human endothelial cells. *FASEB J* 2001;**15**:2121–2130.
55. Nofer JR. Estrogens and atherosclerosis: insights from animal models and cell systems. *J Mol Endocrinol* 2012;**48**:R13–R29.
56. Ueda K, Lu Q, Baur W, Aronovitz MJ, Karas RH. Rapid estrogen receptor signaling mediates estrogen-induced inhibition of vascular smooth muscle cell proliferation. *Arterioscler Thromb Vasc Biol* 2013;**33**:1837–1843.
57. Ma Y, Qiao X, Falone AE, Reslan OM, Sheppard SJ, Khalil RA. Gender-specific reduction in contraction is associated with increased estrogen receptor expression in single vascular smooth muscle cells of female rat. *Cell Physiol Biochem* 2010;**26**: 457–470.
58. Camper-Kirby D, Welch S, Walker A, Shiraishi I, Setchell KD, Schaefer E, Kajstura J, Anversa P, Sussman MA. Myocardial Akt activation and gender: increased nuclear activity in females versus males. *Circ Res* 2001;**88**:1020–1027.
59. Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C, Force T, Mendelsohn ME, Karas RH. 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. *Circ Res* 2004;**95**:692–699.
60. Skavdahl M, Steenbergen C, Clark J, Myers P, Demianenko T, Mao L, Rockman HA, Korach KS, Murphy E. Estrogen receptor-beta mediates male-female differences in the development of pressure overload hypertrophy. *Am J Physiol Heart Circ Physiol* 2005;**288**:H469–H476.
61. Iorga A, Li J, Sharma S, Umar S, Bopassa JC, Nadadur RD, Centala A, Ren S, Saito T, Toro L, Wang Y, Stefani E, Eghbali M. Rescue of pressure overload-induced heart failure by estrogen therapy. *J Am Heart Assoc* 2016;**5**:e002482.
62. O'Donnell E, Floras JS, Harvey PJ. Estrogen status and the renin angiotensin aldosterone system. *Am J Physiol Regul Integr Comp Physiol* 2014;**307**:R498–R500.
63. Zimmerman MA, Sullivan JC. Hypertension: what's sex got to do with it? *Physiology (Bethesda)* 2013;**28**:234–244.
64. Hernandez Schulman I, Raij L. Salt sensitivity and hypertension after menopause: role of nitric oxide and angiotensin II. *Am J Nephrol* 2006;**26**:170–180.
65. Sandberg K, Ji H. Sex differences in primary hypertension. *Biol Sex Differ* 2012;**3**:7.
66. Knopp RH, Paramsothy P, Retzlaff BM, Fish B, Walden C, Dowdy A, Tsunehara C, Aikawa K, Cheung MC. Sex differences in lipoprotein metabolism and dietary response: basis in hormonal differences and implications for cardiovascular disease. *Curr Cardiol Rep* 2006;**8**:452–459.
67. Gardner G, Banka CL, Roberts KA, Mullick AE, Rutledge JC. Modified LDL-mediated increases in endothelial layer permeability are attenuated with 17 beta-estradiol. *Arterioscler Thromb Vasc Biol* 1999;**19**:854–861.
68. Sulistiyani, St Clair RW. Effect of 17 beta-estradiol on metabolism of acetylated low-density lipoprotein by THP-1 macrophages in culture. *Arterioscler Thromb Vasc Biol* 1997;**17**:1691–1700.
69. Herrington DM, Howard TD, Hawkins GA, Reboussin DM, Xu J, Zheng SL, Brosnihan KB, Meyers DA, Bleecker ER. Estrogen-receptor polymorphisms and effects of estrogen replacement on high-density lipoprotein cholesterol in women with coronary disease. *N Engl J Med* 2002;**346**:967–974.
70. Badeau RM, Metso J, Wahala K, Tikkanen MJ, Jauhiainen M. Human macrophage cholesterol efflux potential is enhanced by HDL-associated 17beta-estradiol fatty acyl esters. *J Steroid Biochem Mol Biol* 2009;**116**:44–49.
71. Bryzgalova G, Gao H, Ahren B, Zierath JR, Galuska D, Steiler TL, Dahlman-Wright K, Nilsson S, Gustafsson JA, Efendic S, Khan A. Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. *Diabetologia* 2006;**49**:588–597.
72. Lundholm L, Bryzgalova G, Gao H, Portwood N, Falt S, Berndt KD, Dicker A, Galuska D, Zierath JR, Gustafsson JA, Efendic S, Dahlman-Wright K, Khan A. The estrogen receptor {alpha}-selective agonist propyl pyrazole triol improves glucose tolerance in ob/ob mice; potential molecular mechanisms. *J Endocrinol* 2008;**199**: 275–286.
73. Lindheim SR, Buchanan TA, Duffy DM, Vijod MA, Kojima T, Stanczyk FZ, Lobo RA. Comparison of estimates of insulin sensitivity in pre- and postmenopausal women using the insulin tolerance test and the frequently sampled intravenous glucose tolerance test. *J Soc Gynecol Investig* 1994;**1**:150–154.
74. Walton C, Godsland IF, Proudler AJ, Wynn V, Stevenson JC. The effects of the menopause on insulin sensitivity, secretion and elimination in non-obese, healthy women. *Eur J Clin Invest* 1993;**23**:466–473.
75. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA* 2002; **288**:321–333.
76. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and estrogen/progestin replacement study (HERS) research group. *JAMA* 1998;**280**:605–613.
77. Karim R, Mack WJ, Hodis HN, Roy S, Stanczyk FZ. Influence of age and obesity on serum estradiol, estrone, and sex hormone binding globulin concentrations following oral estrogen administration in postmenopausal women. *J Clin Endocrinol Metab* 2009;**94**:4136–4143.
78. Ying SY, Lin SL. Current perspectives in intronic micro RNAs (miRNAs). *J Biomed Sci* 2006;**13**:5–15.
79. Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, Thiruchelvam P, Barton G, Jiao LR, Wait R, Waxman J, Hannon GJ, Stebbing J. The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. *Proc Natl Acad Sci U S A* 2009;**106**:15732–15737.
80. Maillot G, Lacroix-Triki M, Pierredon S, Gratadou L, Schmidt S, Benes V, Roche H, Dalenc F, Auboeuf D, Millevoi S, Vagner S. Widespread estrogen-dependent repression of micrornas involved in breast tumor cell growth. *Cancer Res* 2009;**69**: 8332–8340.
81. Bhat-Nakshatri P, Wang G, Collins NR, Thomson MJ, Geistlinger TR, Carroll JS, Brown M, Hammond S, Srour EF, Liu Y, Nakshatri H. Estradiol-regulated microRNAs control estradiol response in breast cancer cells. *Nucleic Acids Res* 2009; **37**:4850–4861.
82. Manavalan TT, Teng Y, Appana SN, Datta S, Kalbfleisch TS, Li Y, Klinge CM. Differential expression of microRNA expression in tamoxifen-sensitive MCF-7 versus tamoxifen-resistant LY2 human breast cancer cells. *Cancer Lett* 2011;**313**:26–43.
83. Lin CY, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, Chiu KP, Lipovich L, Barnett DH, Stossi F, Yeo A, George J, Kuznetsov VA, Lee YK, Charn TH, Palanisamy N, Miller LD, Cheung E, Katzenellenbogen BS, Ruan Y, Bourque G, Wei CL, Liu ET. Whole-genome cartography of estrogen receptor alpha binding sites. *PLoS Genet* 2007;**3**:e87.
84. Adams BD, Claffey KP, White BA. Argonaute-2 expression is regulated by epidermal growth factor receptor and mitogen-activated protein kinase signaling and correlates with a transformed phenotype in breast cancer cells. *Endocrinology* 2009; **150**:14–23.
85. Li P, Wei J, Li X, Cheng Y, Chen W, Cui Y, Simoncini T, Gu Z, Yang J, Fu X. 17beta-estradiol enhances vascular endothelial Ets-1/miR-126-3p expression: the possible mechanism for attenuation of atherosclerosis. *J Clin Endocrinol Metab* 2017;**102**: 594–603.
86. Schweisgut J, Schutt C, Wust S, Wietelmann A, Ghesquiere B, Carmeliet P, Drose S, Korach KS, Braun T, Boettger T. Sex-specific, reciprocal regulation of ERalpha

and miR-22 controls muscle lipid metabolism in male mice. *EMBO J* 2017;**36**:1199–1214.
87. Olivieri F, Ahtiainen M, Lazzarini R, Pollanen E, Capri M, Lorenzi M, Fulgenzi G, Albertini MC, Salvioli S, Alen MJ, Kujala UM, Borghetti G, Babini L, Kaprio J, Sipila S, Franceschi C, Kovanen V, Procopio AD. Hormone replacement therapy enhances IGF-1 signaling in skeletal muscle by diminishing miR-182 and miR-223 expressions: a study on postmenopausal monozygotic twin pairs. *Aging Cell* 2014;**13**:850–861.
88. Shi Z, Zhao C, Guo X, Ding H, Cui Y, Shen R, Liu J. Differential expression of microRNAs in omental adipose tissue from gestational diabetes mellitus subjects reveals miR-222 as a regulator of ERalpha expression in estrogen-induced insulin resistance. *Endocrinology* 2014;**155**:1982–1990.
89. Arnal JF, Fontaine C, Billon-Gales A, Favre J, Laurell H, Lenfant F, Gourdy P. Estrogen receptors and endothelium. *Arterioscler Thromb Vasc Biol* 2010;**30**:1506–1512.
90. Hahn C, Schwartz MA. Mechanotransduction in vascular physiology and atherogenesis. *Nat Rev Mol Cell Biol* 2009;**10**:53–62.
91. Lin Z, Kumar A, SenBanerjee S, Staniszewski K, Parmar K, Vaughan DE, Gimbrone MA Jr, Balasubramanian V, Garcia-Cardena G, Jain MK. Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function. *Circ Res* 2005;**96**:e48–e57.
92. Hamik A, Lin Z, Kumar A, Balcells M, Sinha S, Katz J, Feinberg MW, Gerzsten RE, Edelman ER, Jain MK. Kruppel-like factor 4 regulates endothelial inflammation. *J Biol Chem* 2007;**282**:13769–13779.
93. Cleaver O, Melton DA. Endothelial signaling during development. *Nat Med* 2003;**9**:661–668.
94. Seferovic PM, Paulus WJ. Clinical diabetic cardiomyopathy: a two-faced disease with restrictive and dilated phenotypes. *Eur Heart J* 2015;**36**:1718–1727.
95. van Empel VP, Mariani J, Borlaug BA, Kaye DM. Impaired myocardial oxygen availability contributes to abnormal exercise hemodynamics in heart failure with preserved ejection fraction. *J Am Heart Assoc* 2014;**3**:e001293.
96. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004;**84**:767–801.
97. Trochu JN, Bouhour JB, Kaley G, Hintze TH. Role of endothelium-derived nitric oxide in the regulation of cardiac oxygen metabolism: implications in health and disease. *Circ Res* 2000;**87**:1108–1117.
98. Seddon M, Shah AM, Casadei B. Cardiomyocytes as effectors of nitric oxide signalling. *Cardiovasc Res* 2007;**75**:315–326.
99. Kazakov A, Hall R, Jagoda P, Bachelier K, Müller-Best P, Semenov A, Lammert F, Böhm M, Laufs U. Inhibition of endothelial nitric oxide synthase induces and enhances myocardial fibrosis. *Cardiovasc Res* 2013;**100**:211–221.
100. Brutsaert DL. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiol Rev* 2003;**83**:59–115.
101. Zhao J, Imbrie GA, Baur WE, Iyer LK, Aronovitz MJ, Kershaw TB, Haselmann GM, Lu Q, Karas RH. Estrogen receptor-mediated regulation of microRNA inhibits proliferation of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2013;**33**:257–265.
102. Queiros AM, Eschen C, Fliegner D, Kararigas G, Dworatzek E, Westphal C, Sanchez Ruderisch H, Regitz-Zagrosek V. Sex- and estrogen-dependent regulation of a miRNA network in the healthy and hypertrophied heart. *Int J Cardiol* 2013;**169**:331–338.
103. Kararigas G, Dworatzek E, Petrov G, Summer H, Schulze TM, Baczko I, Knosalla C, Golz S, Hetzer R, Regitz-Zagrosek V. Sex-dependent regulation of fibrosis and inflammation in human left ventricular remodelling under pressure overload. *Eur J Heart Fail* 2014;**16**:1160–1167.
104. Wang N, Sun LY, Zhang SC, Wei R, Xie F, Liu J, Yan Y, Duan MJ, Sun LL, Sun YH, Niu HF, Zhang R, Ai J. MicroRNA-23a participates in estrogen deficiency induced gap junction remodeling of rats by targeting GJA1. *Int J Biol Sci* 2015;**11**:390–403.
105. Sun LY, Wang N, Ban T, Sun YH, Han Y, Sun LL, Yan Y, Kang XH, Chen S, Sun LH, Zhang R, Zhao YJ, Zhang H, Ai J, Yang BF. MicroRNA-23a mediates mitochondrial compromise in estrogen deficiency-induced concentric remodeling via targeting PGC-1alpha. *J Mol Cell Cardiol* 2014;**75**:1–11.
106. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A* 1986;**83**:2496–2500.
107. Rosano GM, Vitale C, Marazzi G, Volterrani M. Menopause and cardiovascular disease: the evidence. *Climacteric* 2007;**10(Suppl. 1)**:19–24.
108. Nguyen DK, Disteche CM. Dosage compensation of the active X chromosome in mammals. *Nat Genet* 2006;**38**:47–53.
109. Augui S, Nora EP, Heard E. Regulation of X-chromosome inactivation by the X-inactivation centre. *Nat Rev Genet* 2011;**12**:429–442.
110. Lee JT, Bartolomei MS. X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell* 2013;**152**:1308–1323.
111. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. *Nat Rev Immunol* 2010;**10**:594–604.
112. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* 2016;**16**:626–638.
113. van Zonneveld AJ, de Boer HC, van der Veer EP, Rabelink TJ. Inflammation, vascular injury and repair in rheumatoid arthritis. *Ann Rheum Dis* 2010;**69(Suppl. 1)**:i57–i60.
114. Gianturco L, Bodini BD, Atzeni F, Colombo C, Stella D, Sarzi-Puttini P, Drago L, Galaverna S, Turiel M. Cardiovascular and autoimmune diseases in females: the role of microvasculature and dysfunctional endothelium. *Atherosclerosis* 2015;**241**:259–263.
115. Solomon DH, Karlson EW, Rimm EB, Cannuscio CC, Mandl LA, Manson JE, Stampfer MJ, Curhan GC. Cardiovascular morbidity and mortality in women diagnosed with rheumatoid arthritis. *Circulation* 2003;**107**:1303–1307.
116. Arnold AP, Chen X. What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues? *Front Neuroendocrinol* 2009;**30**:1–9.
117. Ji H, Zheng W, Wu X, Liu J, Ecelbarger CM, Watkins R, Arnold AP, Sandberg K. Sex chromosome effects unmasked in angiotensin II-induced hypertension. *Hypertension* 2010;**55**:1275–1282.
118. Li J, Chen X, McClusky R, Ruiz-Sundstrom M, Itoh Y, Umar S, Arnold AP, Eghbali M. The number of X chromosomes influences protection from cardiac ischaemia/reperfusion injury in mice: one X is better than two. *Cardiovasc Res* 2014;**102**:375–384.
119. Chen X, McClusky R, Itoh Y, Reue K, Arnold AP. X and Y chromosome complement influence adiposity and metabolism in mice. *Endocrinology* 2013;**154**:1092–1104.
120. Meunier J, Lemoine F, Soumillon M, Liechti A, Weier M, Guschanski K, Hu H, Khaitovich P, Kaessmann H. Birth and expression evolution of mammalian microRNA genes. *Genome Res* 2013;**23**:34–45.
121. Boonman-de Winter LJ, Rutten FH, Cramer MJ, Landman MJ, Liem AH, Rutten GE, Hoes AW. High prevalence of previously unknown heart failure and left ventricular dysfunction in patients with type 2 diabetes. *Diabetologia* 2012;**55**:2154–2162.
122. Eaton CB, Pettinger M, Rossouw J, Martin LW, Foraker R, Quddus A, Liu S, Wampler NS, Hank Wu WC, Manson JE, Margolis K, Johnson KC, Allison M, Corbie-Smith G, Rosamond W, Breathett K, Klein L. Risk factors for incident hospitalized heart failure with preserved versus reduced ejection fraction in a multiracial cohort of postmenopausal women. *Circ Heart Fail* 2016;**9**:e002883.
123. Li Y, Song YH, Li F, Yang T, Lu YW, Geng YJ. MicroRNA-221 regulates high glucose-induced endothelial dysfunction. *Biochem Biophys Res Commun* 2009;**381**:81–83.
124. Li XX, Liu YM, Li YJ, Xie N, Yan YF, Chi YL, Zhou L, Xie SY, Wang PY. High glucose concentration induces endothelial cell proliferation by regulating cyclin-D2-related miR-98. *J Cell Mol Med* 2016;**20**:1159–1169.
125. Caporali A, Meloni M, Vollenkle C, Bonci D, Sala-Newby GB, Addis R, Spinetti G, Losa S, Masson R, Baker AH, Agami R, Le Sage C, Condorelli G, Madeddu P, Martelli F, Emanueli C. Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. *Circulation* 2011;**123**:282–291.
126. Caporali A, Meloni M, Nailor A, Mitic T, Shantikumar S, Riu F, Sala-Newby GB, Rose L, Besnier M, Katare R, Voellenkle C, Verkade P, Martelli F, Madeddu P, Emanueli C. p75(NTR)-dependent activation of NF-kappaB regulates microRNA-503 transcription and pericyte-endothelial crosstalk in diabetes after limb ischaemia. *Nat Commun* 2015;**6**:8024.
127. Coleman CB, Lightell DJ Jr, Moss SC, Bates M, Parrino PE, Woods TC. Elevation of miR-221 and -222 in the internal mammary arteries of diabetic subjects and normalization with metformin. *Mol Cell Endocrinol* 2013;**374**:125–129.
128. Reddy MA, Das S, Zhuo C, Jin W, Wang M, Lanting L, Natarajan R. Regulation of vascular smooth muscle cell dysfunction under diabetic conditions by miR-504. *Arterioscler Thromb Vasc Biol* 2016;**36**:864–873.
129. Kalogeropoulos A, Georgiopoulou V, Psaty BM, Rodondi N, Smith AL, Harrison DG, Liu Y, Hoffmann U, Bauer DC, Newman AB, Kritchevsky SB, Harris TB, Butler J. Inflammatory markers and incident heart failure risk in older adults: the Health ABC (Health, Aging, and Body Composition) study. *J Am Coll Cardiol* 2010;**55**:2129–2137.
130. Fang Y, Davies PF. Site-specific microRNA-92a regulation of Kruppel-like factors 4 and 2 in atherosusceptible endothelium. *Arterioscler Thromb Vasc Biol* 2012;**32**:979–987.
131. Loyer X, Potteaux S, Vion AC, Guerin CL, Boulkroun S, Rautou PE, Ramkhelawon B, Esposito B, Dalloz M, Paul JL, Julia P, Maccario J, Boulanger CM, Mallat Z, Tedgui A. Inhibition of microRNA-92a prevents endothelial dysfunction and atherosclerosis in mice. *Circ Res* 2014;**114**:434–443.
132. Chen Z, Wen L, Martin M, Hsu CY, Fang L, Lin FM, Lin TY, Geary MJ, Geary GG, Zhao Y, Johnson DA, Chen JW, Lin SJ, Chien S, Huang HD, Miller YI, Huang PH, Shyy JY. Oxidative stress activates endothelial innate immunity via sterol regulatory element binding protein 2 (SREBP2) transactivation of microRNA-92a. *Circulation* 2015;**131**:805–814.
133. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol* 2011;**13**:423–433.
134. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, Catherinet C, Prado-Lourenco L, Levin MG, Thacker S, Sethupathy P, Barter PJ, Remaley AT, Rye KA. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun* 2014;**5**:3292.
135. Li S, Chen H, Ren J, Geng Q, Song J, Lee C, Cao C, Zhang J, Xu N. MicroRNA-223 inhibits tissue factor expression in vascular endothelial cells. *Atherosclerosis* 2014;**237**:514–520.

136. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 2011;**473**:298–307.
137. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;**9**:669–676.
138. Ushio-Fukai M. Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res* 2006;**71**:226–235.
139. Jeansson M, Gawlik A, Anderson G, Li C, Kerjaschki D, Henkelman M, Quaggin SE. Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. *J Clin Invest* 2011;**21**:2278–2289.
140. Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. *Nat Med* 2006;**12**:235–239.
141. Boodhwani M, Sodha NR, Mieno S, Xu SH, Feng J, Ramlawi B, Clements RT, Sellke FW. Functional, cellular, and molecular characterization of the angiogenic response to chronic myocardial ischemia in diabetes. *Circulation* 2007;**116**:I31–I37.
142. Mohammed SF, Majure DT, Redfield MM. Zooming in on the Microvasculature in Heart Failure With Preserved Ejection Fraction. *Circ Heart Fail* 2016;**9**:e003272.
143. Kuehbacher A, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* 2007;**101**:59–68.
144. Ghosh G, Subramanian IV, Adhikari N, Zhang X, Joshi HP, Basi D, Chandrashekhar YS, Hall JL, Roy S, Zeng Y, Ramakrishnan S. Hypoxia-induced microRNA-424 expression in human endothelial cells regulates HIF-alpha isoforms and promotes angiogenesis. *J Clin Invest* 2010;**120**:4141–4154.
145. Chamorro-Jorganes A, Araldi E, Penalva LO, Sandhu D, Fernandez-Hernando C, Suarez Y. MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. *Arterioscler Thromb Vasc Biol* 2011;**31**:2595–2606.
146. Shi L, Fisslthaler B, Zippel N, Fromel T, Hu J, Elgheznawy A, Heide H, Popp R, Fleming I. MicroRNA-223 antagonizes angiogenesis by targeting beta1 integrin and preventing growth factor signaling in endothelial cells. *Circ Res* 2013;**113**:1320–1330.
147. Yang Q, Jia C, Wang P, Xiong M, Cui J, Li L, Wang W, Wu Q, Chen Y, Zhang T. MicroRNA-505 identified from patients with essential hypertension impairs endothelial cell migration and tube formation. *Int J Cardiol* 2014;**177**:925–934.
148. Dentelli P, Rosso A, Orso F, Olgasi C, Taverna D, Brizzi MF. microRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression. *Arterioscler Thromb Vasc Biol* 2010;**30**:1562–1568.
149. Chen CF, Huang J, Li H, Zhang C, Huang X, Tong G, Xu YZ. MicroRNA-221 regulates endothelial nitric oxide production and inflammatory response by targeting adiponectin receptor 1. *Gene* 2015;**565**:246–251.
150. Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circ Res* 2007;**100**:1164–1173.
151. Rippe C, Blimline M, Magerko KA, Lawson BR, LaRocca TJ, Donato AJ, Seals DR. MicroRNA changes in human arterial endothelial cells with senescence: relation to apoptosis, eNOS and inflammation. *Exp Gerontol* 2012;**47**:45–51.
152. Urbich C, Kuehbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008;**79**:581–588.
153. Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, Mercatanti A, Hammond S, Rainaldi G. MicroRNAs modulate the angiogenic properties of HUVECs. *Blood* 2006;**108**:3068–3071.
154. Chen Y, Banda M, Speyer CL, Smith JS, Rabson AB, Gorski DH. Regulation of the expression and activity of the antiangiogenic homeobox gene GAX/MEOX2 by ZEB2 and microRNA-221. *Mol Cell Biol* 2010;**30**:3902–3913.
155. Hinkel R, Penzkofer D, Zuhlke S, Fischer A, Husada W, Xu QF, Baloch E, van Rooij E, Zeiher AM, Kupatt C, Dimmeler S. Inhibition of microRNA-92a protects against ischemia/reperfusion injury in a large-animal model. *Circulation* 2013;**128**:1066–1075.
156. Huss JM, Kelly DP. Mitochondrial energy metabolism in heart failure: a question of balance. *J Clin Invest* 2005;**115**:547–555.
157. Chen Y, Liu Y, Dorn GW. Mitochondrial fusion is essential for organelle function and cardiac homeostasis. *Circ Res* 2011;**109**:1327–1331.
158. Shenouda SM, Widlansky ME, Chen K, Xu G, Holbrook M, Tabit CE, Hamburg NM, Frame AA, Caiano TL, Kluge MA, Duess MA, Levit A, Kim B, Hartman ML, Joseph L, Shirihai OS, Vita JA. Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. *Circulation* 2011;**124**:444–453.
159. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000;**404**:787–790.
160. Falcao-Pires I, Hamdani N, Borbely A, Gavina C, Schalkwijk CG, van der Velden J, van Heerebeek L, Stienen GJ, Niessen HW, Leite-Moreira AF, Paulus WJ. Diabetes mellitus worsens diastolic left ventricular dysfunction in aortic stenosis through altered myocardial structure and cardiomyocyte stiffness. *Circulation* 2011;**124**:1151–1159.
161. Wang K, Liu CY, Zhang XJ, Feng C, Zhou LY, Zhao Y, Li PF. miR-361-regulated prohibitin inhibits mitochondrial fission and apoptosis and protects heart from ischemia injury. *Cell Death Differ* 2015;**22**:1058–1068.
162. Wang K, Zhou LY, Wang JX, Wang Y, Sun T, Zhao B, Yang YJ, An T, Long B, Li N, Liu CY, Gong Y, Gao JN, Dong YH, Zhang J, Li PF. E2F1-dependent miR-421 regulates mitochondrial fragmentation and myocardial infarction by targeting Pink1. *Nat Commun* 2015;**6**:7619.
163. Palomer X, Álvarez-Guardia D, Davidson MM, Chan TO, Feldman AM, Vázquez-Carrera M, Eltzschig HK. The interplay between NF-kappaB and E2F1 coordinately regulates inflammation and metabolism in human cardiac cells. *PLoS One* 2011;**6**:e19724.
164. Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, Murphy E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res* 2010;**106**:1681–1691.
165. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* 2011;**147**:728–741.
166. Kobayashi S, Liang Q. Autophagy and mitophagy in diabetic cardiomyopathy. *Biochim Biophys Acta* 2015;**1852**:252–261.
167. Shirakabe A, Ikeda Y, Sciarretta S, Zablocki DK, Sadoshima J. Aging and autophagy in the heart. *Circ Res* 2016;**118**:1563–1576.
168. Wang K, Liu CY, Zhou LY, Wang JX, Wang M, Zhao B, Zhao WK, Xu SJ, Fan LH, Zhang XJ, Feng C, Wang CQ, Zhao YF, Li PF. APF lncRNA regulates autophagy and myocardial infarction by targeting miR-188-3p. *Nat Commun* 2015;**6**:6779.
169. Bo L, Su-Ling D, Fang L, Lu-Yu Z, Tao A, Stefan D, Kun W, Pei-Feng L. Autophagic program is regulated by miR-325. *Cell Death Differ* 2014;**21**:967–977.
170. Su M, Wang J, Wang C, Wang X, Dong W, Qiu W, Wang Y, Zhao X, Zou Y, Song L, Zhang L, Hui R. MicroRNA-221 inhibits autophagy and promotes heart failure by modulating the p27/CDK2/mTOR axis. *Cell Death Differ* 2015;**22**:986–999.
171. Melenovsky V, Hwang SJ, Redfield MM, Zakeri R, Lin G, Borlaug BA. Left atrial remodeling and function in advanced heart failure with preserved or reduced ejection fraction. *Circ Heart Fail* 2015;**8**:295–303.
172. Shah AM, Shah SJ, Anand IS, Sweitzer NK, O'Meara E, Heitner JF, Sopko G, Li G, Assmann SF, McKinlay SM, Pitt B, Pfeffer MA, Solomon SD. Cardiac structure and function in heart failure with preserved ejection fraction: baseline findings from the echocardiographic study of the treatment of preserved cardiac function heart failure with an aldosterone antagonist trial. *Circ Heart Fail* 2014;**7**:104–115.
173. Melenovsky V, Borlaug BA, Rosen B, Hay I, Ferruci L, Morell CH, Lakatta EG, Najjar SS, Kass DA. Cardiovascular features of heart failure with preserved ejection fraction versus nonfailing hypertensive left ventricular hypertrophy in the urban Baltimore community: the role of atrial remodeling/dysfunction. *J Am Coll Cardiol* 2007;**49**:198–207.
174. Bernardo BC, Nguyen SS, Winbanks CE, Gao XM, Boey EJ, Tham YK, Kiriazis H, Ooi JY, Porrello ER, Igoor S, Thomas CJ, Gregorevic P, Lin RC, Du XJ, McMullen JR. Therapeutic silencing of miR-652 restores heart function and attenuates adverse remodeling in a setting of established pathological hypertrophy. *FASEB J* 2014;**28**:5097–5110.
175. Wang YS, Zhou J, Hong K, Cheng XS, Li YG. MicroRNA-223 displays a protective role against cardiomyocyte hypertrophy by targeting cardiac troponin I-interacting kinase. *Cell Physiol Biochem* 2015;**35**:1546–1556.
176. Wang C, Wang S, Zhao P, Wang X, Wang J, Wang Y, Song L, Zou Y, Hui R. MiR-221 promotes cardiac hypertrophy in vitro through the modulation of p27 expression. *J Cell Biochem* 2012;**113**:2040–2046.
177. Yang Y, Ago T, Zhai P, Abdellatif M, Sadoshima J. Thioredoxin 1 negatively regulates angiotensin II-induced cardiac hypertrophy through upregulation of miR-98/let-7. *Circ Res* 2011;**108**:305–313.
178. Tschope C, Paulus WJ. Is echocardiographic evaluation of diastolic function useful in determining clinical care? Doppler echocardiography yields dubious estimates of left ventricular diastolic pressures. *Circulation* 2009;**120**:810–820; discussion 820.
179. Franssen C, Paulus WJ. The future diagnosis of heart failure with normal ejection fraction: less imaging, more biomarkers? *Eur J Heart Fail* 2011;**13**:1043–1045.
180. Mottram PM, Leano R, Marwick TH. Usefulness of B-type natriuretic peptide in hypertensive patients with exertional dyspnea and normal left ventricular ejection fraction and correlation with new echocardiographic indexes of systolic and diastolic function. *Am J Cardiol* 2003;**92**:1434–1438.
181. Meyer S, van der Meer P, van Deursen VM, Jaarsma T, van Veldhuisen DJ, van der Wal MH, Hillege HL, Voors AA. Neurohormonal and clinical sex differences in heart failure. *Eur Heart J* 2013;**34**:2538–2547.
182. Wong LL, Armugam A, Sepramaniam S, Karolina DS, Lim KY, Lim JY, Chong JP, Ng JY, Chen YT, Chan MM, Chen Z, Yeo PS, Ng TP, Ling LH, Sim D, Leong KT, Ong HY, Jaufeerally F, Wong R, Chai P, Low AF, Lam CS, Jeyaseelan K, Richards AM. Circulating microRNAs in heart failure with reduced and preserved left ventricular ejection fraction. *Eur J Heart Fail* 2015;**17**:393–404.
183. Watson CJ, Gupta SK, O'Connell E, Thum S, Glezeva N, Fendrich J, Gallagher J, Ledwidge M, Grote-Levi L, McDonald K, Thum T. MicroRNA signatures differentiate preserved from reduced ejection fraction heart failure. *Eur J Heart Fail* 2015;**17**:405–415.
184. Schmitter D, Voors AA, van der Harst P. HFpEF vs. HFrEF: can microRNAs advance the diagnosis? *Eur J Heart Fail* 2015;**17**:351–354.
185. Nair N, Kumar S, Gongora E, Gupta S. Circulating miRNA as novel markers for diastolic dysfunction. *Mol Cell Biochem* 2013;**376**:33–40.
186. van Veldhuisen DJ, Linssen GC, Jaarsma T, van Gilst WH, Hoes AW, Tijssen JG, Paulus WJ, Voors AA, Hillege HL. B-type natriuretic peptide and prognosis in heart

failure patients with preserved and reduced ejection fraction. *J Am Coll Cardiol* 2013;**61**:1498–1506.
187. Nakada Y, Kawakami R, Nakano T, Takitsume A, Nakagawa H, Ueda T, Nishida T, Onoue K, Soeda T, Okayama S, Takeda Y, Watanabe M, Kawata H, Okura H, Saito Y. Sex differences in clinical characteristics and long-term outcome in acute decompensated heart failure patients with preserved and reduced ejection fraction. *Am J Physiol Heart Circ Physiol* 2016;**310**:H813–H820.
188. Wang YT, Tsai PC, Liao YC, Hsu CY, Juo SH. Circulating microRNAs have a sex-specific association with metabolic syndrome. *J Biomed Sci* 2013;**20**:72.
189. Carreras-Badosa G, Bonmatí A, Ortega F-J, Mercader J-M, Guindo-Martínez M, Torrents D, Prats-Puig A, Martinez-Calcerrada J-M, Platero-Gutierrez E, De Zegher F, Ibáñez L, Fernandez-Real J-M, Lopez-Bermejo A, Bassols J. Altered circulating miRNA expression profile in pregestational and gestational obesity. *J Clin Endocrinol Metab* 2015;**100**:E1446–E1456.
190. Hromadnikova I, Kotlabova K, Hympanova L, Krofta L. Gestational hypertension, preeclampsia and intrauterine growth restriction induce dysregulation of cardiovascular and cerebrovascular disease associated microRNAs in maternal whole peripheral blood. *Thromb Res* 2016;**137**:126–140.
191. Zeng Y, Zhang X, Kang K, Chen J, Wu Z, Huang J, Lu W, Chen Y, Zhang J, Wang Z, Zhai Y, Qu J, Ramchandran R, Raj JU, Wang J, Gou D. MicroRNA-223 attenuates hypoxia-induced vascular remodeling by targeting RhoB/MLC2 in pulmonary arterial smooth muscle cells. *Sci Rep* 2016;**6**:24900.
192. Tsai PC, Liao YC, Wang YS, Lin HF, Lin RT, Juo SH. Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease. *J Vasc Res* 2013;**50**:346–354.
193. Jiang Y, Wang HY, Cao HM, Wang CY, Zhang L, Wang H, Liu L, Li Y, Cai JH. Peripheral blood miRNAs as a biomarker for chronic cardiovascular diseases. *Sci Rep* 2015;**4**:5026.
194. Ortega FJ, Mercader JM, Moreno-Navarrete JM, Rovira O, Guerra E, Esteve E, Xifra G, Martínez C, Ricart W, Rieusset J, Rome S, Karczewska-Kupczewska M, Straczkowski M, Fernández-Real JM. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care* 2014;**37**:1375–1383.
195. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res* 2010;**107**:810–817.
196. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S, Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol* 2012;**60**:290–299.
197. Zhang YY, Zhou X, Ji WJ, Shi R, Lu RY, Li JL, Yang GH, Luo T, Zhang JQ, Zhao JH, Jiang TM, Li YM. Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome. *J Thromb Thrombolysis* 2014;**38**:65–72.
198. Bijkerk R, Duijs JM, Khairoun M, Ter Horst CJ, van der Pol P, Mallat MJ, Rotmans JI, de Vries AP, de Koning EJ, de Fijter JW, Rabelink TJ, van Zonneveld AJ, Reinders ME. Circulating microRNAs associate with diabetic nephropathy and systemic microvascular damage and normalize after simultaneous pancreas-kidney transplantation. *Am J Transplant* 2015;**15**:1081–1090.
199. Ventura-Clapier R, Dworatzek E, Seeland U, Kararigas G, Arnal J-F, Brunelleschi S, Carpenter TC, Erdmann J, Franconi F, Giannetta E, Glezerman M, Hofmann SM, Junien C, Katai M, Kublickiene K, König IR, Majdic G, Malorni W, Mieth C, Miller VM, Reynolds RM, Shimokawa H, Tannenbaum C, D'Ursi AM, Regitz-Zagrosek V. Sex in basic research: concepts in the cardiovascular field. *Cardiovasc Res* 2017;**113**:711–724.