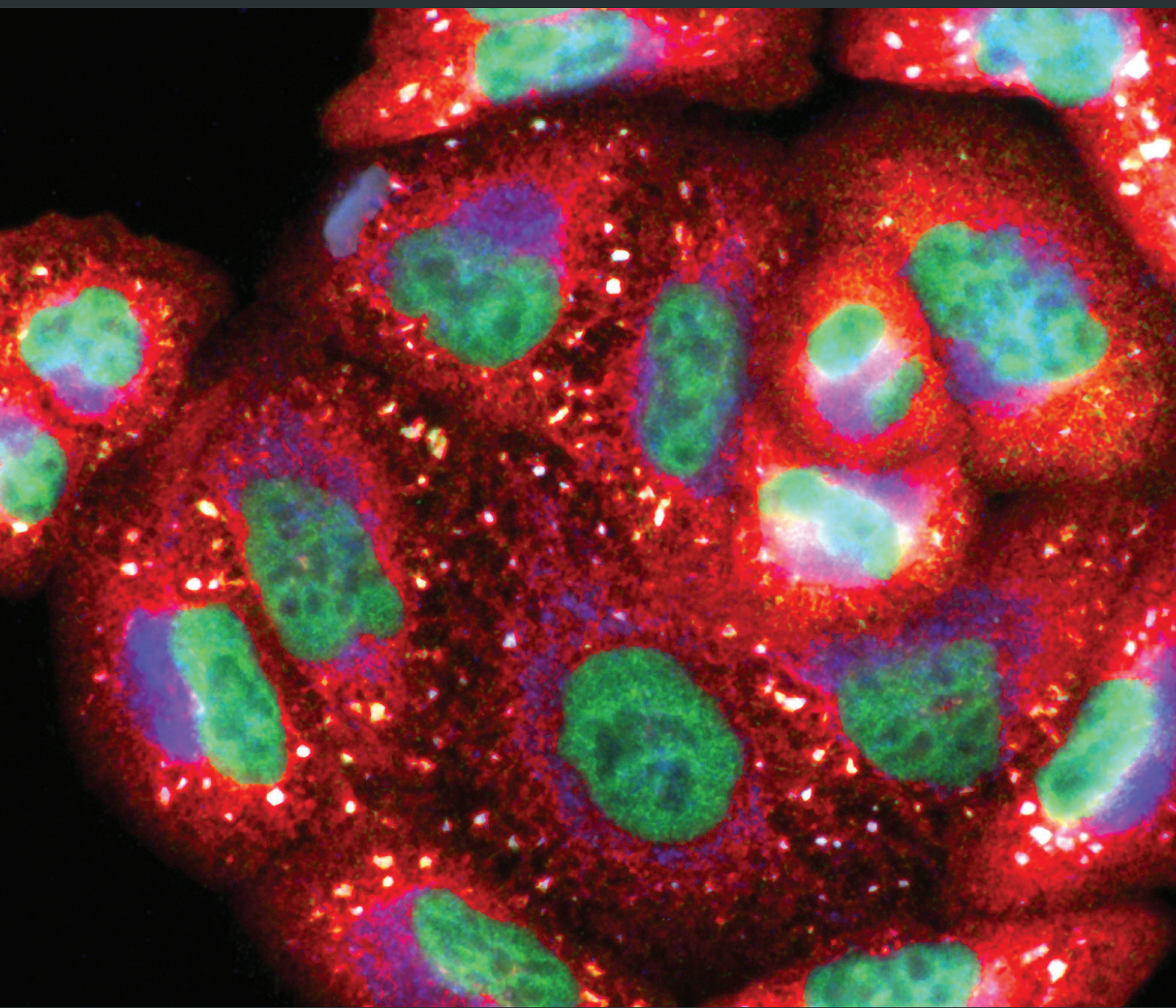
Oxidative Medicine and Cellular Longevity

Regulatory Mechanisms of Estrogen on Vascular Ageing

Lead Guest Editor: Susana Novella
Guest Editors: Ana P. Dantas, Carlos Hermenegildo, and Ylva Hellsten



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Editorial Board

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Editorial

Regulatory Mechanisms of Estrogen on Vascular Ageing

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Women can be considered hemodynamically younger than men of the same age, based on epidemiological studies establishing that the incidence of vascular diseases in women is relatively lower compared to that in aged-matched men. However, after menopause, these numbers increase to values that are close to those found in men. Vascular ageing is associated with structural and functional changes of the vascular wall, including endothelial dysfunction, arterial stiffening, and remodelling, as well as impaired angiogenesis, which become major risk factors in the development of cardiovascular disease. Data from clinical and basic research have established that vascular ageing does not follow the same timeline in women as it does in men. Although vascular dysfunction and cardiovascular risk factors are comparable in elderly men and women, the rate of change is lower in women during their fertile years. This age- and sex-related difference in the progression of vascular dysfunction is not completely understood, although experimental and observational clinical studies have attributed most of the vascular protection in women to estrogen and its modulatory action in the vascular system via interaction with its receptors ERα, ERβ, and G protein-coupled estrogen receptor (GPER).

Nevertheless, there is much controversy surrounding the clinical use of estrogen as a therapeutic strategy in primary and secondary prevention of vascular disease in women. Contrary to the observational studies suggesting the value of estrogen to vascular health in women, clinical trials such as the Women's Health Initiative (WHI) reported an increase in cardiovascular events in women treated with estrogen replacement therapy compared with those treated with placebo. A main reason for the discrepancy among studies is that women enrolled in the clinical trials were on average 10 years older than those included in the observational studies. When analyses are done by the age group, it is revealed that estrogen therapy produces more favourable results in younger women (closer to onset of menopause) than in the late average onset enrolled in most trials. These analyses led scientists to conceive the so-called "timing hypothesis," which relies on the concept that estrogen has beneficial effects only if taken before, or close to, the onset of menopause, at which time the detrimental effects of ageing have not yet been established in the vasculature.

Among the mechanisms underlying the estrogen-mediated effects on vascular function are anti-inflammatory and antioxidative actions, as well as epigenetic modifications, all of which are influenced by ageing. In fact, a great burden of oxidative stress is seen during ageing and menopause which can contribute to vascular dysfunction. That way, oxidative stress together with the inflammatory activation of the vascular endothelium induces a wide range of local and systemic responses, such as the expression of adhesion molecules, the production of chemotactic factors, and free radicals that could regulate the vascular effects of estrogen. Thus, the vasoprotective effects seen in younger women, in the perimenopause or close to menopause onset, could become detrimental when women get older.

This special issue presents novel and updated research regarding the mechanisms by which estrogen modulates metabolism and vascular homeostasis in different stages of women's health including menarche, pregnancy, and

menopause. With special focus on oxidative stress, these mechanisms could be pivotal for the development of novel therapeutic strategies to prevent and treat vascular disease in women. The manuscripts within this special issue are all equally recommended by the editors, and they contain especially interesting points worth commenting on.

Estrogens are important modulators of lipid metabolism and contribute to the low prevalence of atherosclerotic vascular disease. However, estrogen promotes the synthesis of triglycerides (TG) in the liver and secretes TG into the circulation as very-low-density lipoprotein (VLDL) particles, being the hypertriglyceridemia one of the five pathological traits that characterize metabolic syndrome. In fact, an association exists between early menarche and an increased risk of metabolic syndrome. To better understand this relation, H.-S. Lee et al. identified through a GWAS-based pathway analysis an association among type 2 diabetes mellitus, stress-activated protein kinase (SAPK) signalling, and Jun amino-terminal kinase (JNK) cascade pathways with TG including the genetic interaction with age at menarche.

Type 2 diabetes is an age-associated disease in which oxidative stress is involved, the prevalence is higher in men than in women, and therefore, there are sex-associated differences. In the original study developed by A. Díaz et al., the role of estrogens in protecting against oxidative stress in type 2 diabetic Goto-Kakizaki male and female rats is evaluated. Performing an in-depth study in glucose metabolism, by positron emission tomography, metabolomics, and mitochondrial activity measurements, these authors provide evidence for the beneficial effects of estrogen in ovariectomized type 2 diabetes female rats.

Estrogen also plays a pivotal role in the control of vascular function during pregnancy. Normal gestation is associated with a mild oxidative stress, which is increased in pregnancy complications, such as preeclampsia and intrauterine growth restriction. Estrogen regulates uterine artery function via upregulation of endothelial nitric oxide synthase (eNOS) in endothelial cells and the large-conductance Ca^{2+}-activated K^+ (BKCa) channel in vascular smooth muscle cell. The review presented by X.-Q. Hu et al. provides an updated understanding of the regulation of the estrogen-NOS-NO-KCa pathway by reactive oxygen species in uteroplacental tissues and gives an overview of a link between oxidative stress and uteroplacental dysfunction in pregnancy complications.

Female sex hormones also play a predominant role in varicose physiopathology, especially considering the influence of sex and pregnancy on varicose vein development. The original study proposed by N. García-Honduvilla et al. is aimed at verifying the involvement of steroid receptors and progesterone, estrogen, and androgen receptors in varicose vein development, focusing on sex differences. In both sexes, they observed an increase in sex hormone receptors under varicose vein conditions and highlight the importance of the redistribution of these receptors.

Finally, estrogen also modifies cardiovascular function by modulating gene expression, either directly acting as transcriptional factors or indirectly via epigenetic regulation. In this sense, miRNAs have emerged as a new regulatory mechanism of both physiological and pathological processes, as they regulate gene expression profiles at the posttranscriptional level. Epigenetic changes in the vascular action of estrogens in ageing are deeply reviewed in the work by D. Pérez-Cremades et al. Emerging studies propose a role for miRNA in the vascular effects mediated by estrogens, and in this work, the authors highlight the current knowledge on the role of estrogen-sensitive miRNAs and their influence in regulating vascular ageing. This review sheds light on the potential of translating miRNA research into the clinic, using miRNAs as potential tools for the diagnoses and/or prognoses of cardiovascular disease, as affordable and noninvasive biomarkers, and as a therapeutic tool for regulating (silencing or increasing) miRNA levels.

Conflicts of Interest

The editors declare that there is no conflict of interest regarding the publication of this special issue.

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Research Article

Sex Differences in Age-Associated Type 2 Diabetes in Rats—Role of Estrogens and Oxidative Stress

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Guest Editor: Ana P. Dantas

Females live longer than males, and the estrogens are one of the reasons for this difference. We reported some years ago that estrogens are able to protect rats against oxidative stress, by inducing antioxidant genes. Type 2 diabetes is an age-associated disease in which oxidative stress is involved, and moreover, some studies show that the prevalence is higher in men than in women, and therefore there are sex-associated differences. Thus, the aim of this study was to evaluate the role of estrogens in protecting against oxidative stress in type 2 diabetic males and females. For this purpose, we used Goto-Kakizaki rats, which develop type 2 diabetes with age. We found that female diabetic rats showed lower glycaemia levels with age than did diabetic males and that estrogens enhanced insulin sensitivity in diabetic females. Moreover, glucose uptake, measured by positron emission tomography, was higher in the female brain, cerebellum, and heart than in those from male diabetic rats. There were also sex-associated differences in the plasma metabolic profile as determined by metabolomics. The metabolic profile was similar between estrogen-replaced and control diabetic rats and different from ovariectomized diabetic rats. Oxidative stress is involved in these differences. We showed that hepatic mitochondria from females produced less hydrogen peroxide levels and exhibited lower xanthine oxidase activity. We also found that hepatic mitochondrial glutathione oxidation and lipid oxidation levels were lower in diabetic females when compared with diabetic males. Ovariectomy induced oxidative stress, and estrogen replacement therapy prevented it. These findings provide evidence for estrogen beneficial effects in type 2 diabetes and should be considered when prescribing estrogen replacement therapy to menopausal women.

1. Introduction

Diabetes mellitus is a chronic disease of high prevalence. According to the World Health Organization, there are currently approximately 143 million people with diabetes worldwide. This figure is expected to rise to 300 million by the year 2025 due, above all, to the increase, aging, and urbanization of the population [1]. It is estimated that its prevalence is around 5%, increasing significantly in relation to age: it reaches figures between 10 and 15% in the population over 65 years of age and 20% if we consider only those older than 65 to 80 years [2]. This happens because aging leads to an increase in fasting plasma glucose levels of 1 to 2 mg % per year and postprandial glycaemia increases from 8 to 20 mg %. These changes would be linked to an alteration of the peripheral or hepatic sensitivity to insulin or to an alteration of pancreatic islet function [3]. Of the two basic forms of diabetes, type 2 occurs mainly in adults and is, with much, the most common form. It represents between 85 and 90% of all cases of diabetes [1]. There are sex-specific differences in the prevalence of type 2 diabetes, and it is lower in women compared with men [4]. However, when women reach menopause, the risk of developing diabetes (type 2) increases to levels between 6 and 20% [5]. Type 2 diabetes

is caused by the combination of a decrease in the effect of insulin acting in the body, associated with the inability of the β-pancreatic cells to produce adequate amounts of insulin. It has been shown that estradiol exerts favorable effects on insulin and glucose metabolism *in vivo*, such as increase in pancreatic insulin secretion [6], improvement of peripheral sensitivity to insulin [7], reduction of blood glucose and basal insulinemia [8], increased afterload blood glucose [9], improvement of the lipid effects of insulin [10], prevention of β-pancreatic cell apoptosis [11], and prevention of obesity [12].

After menopause, pancreatic insulin secretion decreases and insulin resistance increases, changes that may be due to the combination of aging [13] and deficiency of estrogen. This deficiency also affects the blood flow to the muscle further limiting the already reduced glucose intake. Currently, clinical studies have been conducted to assess the effect of hormone replacement therapy (HRT) on the development of diabetes disease [14–16]. The following conclusions are derived from them: administration of estrogen combined with progestin (E + G) reduces the risk of developing diabetes by 21% compared to placebo. After one year of treatment, E + G slightly reduces blood glucose and insulin levels, which is probably the reason why the number of diabetes is minor in this group. Although E + G slightly reduces body weight and waist circumference in women after one year, this factor has no influence on the development or not of diabetes.

Projections made indicate that by 2020, approximately 124 million of people will live more than 80 years and the majority of that population will be made up of menopausal women, so women will live more than a third of their lives with estrogen deficiency. The study of the role of estrogen in the development of diabetes is therefore of great importance.

It has already been mentioned that Type 2 diabetes is associated with aging, and we must mention that it is also associated with oxidative stress: glucose is prone to oxidation even under physiological conditions [17, 18]. It has also been shown that the activity of xanthine oxidase, an enzyme that generates superoxide radical, increases in diabetic rats (type I diabetes) [19, 20]. The antioxidant role of estrogens has been amply demonstrated [21]. We studied the mechanisms by which females live longer than males and demonstrate the role of the different rates of free radical production in this process [22–26]. We also observed that estradiol was responsible for this protection against oxidative stress [27–29]. Given that there is a considerable difference in the prevalence of diabetes mellitus between men and women and that this is also related to oxidative stress, our aim was to study the role of oxidative stress in the different prevalence of this disease between genders and to determine the possible protective role of estrogens, as well as the mechanisms by which this possible action exerts. This would allow a deeper knowledge about the effects of HRT on diabetes, since few authors have related the protective role of estrogen in diabetes due to its antioxidant effect [9, 11, 30]. In addition, we cannot fail to mention the complications associated with diabetes at various levels (eye disease, kidney, neuropathy, and cardiovascular). Regarding the relationship between diabetes and cardiovascular disease, this is the main cause of diabetes-related deaths, so that mortality from this disease in adults is 2 (in men) to 4 (in women) greater than mortality in adults without diabetes [31]. This worse prognosis of diabetic women is correlated with an increase in oxidative stress and decreased antioxidant defenses, and these are postulated as a cause probable of the greater susceptibility in the female sex to cardiovascular complications, with more frequent and diffuse coronary lesions. So, the study of oxidative stress in diabetes, and specifically its importance in the difference between males and females, is of great interest.

2. Material and Methods

2.1. Experimental Animals. Male and female Wistar and Goto-Kakizaki (GK) rats were used. GK rats are an excellent model of type 2 diabetes, which also does not present obesity like other models. In this way, we can rule out obesity as experiment variable. They also show similar metabolic, hormonal, and vascular disorders than in human diabetes. Its characteristics include hyperglycemia, defects in the secretion of insulin in response to glucose both *in vivo* and in isolated pancreatic cells, and insulin resistance, both hepatic and peripheral. Complications derived from the diabetes that develops this model are retinopathy, microangiopathy, neuropathy, and nephropathy. The control of the diabetization was carried out through clinical observation (polydipsia, polyphagia, and thinning) and by determining biochemical parameters as the measurement of fasting glucose, using a glucosimeter (Accu-Chek Aviva de Roche). We established an endpoint criterion for the sacrifice of rats with 200 mg/dL of blood glucose. Thus, the GK males were sacrificed at 11-15 months of age and the GK females at 20-22 months of age. As Wistar controls, we used rats of similar ages to those corresponding to their sex.

Rats were stored at the animal house of the Faculty of Medicine of the University of Valencia. Handling, supervision, and experimentation with rats were done in accordance to the recommendations of the Federation of European Laboratory Animal Science Associations. All the work complies with both national and EU legislation—Spanish Royal Decree RD 1201/2005 and EU Directive 86/609/CEE as modified by 2003/65/CE—for the protection of animals used for research experimentation and other scientific purposes. All protocols were previously subjected and approved by the Ethical Committee of the University of Valencia.

Animals were housed at constant temperature and humidity and with a 12 h light/12 h dark cycle. They were fed on a standard laboratory diet (containing 590 g carbohydrates, 30 g lipids, and 160 g protein per kilogram of diet) and tap water *ad libitum*.

2.2. Ovariectomy Procedure. Ovariectomy was performed as follows: Rats were anesthetized with ketamine (100 mg/kg) and acepromazine (2.5 mg/kg). Abdominal skin was cut, the peritoneum was opened, both ovarian arteries were ligated, and both ovaries were removed. Sham-operated rats underwent the same procedure, but ovarian arteries were not

ligated and ovaries were not removed. Animals were allowed to recover for at least three weeks before experiments were performed. Estrogen levels after ovariectomy fall to about 10% of the controls [32]. Where indicated, an estrogen replacement therapy of 17β-estradiol was given subcutaneously at a daily dose of 1 μg/kg body weight for three weeks. This results in an estrogen level similar to controls. This dose was chosen because it was shown that this estrogen dose does not cause changes in liver weight or increases in DNA content [33].

Female rats we divided into 3 groups: sham-operated (Sham), ovariectomized for 3 weeks (OVX), and ovariectomized for 3 weeks and immediately replaced with estradiol (OVX+E).

We have used different tissues in our studies. We measured radical production and antioxidants in liver because we have previously done so in basic experiments trying to understand why females live longer than males and why they produce lower oxidant production, and we did this in the liver because mitochondria are much more uniform than those of the brain. This is the reason for our studies in antioxidant production in liver. However, when trying to measure glucose uptake, the brain is a much better tissue. The liver is characterized (classical as well as PET studies confirm this) by changing the glucose output dramatically from one situation to another. For instance, the liver can take up glucose or release it, depending on the particular metabolic moment of the animal. Thus, the liver (together with the kidney cortex) is the only tissue that can either release glucose or take it up. Glucose uptake by the liver is low, and the PET signal is very low. This contrasts sharply with the case of the brain. Therefore, the brain was a better choice to measure glucose uptake. We also determined heart glucose uptake for the importance of cardiovascular disorders in type 2 diabetes.

Finally, metabolomics is usually performed in serum/plasma. The reason for using plasma is twofold: firstly, this is the milieu used for human studies for obvious ethical reasons, and secondly, plasma gives a general view of the metabolic situation of the animal.

2.3. Glucose Tolerance Test. We kept our rats in a 6-hour fast and took a sample of the basal glycaemia (time 0); after this, they were given a dose of 2 g glucose/kg live weight orally by an esophageal tube (18 G-40 mm). We subsequently carried out glycaemia measurements at times 15, 30, 60, 120, and 180 minutes of basal. The blood samples were obtained from the saphenous vein, according to the method described above, and blood glucose was measured using the glucose meter (Accu-Chek revives the Roche brand).

2.4. Determination of Brain and Heart Glucose Uptake In Vivo. Rats were deprived of food for 8–14 h before 18F-2-fluor-2-deoxiglucose (18F-FDG) injection. 18F-FDG (5.8–11.1 MBq) was injected intraperitoneally after anesthesia with isoflurane (1.5–2% in 100% oxygen, IsoFlo; Abbott Laboratories). PET was started 60 min after 18F-FDG injection as described in [34]. 18F-FDG was synthesized as previously described [35]. The administered dose (FDG activity) was indeed corrected for body weight. We acquired 20 min static images 60 min after injection of 18F-FDG. The biodistribution of 18F-FDG by the heart was compared between all the studied groups. The PET images were obtained with the ALBIRA small animal PET (ONCOVISION, GEM Imaging). Regions of interest were manually drawn over the brain with PMOD software. Tracer uptake by heart was quantified as SUV (Standardized Uptake Value, Total Sum) with a methodology employed before in [36].

2.5. Storage, Preparation, and ^{1}H NMR Spectroscopic Analysis of Blood Plasma. Rats' blood plasmas were stored at -80C and thawed before use. For NMR analysis, as shown in [36], 20 μL of plasma was mixed with 2 μL of D_2O (as a field lock). A total of 20 μL of the mixture of each sample was individually transferred into a 1 mm high-quality NMR capillary. All ^{1}H NMR spectra were acquired using a standard one-dimensional pulse sequence with water suppression (Bruker Avance 600 spectrometer operating at 600.13 MHz with a 1 mm ^{1}H/^{13}C/^{15}N TXI probe). A total of 256 FIDs, free induction decay, were collected into 64 k data points with a spectral width of 14 ppm and a recycle delay (RD) of 1 s. Water signal was saturated with a weak irradiation during the recycle delay. Before Fourier transformation, the FID was multiplied by a 0.3 Hz exponential line broadening. Spectral chemical shift referencing on the alanine CH_3 doublet signal at 1.475 ppm was performed in all spectra. Spectral regions between 0.5 and 4.5 ppm, and between 5.5 and 9.5 ppm, were binned in segments of 0.01 ppm width (6 Hz) for multivariate analysis. We normalized the binned data to the total spectral area. Available spectral databases and 2D NMR experiments were used to aid structural identification of relevant metabolites. Finally, using in-house scripts for data analysis, all spectra were processed using Mnova (MestreLab, Santiago de Compostela, Spain) and transferred to MATLAB® (MathWorks Inc., 2006) as shown in [36].

2.6. Multivariate Analysis of NMR Spectra. In multivariate data, as explained in [36], the representation of any two variables against each other is not sufficient to obtain global correlations. Principal component analysis (PCA) is a method for low-dimensional representation of multivariate data, which optimally preserves the structure of the data. PCA produces a transformation of the variables in a data set into new latent variables called principal components (PCs). These PCs do not correlate to each other and explain decreasing proportions of the total variance of the original variables. Considering that larger variances express larger amounts of information, a compact description of the data sets and the relationships between samples can be generated. Scores for each sample are calculated in these new PCs and plotted in score plots. The loadings of the different PCs help in determining which variables contain more information [37]. We used PLS_Toolbox 6.7 (Eigenvector Research, WA, USA) for MATLAB® to build the PCA models. The PCA model was cross-validated by the leave-one-out method providing a cross-validation RMS of 0.17. Hotelling's T2 for 95% interval of confidence was 12.15.

2.7. Isolation of Mitochondria. Animals were sacrificed by cervical dislocation, and then their livers were quickly removed. Mitochondria from the liver were obtained by differential centrifugation, as described by Rickwood et al. [38].

2.8. Peroxide Production Determination. The rate of peroxide production was determined using a modification of the method described by Barja [39] in isolated mitochondria. Concisely, we incubated the mitochondria at 37°C with 10 mM succinate in 2 mL of phosphate buffer, pH 7.4, containing 5 mM KH_2PO_4, 0.1 mM EGTA, 3 mM $MgCl_2$, 30 mM HEPES, 145 mM KCl, 0.1 mM homovanillic acid, and 6 U/mL horseradish peroxidase. The incubation was stopped at 5, 10, and 15 min with 1 mL of cold 2 M glycine buffer containing 2.2 M NaOH and 50 mM EDTA. Supernatant fluorescence was measured using 312 nm as excitation wavelength and 420 nm as emission wavelength. The peroxide production rate was then calculated using a standard curve of H_2O_2 [40].

2.9. Xanthine Oxidase Determination. XO activity was measured in plasma and soleus muscle by the fluorimetric method described in Beckman et al. [41]. Briefly, isoxanthopterin formation from pterine was followed fluorometrically as previously described (excitation at 345 nm and emission at 390 nm) [42, 43].

2.10. Measurement of Oxidative Stress Parameters. Lipid peroxidation was determined as accumulation of MDA, which was detected by HPLC as an MDA–thiobarbituric acid adduct [44].

Determination of GSH and GSSG was carried out using the high-performance liquid chromatography method, with UV–visible detection, which we developed to measure GSSG in the presence of a large excess of GSH [18, 45]. The essence of this method consists of minimizing GSH oxidation, which then would result in a large increase in GSSG. To measure GSH, isolated mitochondria were treated at 4°C with 12% ice-cold perchloric acid containing 2 mM bathophenanthroline disulfonic acid (BPDS). For GSSG determination, isolated mitochondria were treated at 4°C with 12% ice-cold perchloric acid containing 2 mM BPDS (Sigma Chemical Co.) and 40 mM N-ethylmaleimide (Sigma Chemical Co., St. Louis, MO, USA), in order to prevent GSH oxidation. Samples were then centrifuged at 15,000g for 15 min at 4°C, and we used the acidic supernatants for total glutathione and GSSG measurements.

2.11. Statistical Analysis. Data were represented by mean ± standard deviation (SD). Normality of distribution was checked with the Shapiro–Wilk test, and homogeneity of variance was tested by Levene's statistics. Comparison between groups was performed with a one-way ANOVA and two-tailed t-test. P values <0.05 were considered statistically significant.

3. Results and Discussion

3.1. Males Are More Prone to Develop Type 2 Diabetes with Age than Females Are—Role of Estrogen Replacement Therapy. We first studied the evolution of glycaemia levels in male and female GK controls, and as shown in Figure 1(a), glycaemia levels in males started to increase at 37 weeks of age, but it was not increased in females with age. To deepen this fact, we established an endpoint criterion of glycaemia above 200 mg/dL for sacrificing the rats. Figure 1(b) shows that female GK rats lived longer than did GK male rats, meaning that males reached the endpoint criteria before females did. Indeed, the half span was 52 weeks for males and 80 for females.

It is very well-known that estrogens protect females against many physio-pathological processes [46–51], and it is even involved in the longer longevity of females versus males [24–26, 40]. Moreover, it has been demonstrated its protective role against diabetes [11, 12, 52–56]. Therefore, we ovariectomized females to check the role of estrogens in protecting females against type 2 diabetes development. We also included a group with estrogen replacement therapy to better demonstrate the protective role of estrogens. When we measured glycaemia level evolution with age, we did not find any difference between the GK groups (control, ovx, and ovx+E2) (see Figure 1(c)). However, as shown in Figure 1(d), we found that ovariectomized GK rats gained more weight with the evolution of the disease than the other two groups did. This is very important as obesity is one of the factors that can worsen the disease and its complications [57, 58]. Moreover, we assessed insulin sensitivity by a glucose tolerance test, and we clearly showed that estrogen replacement therapy prevented the increase in glucose levels shown in control and ovariectomized GK female rats. We also included a nondiabetic group of rats (Wistar rats) as control of the diabetic rats (see Figure 1(e)). The protective role of estrogens against insulin resistance has been widely demonstrated [11, 55, 56, 59–61]. Many of the studies have evaluated the insulin resistance in obese models of diabetes, or in combination with a high-fat diet [55, 62, 63]. In our study, we show that in rats which are not obese and develop type 2 diabetes with age, estradiol is also able to enhance insulin sensitivity, allowing its protective role against this age-associated disease.

3.2. Glucose Uptake Is Higher in Female Brain, Cerebellum, and Heart than in Those from Male Diabetic Rats. Insulin resistance is associated with significantly lower regional cerebral glucose metabolism, which in turn may predict worse memory performance [64]. Moreover, several groups have shown that insulin-mediated myocardial glucose uptake is reduced in type 2 diabetic patients in comparison with healthy control subjects [65, 66]. This may play a role in the known cardiovascular differences between men and women with diabetes.

In our model, we determined glucose consumption by PET in different tissues of male and female diabetic rats. Figure 2(a) show representative PET images of glucose consumption in the brain, cerebellum, and heart demonstrating

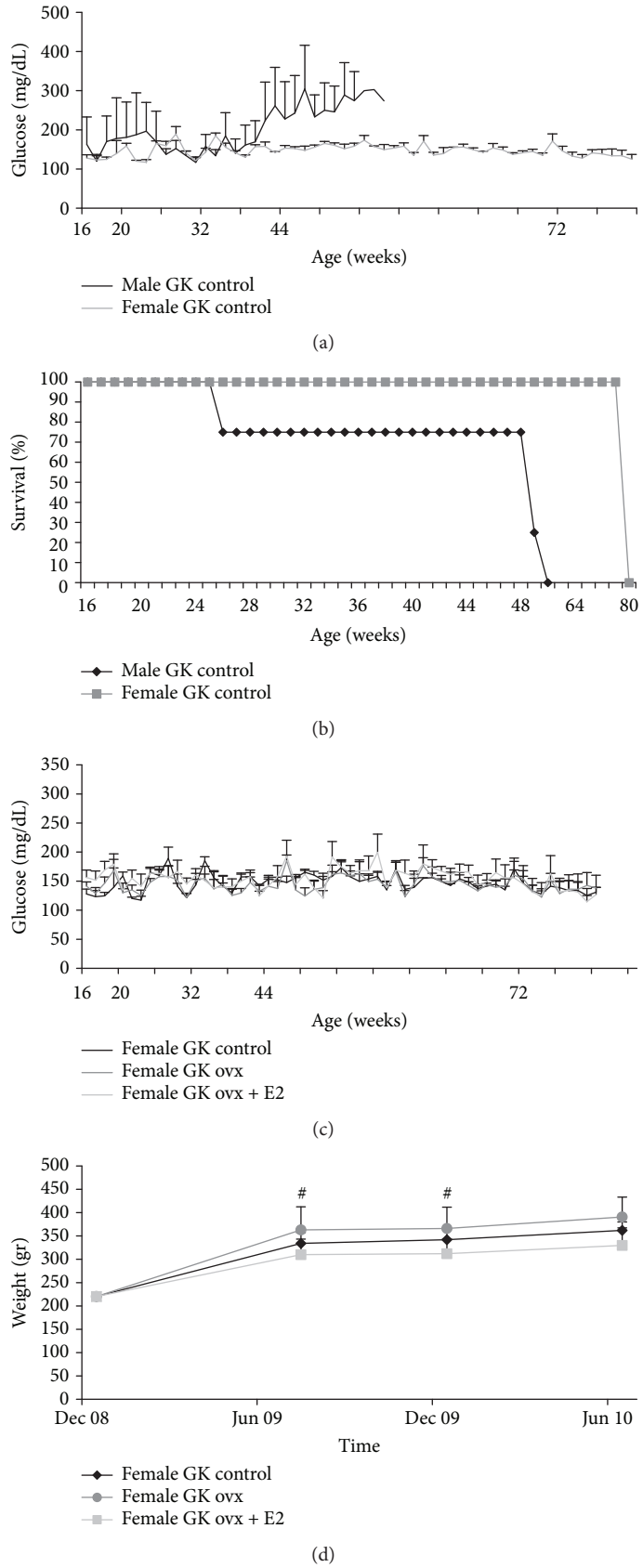
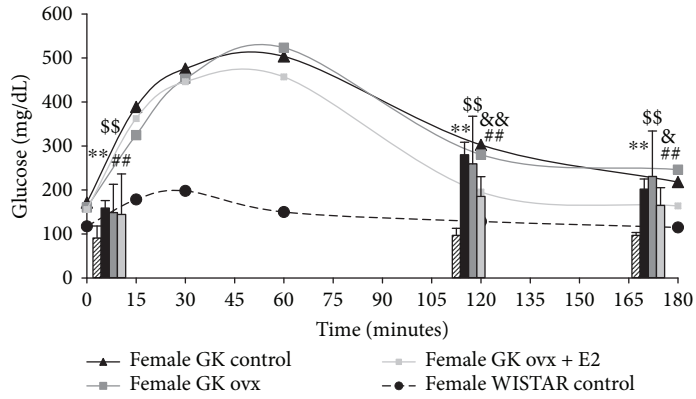


Figure 1: Continued.



(e)

Figure 1: Males are more prone to develop type 2 diabetes with age than females. Role of estrogen replacement therapy. (a) Evolution of glycaemia levels in male and female GK controls. The data are shown as means ± SD ($n = 4$ for both groups). The statistical significance is expressed as $^{*}p < 0.05$. (b) Survival curve in male and female GK controls ($n = 4$ for both groups). (c) Evolution of glycaemia levels in female GK controls ($n = 4$), ovariectomized female GK ($n = 6$), and ovariectomized female GK + estradiol replacement ($n = 6$). The data are shown as means ± SD. (d) Evolution of weight in female GK controls ($n = 4$), ovariectomized female GK ($n = 6$), and ovariectomized female GK + estradiol replacement ($n = 6$). The data are shown as means ± SD. The statistical significance is expressed as $^{\#}p < 0.05$ for GK OVX versus GK OVX + E2. (e) Glucose tolerance test in female WISTAR controls ($n = 6$), female GK controls ($n = 4$), ovariectomized female GK ($n = 6$), and ovariectomized female GK + estradiol replacement ($n = 6$). The data are shown as means ± SD. The statistical significance is expressed as $^{**}p < 0.01$ for WISTAR CTL versus GK CONTROL, $^{\#\#}p < 0.01$ for WISTAR CTL versus GK OVX + E2, $^{\$\$}p < 0.01$ for WISTAR CTL versus GK OVX, $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ for GK OVX versus GK OVX + E2.

that in all tissues, it was lower in males than in females. Figures 2(b)–2(d) show the analysis (SUV (standardized uptake value)) of the PET images in the respective tissues, which indicate that there are statistically significant differences between male and female diabetic rats.

Therefore, the diabetic brain and heart from males are characterized by a reduced capacity to uptake glucose and insulin resistance in comparison with those of females.

3.3. Metabolic Profile Shows that Type 2 Diabetes Has a Very High Impact in the Metabolism, and There Are Sex Differences—Role of Estrogen Replacement Therapy. Metabolomics offers a unique opportunity to study global metabolic profiles in animals (or persons) in different physiological and pathophysiological situations [67]. The principal component analysis represents scores plot of plasma metabolomic data. Distance between samples in the PCA space represents differences in global metabolic profiles.

Figure 3(a) shows that type 2 diabetes has a very high impact in the plasma metabolic profile. Samples from male (black triangle) and female (gray triangle) diabetic rats are well separated in the PCA space, representing a different metabolic profile, to those from young and old control male and female rats (young male (black circle), young female (gray circle), old male (black square), and old female (gray square)). This fact has been previously demonstrated by Knebel et al., who showed that metabolome analyses from diabetic patients enable identification of defined diabetes type-specific differences and detection of biomarkers of insulin sensitivity [68]. When analyzing diabetic rats specifically (Figure 3(b)), we find that there are also sex differences in the metabolic profile. Samples from male diabetic rats (black triangle) are clearly separated from the female diabetic rats (gray triangle), showing a different metabolic profile.

To check the role of estrogens in this difference (see Figure 3(c)), we also determined the metabolic profile in ovariectomized and estrogen-replaced diabetic rats. Samples from ovariectomized rats (gray triangle) are separated in the PCA space in the vertical direction, representing a different metabolic profile, to those from female diabetic rats. On the contrary, rats treated with estrogen replacement therapy (white triangle) are closer in the vertical direction in the PCA space to those of female diabetic rats (black triangle), representing a similar global metabolic profile. Therefore, estrogen replacement therapy restores in part the metabolic profile of female diabetic rats.

Studies performed with diabetic postmenopausal women have shown clear alterations in the lipid metabolic profile and that estrogen replacement therapy can modulate these menopause-associated changes [69]. Mosnier-Pudar et al. conclude that postmenopausal replacement therapy appears preferable in this vascular high-risk type 2 diabetic population, particularly since estrogens may have an antiatherogenic effect by direct action on the vessel walls. However, more studies are needed to establish the possible protective role of estrogen replacement therapy in postmenopausal women suffering from type 2 diabetes [70].

Therefore, we find that type 2 diabetes has a profound impact in the metabolic profile in plasma and that indeed there are sex differences involved, which in part are due to the presence of estrogens.

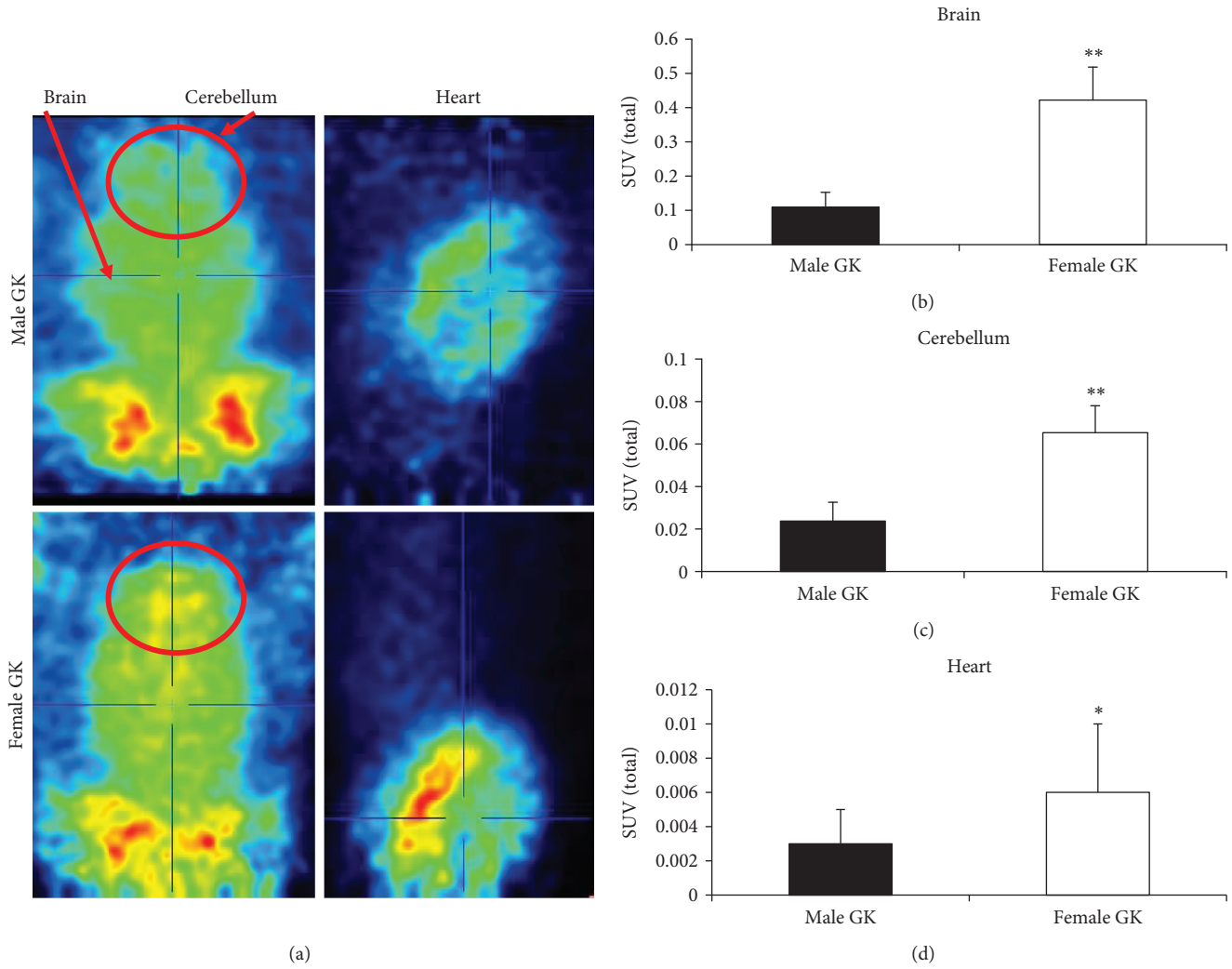


Figure 2: Glucose uptake is higher in the female brain, cerebellum, and heart than in those from male diabetic rats. (a) Representative PET images showing glucose consumption in the brain, cerebellum, and heart in male GK and female GK. (b) Glucose consumption in the brain, (c) cerebellum, and (d) heart in male GK and female GK. The data are shown as means ± SD ($n = 4$ in all cases). The statistical significance is expressed as $^{**}p < 0.01$.

3.4. Type 2 Diabetic Female Rats Are Protected against Free Radical Production Compared with Type 2 Diabetic Male Rats—Protective Role of Estrogen Replacement Therapy. Oxidative stress is one of the mechanisms involved in the pathophysiology of type 2 diabetes [71]. Some years ago, we showed that there are sex-associated differences in oxidative stress and that estrogens protect females against its burden [27–29, 40]. This prompted us to study possible sex-associated oxidative stress differences in diabetic rats, which could explain in part the mechanism underlying why females are protected against the disease when compared with males. For this reason, we measured hydrogen peroxide production in hepatic mitochondria from male and female diabetic rats and, as shown in Figure 4(a), it was higher in those of males when compared with females. Moreover, when we compared with nondiabetic rats, we noted that diabetic males produced higher hydrogen peroxide levels than nondiabetic males did, but, on the contrary, diabetic females produce similar levels of peroxides when compared with nondiabetic rats. This means that female diabetic rats were protected against oxidative stress, but males do not. To check again the role of estrogens, we determined hydrogen peroxide production in ovariectomized and estrogen-replaced diabetic rats (Figure 4(b)), and we found that ovariectomized diabetic female rats showed an increased production compared with female diabetic rats and that estrogen replacement therapy prevented it.

The protection against oxidative stress in females versus males occurred even when females were older (20-22 months old) than males (11-15 months old). This means that females are protected by estrogens against oxidative stress even if they are old. The reason for this effect could be related with the different pattern of hormonal changes with age between humans and rodents. For example, rats and mice do not experience complete follicle loss and maintain estrogens levels until older ages than humans do [72].

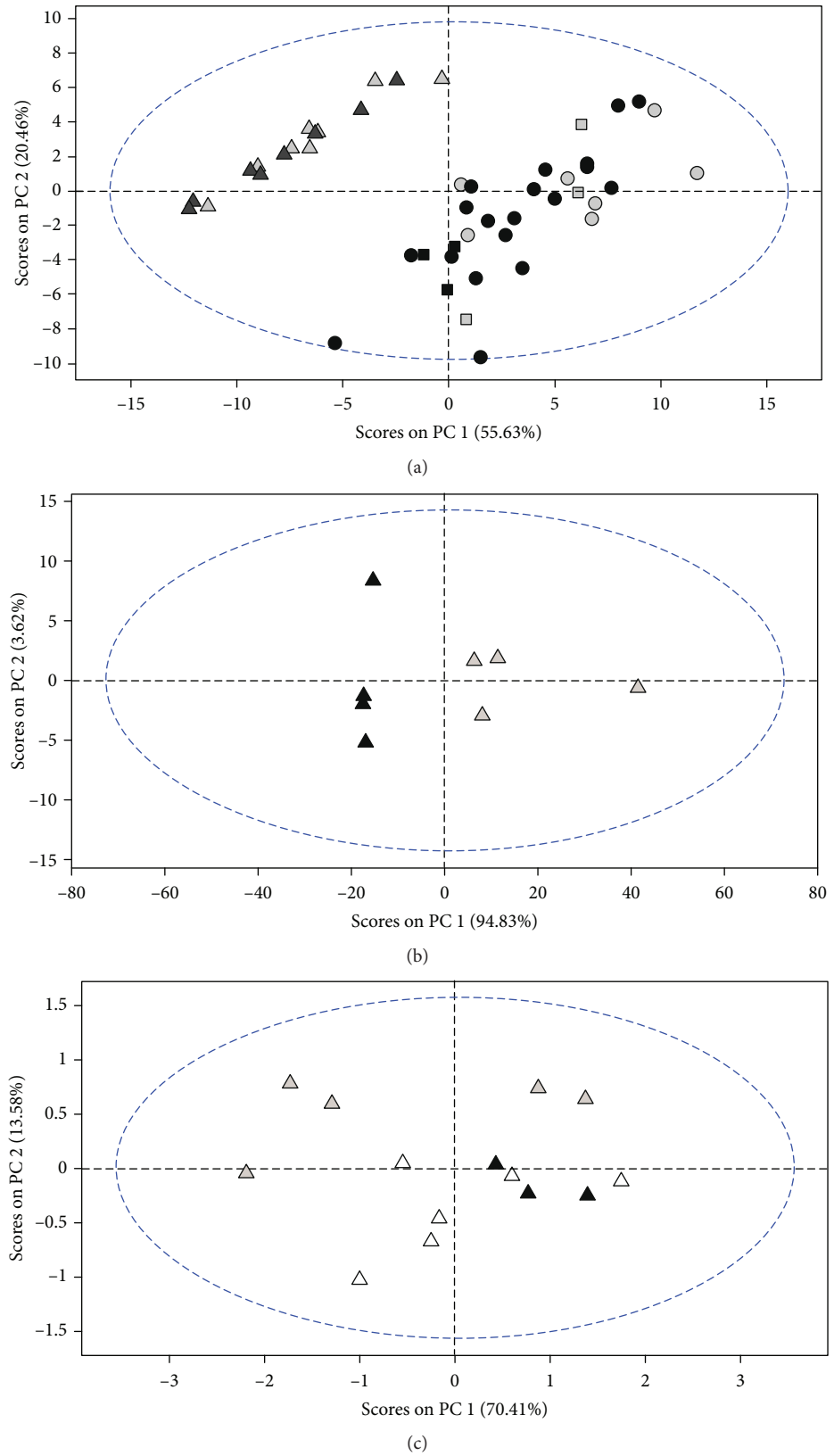


Figure 3: Metabolic profile shows that type 2 diabetes has a very high impact in the metabolism, and there are sex differences. Role of estrogen replacement therapy. (a) Metabolic profile of male GK (black triangle), female GK (gray triangle), young male (black circle), young female (gray circle), old male (black square), and old female (gray square). (b) Comparison of the metabolic profile of male GK (black triangle) and female GK (gray triangle). (c) Comparison of the metabolic profile of female GK controls (dark triangle) ($n = 3$), ovariectomized female GK (gray triangle) ($n = 6$), and ovariectomized female GK + estradiol replacement (white triangle) ($n = 5$).

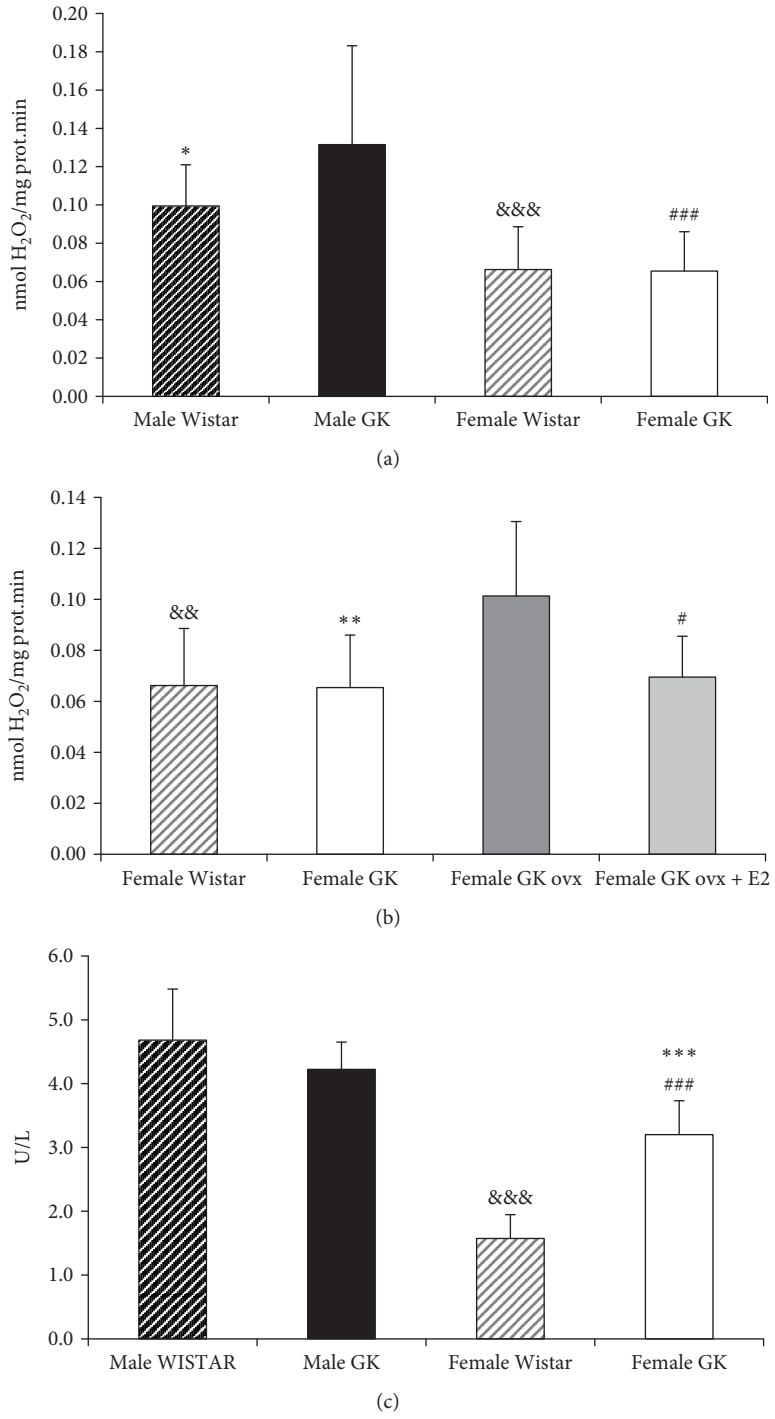


Figure 4: Type 2 diabetic female rats are protected against free radical production compared with type 2 diabetic male rats. Protective role of estrogen replacement therapy. (a) Mitochondrial hydrogen peroxide production in male WISTAR ($n = 12$), male GK ($n = 8$), female WISTAR ($n = 14$), and female GK ($n = 8$). The data are shown as means ± SD. The statistical significance is expressed as $^{*}p < 0.05$ for male WISTAR versus male GK, $^{\#\#\#}p < 0.001$ for male GK versus female GK, and $^{\&\&\&}p < 0.001$ for male WISTAR versus female WISTAR. (b) Mitochondrial hydrogen peroxide production in female WISTAR ($n = 14$), female GK ($n = 4$), ovariectomized female GK ($n = 6$), and ovariectomized female GK + estradiol replacement ($n = 6$). The data are shown as means ± SD. The statistical significance is expressed as $^{\&\&}p < 0.01$ for WISTAR versus GK OVX, $^{**}p < 0.01$ for GK versus GK OVX, and $^{\#}p < 0.05$ for GK OVX versus GK OVX + E2. (c) Xanthine oxidase activity levels in plasma of male WISTAR ($n = 7$), male GK ($n = 8$), female WISTAR ($n = 5$), and female GK ($n = 8$). The data are shown as means ± SD. The statistical significance is expressed as $^{\#\#\#}p < 0.001$ for female GK versus male GK, $^{***}p < 0.001$ for female GK versus female WISTAR, and $^{\&\&\&}p < 0.01$ for female WISTAR versus male WISTAR.

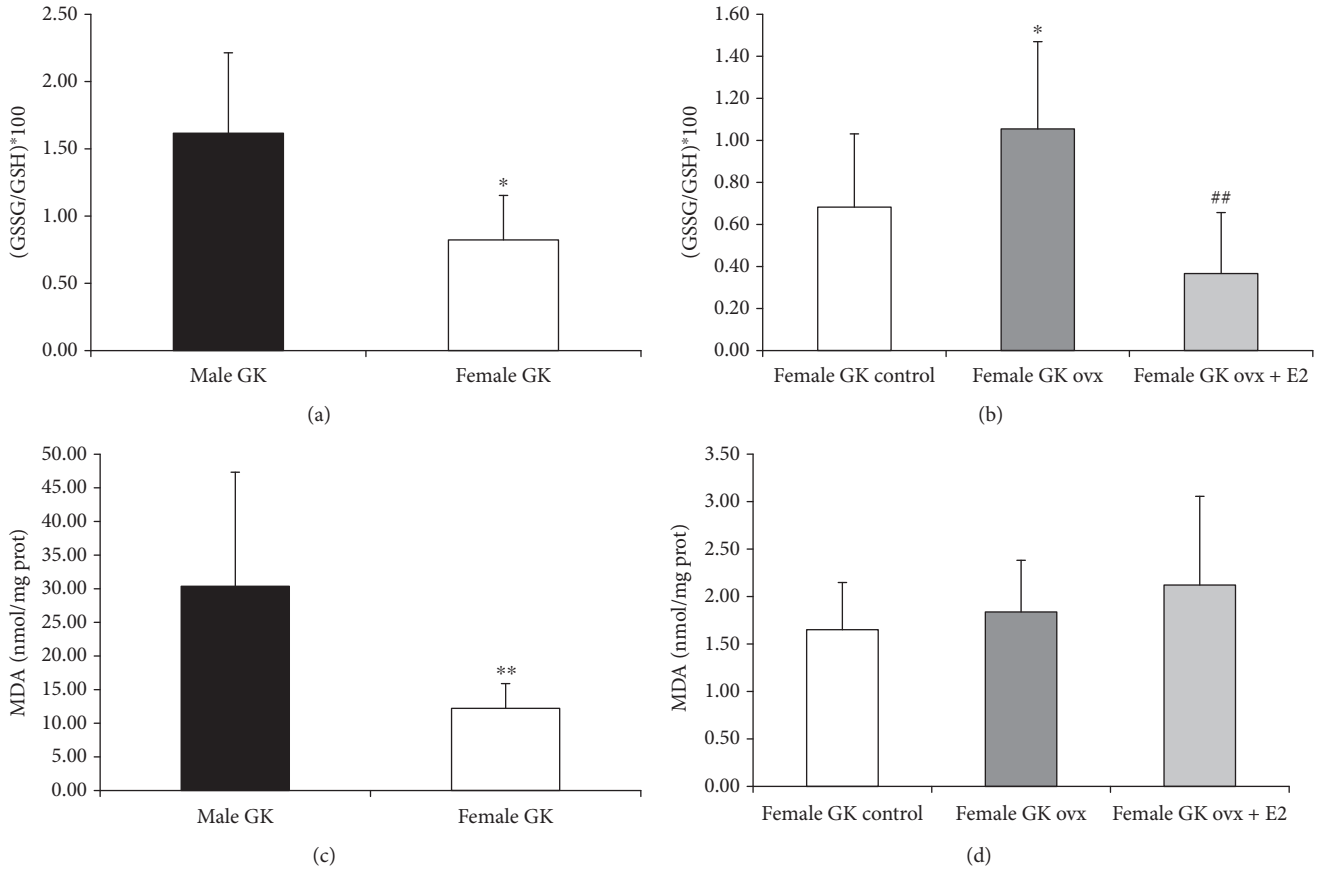


Figure 5: Type 2 diabetic female rats show lower levels of mitochondrial oxidative stress-related parameters than type 2 male diabetic rats. Protective role of estrogen replacement therapy (a) GSSG/GSH*100 levels in hepatic mitochondria of male GK and female GK. The data are shown as means ± SD ($n = 8$ for both groups). The statistical significance is expressed as $^{*}p < 0.05$. (b) GSSG/GSH*100 levels in hepatic mitochondria of female GK ($n = 4$), ovariectomized female GK ($n = 6$), and ovariectomized female GK + estradiol replacement ($n = 6$). The data are shown as means ± SD. The statistical significance is expressed as $^{*}p < 0.05$ for GK ovx versus GK OVX + E2 and $^{\#\#}p < 0.01$ for GK versus GK OVX. (c) MDA levels in hepatic mitochondria of male GK and female GK. The data are shown as means ± SD ($n = 8$ for both groups). The statistical significance is expressed as $^{**}p < 0.01$. (d) MDA levels in hepatic mitochondria of female GK ($n = 4$), ovariectomized female GK ($n = 6$), and ovariectomized female GK + estradiol replacement ($n = 6$). The data are shown as means ± SD.

Another source of oxidative stress which has been involved in diabetes is xanthine oxidase activity [19]. Figure 5 shows that it was similar in nondiabetic and diabetic males, but was higher in diabetic females compared with nondiabetic females. Nevertheless, again it was higher in males when compared with females, either nondiabetic or diabetic rats.

In order to clarify better these results, we decided to measure more oxidative stress-related parameters. Figure 5(a) shows that the glutathione ratio (GSSG/GSH) in hepatic mitochondria was higher in diabetic males compared with diabetic females. In Figure 5(b), we show that ovariectomy increases this ratio, and estrogen-replacement therapy prevents the increase. We also measured an index of lipid peroxidation, which is malondialdehyde (MDA), and we showed again that it was higher in hepatic mitochondria from diabetic males, compared with those of diabetic females (Figure 5(c)). In this case, we did not find any effect of ovariectomy or estrogen replacement therapy (Figure 5(d)).

4. Conclusion

There are sex-associated differences in type 2 diabetes. Females are protected against the disease in part because of the presence of estrogens. Oxidative stress could be one of the mechanisms underlying the protective effect of them. More studies are needed to elucidate the role of estrogens in protecting against type 2 diabetes, as it is very important for recommending estrogen replacement therapy to diabetic postmenopausal women with no contraindications to the therapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Effect of Interaction between Early Menarche and Genetic Polymorphisms on Triglyceride

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Early menarche has been associated with increased risk of metabolic syndrome. Therefore, investigating the association of each component of metabolic syndrome with age at menarche, and interactions between them, might lead to a better understanding of metabolic syndrome pathogenesis. In this study, we evaluated age at menarche for risk of metabolic syndrome and associations with its components. As a result, the risk of MetS incidence was significantly increased only at ≤12 years of age at menarche (OR = 1.91, $P < 0.05$). Women with early menarche (≤12 years) had significantly higher levels of triglycerides (β coefficient = 37.83, $P = 0.02$). In addition, hypertriglyceridemia was significantly increased at early menarche with 1.99 (95% CI: 1.16–3.41, $P < 0.01$). With GWAS-based pathway analysis, we found the type 2 diabetes mellitus, stress-activated protein kinase signaling, and Jun amino-terminal kinase cascade pathways (all nominal $P < 0.001$, all FDR < 0.05) to be significantly involved with early menarche on triglyceride levels. These findings may help us understand the role of early menarche on triglyceride and interaction between gene and early menarche on triglyceride for the development of metabolic syndrome.

1. Introduction

Menarche, defined as the first menstrual period in a woman's life, is a marker of female puberty and the onset of ovarian and other endocrine functions related to reproductive capacity. Age of menarche has declined over the last several decades through improvement of socioeconomic conditions or exposure to environmental chemicals and received a great deal of attention as having important health implications [1]. Age at menarche (AAM) can be a risk factor for disease, and there were several studies on the interaction between AAM and genetic factors especially for breast cancer susceptibility [2–4]. Early menarche is often defined as menarche before the age of 12 years (≤11 years old), but some investigators base definition on menarche at ≤12 years [5]. A number of studies have reported associations between early menarche onset and intermediate quantitative traits such as increased blood glucose levels [6], impaired glucose tolerance [7], and insulin resistance [8], further supporting a link between early pubertal development and an adverse metabolic profile. Others have reported adverse metabolic consequences (e.g., diabetes) arising from early sexual maturation [9, 10]. One possible mechanism for this association is a direct link between puberty timing and glucose regulation, supported by the insulin-sensitizing agent metformin delay of menarche in girls with precocious puberty [11]. It is conceivable that part of the association between menarche onset and type 2 diabetes risk is explained by increased adiposity [9, 10]. However, these data are not consistent, and the relationship between AAM and metabolic components remains poorly understood. Metabolic syndrome (MetS) is considered a worldwide epidemic. In general, the International Diabetes Federation estimates that one-quarter of the world's adult population has MetS [12]. Higher socioeconomic status, sedentary lifestyle, and high body mass index (BMI) significantly associate with MetS. Data from the National Health and Nutrition Examination Survey (NHANES, 1999–2006) reported that nearly 34% of all U.S. adults, and 50% of those aged 60 years or older, were estimated to have MetS [12]. The prevalence of MetS in Korea has also been steadily increasing

in recent years, from 24.9%, in 1998, to 31.3%, in 2007; this prevalence is relatively high compared to those of other Asian countries [13]. MetS is characterized by the clustering of five pathological traits, including large waist circumference, hypertriglyceridemia, low-density lipoprotein (LDL) cholesterol level, hypertension, and hyperglycemia. However, mechanisms and differences, correlated with the relative risk of susceptibility to MetS, remain poorly characterized.

To date, genome-wide association studies (GWAS) have identified several susceptibility regions and genes for MetS and its component phenotypes [14, 15]. Since the first report from a GWA study of obesity, an increasing number of genetic factors have been shown to associate with several traits of obesity and MetS. Despite the success of recent GWAS, the identified variants explain only a small proportion of the heritability of most complex diseases [16]. Heritability estimates of MetS range from approximately 10 to 30% [17] among individuals; therefore, implicated genes may interact synergistically with environmental factors, in the pathogenesis of MetS.

Here, we investigated the role of AAM on MetS components for MetS risk. Specifically, we examined the association between AAM and levels of individual component of MetS such as triglyceride (TG). Furthermore, we evaluated GWAS-identified genetic variants for interaction effects with MetS components using a pathway-based analysis and characterized the role of AAM in the pathogenesis of MetS.

2. Methods

2.1. Study Design and Participants. This study, an ongoing prospective investigation, is a population-based cohort included in the Korean Genome and Epidemiology Study (KoGES) [18]. Originally, 5018 woman participants, aged 40 to 69 years, were recruited from the Korean rural (Ansung) and urban (Ansan) communities at baseline, as part of the Korean Association Resource Project (KARE). From 2007 to 2008, the samples were scrutinized for quality control purposes, and 3493 participants remained. We excluded those with reported AAM of ≤10 years ($n = 3$) or ≥19 years ($n = 272$) or missing ($n = 41$), probably due to recall error or the presence of a comorbid pathological condition. Finally, information was available from 3180 women for the study of AAM.

These cohorts included a standardized health interview, using well-established questions, to determine the demographic and socioeconomic characteristics of all subjects, including menstruation history. AAM was defined as the age of the first menstrual period. Women reported their AAM in single years and were classified for this study as having an AAM of ≤12, 13, 14, 15, 16, or ≥17 years [19]. Comprehensive health examinations, including evaluation of anthropometric indexes, measurement of blood pressure, questionnaire-based interviews, and collection of biospecimens for assays (e.g., C-reactive protein), were conducted biennially by health professionals who were trained with a standardized protocol. Women who smoked regularly during the previous 12 months were classified as current smokers. Levels of physical activity were semiquantitatively assessed using a questionnaire previously validated against objective methods [20] and were coded as inactive, moderately inactive, moderately active, and active. Educational attainment was categorized into three groups: less than 7 years (elementary school graduates), 7–9 years (middle school graduates), and more than 10 years (high school graduates). Monthly household income was also categorized into three groups: less than $1000 USD (in 2014), $1000–2000, and ≥$2000 [21]. In addition, we performed the additional analysis for oxidative stress-related exposure with OBS score and inflammatory marker with C-reactive protein (CRP) at each AAM [22, 23]. Written informed consent was obtained from all participants at the KoGES. This study was approved by the Institutional Review Board of Seoul National University.

2.2. Definition of the Metabolic Syndrome and Its Components. We used the definition of MetS proposed by the criteria of the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) guidelines [24]. As detailed in our study, participants having three or more of the following criteria were defined as having MetS, except for the determination of central obesity. Waist circumference cut-off values were used based on guidelines from the Korean Society for the Study of Obesity [25]: (1) central obesity, given as waist high circumference (≥85 cm); (2) high concentration of serum triglycerides (≥150 mg/dl); (3) low concentrations of serum HDL cholesterol (<50 mg/dl); (4) hypertension (systolic/diastolic pressure ≥ 130/85 mmHg) or antihypertensive medication; and (5) high concentrations of fasting glucose (≥100 mg/dl) or antidiabetic medication.

2.3. Genotyping. The data used for GWAS was obtained from KoGES, which was conducted by the Korean National Institute of Health. DNA samples were isolated from the peripheral blood of all participants and genotyped, using the Affymetrix Genome-Wide Human SNP array 5.0 (Affymetrix Inc., Rockville, MD, USA). Quality control procedures were adopted, such as missing genotype frequency > 0.5% and minor allele frequency (MAF) ≤ 0.05. After sample and genotype quality controls, 344,396 SNPs for 3180 individuals were available in the KARE database [26, 27]. The KARE data used to support the findings of this study are restricted by the Institutional Review Board of the Korean National Institute of Health, who can contact at National Biobank of Korea (http://koreabiobank.re.kr, 82–1661-9070).

2.4. Statistical Analysis. For demographics and characteristics of subjects, data were presented as means and standard deviations for continuous variables, or a percentage (%), for categorical variables (Table 1). Differences among subjects in different groups were detected using the Kruskal-Wallis test, for continuous variables, and the chi-square test, for categorical variables.

At first, logistic regression analyses were performed to determine the odds ratios (ORs) of MetS (Figure 1), and its five components, depending on each year of menarche, with 16 years of age as a reference group (Figure 2). Logistic regression models were adjusted for age, area, income, education,

Table 1: Characteristics of study subjects.

	Age at menarche (years)						All	P^b
	≤12	13	14	15	16	≥17		
Women, *n* (%)	77 (2.4)	269 (8.5)	531 (16.6)	669 (21.1)	689 (21.7)	945 (29.8)	3180	
Age at enrolment (years)	51.25 ± 6.92	52.58 ± 7.74	53.33 ± 8.47	54.92 ± 8.80	56.94 ± 8.82	59.18 ± 8.49	56.07 ± 8.88	<0.001
Area, *n* (%)								
Rural (Ansung)	26 (34)	83 (31)	199 (37)	339 (51)	412 (60)	640 (68)	1699	<0.001
Urban (Ansan)	51 (66)	186 (69)	332 (63)	330 (49)	277 (40)	305 (32)	1481	
BMI (kg/m^2)	25.55 ± 3.37	24.62 ± 3.42	24.78 ± 3.07	24.72 ± 3.20	24.86 ± 3.16	24.89 ± 3.12	24.80 ± 3.16	0.848
Current smokers, *n* (%)	0 (0)	3 (1.1)	9 (1.7)	11 (1.6)	9 (1.3)	27 (2.9)	59 (1.8)	0.177
Alcohol consumption (g/l)	3.52 ± 11.18	1.51 ± 5.36	1.49 ± 5.28	1.40 ± 6.85	1.41 ± 6.16	1.28 ± 4.96	1.44 ± 5.96	0.068
Age at menopause	48.26 ± 3.83	47.52 ± 5.61	47.60 ± 4.62	47.87 ± 5.40	47.83 ± 5.11	47.90 ± 5.24	47.82 ± 5.16	0.718
OBS score	9.34 ± 2.41	9.39 ± 2.43	9.53 ± 2.56	9.19 ± 2.47	8.85 ± 2.53	8.91 ± 2.50	9.10 ± 2.51	0.698
CRP (mg/dl)	1.04 ± 1.34	1.22 ± 1.10	1.33 ± 1.24	1.35 ± 1.23	1.40 ± 1.27	1.53 ± 1.44	1.41 ± 1.30	0.07
Education, *n* (%)								<0.001
Less than elementary school	7 (9.1)	20 (7.4)	55 (10.4)	95 (14.2)	138 (20.1)	225 (23.8)	540 (17)	
Middle school graduate	24 (31.2)	111 (41.3)	185 (35)	305 (45.6)	384 (55.8)	575 (60.8)	1584 (50)	
High school and above	46 (59.7)	138 (51.3)	289 (54.6)	269 (40.2)	166 (24.1)	141 (24.5)	1049 (33)	
Income, *n* (%)								<0.001
<100[a]	16 (20.8)	61 (22.7)	138 (26.4)	243 (36.6)	305 (44.8)	502 (53.1)	1265 (40.2)	
100–200	23 (29.9)	59 (21.9)	112 (21.5)	144 (21.7)	156 (22.9)	220 (23.3)	714 (22.7)	
≥200	38 (49.4)	149 (55.4)	272 (52.1)	277 (41.7)	220 (32.3)	214 (22.6)	1170 (37.1)	

Values are expressed as means ± SDs (standard deviations) or number (%). BMI: body mass index; OBS: oxidative balance score; CRP: C-reactive protein; [a]10^4 KRW: equivalent with 1000 US dollar in 2014. [b]*P* value was examined by the Kruskal-Wallis test or chi-square test.

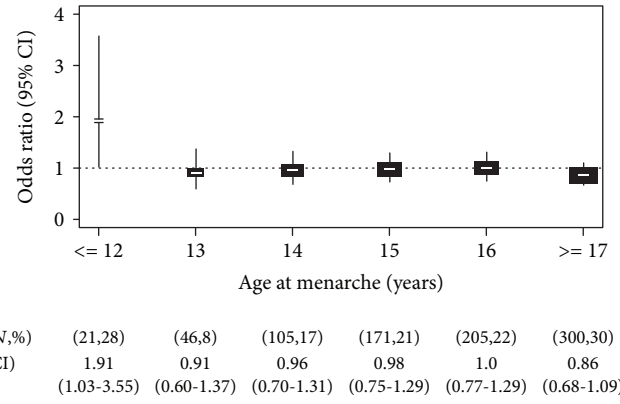


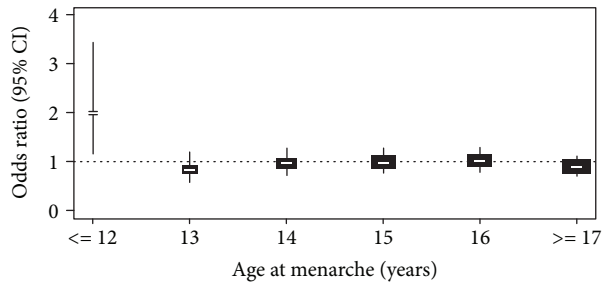
Figure 1: Risk of MetS in each age of menarche. Odds ratio (OR) and 95% confidence interval (CI) of Mets by each age at menarche. *N* is the number of incidence in each age of menarche; % is the percent of incidence in each age of menarche. The size of rectangle represents the number of samples. The vertical line indicates the overall OR on each age of menarche. ORs were adjusted for area, age, income, education, and C-reactive protein levels. The reference category was menarche at 16 years of age.

and CRP levels. Next, we evaluated beta coefficients for each year of menarche, using linear regression analyses for individual components of MetS, with 16 years of age as a reference group (Table 2). This reference group was chosen because it covers the median of AAM (median age = 16.0) [28].

To test the interactions between SNPs and AAM, we performed interaction tests for identifying genetic variants associated with MetS and its characteristic components. In addition, we performed the joint test of the main and interaction effects with one degree of freedom (df). We then used the following equation:

$$Y_i(\text{or log it}\, p_i) = \beta_0 + \beta_1 E_i + \beta_{\text{main}} G_{\text{i}} + \beta_{\text{int}} GE_{\text{i}}, \quad (1)$$

where Y_i is the MetS component for individual i and $p_i = P_r$ (Y_i=1) of MetS for a binary trait. We then used the Wald test statistic to report the results of the joint (P_{joint}) test, with two dfs and the gene-risk factor (P_{int}) with one df, referring to their



Incident (*N*,%)	(27,35)	(57,21)	(131,25)	(175,26)	(198,29)	(271,29)
OR (95% CI)	1.99 (1.16-3.41)	0.83 (0.58-1.19)	0.96 (0.73-1.26)	0.98 (0.76-1.26)	1.0 (0.79-1.28)	0.89 (0.71-1.11)

Figure 2: Risk of hypertriglyceridemia in each age at menarche. Odds ratio (OR) and 95% confidence interval (CI) of hypertriglyceridemia by each age at menarche. *N* is the number of incidence in each age of menarche; % is the percent of incidence in each age of menarche. The size of rectangle represents the number of samples. The vertical line indicates the overall OR on each age of menarche. ORs were adjusted for area, age, income, education, and C-reactive protein levels. The reference category was menarche at 16 years of age.

respective null hypotheses of the joint effects ($\beta_{\text{main}} = 0$ and $\beta_{\text{int}} = 0$) and of the interaction effect ($\beta_{\text{int}} = 0$), respectively.

The critical P values for accessing the significance of interaction were calculated by Bonferroni correction ($P < 1.45 \times 10^{-7}$) or the false discovery rate (FDR), with a q value less than 0.05. Quantile-quantile (QQ) plots of the P values for the joint test and interaction analysis suggested a moderate inflation of the genome-wide analysis only for TG. The inflation factors were 1.32 and 1.24 for interaction and joint effects, respectively, for TG in KARE dataset (Supplemental Figure S2). There were no genomic inflations for other components ($\lambda < 1.05$). Therefore, for genomic controls of TG, the inverse normal transformed TG was used as a response, adjusting for age, area, income, education, and CRP levels for KARE. All analyses were performed using the R software version 2.11.1 (http://www.r-project.org/). Data management, descriptive statistics for the covariates and outcome variates, and the regression analyses were conducted using the *Stats* R package.

2.5. Pathway-Based Analysis. Pathway-based approaches using GWAS data are now used routinely to study complex diseases [29]. To analyze pathways interacting AAM with TG genetic polymorphism, we used the improved gene set enrichment analysis (GSEA) [29]. This approach has the advantage that genetic variant associations, mapping to any genes, provide insight into biological functions, signal pathways, and mechanisms of disease.

In our study, 344,396 SNPs were mapped to genes within 20 kb boundaries. Pathways consisting of <20 or >200 genes were excluded from further analysis, to reduce the multiple testing issue and avoid testing overly narrow or broad functional categories [22, 27]. A false discovery rate (FDR) was used for multiple testing correction, with q values < 0.05 considered significant. Improved GSEA approaches use a comprehensive pathway and gene set database from SNP data. Pathway-related information is obtained from the KEGG (Kyoto Encyclopedia of Genes and Genomes pathway database), BioCarta, and GO (gene ontology) databases [27].

3. Results

3.1. Characteristics of Participants. A total of 3180 women (mean age: 56.07 years; SD: 8.88 years) were included in this study. Demographic characteristics of the women are presented in Table 1, which shows the baseline characteristics of the study population stratified by AAM. The mean age of menarche was 15.83 (SD of 1.89) years. Because of the small group numbers for each subject having earlier ages of menarche, we combined subjects aged 11 and 12 years of menarche. At baseline, women with earlier menarche were slightly younger, had higher BMI and higher alcohol consumption, and more frequently lived in urban areas (Table 1). The individuals with early menarche were more likely higher educated and had higher monthly incomes. However, we observed no association of OBS scores as an oxidative stress-related exposure and CRP levels as an inflammation marker with AAM. All women with early menarche had never regularly smoked.

3.2. Risks of Metabolic Syndrome and Its Association with Triglyceride by Age at Menarche. Figure 1 shows the ORs of MetS by each year of AAM (from 12 to 17 years). Compared with the reference group (age at 16 years), the risk of incident MetS was significantly increased only at ≤12 years of AAM with 1.91 (95% CI, 1.03–3.55, $P < 0.05$), after adjustment for area, age, income, education, and CRP levels. We also investigated ORs between AAM and five individual components of MetS. Only hypertriglyceridemia was significantly increased at ≤12 years of AAM with 1.99 (95% CI, 1.16–3.41, $P < 0.01$, Figure 2). However, other phenotypes did not significantly associate with AAM (Supplemental Figure S1).

We then examined the association between AAM and each component of MetS, separately. Linear regression analysis (Table 2) showed that women with earlier AAM had higher waist circumference and TG, compared with those with average AAM, after adjusting for the same covariates ($P < 0.05$). The corresponding regression coefficients of waist circumference and TG were 3.54 (95%

Table 2: Association of age at menarche with metabolic traits (data are expressed as beta coefficients, with 95% confidence intervals, for linear regression adjusted for age, area, education, income, and CRP level) in KARE.

Metabolic traits	Age at menarche (years)						
	≤12	13	14	15	16	≥17	P^a
	Beta coefficient (95% CI)						
SBP (mmHg)	2.22 (−1.65–6.10)	−1.51 (−3.82–0.80)	1.17 (−0.64–2.99)	0.54 (−1.11–2.18)	0 (−1.66–1.66)	−0.004 (−1.53–1.52)	0.27
DBP (mmHg)	1.26 (−0.98–3.50)	−0.34 (−1.71–1.03)	0.44 (−0.63–1.51)	0.42 (−0.56–1.39)	0 (−0.96–0.96)	0.58 (−0.32–1.49)	0.44
FI (mg/dl)	−0.02 (−0.92–0.88)	0.07 (−0.51–0.65)	−0.002 (−0.47–0.46)	−0.24 (−0.64–0.17)	0 (−0.40–0.40)	−0.40 (−0.77–0.03)	0.91
FG (μU/ml)	2.19 (−1.13–5.51)	−0.50 (−2.42–1.42)	0.71 (−0.94–2.37)	0.63 (−0.94–2.19)	0 (−1.44–1.44)	0.97 (−0.58–2.5)	0.12
WC (cm)	3.54 * (1.53–5.55)	0.79 (−0.42–2.00)	0.55 (−0.40–1.49)	0.26 (−0.63–1.15)	0 (−0.86–0.86)	−0.52 (−1.33–0.29)	<0.01
TG (mg/dl)	37.83* (16.00–59.66)	4.67 (−6.93–16.26)	3.15 (−6.60–12.90)	1.80 (−6.29–9.90)	0 (−8.24–8.24)	−0.004 (−7.81–7.80)	0.02
HDL (mg/dl)	−0.07 (−2.48–2.33)	0.52 (−0.94–1.99)	0.81 (−0.38–2.00)	0.11 (−0.95–1.17)	0 (−1.03–1.03)	0.66 (−0.31–1.64)	0.71

SBP: systolic blood pressure; DBP: diastolic blood pressure; FI: fasting insulin; FG: fasting glucose; WC: waist circumference; TG: triglyceride; HDL: high-density lipoprotein. [a]Age at menarche as a dummy variable (≤12 years: 0, >12 years: 1), *$P < 0.01$.

Table 3: Discovery GWAS top hits for joint and 1df interaction of gene age at menarche on triglyceride.

SNP ID	Nearest gene	Chr	Ref/var	Position	MAF	Test of interaction	
						P_{joint}	P_{int}
rs6589566	*ZPR1*	11	A/C	116,781,707	0.22	2.92×10^{-12}	0.15
rs10503669	*LPL*	8	A/C	19,990,179	0.12	3.87×10^{-9}	0.03
rs603446	*ZPR1*	11	C/T	116,783,719	0.23	4.82×10^{-9}	0.67
rs17482753	*LPL*	8	G/T	19,975,135	0.13	1.40×10^{-8}	0.02
rs17410962	*LPL*	8	A/G	19,990,569	0.13	1.48×10^{-8}	0.02
rs11216186	*SIK3*	11	C/T	116,913,976	0.13	2.41×10^{-8}	0.03
rs17120157	*SIK3*	11	C/T	116,941,912	0.13	6.43×10^{-8}	0.03
rs10892068	*SIK3*	11	C/T	117,057,830	0.13	7.34×10^{-8}	0.03
rs17120293	*SIK3*	11	C/T	117,074,130	0.13	7.44×10^{-8}	0.03
rs12292858	*SIK3*	11	A/C	116,943,263	0.18	9.76×10^{-8}	0.09
rs2044426	*SIK3*	11	C/T	116,885,467	0.10	1.52×10^{-7}	0.02
rs6073350	*JPH2*	20	A/T	44,145,104	0.04	1.61×10^{-7}	7.14
rs12279433	*SIK3*	11	G/T	116,877,505	0.10	1.69×10^{-7}	0.02
rs1501675	*ARHGEF28*	5	A/C	73,695,382	0.02	1.77×10^{-7}	6.12×10^{-8}
rs1014492	*SCG2*	2	A/G	223,633,665	0.10	2.23×10^{-7}	4.49×10^{-4}
rs11216315	*PCSK7*	11	C/G	117,209,924	0.13	2.94×10^{-7}	0.04
rs11216126	*BUD13*	11	A/C	116,746,524	0.20	3.60×10^{-7}	0.12
rs7513082	*LOC10537172 (UBQLN4)*	1	A/C	156,031,555	0.02	4.01×10^{-7}	5.82×10^{-8}
rs13046	*TTT4*	1	A/C	54,742,081	0.01	6.18×10^{-7}	1.83×10^{-5}
rs16875865	*LOC107986425 (LHFPL2)*	5	A/G	78,746,592	0.01	6.40×10^{-7}	4.60×10^{-7}

CI = 1.53–5.55, $P = 0.01$) and 37.83 (95% CI = 16.00–59.66, $P = 0.02$), respectively. From these results (Figure 2 and Table 2), early menarche (12 years and before) is associated with increased risk of MetS, and this association appeared to be mediated mainly by increased TG of MetS.

3.3. Interaction between Genotypes and Age at Menarche on Triglyceride. As we considered that AAM was a modulator for the development of MetS, we conducted the joint analysis of the main SNP effect and its interaction effect with AAM interaction on MetS, and its individual traits, for all 352,228 SNPs. A list of SNPs associated with AAM-TG interactions, at $P < 7 \times 10^{-7}$, by joint test, is provided in Table 3. The strongest statistical evidence for SNP × AAM joint interaction was an SNP of rs6589566, located on the chromosome 11, in zinc finger protein 1 (ZPR1) ($P = 9.54 \times 10^{-7}$). We found that main genetic effects of SNPs located chromosomes 11 and 8 were the primary contributors to these joint associations. Specifically, we identified SNP rs1501675, located within an intronic region of *ARHGEF28*, as the most significant SNP, with $P = 6.12 \times 10^{-8}$ for AAM-to-TG. Other interesting SNPs were located near (±2 kb) or within the loci *LOC10537172*, *SCG2*, *TTT4*, and *LOC107986425* (all nominal P values $< 5 \times 10^{-4}$).

Based on these findings, we tested pathway level-based interaction between AAM and genetic variation, according to SNPs with significant P values for enriched biological processes, in TG. When mapping SNPs were limited to 20 kb regions flanking a gene, three pathways, type 2 diabetes mellitus, stress-activated protein kinase (SAPK) signaling pathway, and Jun amino-terminal kinases (JNK) cascade, were significantly enriched, with association signals and FDR < 0.05 (Table 4).

4. Discussion

In this study, we observed that early menarche (at or before 12 years of age) was associated with high prevalences of MetS; however, later AAM showed no significant association. In particular, this trend appeared to be mediated by increased TG, with earliest menarche ages associated with increased TG, in our study population. A number of studies have previously investigated the association between menarcheal age and MetS with elevated blood glucose or BMI or TG [30–32]. Recently, two studies have examined the association between AAM and risk of MetS in the Korean population. One study, of 1464 Korean women, showed that the relative risk of MetS was 3.84 (96% CI: 1.52–9.70) for menarche at age <12 years in premenopausal women (from the KNHANES database) from 2007 to 2009, along with higher blood pressure [33]. Won et al. also recently reported that 12,336 participants with early menarche (age < 12 years) were at increased risk of MetS (OR = 1.35, 95% CI: 1.03–2.12), with higher prevalence of hypertension and diabetes in KNHANES from 2010 to 2013 [19].

Puberty is the transition to adulthood that culminates in the production of mature gametes and the initiation of reproductive activity. The process begins within the central nervous system, where gonadotropin-releasing hormone (GnRH) neurons are activated to release the neurohormone, stimulating pituitary gonadotropic hormone secretions that in turn direct gonadal steroid hormone production.

Table 4: Pathway-based analysis of interaction between early menarche and genetic variation for triglyceride in Korean women.

Pathways	Description	FDR	Significant genes	Selected genes	All genes
Type 2 diabetes mellitus	KEGG type 2 diabetes mellitus	0.016	17	33	44
Stress-activated protein kinase signaling pathway (GO: 0031098)	Stress-activated protein kinase (SAPK) cascade	0.019	14	38	49
JNK cascade (GO: 0007254)	A cascade of protein kinase activities, culminating in the phosphorylation and activation of a member of the JUN kinase subfamily of stress-activated protein kinases	0.021	14	37	47

Therefore, a younger AAM is associated with higher cumulative exposure to ovarian hormones [34]. Estrogen increases TG by promoting synthesis of TG in the liver and secreting TG into the circulation as very-low-density lipoprotein (VLDL) particles [35]. TG levels correlatively increase during pregnancy with estrogen-induced stimulated secretion of hepatic TG-rich lipoprotein [36]. However, the etiology of MetS, with regard to its association with TG, has yet to be unraveled completely with consideration of AAM.

Several studies have also demonstrated the importance of environmental triggers (including endogenous and exogenous exposure to hormone) in the development of chronic disease. Therefore, assay of gene-environmental interactions helps to understand the etiology of disease. Although several GWA studies have investigated AAM and breast cancer risk [4, 37], few GWA studies have focused on AAM and MetS with its components. Therefore, we conducted pathway analysis for investigating interaction of genetic variants and TG using GSEA. We found that T2 diabetes mellitus, stress-activated protein kinase, and JNK cascade pathways were associated with AAM on TG levels.

During puberty, there are proinflammatory and prooxidative changes and relative insulin resistance, which also play a role in the development of T2D [38, 39]. Early puberty may be causally related to lower insulin sensitivity and inflammatory changes [40]. Reductions of insulin sensitivity and compensatory hyperinsulinemia are physiological during puberty, and this partly reflects the effects of increased growth hormone and IGF-1. Recent study reported that GnRH signaling may regulate T2D using pathway enrichment analysis [41]. However, it was reported that obesity drives metabolic risk in the prepubertal population rather than premature adrenarche [42]. More detailed mechanisms of the synergistic effects of early menarche and TG require further investigation.

SAPK/JNK are members of the MAPK family and are activated by a variety of environmental stresses, inflammatory cytokines, and growth factors. GnRH can activate the JNK/SAPK, p38, and ERK5 cascades in different cell models with varying kinetics [43]. In addition, JNKs may be a central mediator of impaired glucose metabolism and insulin resistance [44]. JNKs are also activated in obesity in numerous metabolically important cells and tissues such as adipose tissue, macrophages, the liver, skeletal muscle, and regions of the brain and pituitary. Recent studies have clearly established the important roles JNK signaling fulfils in macrophages, the liver, and cells of the anterior pituitary. Macrophage TG accumulation upregulates PON2 expression via the MEK/JNK/c-Jun pathway, and these effects could be related, at least in part, to cellular TG-induced ROS formation [45]. Collectively, these studies place JNKs as important mediator of disruptions to metabolic homeostasis. In addition, 2-methoxyestradio activated SAPK/JNK in endothelial cells in a concentration-dependent manner [46]. Although several studies shed light on the associations of stress-related pathway and TG, assessing interaction of AAM is still challenging.

Our study provides some interesting results on the interplay between AAM and the genome for TG; however, some limitations need to be considered. One of them is the relatively small sample size of women with early menarche, which may provide a lack of statistical power, limiting the magnitude of interaction effects. In addition, another weakness could be the cross-sectional design of the study, with self-reported medical histories and recalled AAM. Even though AAM can be recalled by women with moderate accuracy, the use of self-reported menarcheal age may lead to some degree of recall bias [47], and recall can be influenced by the individual's current health condition [48]. Since TG might be associated with other chronic diseases such as cardiovascular disease or hyperlipidemia [49], we tried to investigate the association between TG and other chronic diseases such as hyperlipidemia and coronary artery disease (CAD) in the study participants. Based on self-reported data to chronic diseases, we could not investigate the association between TG and other chronic diseases such as hyperlipidemia and CAD due to this small sample size lack of information. Considering confounding factors on TG, we could not investigate the effect of lipid-lowering drug and estrogen use on TG due to the small size. Due to lack of relevant information, the confounding factors mentioned above are excluded from our analysis, which may limit the strength of our findings.

This is a first attempt of a genome-wide gene-AAM interaction study on MetS components in Korean women. We identified that type 2 diabetes mellitus, the SAPK signaling pathway, and JNK cascade were associated with TG including the genetic interaction with AAM. These findings may help us understand the role of AAM on the development of MetS and gene-environment interactions that confer MetS susceptibility.

Data Availability

GWAS dataset and epidemiological data for KARE project are third party data and are available under the approval of the data access committee of the National Biobank of Korea, who can be contacted at biobank@korea.kr.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Supplemental Figure S1. Risk of components of MetS in each age of menarche. ORs are adjusted by area, age, income, education, and C-reactive protein levels. Reference category is menarche at 16 years of age. Size of rectangle represents number of samples. A: high blood pressure; B: high fasting glucose; C: abdominal obesity; D: low HDL cholesterol. Supplemental Figure S2. Quantile-quantile plot of observed P values vs. expected P values (on a −log10 scale) for joint and interaction analyses of SNP and age at menarche on TG. A: interaction test; B: joint test; C: interaction test by reverse normal distribution; D: joint test by reverse normal distribution. *(Supplementary Materials)*

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Review Article

Effect of Oxidative Stress on the Estrogen-NOS-NO-K_{Ca} Channel Pathway in Uteroplacental Dysfunction: Its Implication in Pregnancy Complications

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During pregnancy, the adaptive changes in uterine circulation and the formation of the placenta are essential for the growth of the fetus and the well-being of the mother. The steroid hormone estrogen plays a pivotal role in this adaptive process. An insufficient blood supply to the placenta due to uteroplacental dysfunction has been associated with pregnancy complications including preeclampsia and intrauterine fetal growth restriction (IUGR). Oxidative stress is caused by an imbalance between free radical formation and antioxidant defense. Pregnancy itself presents a mild oxidative stress, which is exaggerated in pregnancy complications. Increasing evidence indicates that oxidative stress plays an important role in the maladaptation of uteroplacental circulation partly by impairing estrogen signaling pathways. This review is aimed at providing both an overview of our current understanding of regulation of the estrogen-NOS-NO-K_{Ca} pathway by reactive oxygen species (ROS) in uteroplacental tissues and a link between oxidative stress and uteroplacental dysfunction in pregnancy complications. A better understanding of the mechanisms will facilitate the development of novel and effective therapeutic interventions.

1. Introduction

During pregnancy, maternal circulation undergoes significant physiological changes to meet the increased metabolic demand of the growing fetus and the well-being of the mother [1]. Throughout pregnancy, cardiac output rises by increasing heart rate and stroke volume, reaching ~50% above prepregnancy baseline in the third trimester. Systemic vascular resistance decreases by ~20% in the second trimester, leading to reduced mean arterial blood pressure. In addition, blood volume increases by 40-50%. Nevertheless, marked changes also occur at the maternal-fetal interface. The placenta formation and structural and physiological remodeling of uterine arteries lead to the establishment of the low-resistance uteroplacental circulation. In human and sheep, uterine blood flow increases from 20 to 50 ml/min in nonpregnant state to ≥1000 ml/min at near-term pregnancy. Elevated steroid hormones such as 17β-estradiol ($E_2\beta$) and progesterone are believed to play an important role in the cardiovascular adaptation during pregnancy [2–4].

Aberrant uteroplacental adaptation leads to pregnancy complications such as preeclampsia and intrauterine (fetal) growth restriction (IUGR). These complications are associated with diminished uteroplacental blood flow [5, 6]. Both preeclampsia and IUGR are major causes of maternal and/or fetal morbidity and mortality. Accumulating evidence suggests that preeclampsia and IUGR also have detrimental effects on the health of both the mother beyond pregnancy and offspring. Women with a history of preeclampsia have increased risk of cardiovascular disease [7]. Moreover, offspring born from preeclamptic pregnancy also have high incidence of high blood pressure and stroke later in life [8, 9]. Similarly, IUGR is associated with increased prevalence of metabolic syndrome, diabetes, and cardiovascular disease in later life of offspring [10, 11].

Although the etiologies of preeclampsia and IUGR are not fully elucidated, placental insufficiency (or uteroplacental

vascular insufficiency), the inability to deliver an adequate supply of oxygen and nutrients to the fetus due to reduced blood flow to the placenta, is generally considered as a major contributor to the development of these disorders. Soleymanlou et al. revealed a remarkable similarity of global gene expression in hypoxia-treated placenta explants, high-altitude placentas, and preeclamptic placentas [12], implying an important causative role of hypoxia in these complications. This notion is further substantiated by observations in animal models in which gestational hypoxia imitated placental insufficiency, reduced fetal growth, and induced preeclampsia-like symptoms [13–15].

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants [16]. Prolonged hypoxia is shown to elicit oxidative stress [17]. Consistently, placental insufficiency also promotes oxidative stress in preeclampsia, IUGR, and high-altitude pregnancy [18, 19]. Accumulating evidence suggests a critical role of reactive oxygen species (ROS) in the pathogenesis of pregnancy complications [20, 21]. However, the mechanistic insights into ROS-induced maladaptation of uteroplacental circulation remain largely elusive. In this article, we provide a succinct review of effects of oxidative stress on $E_2\beta$ signaling pathways in the uteroplacental circulation in pregnancy complications.

2. $E_2\beta$ Signaling and Uteroplacental Circulation in Physiological and Pathophysiological Conditions

2.1. Estrogen and Estrogen Receptors (ERs) in Normal Pregnancy and Pregnancy Complications. Both $E_2\beta$ and its metabolites are essential for the success of pregnancy. Starting from approximately week 9 of gestation, the placenta becomes the primary site of estrogen synthesis involving enzymes such as aromatase (CYP19) and hydroxysteroid 17β-dehydrogenases 1 (HSD17B1, 17β-HSD1) [22]. Circulating estrogen rises progressively throughout pregnancy, and plasma 17β-estradiol (E2β) level at term is ~100-fold higher than that in nonpregnant subjects. Similarly, E2β metabolites produced by cytochrome P450s and catechol-O-methyltransferase (COMT) such as catecholestradiols also elevated during pregnancy [23]. However, estrogen biosynthesis and metabolism are apparently impaired in pregnancy complications. Maternal plasma E2β levels are significantly lower in preeclamptic [24–26] and IUGR [27] pregnancies. Low circulating E2β was also observed in high-altitude human and sheep pregnancy [28–30], although one study showed an increase in plasma estrogen [31]. The metabolism of E2β is also impaired in preeclampsia, leading to reduced 2-methoxyestrone and 2-methoxyestradiol [25, 32]. It appears that the reduced circulating levels of E2β and its metabolites in pregnancy complications are the result of dysregulation of steroidogenic enzyme expression in the placenta. Preeclamptic placenta displayed deficiency of aromatase, HSD17B1, and COMT [24, 25, 32–34]. The impaired estrogen steroidogenesis and metabolism in these disorders are evidently caused by placental insufficiency. Aromatase in cultured human trophoblast cells and in trophoblast cell line JEG-3 was downregulated by hypoxia [24, 35], and the expression of placental aromatase was reduced in a rabbit model of placental ischemia [24]. Aberrant production of E2β and its metabolites could contribute to the pathogenesis of pregnancy complications due to their key roles in regulating trophoblast invasion, angiogenesis, and uterine vascular tone, which will be discussed in later sections.

Estrogen produces its plethoric effects *via* interacting with its receptors involving both nongenomic and genomic mechanisms. To elicit genomic actions, estrogen binds to the nuclear estrogen receptor α (ERα) or estrogen receptor β (ERβ). The receptors become dimerized and bind to the estrogen response element (ERE) located in the target gene promoter, triggering or suppressing gene expression [36]. Estrogen can also activate membrane G-protein-coupled estrogen receptor (GPER, or GPR30) and membrane-associated ERα and ERβ, which in turn stimulate adenylate cyclase to generate cAMP or activate kinases such as tyrosine kinase Src, phosphoinositide 3-kinase (PI3K), extracellular-signal-regulated kinase (ERK), and protein kinase B (PKB or AKT) [37]. Activation of membrane or membrane-associated estrogen receptors can lead both acute and long-term effects. The presence of ERα, ERβ, and GPER in uterine arteries and the placenta has been demonstrated by real-time polymerase chain reaction (PCR), Western blot, and immunohistochemistry [38–41]. The expression of all forms of estrogen receptors in uterine arteries and the placenta increases as pregnancy advances [38–40, 42]. The maintenance or upregulation of ERs in the uteroplacental tissues apparently requires continuous estrogen stimulation. Ovariectomy in sheep reduced ERβ expression in the endothelium of uterine arteries [42]. In addition, chronic treatment with E2β *in vivo* and ex vivo significantly increased ERα expression in uterine arteries [40, 42]. The expression of GPER in HTR8/SVneo cells derived from first trimester extravillous trophoblast and placental extravillous explants was also upregulated by E2β [43].

Information on estrogen receptor expression in pregnancy complications is scant, and conflicting observations have been reported. ERα expression was described as increased, decreased, or unchanged in the preeclamptic placenta [44–46]. No conclusion could be drawn currently, and more rigorous studies are needed to clarify the discrepancy. The expression of ERα in uteroplacental tissues was suppressed in high-altitude pregnancy [40], and hypoxia appeared to be the causative factor responsible for ERα downregulation [45, 47]. Defective expression of ERα could have profound effects on uteroplacental function including gene expression. Intriguingly, the placental expression of ERβ appears to be differently affected in preeclampsia and IUGR. Whereas ERβ expression was reduced in the IUGR placenta [44], an upregulation of ERβ was observed in preeclamptic placentas [44, 45]. These observations suggest that the etiologies of preeclampsia and IUGR may differ. It remains to be determined whether/how the distinct regulations of ERβ contribute to the pathogenesis of these two complications. The placental expression of GPER was reduced in preeclamptic pregnancy [43, 48], which may lead to dysfunction of uteroplacental vessels.

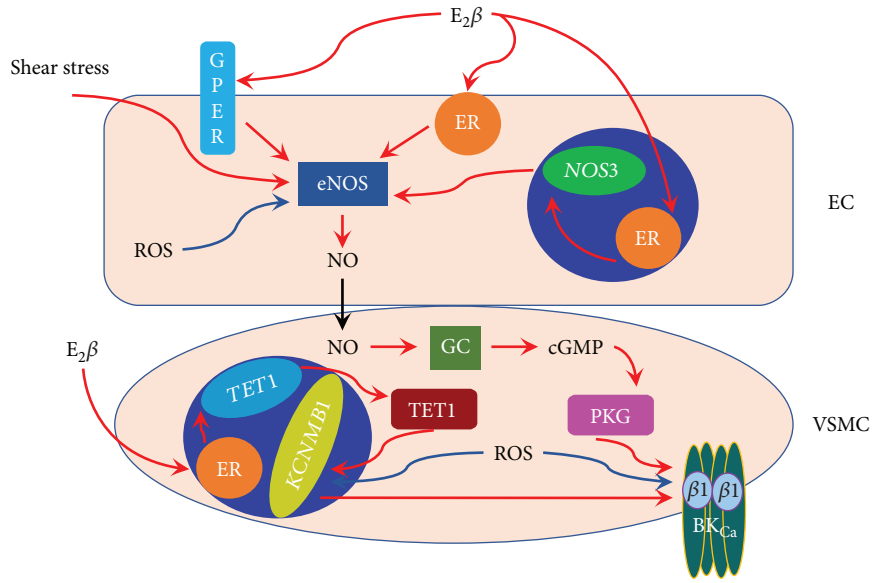

Figure 1: Estrogen ($E_2\beta$) regulates uterine artery function partly *via* its actions on endothelial nitric oxide synthase (eNOS) in the endothelial cell (EC) and the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel in vascular smooth muscle cell (VSMC) during pregnancy. Shear stress stimulates eNOS activity, leading to increased NO production. $E_2\beta$ could increase the expression of eNOS in ECs via interacting with nuclear estrogen receptors (ERs) and/or elevate eNOS activity via interacting with the G protein-coupled estrogen receptor (GPER, GPR30) or membrane-associated ERα and ERβ. In addition, $E_2\beta$ increases the expression of the BK_{Ca} channel $\beta1$ subunit encoded by *KCNMB1* and channel activity via upregulating ten-eleven translocation methylcytosine dioxygenase 1 (TET1, encoded by *TET1*) in VSMCs. Moreover, the activity of the BK_{Ca} channel can be enhanced by NO-PKG signaling. In pregnancy complications, excessive oxygen species (ROS) impair the estrogen-NOS-NO-BK_{Ca} channel pathway.

2.2. Estrogen and the Regulation of Uteroplacental Circulation. Several lines of evidence have implicated a critical role of estrogen in the adaptation of the uteroplacental circulation. First, the high ratio of E2β to progesterone in the follicular phase was associated with increased blood to the uterus [49, 50]. Second, reduced uterine vascular resistance and increased uterine blood flow concurred with progressively rising plasma E2β levels during pregnancy [51–53]. Third, acute treatment with exogenous E2β markedly increased uterine blood flow and/or reduced uterine vascular resistance in nonpregnant animals [54–56]. Fourth, chronic administration of E2β into nonpregnant sheep also significantly increased uterine blood flow and/or reduced uterine vascular resistance [57, 58]. *Ex vivo* treatment of uterine arteries from nonpregnant sheep with E2β reduced uterine arterial myogenic tone [59]. The chronic effects of E2β simulated pregnancy-induced hemodynamic changes in the uterine circulation. Fifth, the nonselective ERα/ERβ antagonist ICI 182,780 reduced the increase in uterine blood flow induced by exogenous E2β in nonpregnant sheep and by endogenous E2β in the follicular phase of nonpregnant sheep by ~60% [53]. Intriguingly, the same antagonist also lowered basal uterine blood flow in pregnant sheep by 37% [53]. Importantly, $E_2\beta$ and its metabolites also play an important role in uteroplacental adaptation. $E_2\beta$, 2-hydroxyestradiol, 4-hydroxyestradiol, and 4-methoxyestradiol were implicated in angiogenesis by promoting endothelial cell proliferation [60], whereas 2-methoxyestradiol promoted the differentiation of the cytotrophoblast to an invasive phenotype [61].

2.3. NO and Ca^{2+}-Activated K^+(K_{Ca}) Channels in Regulating Uteroplacental Function. Nitric oxide (NO) is a gaseous messenger-generated nitric oxide synthase (NOS). NO contributes to the maintenance of cardiovascular homeostasis by regulating vasocontractility [62]. The large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel is primarily expressed in vascular smooth muscle cells (VSMCs) and plays a pivotal role in regulating myogenic tone [63]. In VSMCs, the BK_{Ca} channel is a heteromeric assembly of the pore-forming α subunit and accessary $\beta1$ subunits [64]. The $\beta1$ subunit encoded by *KCNMB1* increases the channel's Ca^{2+}/voltage sensitivity. Importantly, the BK_{Ca} channel is one of many targets of NO in the cardiovascular system [64]. Not surprisingly, the NO-cGMP-PKG-BK_{Ca} channel axis is implicated in the adaptation of uteroplacental circulation during pregnancy [65] (Figure 1).

Activation of either of ERα, ERβ, or GPER induced acute vasorelaxation of uterine arteries [66]. The acute estrogen effects in regulating uterine hemodynamics involved stimulation of endothelial NOS (eNOS) activity and increased NO release in endothelial cells (ECs) [38, 67] and activation of BK_{Ca} channels in VSMCs [67]. Stimulation of eNOS activity by estrogen in uterine arterial ECs required phosphorylation of the enzyme at serine 635 and serine 1177 mediated by ERα [68]. E2β could also directly activate BK_{Ca} channels in uterine arterial VSMCs [67], possibly *via* interacting with the accessory $\beta1$ subunit [69].

E2β could also exert its genomic effect to regulate the expression of both NOS and BK_{Ca} channels in uteroplacental tissues. Expression and function of eNOS [70–72] and the

BK_{Ca} channel β1 subunit [65, 73, 74] in uterine arteries were increased in the follicular phase and during pregnancy. The upregulation of eNOS and the BK_{Ca} channel β1 subunit in uteroplacental circulation during these two physiological states was apparently stimulated by estrogen as chronic treatment with exogenous E2β in intact nonpregnant animals [58, 75, 76] and in ex vivo cultured uterine arteries [73] elevated their abundance and activity.

In vivo studies revealed distinct contributions of eNOS and the BK_{Ca} channel to basal uterine blood flow in nonpregnant and pregnant sheep. Intrauterine arterial infusion of the NO synthase inhibitor L-nitro-arginine methyl ester (L-NAME) demonstrated minimal contribution of NO to basal uterine blood flow in both nonpregnant and pregnant sheep [77]. However, infusion of the BK_{Ca} channel blocker tetraethylammonium into uterine arteries revealed that at least half of the basal uterine blood flow is maintained by the BK_{Ca} channel in pregnant sheep, whereas the channel did not contribute to basal uterine blood flow in nonpregnant animals [67, 78]. These findings are reinforced by the observations that uterine arterial myogenic tone (i.e., the major constituent of vascular tone) of pregnant subjects was regulated by the BK_{Ca} channel [73], but not by the endothelium [79, 80]. Thus, estrogen-induced eNOS expression and activity during pregnancy are probably responsible for enhanced endothelium-dependent vasorelaxation in uterine arteries in response to given vasodilators [81, 82] and uterine artery remodeling [83], but not for regulating basal uterine vascular tone. In contrast, the upregulation of the BK_{Ca} channel is essential for the reduced uterine vascular tone during pregnancy. In addition, the upregulated BK_{Ca} channel also contributed to blunted vasoconstrictor responses in uterine arteries during pregnancy [65, 84]. Thus, the BK_{Ca} channel in uteroplacental circulation functions as a negative feedback control mechanism to prevent excessive vasoconstriction. Together, these findings reinforced the notion that E2β, through its acute and chronic actions on eNOS and BK_{Ca} channels, plays a pivotal role in uteroplacental adaptation.

Expression/activity of placental eNOS in preeclamptic and IUGR pregnancies was reported as either unaltered [85, 86], decreased [44, 87], or increased [88, 89]. Whereas eNOS in placental chorionic plate arteries was downregulated in preeclampsia [90], this enzyme in uterine arteries was upregulated in high-altitude pregnancy [91]. Regardless of uteroplacental eNOS expression status, NO bioavailability in pregnancy complications appeared to be reduced due to substrate deficiency and enzyme inhibition. Both plasma and placental L-arginine levels were reduced in preeclampsia [86, 92]. In addition, the expression of arginase-2, which consumes eNOS's substrate L-arginine, was increased in the placenta and in omental vessels of women with preeclampsia [86, 93]. The increased arginase-2 expression could be imitated by treating human umbilical vein endothelial cells (HUVECs) with preeclamptic plasma [93]. Moreover, HUVECs from IUGR pregnancy also displayed increased arginase-2 expression and activity and placental vessels exhibited impaired eNOS-dependent relaxation [89]. A deficiency of L-arginine would not only reduced eNOS-derived NO but also increase eNOS-mediated superoxide production leading to peroxynitrite ($ONOO^-$) formation, evidenced by increased nitrotyrosine staining in villi and maternal vasculature of preeclamptic women [86, 94]. Similarly, nitrotyrosine staining was increased in the syncytiotrophoblast and extravillous trophoblast of high-altitude placenta [95]. Intriguingly, the circulating level of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, also increased in preeclamptic and IUGR pregnancies [96, 97]. Not surprisingly, NOS-dependent relaxation of placental chorionic arteries from IUGR pregnancy was impaired [98]. Moreover, both chronic blockade of NOS with L-NAME or knockout of eNOS in rodents increased maternal blood pressure and reduced fetal growth [99, 100], partly due to impaired uteroplacental vessel remodeling [101].

The expression and function of the BK_{Ca} channel in uteroplacental vessels are also impaired in pregnancy complications. It appears that the β1 subunit of the channel is selectively targeted, whereas the α subunit remains unaffected in these disorders. The BK_{Ca} channel β1 subunit was downregulated in placental chorionic plate arteries in preeclampsia [102] and uterine arteries in high-altitude pregnancy [103]. High-altitude pregnancy also suppressed the ability of estrogen to upregulate the expression of the BK_{Ca} channel β1 subunit in uterine arteries [103], leading to increased uterine arterial myogenic tone. BK_{Ca} channel-mediated vasorelaxation was also reduced in both pathological conditions. The impact of high-altitude pregnancy on the BK_{Ca} channel was simulated by ex vivo hypoxia [104], implicating a causative role of hypoxia in the downregulation of the BK_{Ca} channel β1 subunit. In a preeclampsia-like murine model induced by autoantibodies against angiotensin II type 1 receptor (AT1-AA), the expression of the BK_{Ca} channel β1 subunit and channel activity in mesenteric arteries was also reduced [105].

The intermediate-conductance (IKs) and small-conductance (SKs) Ca^{2+}-activated K^+ channels are predominantly expressed in ECs and also mediate endothelium-dependent vasodilation [106]. The endothelium-derived hyperpolarizing factor (EDHF) causes hyperpolarization of VSMCs by activating IKs and SKs. Both IKs and SKs are expressed in uteroplacental tissues [90, 107, 108]. IKs and SKs are also expressed in VSMCs of uterine and placental chorionic plate arteries in addition to their expression in ECs [90, 107]. In the uteroplacental system, IKs and SKs participated in the regulation of contractility of uterine and placental vessels [90, 107, 109]. Moreover, SK3 was also involved in regulating uterine vascular remodeling and placental vascularization [110, 111]. Like BK_{Ca} channels, $E_2\beta$ is required to maintain and to upregulate the expression and function of SKs in vasculature. Pregnancy *via* estrogen's action upregulated the expression of SK2 and SK3 in uterine arteries [107]. Ovariectomy reduced SK3 activity in ECs and ablated the channel's role in EDHF-mediated vasorelaxation in non-uterine arteries [112].

The expression and function of IK1, SK2, or SK3 in uteroplacental vessels and umbilical vessels were downregulated in high-altitude pregnancy and preeclampsia [90, 107, 113] as well as in a rat model of preeclampsia induced by testosterone [108]. Given the important role of estrogen in the

regulation of IKs and SKs in uteroplacental circulation, it is anticipated that impaired $E_2\beta$-ER signaling could contribute to the downregulation of these ion channels in high-altitude and preeclamptic pregnancies.

Together, evidence presented in this section demonstrated critical roles of both estrogen synthesis and metabolism in the adaptation of uteroplacental circulation. Preeminently, $E_2\beta$ and its metabolites contribute to this adaptive process by promoting angiogenesis, trophoblast invasion, and remodeling and by lowering uterine vascular tone through upregulating activity and/or expression of both eNOS and K_{Ca} channels. However, the $E_2\beta$-NOS-NO-K_{Ca} channel pathway is disrupted in pregnancy complications, which could contribute to the pathogenesis of these disorders.

3. Oxidative Stress and Pregnancy Complications

3.1. Cellular Sources of ROS and Antioxidant Defense. ROS are oxidants formed during oxygen metabolism, primarily produced during oxidative phosphorylation in the mitochondria and by oxidases such as NADPH oxidases (NOXs) and xanthine oxidase (XO) as well as uncoupled NOS [114, 115]. ROS include free radicals such as superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) and nonradical hydrogen peroxide (H_2O_2). In order to maintain redox hemostasis, mammalian cells have developed enzymatic and nonenzymatic defense mechanisms to balance the oxidative state. The major antioxidant enzymes involved in detoxifying ROS include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and peroxiredoxin (Prx) [116]. Nonenzymatic antioxidants include metabolic products such as glutathione (GSH), uric acid, and melatonin [117, 118].

ROS at low levels can act as intracellular second messengers to modulate cellular responses. The very short lifetime and diffusion distance of $O_2^{\cdot-}$ and $^{\cdot}OH$ make them unsuitable to function as signaling molecules. In contrast, H_2O_2 mediates reversible oxidation of cysteine residues in proteins, which can alter protein activities and functions [119]. These proteins include enzymes (i.e., mitogen-activated protein kinases (MAPKs), tyrosine kinases, and protein tyrosine phosphatases) and transcription factors (i.e., activator protein-1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and hypoxia-inducible factor 1 (HIF-1)). When ROS production overwhelms the intrinsic antioxidant defense, due to either increased ROS formation or reduced ability to neutralize ROS or both, oxidative stress arises. As a consequence, ROS attack cellular components, leading to potential cell/tissue damage.

3.2. Normal Pregnancy Is a Mild State of Oxidative Stress. The metabolic activity of the placenta is high in order to meet the growth of both the placenta and the fetus, leading to increased ROS production during normal pregnancy. It has long been proposed that pregnancy is a state of oxidative stress [120, 121]. This notion is supported by the following observations: (1) increased levels of superoxide $O_2^{\cdot-}$, 8-iso-prostaglandin $F_2\alpha$ (8-iso-$PGF_2\alpha$), and malondialdehyde (MDA) in the circulation and the placenta [122–124]; (2) reduced circulating expression and activity of enzymatic antioxidants such as SOD, GPx, and catalase [123, 125]; and (3) decreased levels of nonenzymatic antioxidants including uric acid, vitamin C, vitamin E, and GSH [123, 126]. Notably, the increased ROS production in early pregnancy plays an important role in trophoblast proliferation, differentiation, invasion, and angiogenesis [127, 128]. As gestation advances, placental SODs and catalase as well as total antioxidant capacity also increase [129, 130], which counters the increased ROS generation. Thus, a relatively physiological balance between oxidants and antioxidants is maintained in normal pregnancy.

3.3. Pregnancy Complications Are Associated with Heightened Oxidative Stress. Both acute and chronic hypoxia has been shown to elevate ROS [17, 131]. Mitochondria and NOXs are the major sources of ROS in response to oxygen deprivation [132–134]. Placental insufficiency is believed to be a critical element in the pathogenesis of preeclampsia and IUGR. Not surprisingly, these disorders display heightened oxidative stress compared to normal pregnancy. Apparently, both overproduction of ROS and reduction of antioxidant defense contribute to the heightened oxidative stress in pregnancy complications. The activity and expression of oxidant enzymes such as NOXs and XO increased in the preeclamptic placenta and/or circulation [135–137]. In contrast, levels and activity of circulating and placental antioxidant enzymes such as SOD, catalase, and GPx as well as thioredoxin (Trx) were decreased in preeclamptic and IUGR pregnancies [135, 138, 139]. Similarly, activities of SOD, GPx, and Trx reductase (TrxR) were reduced in placentas from high-altitude pregnancy [95]. Moreover, mitochondria in the placenta became dysfunctional in pregnancy complication. Mitochondria appear to be damaged as evidenced by swelling and broken cristae in the preeclamptic placenta [140]. Respiratory chain enzyme expression and activity of mitochondrial complexes were suppressed in preeclamptic and IUGR placentas as well as in placentas from high-altitude pregnancy [140–142], uncoupling respiration from oxidative phosphorylation. Furthermore, circulating and placental nonenzymatic antioxidants including GSH, vitamin C, and melatonin were lower in preeclampsia and IUGR [139, 143, 144]. Concomitantly, both complications exhibited higher ROS [124, 145, 146] and oxidative stress markers [147–149] in the circulation and the placenta, leading to lipid peroxidation and oxidative DNA damage [144, 149]. Increased nitrotyrosine immunostaining was observed in villous vessels of the placenta [94, 135] and systemic vessels [150] in preeclamptic pregnancy, suggesting that preeclampsia promotes NOS uncoupling and $OONO^-$ generation.

3.4. Animal Models Replicate Oxidative Stress in Pregnancy Complications. The elevated oxidative stress in preeclampsia and IUGR has been imitated in animal models. Increased placental $O_2^{\cdot-}$ was observed in an $eNOS^{-/-}$ mouse model of fetal growth restriction [151]. The reduced uterine perfusion pressure model reduced levels of SODs and GPx, increased levels of MDA, and decreased mitochondrial complexes I and II expression [152–154] in rat placentas. Rodent models

of preeclampsia and/or IUGR also promoted eNOS uncoupling in the aorta and placenta [14, 155] and decreased placental GSH content [156]. Therefore, similar to human pregnancy complications, an imbalance between oxidant and antioxidant systems apparently accounts for the heightened oxidative stress in these animal models and hypoxia appeared to be a major cause of the heightened oxidative stress in these models. Increased uterine arterial ROS generation was detected in a sheep model of high-altitude pregnancy due to increased NOX2 expression, which could be replicated by ex vivo hypoxic treatment of uterine arteries [157]. Naïve high-altitude pregnant sheep exhibited higher circulating MDA than low-altitude pregnant sheep and native high-altitude pregnant sheep [30]. In addition, gestational hypoxia increased levels of 4-hydroxynonenal (4-HNE), a lipid peroxidation product, in rat placentas [158].

In conclusion, oxidative stress is an inherent feature of normal pregnancy and plays an important role in the development of the placenta. The uteroplacental system is particularly vulnerable to oxidative stress. When unchecked, oxidative stress becomes augmented and could give rise to pathological conditions such as preeclampsia and IUGA, harming both the mother and the fetus. Therefore, oxidative can play both physiological and pathological roles in the progression and outcome of pregnancy.

4. Regulation of $E_2\beta$ Production and $E_2\beta$ Signaling Pathway by ROS in Pregnancy Complications

As aforementioned, $E_2\beta$ is an essential element in the adaptation of the uteroplacental circulation during pregnancy. Given heightened oxidative stress in preeclampsia, IUGR and high-altitude pregnancy, and diverse effects of ROS on macromolecules, it is not surprising that excessive ROS plays a critical role in the pathogenesis of these complications by disrupting the $E_2\beta$ signaling pathway. ROS could directly or indirectly exert their detrimental effects on the targets, and their actions could be acute or chronic. Unfortunately, there is limited information regarding the impacts of ROS on the $E_2\beta$ signaling pathway in uteroplacental circulation under pathophysiological conditions. In this section, findings from both uteroplacental and nonuteroplacental tissues/cells will be discussed.

4.1. ROS and Estrogen Synthesis. Aromatase and HSD17B1, two key enzymes in estrogen biosynthesis catalyze the interconversion between testosterone and E2β and between estrone and E2β, respectively, using cofactors NADPH [159, 160]. In fact, NADPH is a key component against cellular oxidation. Maintaining an adequate NADPH/$NADP^+$ ratio is essential to activities of these enzymes and E2β generation. In HUVECs, high glucose elevated ROS [161] but reduced the NADPH level [162]. Lowering the NADPH/$NADP^+$ ratio markedly reduced the conversion of estrone to E2β in HEK293 cells [163]. Interestingly, the reduced E2β level in preeclamptic placental explants was mimicked by the treatment of placental explants from normal pregnancy with H_2O_2 [164]. Moreover, H_2O_2 treatment of the homogenate of the human ovary suppressed aromatase activity, which could be prevented by GPx [165]. These observations suggest that oxidative stress could impair estrogen synthesis by suppressing key enzyme activities.

4.2. ROS and Estrogen Receptor Expression. ERα expression is also subject to ROS modulation. In general, the expression of ERα is negatively regulated by ROS. The following observations were made in cancer cell lines. In MCF-7 cells, a brief treatment with glucose oxidase, which catalyzes the oxidation of glucose to H_2O_2 and D-glucono-δ-lactone, resulted in marked ERα level reduction 24 hours after the treatment [166]. Chronic (16 hours) H_2O_2 treatment of ZR-75-1 cells also decreased ERα protein level [167]. The detrimental effect of H_2O_2 on ERα expression could be normalized by increasing antioxidant capacity. Overexpression of Prx-1, a H_2O_2 scavenger, ablated H_2O_2-induced downregulation of ERα, whereas inhibition of Prx-1/2 activity with adenanthin promoted ERα downregulation [167].

4.3. ROS and NO Production. NOS are also regulated by ROS. ROS affect NO production apparently through altering eNOS expression/activity and eNOS cofactors. In HUVECs, H_2O_2 treatment for 2 hours increased eNOS phosphorylation of serine 1177 and enzyme activity, whereas catalase did the opposite [168]. However, H_2O_2 was found to decrease NO bioavailability in porcine aortic ECs by inactivation of eNOS cofactors without altering enzyme activity [169]. Long-term treatment with H_2O_2 or superoxide treatment resulted in downregulation of eNOS in HUVECs [170, 171]. NOXs appeared to be major sources of ROS responsible for eNOS downregulation. HUVECs from women with preeclampsia exhibited NOX2 upregulation and eNOS downregulation [113]. In addition, the upregulation of NOX4 by angiotensin II and high glucose promoted eNOS uncoupling, leading to increased generation of $O_2^{\cdot-}$ and $OONO^-$ in glomerular mesangial cells [172, 173]. Thus, it is expected that inhibiting oxidant generation or enhancing antioxidant defense could potentially normalize the adverse effect of ROS on eNOS. As expected, eNOS expression was partially rescued or restored by NOX inhibitor apocynin or overexpression of SOD2 [113, 174]. Administration of the GSH synthase inhibitor buthionine sulfoximine into rats decreased total GSH level in the liver, reduced urinary excretion of NOx, and increased nitrotyrosine staining in the kidney without altering renal eNOS level [175].

4.4. ROS and K_{Ca} Channels. ROS display complex actions toward the BK_{Ca} channel. H_2O_2 could be stimulatory or inhibitory on BK_{Ca} channel activity depending on experimental conditions. H_2O_2 increased BK_{Ca} channel activity in human and porcine artery VSMCs and HUVECs [176–178], whereas it decreased BK_{Ca} channel-mediated currents in porcine renal artery ECs and vascular smooth muscle-type BK_{Ca} channel reconstituted in HEK293 cells [179]. A study by Tang et al. revealed that both cysteine and methionine residues of the BK_{Ca} channel were subject to redox modulation [180]. Interestingly, oxidation of cysteine and methionine produced opposite regulations of BK_{Ca} channel activity.

Whereas cysteine oxidation decreased BK_{Ca} channel currents, methionine oxidation increased channel activity. Moreover, oxidation of a cysteine residue near the Ca^{2+} bowl of the BK_{Ca} channel α subunit by H_2O_2 almost abolished physiological activation of the channel [181]. It is likely that distinct actions of H_2O_2 on the BK_{Ca} channel resulted from selectively targeting cysteine and methionine residues. Whereas $O_2^{\cdot-}$ did not alter currents mediated by the BK_{Ca} channel, $ONOO^-$ exhibited an inhibitory effect on BK_{Ca} channel activity [182, 183]. It appears that the BK_{Ca} channel in uterine artery VSMCs of high-altitude pregnant sheep is under tonic inhibition by ROS. An acute application of antioxidants such as N-acetylcysteine (NAC), the NOX inhibitor apocynin, and the synthetic SOD/catalase mimetic EUK-134 partially reversed gestational hypoxia-induced suppression of BK_{Ca} channel-mediated currents and vasorelaxation [104, 184]. As NOX2 was upregulated in gestational hypoxia, the superoxide generated by this enzyme and its dismutation product H_2O_2 probably contributed to the gestational hypoxia-induced suppression of BK_{Ca} channel activity/-function in uterine arteries [157]. IK channel-mediated currents in HUVECs were also inhibited by the superoxide donors, xanthine/xanthine oxidase (X/XO) mixture [185].

In addition to direct modulation of K_{Ca} channel activity, ROS also exert a significant impact on the expression of K_{Ca} channels. High-altitude pregnancy increased uterine vascular tone owing to NOX2 overexpression and *KCNMB1* downregulation as well as decreased BK_{Ca} channel activity [103, 157]. These detrimental effects could be simulated by ex vivo hypoxic treatment of uterine arteries of low-altitude pregnancy [104]. A cause-and-effect relationship was established by the observation that antioxidants apocynin and NAC largely eliminated gestational hypoxia-induced reduction of *KCNMB1* expression and channel activity [104, 157]. In addition, estrogen-induced upregulation of the BK_{Ca} channel β1 subunit and channel activity in uterine arteries was eradicated by gestational hypoxia, which was restored by NAC in ex vivo experiments [104, 184]. Similarly, preeclampsia reduced the expression of *KCNMB1* along with upregulation of NOX2 and superoxide in HUVECs [113]. Importantly, the *KCNMB1* downregulation was partially rescued by treating cultured HUVECs with apocynin [113]. The *KCNMB1* downregulation appeared to be directly induced by ROS. Exposure of the cultured human coronary artery VSMCs to H_2O_2 for 12 hours led to reduced *KCNMB1* expression [186]. These observations signal a contributing role of ROS in the dysfunction of the BK_{Ca} channel in uteroplacental circulation. Targeting *KCNMB1* expression by ROS is also observed in diabetes. The BK_{Ca} channel β1 subunit protein level was downregulated in diabetic mouse aorta, which was accompanied by increased expression of NOX1 and NOX4, decreased expression of SOD and catalase, and elevated $O_2^{\cdot-}$ generation [186].

The expression of SK and IK channels is also regulated by ROS in pregnancy complications. Pregnancy/estrogen--induced upregulation of SK2 ($K_{Ca}2.2$) and SK3 ($K_{Ca}2.3$) channel expression/activity in ovine uterine arteries was diminished at high altitude [107], and a causative role of ROS was evidenced by the reversal of gestational hypoxia-induced detrimental effects with NAC [184]. Treatment of human uterine microvascular ECs with serum from preeclamptic women also reduced SK3 and IK1 expression, which was reversed by silencing NOX4 with siRNA or treatment with a membrane-permeable SOD [187]. The reduced expression of SK3 and IK1 ($K_{Ca}3.1$) in the placenta, umbilical vessels, and HUVECs was also associated with the upregulation of NOX2 or NOX4 and heightened oxidative stress in preeclamptic pregnancy [113, 187, 188]. The contributing role of ROS to the downregulation of SK_{Ca} and IK_{Ca} channels was substantiated based on the following findings: (1) restoration of channel expression by antioxidants such as apocynin, tempol, and tiron and (2) simulation of the downregulation by oxidants such as superoxide generated by exogenous X/XO mixture and H_2O_2 [113, 188, 189].

Overwhelming evidence suggests that the $E_2\beta$-NOS--NO-K_{Ca} channel pathway in uteroplacental tissue is a target of oxidative stress in pregnancy complications. Overall, excessive ROS inhibited $E_2\beta$ synthesis and estrogen receptor expression. In addition, NOS and K_{Ca} channel expression/activity could also be suppressed by oxidative stress, leading to reduced NO bioavailability and impaired K_{Ca} functions.

5. The Interplay among Hypoxia, ROS, and Epigenetic Modifications in Pregnancy Complications

Although it is now well-recognized that placental insufficiency and oxidative stress are important contributors to the pathogenesis of preeclampsia and IUGR, the mechanisms underlying their actions in these complications are not fully resolved. Recent studies have identified epigenetic modifications as important mechanisms underlying various human diseases [190]. In this section, we will try to establish a link among hypoxia, ROS, and epigenome in preeclampsia and IUGR.

5.1. ROS in O_2 Sensing. HIFs are transcription factors and function as master regulators of cellular responses to hypoxia. HIFs are heterodimers composed of a HIF-α subunit (HIF-1α and HIF-2α) and a constitutively expressed HIF-1β subunit. Under normoxia, HIF-α subunits are hydroxylated on proline residues by the O_2-dependent prolyl hydroxylases (PHDs), resulting in ubiquitination and successive proteasomal degradation by the von Hippel–Lindau protein (pVHL) E3-ubiquitin ligase. In hypoxia, PHD activity is suppressed. Subsequently, HIF-α is accumulated, translocated into the nucleus, and dimerized with HIF-1β, leading to gene expression by binding to hypoxia-responsive element (HRE) in the promoter of the target gene. Interestingly, ROS appear to participate in cellular oxygen sensing and hypoxic activation of HIFs. ROS generated by mitochondrial complex III in response to hypoxia were found to stabilize HIF-1α [132, 191]. The stabilization of HIF-1α was mimicked by exogenous H_2O_2 and by genetic suppression of SOD2 under normoxia [191, 192]. However, HIF-1α stabilization was attenuated by silencing Rieske iron-sulfur protein of complex III and by enzymatic and nonenzymatic antioxidants

[193–195]. ROS produced by NOXs could also led to accumulation of HIF-1α [196, 197] and HIF-2α [198, 199]. ROS stabilized HIF-α apparently through suppressing the ability of the PHDs to hydroxylate HIF-α protein [200]. ROS-mediated stabilization of HIFs thus constitutes an important mechanism for hypoxia to stimulate gene expression.

5.2. Crosstalk between ROS and Epigenome. Whereas genome confers genetic information for making and maintaining an organism, the epigenome describes all the chemical modifications to DNA and histone proteins. Epigenetic modifications of the genome determine how the information in genes is expressed by switching genes on and off without altering the DNA sequence. The major mechanisms of the epigenetic modification include DNA methylation, histone modifications, and noncoding-RNA-based silencing [201]. Several lines of evidence suggest existence of a crosstalk between ROS and epigenetic modifications. ROS are found to promote DNA hypermethylation by altering DNA methylation/-demethylation machineries and enzyme recruitment. In vitro studies demonstrated that H_2O_2 treatment increased expression/activity of DNA methyltransferases (DNMTs) [202–204], although many of these studies were conducted in cancer cell lines. In addition, H_2O_2 could facilitate DNA methylation by recruiting DNMT1 to the CpG sites in gene promoters [203, 205]. The linking of ROS induced by hypoxia and other stimuli to DNA hypermethylation was further confirmed by findings that antioxidants such as NAC and apocynin were able to prevent both ROS-induced global methylation or specific gene methylation [202, 206, 207] and upregulation of DNMTs [202]. ROS could also impair DNA demethylation. In a cell-free system, H_2O_2 suppressed enzymatic activity of ten-eleven translocation (TET) dioxygenase [208]. The catalytic activity of TETs requires vitamin C and Fe^{2+} as cofactors [209, 210]. To maintain an active dioxygenase enzyme, vitamin C is required to reduce Fe^{3+} to Fe^{2+}. Thus, vitamin C depletion in pregnancy complications [144, 211] would reduce TET activity. Histone modifications are also subject to ROS regulation. It is found that increasing oxidative stress by H_2O_2 upregulated histone deacetylase 1 (HDAC1) in cancer cell lines [204]. Prolonged treatment with H_2O_2 also increased global histone methylation marks H3K4me3 and H3K27me3 in human bronchial epithelial cells [208]. It appears that ROS produced from both mitochondria and NOX promotes microRNA-210 (miR-210) generation. Whereas Nox4 siRNA partially decreased hypoxia-induced miR-210 expression, mitochondrial complexes I and III inhibitors rotenone and antimycin increased miR-210 biogenesis in adipose-derived stem cells [212].

Conversely, ROS production could be altered by epigenetic modifications of genes for enzymatic oxidants and antioxidants. It appears that hypermethylation promotes oxidative stress, whereas demethylation boosts antioxidation. In human pulmonary arterial hypertension, a CpG island in an enhancer region of intron 2 and another in the promoter of SOD2 were hypermethylated in pulmonary arterial smooth muscle cells (PASMCs) owing to upregulation of DNMT1 and DNMT3b, leading to downregulation of the antioxidant enzyme [213]. Similarly, hypoxia also reduced SOD2 expression in the rat carotid body via hypermethylation of a single CpG dinucleotide close to the transcription start site [214]. H_2O_2 promoted methylation of a CpG island in the catalase promoter and downregulated catalase [215]. TET1 deficiency produced by TET1 siRNA enhanced H_2O_2-induced increase apoptosis of cerebellar granule cells [216], suggesting that TET1-mediated demethylation may upregulate antioxidant mechanisms to counter oxidative stress. Histone modification also contributes to the hemostasis of the oxidant-antioxidant system. The expression/activity of SOD3 in the lung from human idiopathic pulmonary arterial hypertension was reduced, and this downregulation could be reversed by the treatment of PASMCs with class I HDAC inhibitors or HDAC3 siRNA [217], suggesting that histone deacetylation negatively regulates SOD3 expression. In contrast, histone deacetylation mediated by HDAC3 upregulated NOX4 in HUVECs as HDAC3 siRNA and pan-HDAC inhibitor scriptaid reduced NOX4 expression [218]. Furthermore, miRs also participate in the regulation of mitochondrial metabolism and function. The downregulation of iron-sulfur cluster assembly enzyme (ISCU) in mitochondria by miR-210 in hypoxia would block electron exit from complex I, promoting its leakage to generation of ROS [219]. Overall, it appears that there exists a positive feed-forward loop between ROS generation and epigenetic modifications.

5.3. Epigenetic Mechanisms in Regulating Uteroplacental Circulation during Normal Pregnancy. In sheep, the upregulation of ERα in uterine arteries was conferred by an epigenetic mechanism [220]. The specificity protein 1- (Sp1-) binding site ($Sp1_{-520}$) at the promoter of the ERα encoding gene *ESR1*, to which Sp1 or Sp1-ERα binds, was essential for E2β-stimulated promoter activity. The CpG dinucleotide of this site was hypermethylated in nonpregnant animals, and the gene is thus kept quiescent. However, the Sp1 site became less methylated in pregnant animals and enabled the expression of the gene, leading to increased ERα mRNA and protein abundance in uterine arteries and subsequent attenuation of uterine vascular tone.

E2β also epigenetically upregulates *KCNMB1* expression in uterine arteries [221, 222]. Similar to ERα, the CpG dinucleotide in the Sp1-binding site (-380) at the promoter of *KCNMB1* was highly methylated in uterine arteries of nonpregnant sheep, resulting in gene silence. During pregnancy, E2β through ERα stimulated *TET1* (TET1 encoding gene) promoter activity and gene expression. The upregulation of *TET1* in turn promoted $Sp1_{-380}$ demethylation of the *KCNMB1* promoter. Consequently, the expression of *KCNMB1* and the activity of the BK_{Ca} channel increased in uterine arteries, leading to reduced myogenic tone.

5.4. Aberrant Epigenetic Modifications in Pregnancy Complications. Epigenetic mechanisms play an important role in the pathophysiological processes of pregnancy complications. Global hypermethylation was observed in preeclamptic placenta [223, 224]. In addition, various genes including *ESR1* and *KCNMB1* in the uterine arteries of

high-altitude pregnant sheep [52, 220, 221, 225] and *IGF1*, *HSD11B2*, *H19*, and *HLA-G* in the placenta from preeclamptic and IUGR pregnancies [224, 226, 227] were hypermethylated. The increased methylation in the uteroplacental tissues was accompanied by upregulation of DNMT1 and DNMT3b expression/activity [224, 225, 227, 228] and downregulation of TET1, TET2, and TET3 expression [52, 227, 229, 230]. Pregnancy complications also alter histone modification in the placenta. JMJD6 histone demethylase activity was suppressed in preeclamptic placenta [231]. Moreover, miR-210 was also upregulated in both uterine arteries and placenta of high-altitude pregnancy [52, 142]. Increased miR-210 level was also observed in preeclamptic and IUGR placenta [140, 230, 232]. These changes undoubtedly would contribute to the aberrant expression of key elements in the $E_2\beta$-NOS-NO-K_{Ca} pathway in uteroplacental circulation.

The aforementioned changes in epigenetic modifications of the uteroplacental system in pregnancy complications are apparently caused by hypoxia/ischemia. HIF-1α overexpression in uteroplacental tissues is a characterized feature in pregnancy complications and high-altitude pregnancy [157, 233, 234]. Both ex vivo hypoxia treatment of tissues or pharmacologically induced hypoxia in intact animal models induced the expression of DNMTs and miR-210 [142, 225, 235] and repressed both histone demethylase activity [231] and TETs expression/activity [235, 236]. Although not investigated in the uteroplacental tissues, studies conducted in other tissues/cells suggest that hypoxia-induced alterations in epigenetic machineries is HIF-1α-dependent. *DNMT1*, *DNMT3b*, and *miR210* all contain hypoxia-responsive element (HRE) in their promoters, and the binding of HIF-1α to HRE stimulates the expression of these genes [237]. Hypoxia *via* HIF-1α also induced the expression of histone demethylases JHDM1B/KDM2B and JARID1B/KDM5B, which demethylate the activating mark H3K4me2/3, leading to gene repression [238]. The $E_2\beta$ metabolite 2-methoxyestradiol is an endogenous HIF inhibitor [239]. The reduced 2-methoxyestradiol level in preeclampsia probably contributes to aberrant epigenetic modifications in uteroplacental tissues due to the relief of HIF inhibition.

Intriguingly, hypoxia-induced *TET1* repression in uterine arteries was mediated by miR-210 and the binding of miR-210 to the 3′-untranslated region (3′UTR) of TET1 mRNA resulted in degradation of the transcript [52]. The overall effects of upregulation of DNMT3b and downregulation of TET1 in uterine arteries promoted *ESR1* and *KCNMB1* hypermethylation and gene repression [52, 220, 221, 225, 235]. ERα and the BK_{Ca} channel are two key elements contributing to reduced uterine vascular tone in pregnancy [59, 73]. Consequently, the downregulation of both ERα and the BK_{Ca} channel impaired pregnancy-induced attenuation of uterine vascular tone, leading to maladaptation of uteroplacental circulation [40, 47, 225] (Figure 2). Increased DNA methylation may also contribute to impaired spiral artery remodeling. The downregulation of TET2 reduced *in vitro* trophoblast migration and invasion [230]. The overexpression of miR-210 in the preeclamptic placenta suppressed ISCU and impaired mitochondrial respiration [140, 142, 232]. It is probably that both the miR-210-mediated mitochondrial dysfunction and DNA hypermethylation (indirectly *via* downregulating TETs) disrupt trophoblast invasion and impair spiral artery remodeling in high-altitude pregnancy and pregnancy complications. In addition, miR-210 also targeted potassium channel modulatory factor 1 (KCMF1) and thrombospondin type I domain-containing 7A (THSD7A), which could also contribute to the impaired trophoblast invasion [240, 241]. The expression of *CYP19A1* and *HSD17B1* is also regulated by DNA methylation. Methylation of CpG islands in the promoters of both genes suppressed their expression [242, 243]. Although not examined in the placenta, it is probably DNA methylation-mediated downregulation of aromatase and HSD17B1 also occurs in preeclampsia, IUGR, and high-altitude pregnancy. Furthermore, the expression of *HSD17B1* was downregulated by miR-210 in preeclamptic placenta [33]. The epigenetic modifications of key enzymes in estrogen biosynthesis could then reduce circulating E2β level in pregnancy complications.

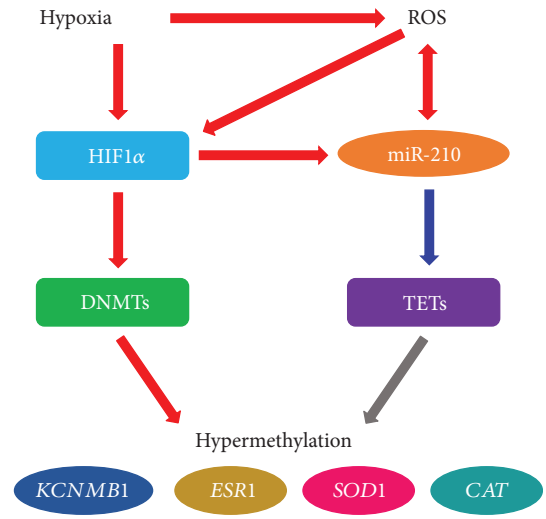

Figure 2: Crosstalk among hypoxia, ROS, and DNA methylation. The cellular responses to hypoxia are primarily mediated by hypoxia-inducible factor-1 (HIF-1). Hypoxia could induce HIF-1 either directly or indirectly through the stabilization of HIF-1α by ROS. HIF-1 could upregulate DNA methyltransferases (DNMTs) and miR-210. ROS may be able directly to induce miR-210. TET1 mRNA is a target of miR-210 and is degraded upon the binding of miR-210 to the 3′UTR of the transcript. The upregulation of DNMTs and downregulation of TET1 result in hypermethylation of *ESR*, *KCNMB1*, *SOD1*, and *CAT* (catalase encoding gene) and subsequent gene repression. The repression of *ESR* and *KCNMB1* ultimately increases uterine vascular tone, whereas the repression of *SOD1* and *CAT* elevates ROS. Moreover, miR-210 could also target iron-sulfur cluster scaffold (ISCU) in the mitochondria leading to increased ROS formation. Red arrow: stimulatory effect, blue arrow: inhibitory effect, and grey arrow: indirect action due to TET1 downregulation.

6. Concluding Remarks

Preeclampsia and IUGR are leading causes of maternal and perinatal mortality and morbidity and have great impacts

on maternal and offspring health. Unfortunately, there is currently no cure for them. Preeclampsia, IUGR, and high-altitude pregnancy all exhibit uteroplacental hypoxia/ischemia and oxidative stress concurrently. Moreover, these pregnancy complications are associated with altered epigenome. There exist interplays among ROS, HIFs, and epigenome. The ROS-HIF pathway appears to be a potential cause in the changes of epigenetic modifications in these complications. In uterine arteries, HIF-1α apparently functions as an important link between ROS and aberrant epigenetic modifications, leading to disrupted $E_2\beta$-BK_{Ca} axis and increased uterine vascular tone. In the placenta, the ROS-HIF-epigenome interplay impairs estrogen synthesis, trophoblast invasion, and spiral artery transformation. Both preeclampsia and IUGR are multifactorial disorders. What we know about these complications is only the tip of the iceberg. Further studies are needed to advance our understanding on the pathogenesis of them in order to develop effective therapeutics.

Abbreviations

3′UTR: 3′-Untranslated region
4-HNE: 4-Hydroxynonenal
8-iso-$PGF_2\alpha$: 8-Iso-prostaglandin $F_2\alpha$
ADMA: Asymmetric dimethylarginine
AP-1: Activator protein-1
AT1-AA: Autoantibodies against angiotensin II type 1 receptor
BK_{Ca}: Large-conductance Ca^{2+}-activated K^+ channel
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
COMT: Catechol-O-methyltransferase
CpG: Cytosine-guanine dinucleotide
CYP19: Aromatase
CYP19A1: The gene encoding aromatase
DNMT: DNA methyltransferase
E2β: 17β-Estradiol
ECs: Endothelial cells
EDHF: Endothelium-derived hyperpolarizing factor
eNOS: Endothelial nitric oxide synthase
ERα: Estrogen receptor α
ERβ: Estrogen receptor β
ERK: Extracellular signal-regulated kinase
ERE: Estrogen response element
ESR1: The gene encoding ERα
GPER (GPR30): G-protein-coupled estrogen receptor
GPx: Glutathione peroxidase
GSH: Glutathione
H19: The gene encoding imprinted maternally expressed transcript
HRE: Hypoxia-responsive element
HDAC: Histone deacetylase
HIF: Hypoxia-inducible factor
HLA-G: The gene encoding major histocompatibility complex, class I, G
H_2O_2: Hydrogen peroxide
HRE: Hypoxia-responsive element
HSD11B2: The gene encoding hydroxysteroid 11β-dehydrogenase 2
HSD17B1 (17β-HSD1): Hydroxysteroid 17β-dehydrogenases 1
HUVEC: Human umbilical vein endothelial cell
IGF1: The gene encoding insulin-like growth factor 1
IK: Intermediate-conductance Ca^{2+}-activated K^+ channel
IUGR: Intrauterine growth restriction
ISCU: Iron-sulfur cluster scaffold
K_{Ca}: Ca^{2+}-activated K^+ channel
KCMF1: Potassium channel modulatory factor 1
KCNMB1: The gene encoding BK_{Ca} channel β subunit 1
L-NAME: L-Nitro-arginine methyl ester or N^{ω}-nitro-L-arginine methyl ester
MDA: Malondialdehyde
MAPKs: Mitogen-activated protein kinases
miR: MicroRNA
NAC: N-Acetylcysteine
NADP: Nicotinamide adenine dinucleotide phosphate
NADPH: Reduced form of $NADP^+$
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NO: Nitric oxide
NOS: Nitric oxide synthases
NOX: NADPH oxidase
O_2: Oxygen
$O_2^{\cdot-}$: Superoxide
$^{\cdot}OH$: Hydroxyl radical
$ONOO^-$: Peroxynitrite
PASMC: Pulmonary arterial smooth muscle cell
PHD: Prolyl hydroxylases
PCR: Polymerase chain reaction
PI3K: Phosphoinositide 3-kinase
PKB (AKT): Protein kinase B
PKG: Protein kinase G
Prx: Peroxiredoxin
pVHL: von Hippel–Lindau protein
ROS: Reactive oxygen species
siRNA: Small interfering RNA
SK: Small-conductance Ca^{2+}-activated K^+ channel
SOD: Superoxide dismutase
Sp1: Specificity protein 1
TET: Ten-eleven translocation dioxygenase
THSD7A: Thrombospondin type I domain containing 7A
Trx: Thioredoxin
TrxR: Thioredoxin reductase
VSMCs: Vascular smooth muscle cells

X: Xanthine
XO: Xanthine oxidase.

Conflicts of Interest

None of the authors has any conflict of interests to disclose.

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Review Article

Role of miRNA in the Regulatory Mechanisms of Estrogens in Cardiovascular Ageing

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Cardiovascular diseases are a worldwide health problem and are the leading cause of mortality in developed countries. Together with experimental data, the lower incidence of cardiovascular diseases in women than in men of reproductive age points to the influence of sex hormones at the cardiovascular level and suggests that estrogens play a protective role against cardiovascular disease and that this role is also modified by ageing. Estrogens affect cardiovascular function via their specific estrogen receptors to trigger gene expression changes at the transcriptional level. In addition, emerging studies have proposed a role for microRNAs in the vascular effects mediated by estrogens. miRNAs regulate gene expression by repressing translational processes and have been estimated to be involved in the regulation of approximately 30% of all protein-coding genes in mammals. In this review, we highlight the current knowledge of the role of estrogen-sensitive miRNAs, and their influence in regulating vascular ageing.

1. Introduction

The prevalence, incidence, and prognosis of cardiovascular diseases differ between genders. Indeed, statistical data reveal that women develop cardiovascular disease later than men [1] and the incidence of cardiovascular diseases in women increases from menopause [2, 3]. Both clinical and experimental data have extensively demonstrated the beneficial effects of estrogens at the cardiovascular level [4, 5], although hormonal replacement therapies (HRTs) in postmenopausal women have been implemented with controversial results [6, 7]. Such studies have led some researchers to conclude that the protective effect of HRT depends on age [8].

Ageing is a physiological and multifactorial process characterized by the progressive loss of anatomical and functional integrity, which leads to an increased risk of different pathologies, including cardiovascular disease. At the molecular level, different mechanisms have been established as crucial in the regulation of the ageing process [9]. Among these, epigenetic mechanisms affect gene expression without causing changes in the DNA sequence and can be influenced by external factors, including the environment and lifestyle [10].

Some evidence indicates that estrogen-dependent regulation of cardiovascular function in ageing is mediated by epigenetic mechanisms. An age-related increase in methylation-associated inactivation of genes encoding estrogen receptors (ERs) has been described, and ER methylation in atherosclerotic plaques is higher than in nonplaque regions in vascular tissues [11, 12], which suggests that estrogen activity in atherogenesis and vascular system aging is epigenetically regulated. Another epigenetic mechanism that has also been associated with estrogens is histone modification. In this regard, in a postmenopausal metabolic syndrome murine model, estradiol prevented cardiovascular dysfunction by suppressing histone H3 acetylation [13].

In addition to DNA methylation and histone modification, the most recently described epigenetic mechanism is RNA-based machinery. Regulatory noncoding RNAs are

classified depending on RNA length, and among them, microRNAs (miRNAs) constitute the dominant class in most tissues. MiRNAs regulate protein translation by targeting their target messenger RNAs (mRNAs) via sequence-specific interaction to repress translation or degrade target mRNA [14]. In addition, emerging evidence suggests that miRNAs have also nuclear functions in the regulation of gene expression at transcriptional level [15]. Most miRNAs are located within cells, although some have also been found circulating in body fluids [16]. Thus, miRNA-mediated regulation is now considered one of the most important posttranscriptional gene regulation mechanisms and is estimated to modulate up to 30% of mammal genes, including important roles in human physiology, ageing, and cardiovascular function [17, 18].

Given the world's increasingly ageing population [19] and that cardiovascular diseases are the leading cause of death in developed countries, it is important to improve our understanding of the regulatory mechanisms underlying the ageing of the cardiovascular system. In addition, although growing evidence has established miRNAs as crucial epigenetic regulators of vascular function, their role in the regulation of estrogen in cardiovascular ageing has not yet been fully elucidated. Therefore, in this review, we summarize current knowledge of the role of estrogens in cardiovascular function and ageing with a special focus on the miRNAs related to estrogens during female vascular ageing.

2. The Involvement of Estrogens in Vascular Function

Estrogens modulate the cardiovascular system directly by acting on vascular and inflammatory cells, which express ERs, or indirectly via systemic effects [20]; estrogen functions through ERs by genomic and nongenomic mechanisms. In the former, also known as the "classical" mechanism, estrogens bind to ERs to form a complex that regulates gene transcription by binding to specific DNA motifs in gene promoter regions [21]. In this sense, ERα and ERβ are the two main ER isoforms, and these form homo- or heterodimers, which can induce changes in gene expression. The involvement of specific ER isoforms in estrogen-mediated effects has been extensively studied [22], and both opposing gene expression regulatory effects [23, 24] and redundant mediatory roles [25, 26] have been described. Estrogen signaling is selectively regulated by the relative balance between ERα and ERβ expression in target organs [27], although the beneficial effects that estrogens have on the vascular system are mainly attributed to ERα [28, 29]. Furthermore, estrogens can also trigger fast responses through plasma membrane ER receptors and G protein-coupled ERs (GPERs) [30].

In general, the vascular protective effects of estrogens are attributed to their role in increasing arterial vasodilation, their action on vasoactive mediator release and smooth muscle contraction, inhibition of inflammatory processes, and regulation of systemic lipid metabolism and oxidative stress balance [2, 31, 32]. The regulation of vascular reactivity by estrogens is mainly related to the maintenance of normal endothelial function [33]. In endothelial cells, estrogens modulate nitric oxide (NO) bioavailability by both genomic and nongenomic effects by increasing endothelial NO synthase (eNOS) expression at the transcriptional level, eNOS activation through phosphorylation, and regulation of its endogenous inhibitors and cellular location [34–36]. Moreover, a role for estrogens in regulating other vascular mediators related to prostanoids and endothelin signaling has also been described. Estradiol increases prostacyclin release by upregulating cyclooxygenase 1 (COX-1) and prostacyclin synthase (PGIS) expression in endothelial cells [37] and by decreasing endothelin-1 release in both basal and stimulated conditions [38, 39].

In addition to the regulation of endothelial-derived factors, estrogens directly regulate the smooth muscle layer by inhibiting the proliferation, migration, and vascular contraction of vascular smooth muscle cells (VSMCs) [40]. Indeed, estrogen-mediated vasorelaxation can also occur in endothelium-denuded segments [41]. Specifically, estradiol decreases smooth muscle constriction by interfering in the mechanisms of Ca^{2+} mobilization and Ca^{2+} entry [42] and by activating K^+ channels [43], leading to membrane hyperpolarization and vascular relaxation. Estrogen can also modulate vasoconstriction by interfering in protein kinase C and Rho-kinase signaling in VSMCs [44, 45].

The renin-angiotensin system (RAS) is another important regulator of vascular contractibility which is regulated by estrogen. Estrogens are implicated in the inhibition of circulating renin, the activity of angiotensin-converting enzymes, and in circulating angiotensin (Ang) II levels [46]. Furthermore, components of RAS are synthesized and act locally in different tissues, including in the vasculature. In this case, estradiol increases the expression of Ang 1–7 by inducing the expression of angiotensin-converting enzymes [47] and of Ang II receptor type 1 expression [48] in endothelial cells, thus promoting vasodilation. In addition, estradiol-dependent NO production is mediated by Ang 1–7-induced activation of the Mas receptor [49], suggesting the presence of a functional interaction between both these pathways.

The beneficial effects of estrogens in the cardiovascular system are also attributed to their role in modulating the inflammatory response [50] and vascular lipid accumulation [32]. Estrogens inhibit monocyte-endothelial interactions by reducing the expression of cell adhesion molecules in the endothelium when exposed to inflammatory stimuli [51, 52]. Moreover, a reduction in neutrophil chemotaxis [53] and leukocyte infiltration [54] have been established as inflammatory regulatory mechanisms which are mediated by estrogens after vascular injury. Estradiol also reduces the expression of superoxide-induced adhesion molecules and cytokines in VSMCs by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression [55]. Moreover, it regulates oxidative stress by decreasing both the expression and activity of superoxide dismutase in VSMCs [56] and endothelial cells [57]. Estrogens can also confer protective effects by modulating systemic lipid metabolism [32], lipid-vascular wall interactions by reducing lipid loading [58, 59], and oxidative stress-mediated LDL modifications [60], thus preventing the formation of foam cells.

3. The Role of Estrogens in Vascular Ageing

Epidemiological data reveal sex differences in the number of deaths caused by cardiovascular disease. These numbers are greater in men than in women under the age of 65 and similar over this age [61], suggesting the importance of ageing in sex-related differences observed in cardiovascular disease. In this regard, sex-specific differences of cardiovascular ageing have been reported, and these patterns have been explained by both hormonal and nonhormonal factors [62].

Considering the beneficial role of estrogens in the cardiovascular systems described above, the use of HRT in postmenopausal women has produced controversial results [6, 7]. The current consensus indicates that the protective effects that estrogens confer on cardiovascular function depend on the prompt initiation of estrogen therapy after menopause [63]. The phenomenon is referred to as the "timing hypothesis" and postulates that estrogen supplementation may only have beneficial effects when initiated before the detrimental effects that ageing has on the cardiovascular system become established [8]. Vascular ageing is associated with endothelial dysfunction and arterial stiffening, vascular remodeling, and increased inflammation [64]. These characteristics can lead to pathological conditions such as myocardial hypertrophy, fibrotic tissue formation, and increased systolic pressure, resulting in a higher risk of atherosclerosis, hypertension, and ischemic cardiovascular disease [65]. Endothelial dysfunction is less prominent in premenopausal women compared to age-matched men and postmenopausal women, which highlights the protective effect of estrogens [66]. Indeed, in a recent study, Nicholson et al. demonstrated that the contractility responses of uterine arteries to estradiol treatment in postmenopausal women were impaired compared to their premenopausal counterparts [67].

The vascular ageing process is associated with a decline in endothelial-dependent relaxation and an increased vasoconstrictor responses mediated by an imbalance between vasodilator and vasoconstriction factors [68–70]. In this regard, NO and prostanoids seem to be the main pathways implicated, and these act by progressively reducing the bioavailability of NO and increasing vasoconstrictor prostanoids in aged vascular tissues. Indeed, relaxation induced by bradykinin in isolated mesenteric microvessels was mainly mediated by the action of NO, although COX-dependent vasodilators were also implicated in young subjects, whereas COX-dependent activity produced vasoconstriction in older subjects [69]. Although some of the data regarding eNOS expression at the molecular level are contradictory, ageing progressively decreases eNOS activity both in animal models and in human samples [71, 72]. Moreover, decreased expression of the eNOS cofactor, tetrahydrobiopterin [73], elevated circulating levels of endogenous inhibitors [74], and enhanced oxidative stress [69] have also been postulated as mechanisms which underlie decreased eNOS activity during ageing. Conversely, rather than altered expression of the COX isoforms, an age-associated switch from vasodilatory to vasoconstrictor prostanoid release has been related to enhanced expression of the thromboxane A2 (TXA2) receptor in the smooth muscle layer in both human mesenteric arteries and in aortic segments from female mice [69, 70, 75].

Oxidative stress is also increased in aged tissues, including the vascular system, and influences endothelial dysfunction by scavenging NO, thus decreasing its bioavailability [76]. Increased oxidative stress levels in vascular ageing are caused by altered antioxidant enzyme expression and enhanced production of reactive oxygen species (ROS) mainly by mitochondria and NADPH oxidase but also by COX and uncoupled eNOS [77]. The role of estrogens in oxidative stress during ageing has been previously reported: in vascular tissues, a decline in NO-mediated vasodilation is associated with increased ROS, especially superoxide, in coronary arterioles [78]. In the same study, the authors describe a decrease in Cu/Zn superoxide dismutase expression in both aged and ovariectomized rats, while estrogen replacement restores its expression to the levels of young intact animals [78]. Furthermore, COX-dependent superoxide production induced by a TXA2 analogue in female aortic segments was enhanced by ageing and estrogen deprivation, findings that were abrogated by estrogen supplementation [79].

Ageing also influences the structural properties of vasculature, and changes in the media thickness and extracellular fiber content contribute to low arterial compliance. Specifically, ageing is associated with the increased production of collagen fibers and decreased elastin fiber synthesis, vascular calcification, and VSMC proliferation [80]. Estrogen administration mitigates progressive age-dependent reductions both in human carotid artery wall thickness in postmenopausal women [81] and in the mesenteric arteries of old female rats [82]. In addition, increased collagen content has been associated with decreased activity and protein levels of specific collagen-degrading metalloproteinase enzymes [82, 83]. Finally, aberrant VSMC growth is also associated with ageing-associated remodeling processes as VSMCs switch from a contractile to synthetic-proliferative phenotype [84]. As previously described, estrogens inhibit the proliferation of cultured VSMCs [40] and it is thought that ageing males are more prone than females to these changes in the VSMC phenotype [85]. In addition, estrogen treatment also attenuates neointimal formation after vascular damage [86]. Finally, in relation to these metalloproteinase and VSMC phenotypes, estrogens may be involved in plaque instability and thus the greater cardiovascular disease risk in postmenopausal women who start HRT late.

Along with endothelial dysfunction and vascular remodeling, the other key player in vascular ageing is inflammation. Ageing is associated to a progressive increment of the tissue's proinflammatory status, phenomenon termed as "inflammageing." The signaling of this low-grade chronic inflammation state is linked to an increased cell death, a metabolic dysfunction and a proteostasis loss, and it is also associated with an age-related functional detriment [87]. At vascular level, the ageing process is characterized by an acquisition of a proinflammatory phenotype with increased release of cytokines and enhanced cell adhesion molecules expression, developing a more adhesive endothelium, phenomenon also known as endothelial activation [64, 88]. The role of estrogens as an anti- and/or proinflammatory factor in this process is

controversial and may be dependent on immune stimuli, the cell types involved in the response, organ target, timing and concentration of their administration, intracellular metabolism of estrogen, and relative expression of the implicated ERs [50]. In a study using uterine arteries from postmenopausal women that were exposed to estradiol, Novella et al. demonstrated that although estradiol exposure decreased most of the inflammatory cytokines assessed in women in the early stages of menopause, ageing was associated with estrogen's effect on the switch from anti-inflammatory to proinflammatory in the arteries of women who had started menopause at least 10 years prior [89], suggesting that the effect of estrogens is timing-dependent. In addition, estradiol attenuates inflammatory responses in macrophages and VSMCs derived from young but not old female mice [90], suggesting that the anti-inflammatory properties of estradiol are lost as ageing continues.

As mentioned above, the relative expression of specific ERs has been related to the role of estrogens in vascular ageing and it has postulated that a change in the balance between ERα and ERβ could explain the lack of vascular protection provided by estrogen. In that sense, an age-dependent increase in ERβ but not in ERα expression was observed in uterine arteries from postmenopausal women [89], thus producing this increased ERβ/ERα ratio. In young ovariectomized mice, although estrogen supplementation downregulated ERs, it did not alter the ERβ/ERα ratio in endothelium or smooth muscle; in contrast, the ERβ/ERα ratio increased after estrogen treatment in aged female mice [91]. Finally, decreased ERα expression was also reported in aged compared to young macrophages [90].

4. Sex and Age Influence in miRNA Expression

Sex and age differences in miRNA expression profiles have been described in different tissues [92–94], providing evidence for the role of sex hormones and ageing in miRNA regulation. Sex differences are usually attributed to the modulation of estrogen transcriptional activity mediated by ERs, and so differences in the miRNA profiles obtained from ER + and ER− breast cancer cells [95, 96] are related to the loss of ER binding sites located near the miRNA sequences which are found less abundance in ER− breast cancer cells [96]. At the vascular cell level, ER binding sites were located within the regulatory region of estradiol-regulated miRNAs [97, 98].

Sex-biased miRNAs are also driven by the expression of miRNAs located in sex chromosomes. It is worth noting that, according to miRBase (March 2018; http://www.mirbase.org), 118 miRNAs are located on the human X chromosome but only 4 were present on the Y chromosome. Most X chromosome miRNAs escape X chromosome inactivation which occurs early in development to compensate for gene dosage imbalances between the sexes [99]. In this respect, the mosaicism resulting from X chromosome inactivation may be involved in some of the increased susceptibility to inflammatory and autoimmune diseases experienced by women [100]. Regarding X-linked miRNAs and cardiovascular diseases, Florijn et al. recently reviewed the implication of miRNAs in women with heart failure with a preserved ejection fraction and concluded that estradiol-induced miRNAs are protective while X-linked miRNAs are associated with deleterious effects [101]. Furthermore, both age and estrogens can modulate miRNA profiles by regulating miRNA biosynthesis pathways.

miRNA production is a two-step process involving nuclear cropping and cytosolic dicing; mature miRNAs are derived from a stem-loop transcript (also known as pri-miRNA) which is cleaved in the nucleus by a microprocessor complex—comprising the RNase III, Drosha, and DiGeorge syndrome critical region 8 (DGCR8)—into a small hairpin-shaped RNA (pre-miRNA) which is transported to the cytoplasm through exportin 5 where maturation can be completed. In a second processing step, the pre-miRNA is cleaved—by the RNase III, Dicer—into approximately 22-nt miRNA duplexes. One strand from the miRNA duplex usually remains as a mature miRNA, while the other is generally selectively degraded via a thermodynamic stability-dependent process. Finally, mature miRNAs are loaded into Argonaute (AGO) proteins to form, along with other components, the RNA-induced silencing complex (RISC) effector. The final miRNAs function as a guide by base pairing—usually at the 3′-untranslated region (UTR)—to target mRNAs, and AGO proteins recruit factors that induce the translational repression of these mRNAs [102].

With ageing, molecules implicated in miRNA expression machinery become downregulated, thus suggesting that miRNA expression is age-dependent: an effect described in old versus young peripheral blood mononuclear cells [103]. The same study showed that the expression of miRNA biogenesis molecules is depressed in octogenarians compared with centenarians, which may indicate that greater expression of miRNA production components is associated with extraordinarily successful ageing. At the vascular level, Dicer1 is downregulated in old versus young cerebral vessels isolated from rats, which these authors associated with altered miRNA expression profiles and impaired endothelial function [104]. Indeed, the impaired vascular formation observed in Dicer1 knockout mice was one of the first pieces of evidence that related miRNA biosynthesis to vascular function [105]. In endothelial cells, Dicer1 depletion revealed that miRNA processing is essential for correct endothelial gene expression and function, including proliferation and angiogenesis [106, 107].

The role of estrogen in regulating key miRNA production molecules has been reviewed elsewhere [108], and of note, most studies relating estrogen activity and miRNA biosynthesis have been performed in reproductive organs. Differences in key miRNA-processing genes have been observed between ER+ and ER− breast cancer cells [95, 109]; specifically, the expression of Dicer1, DGCR8, and Drosha was higher, and that of Ago-2 was lower in ER+ breast tumors [110]. Nevertheless, among the miRNA processing genes, only Dicer1 contains an ERα binding site in its regulatory region [110]. Furthermore, a regulatory ER-Drosha interaction has been reported in breast cancer cells [111] and an estrogen-dependent increase in exportin-5 expression was reported in the mouse uteri [112]. At the vascular level, global transcriptomic analysis data of endothelial cells treated with

estradiol reported the deregulation of key miRNA biosynthesis pathway genes [113]. Specifically, DGCR8 upregulation and Dicer1 and Ago-2 downregulation were observed in estradiol-treated cells, suggesting that estrogens regulate endothelial miRNA production machinery [113].

5. Estrogen-Regulated miRNAs and Vascular Ageing

As described above, sex- and age-specific miRNA profiles are the result of transcriptomic changes, sex chromosome expression, and miRNA biosynthesis regulation. In addition, findings in women receiving HRT have provided insights into the roles of estrogen-associated miRNAs during ageing in different tissues (Table 1). Changes in the miRNA expression profile in bone tissue from both ovariectomy-induced osteoporotic mice and postmenopausal women have been described [114, 115]. Specifically, miR-127 and miR-136 have been described as negative regulators of bone mass [114], whereas the expression level of miR-30b-5p has been proposed as a suitable serum biomarker for osteoporosis and osteopenia in postmenopausal women [115]. Furthermore, Olivieri et al. reported that estrogen has a positive impact in postmenopausal women using HRT as a result of skeletal muscle changes mediated via the suppression of miR-182 and miR-223 expression. These miRNAs are implicated in regulating the insulin/insulin-like growth factor (IGF-1) pathway which is key in muscle mass homeostasis [116]. Moreover, changes in the miRNA expression profile in adipose tissue have recently been described in association with HRT [117]. Specifically, miR-19a-3p was identified as being HRT-sensitive in adipose tissue because its levels in HRT-treated postmenopausal women were similar to those observed in premenopausal women and were higher than in postmenopausal women who did not use HRT. ESR1 is a miR-19a-3p target [118] and, thus, could be involved in the changes in ERα observed in adipose tissue during ageing [119] and may underlie the adverse age-related alterations in adipose metabolism in women [120].

In addition to tissue-specific miRNA expression, estrogen-dependent regulation in circulating miRNAs has also been described both in ovariectomized animals [115] and postmenopausal women receiving HRT [121, 122]. The studies in women were performed in monozygotic twin pairs and showed an association between changes in serum inflammatory markers and inflammatory-related miR-21 and miR-146a [121]. There were also alterations in the miRNAs included in exosomes in postmenopausal women using HRT [122], suggesting that changes in circulating miRNAs are associated with estradiol levels. Therefore, estrogen-sensitive miRNAs could be used both as therapeutic targets and as potential biomarkers for characteristic physiological alterations related to female ageing, such as osteoporosis, sarcopenia, changes in body fat homeostasis, and ageing-associated inflammation.

Specific miRNAs implicated in cardiac and vascular ageing have been reviewed in depth elsewhere [17, 18]. In addition, we have addressed the role of estrogen-regulated miRNAs in cardiovascular function in a recent review [113]. However, how miRNAs are implicated in the action of estrogen in the vasculature during ageing remains to be elucidated. In the following paragraphs, we describe the involvement of specific miRNAs in the effect of estrogens during female vascular ageing, and Tables 2 and 3 summarize the main circulating and tissue-specific miRNAs involved in female vascular ageing, respectively.

miR-126 is highly expressed in vascularized tissues and was the first miRNA reported as being crucial in endothelial biology. It is located within an intron of the epidermal growth factor-like domain multiple 7 *(EGFL7)* gene which is mostly expressed in endothelium and is involved in vascular angiogenesis. This miR-126 is involved in vascular integrity and angiogenesis [123, 124] but also appears to modulate immune cell adhesion and VSMC function [125, 126]. Serum levels of estradiol are positively associated with miR-126-3p expression throughout the menstrual cycle; estradiol also increases miR-126-3p expression in cultured endothelial cells [127] but decreases it in lymphocytes [128], suggesting that it has a cell-specific effect on miR-126-3p expression. In endothelial cells, estradiol-dependent miR-126-3p suppresses the miR-126-3p targets, Spred1 and *VCAM1*, and is related to an increase in cell migration, proliferation, and tube formation, while decreases monocyte adhesion [127]. Moreover, miR-126-3p is also implicated in the estradiol-dependent reduction of plaque size in $ApoE^{-/-}$ mice [127]. Indeed, miR-126 released by endothelial cells controls VSMC behavior [126] and limits neointimal formation [129]. Given that circulating miR-126 expression is altered in several cardiovascular diseases [130], these findings suggest that estradiol's vasculoprotective and antiatherogenic properties could be partly mediated by miR-126. Circulating miR-126 is downregulated in different cardiovascular diseases [131, 132], although its relationship with and ageing is contradictory; compared to young individuals, miR-126 was downregulated in blood samples from centenarians [133], but miR-126-5p expression in circulating exosomes was higher in postmenopausal versus premenopausal women [122] and miR-126-3p was increased in senescent endothelial cells in vitro and in plasma collected from healthy older patients [134]. Olivieri et al. hypothesize that this miR-126 upregulation is a compensatory mechanism to reduce cell dysfunction during normal ageing.

miR-106 expression is sex-specific. miR-106a/b were among the sex-specific miRNAs regulated via ERβ in a murine model of pressure overload-induced cardiac fibrosis mediated by regulation of specific profibrotic MAPK signaling repressors [135], evidence that miR-106 may be involved in sex-related differences in this pathology. In estradiol-treated rat cardiac fibroblasts, miR-106b expression is decreased in both female and male cells while miR-106a expression is downregulated in female cells but upregulated in male cells [135]; furthermore, miR-106b was downregulated in cultured VSMCs treated with estradiol [97], suggesting that estradiol has sex-specific effects in downregulating miR-106b. In contrast, miR-106b-5p expression was lower in postmenopausal women not using HRT compared to those that did use HRT [122], suggesting that estrogens have the opposite effect on miR-106 expression during ageing. The

Table 1: HRT-sensitive miRNAs involved in age-associated diseases in postmenopausal women; miRNAs related to disease and the tissue sample types used in the study are listed and the miRNA-related function and specific miRNA targets are shown.

Disease (tissue)	miRNA	Function (targets)	References
Osteoporosis (bone)	miR-127 and miR-136	Regulation of bone mass (COL1, ALP, RUNX2, and OC)	[114]
Osteoporosis (serum)	miR-30b-5p	Biomarker	[115]
Sarcopenia (skeletal muscle)	miR-182 and miR-223	Regulation of the insulin/IGF-1 pathway (IGF-1R and FOXO3A)	[116]
Obesity (adipose tissue)	miR-19a-3p	Adipocyte cell fate, death, and proliferation (ESR1, AKT1, BCL2, BRAF, and CCND1)	[117]
Inflammageing (Serum)	miR-21 and miR-146a	Biomarker	[121]

Table 2: Circulating miRNAs associated with estrogen levels and/or ageing.

miRNA	Ageing	Estrogen	Sample/model	References
miR-126	−		Blood samples from centenarians	[133]
miR-126-3p	−		Plasma from healthy people	[134]
miR-126-3p		+	Serum from healthy women	[127]
miR-126-5p	+		Serum exosomes from postmenopausal women	[122]
miR-106b	−		Serum from older women	[137]
miR-106b-5p		+	Serum from HRT-treated postmenopausal women	[122]
miR-30b-5p		+	Serum samples from ovariectomized rats	[115]
miR-23a		−	Serum from female rats	[166]
miR-144		+	Serum from HRT-treated postmenopausal women	[122]
miR-146a		−	Serum from HRT-treated postmenopausal women	[121]
miR-21	+		Plasma from elderly people	[183]
	+	−	Serum from HRT-treated and non-HRT-treated postmenopausal women	[121]

Positive or negative associations with ageing and estrogens are represented, and the sample type or model used in each study is shown. HRT: hormone replacement therapy.

downregulation of miR-106a has been also reported in replicative endothelial cell ageing [136] and in serum from older women [137]. In endothelial cells treated with tumor necrosis factor alpha (TNF-α), miR-106b-5p exerts antiapoptotic effects by repressing phosphatase and tensin homolog *(PTEN)* caspase activity [138], and in a rat cardiomyoblast cell line exposed to hypoxia, it suppresses apoptosis by directly targeting p21 [139], findings that concur with the known repressive effects of estrogens on *PTEN* and apoptosis [140, 141].

In *miR-221/222*, the cluster containing the miR-221/222 gene is located on the X chromosome and is regulated both by ageing and estrogen-mediated mechanisms; serum levels of miR-222 increase with ageing in both males and females [137]. Moreover, in human aortic endothelial cells, miR-221/222 expression was upregulated in an in vitro replicative senescence model and correlated with decreased *eNOS* expression and activity [142]. Moreover, endothelial cells transfected with miR-221 and miR-222 showed reduced eNOS protein levels [106]. However, eNOS 3′-UTR mRNA does not contain a target sequence for miR-221 or miR-222, suggesting that an intermediate mechanism may be responsible for this miR-221/222-mediated *eNOS* repression. Indeed, miR-222, that was among the sex-dimorphic miRNAs identified in mice heart tissues and isolated cardiomyocytes, inhibits *eNOS* expression by directly targeting ets-1 mRNA [143], an upstream *eNOS* regulator. These results indicate that miR-222 plays an important role in heart function that may be sex-specific in terms of providing female cardioprotection [144]. Conversely, miR-221/222 regulate and are regulated by ERα activity [145], suggesting the presence of a negative regulatory loop between them. One hypothesis is that before menopause, estrogen-ERα activity limits miR-221/222 levels, thus maintaining *eNOS* expression and cardioprotection. However, ageing and menopause increase miR-221/222 levels and decrease ERα and eNOS expression by downregulating ets-1. In addition, miR-221/222 are strongly upregulated in the carotid artery in a balloon injury model and their depletion suppresses VSMC proliferation and neointimal formation [146] which may explain the role of estrogens in attenuating vascular damage [86].

miR-143/145 activity appears to be essential in regulating VSMCs to prevent the ageing-related switch from a contractile to synthetic-proliferative VSMC phenotype [147], and miR-143/145 dysregulation has been described in different cardiovascular pathologies. These miRNAs maintain VSMCs in a quiescent state and inhibit proliferation by regulating

Table 3: Cardiovascular-related miRNAs associated with estrogens and ageing.

miRNA	Ageing	Estrogen	Tissue/cell	Function (target)	References
miR-126		+	Endothelial cells	Endothelial proliferation, migration, tube formation, and monocyte adhesion (Spred1 and VCAM1)	[127]
	+		Endothelial cells	Senescence-associated compensatory mechanism (Spred1)	[134]
		−	Lymphocytes	Unexplored	[128]
miR-106	−		Endothelial cells	Unexplored (p21/CDKN1A)	[136]
		−	VSMCs	Unexplored	[97]
		−	Cardiac fibroblasts	Regulation of cardiac fibrosis via ERβ (Rasa1 and Rasa2)	[135]
miR-221/222	+		Endothelial cells	Suppression of eNOS and $p^{Ser1177}$-eNOS	[142]
	+		Aorta	Unexplored	[173]
		f < m	Cardiomyocytes	Regulation of eNOS expression (Ets-1)	[143]
miR-143/145		+	PASMCs	SMC and EC cell migratory phenotypes	[150]
miR-30b-5p		+	Endothelial cells	Unexplored	[98]
miR-23a	+		Endothelial cells	Unexplored	[164]
	+		Fibroblasts	Telomere dysfunction (TRF2)	[165]
		−	Myocardium	Loss of cardiac gap junctions (CX43)	[166]
		−	Cardiomyocytes	Ventricular remodeling (PGC1)	[169]
miR-203	+		Aortic SMCs	VSMC stiffness (Src and ERK)	[173]
		+	Aortic SMCs	Inhibition of VSMC proliferation (Abl1 and p63)	[97]
miR-144	+		Endothelial cells	Antioxidant response (NRF2)	[176]
miR-146a	+		Endothelial cells	Proinflammatory status marker (IRAK1)	[180]
	−		Endothelial cells	Senescence-like phenotype (NOX4)	[181]
		−	Lymphocytes	Regulation of LPS-induced IFN-γ (unknown)	[128]
miR-21	+		Heart	Unexplored	[182]
	+		Endothelial cells	Decrease angiogenesis and cell proliferation (NFIB and CDC25A)	[164]
	−		Endothelial cells	Antiproliferative effect (associated with PTEN and p27)	[142]
		−	Cardiac fibroblasts	Regulation of cardiac fibrosis (SPRY1, Rasa1, and Rasa2)	[135]
miR-34	+		Aorta	Unexplored	[173]
	+		Aorta/VSMCs	Promotes VSMC senescence and inflammation (SIRT1)	[192]
	+		Endothelial cells	Cell growth arrest and senescence (SIRT1)	[191]
	+		Cardiomyocytes	Age-associated cell death (PNUTS/PPP1R10)	[190]
		−	Endothelial cells	Regulation of eNOS expression (SIRT1)	[194]
miR-22	+		Cardiac fibroblasts	Induction of cellular senescence and migratory activity (OGN)	[196]
	+		Cardiomyocytes	Inhibition of cardiac autophagy and cell hypertrophy (Akt3, Hdac6, and Ppara)	[197]
		−	Cardiomyocytes/myocardium	Increased antioxidant defense (SP1)	[198]
miR-125	+		Endothelial cells	Impaired angiogenesis (RTEF1)	[202]
		+	Macrophages	Inhibitor of NF-κB signaling (κB-Ras2)	[201]
		−	Lymphocytes	Unexplored	[128]

Positive or negative associations with ageing and estrogens are represented, and the tissue or cell type used, miRNA-related function, and described miRNA target are shown. Sex differences are indicated where appropriate (f: female; m: male). SMC: smooth muscle cell; VSMC: vascular SMC; PASMC: pulmonary artery SMC.

several targets, including angiotensin-converting enzyme [148] which modulates vascular contractibility. It has been reported that miR-143/145 are delivered from endothelial cells to VSMCs where they repress genes associated with dedifferentiation [149] such as myocardin, *ELK1*, and *KLF4*. Estradiol induces miR-143/145 expression in pulmonary artery smooth muscle cells (PASMCs) via specific ER binding sites located in their promoter regions [150]. Moreover, estradiol-treated PASMCs secrete exosomes enriched in miR-143/145 which regulate VSMC-endothelium crosstalk in pulmonary arterial hypertension, regulating vascular remodeling [150] and perhaps playing a role in exerting estrogen-mediated effects on the VSMC phenotype. Although no specific studies on miR-143/145 expression in

cardiovascular cells during ageing have been undertaken, miR-145 expression negatively correlates with age in patients with coronary artery disease [151].

miR-30 is estrogen-sensitive and its expression is related to estrogen levels in different tissues [110, 152, 153]; indeed, ER binding sites have been identified upstream of the miR-30 transcription start site [96, 98]. Reinforcing the role of estrogens in regulating miR-30b-5p, its expression was downregulated in serum from ovariectomized rats and from postmenopausal osteoporotic women [115] and miR-30b-5p was the most upregulated miRNA in endothelial cells treated with estradiol [98]. There is also evidence that miR-30b family members are involved in cardiovascular diseases because their expression is decreased in vascular injury [154], inversely correlates with blood pressure parameters [155], and inhibits VSMC proliferation and neointimal formation [154]. However, the role of miR-30 family members in myocardial infarction remains controversial [156–158]. In endothelial cells, miR-30 is implicated in angiogenic processes [159], has anti-inflammatory effects by decreasing angiopoietin 2-induced *VCAM1* expression [160], and inhibits apoptosis in human coronary artery endothelial cells [161]. In this regard, it is noteworthy that estradiol also has proangiogenic, anti-inflammatory, and antiapoptotic properties in response to vascular injury [162].

miR-23a belongs to the miR-23-24-27 cluster which has been implicated in angiogenic processes and cardiac function [163] and also has ERα binding sites in its regulatory regions. Members of this cluster are upregulated during endothelial replicative ageing [164], and miR-23a overexpression reduces telomere length and induces senescence in human fibroblasts [165]. In serum and myocardium in rats, miR-23a expression inversely correlates with estradiol levels and regulated connexin-43 in a menopausal rat model [166]. In postmenopausal rats, estrogen supplementation rescued blockage of cardiac conduction by decreasing miR-23a, thus revealing potential mechanisms involved in postmenopause-related arrhythmias [167]. Moreover, miR-23a may affect cardiac hypertrophic processes [168]: its increased expression in estrogen-deficient ovariectomized mice causes mitochondrial compromise and ventricular remodeling by directly repressing peroxisome proliferator-activated receptor-γ coactivator 1-α *(PGC-1α)* expression in cardiomyocytes [169]. Moreover, miR-27 is upregulated in senescent endothelial cells [164] and in circulating exosomes in premenopausal versus postmenopausal women [122]. This miRNA has been implicated in LDLR expression [170], angiogenic processes [107], and may be a biomarker for progression in asymptomatic carotid stenosis [171].

miR-203 is upregulated in estradiol-treated mouse aortic VSMCs [97]; miR-203 induction is regulated through an ERα-dependent mechanism and its inhibition abolishes estradiol-mediated inhibition of VSMC proliferation, suggesting a role for miR-203 in the antiproliferative effect of estrogens [97]. In addition, miR-203 can downregulate ERα by direct targeting [172], suggesting its involvement in a regulatory loop. Conversely, increased miR-203 expression found in aged aorta has recently been associated with age-related VSMC stiffness [173].

In *miR-144*, circulating estradiol concentrations were positively correlated with miR-144-5p in a study of women receiving HRT [122], and in the same study, miR-144-5p was inversely correlated with TNF-α levels, together suggesting it has an estrogen-mediated role in regulating inflammatory processes. Indeed, miR-144 is implicated in targeting superoxide-related proteins such as COX-2 [174] and the NADPH oxidase component Rac1 [175]. Conversely, miR-144 is upregulated in cerebral microvascular endothelial cells from aged mice, and its inhibition upregulates the antioxidant transcription factor NRF2 [176]; miR-144 is also involved in lipid metabolism by repressing the expression of cholesterol efflux regulatory protein ABCA1 [177], while the ABCA1 inducer, nuclear factor LXR, increases miR-144 expression, suggesting the presence of a negative regulatory loop. Furthermore, induced LXR and ABCA1 expression has been related to an estradiol-dependent reduction of lipid accumulation in macrophages and VSMCs [58, 59] suggesting that estrogen tightly regulates lipid content via this miRNA-mediated pathway. Finally, the effect of estrogen on cholesterol efflux is modified by age [178] and this may be related with the dysregulation of miR-144 expression during ageing.

miR-146a is one of the key miRNAs associated with vascular inflammation; it regulates endothelial activation by targeting upstream NF-κB pathway regulators such as TNF receptor associated factor *(TRAF)* 6 and IL-1 receptor associated kinase *(IRAK1)* [179] and is described as a marker of senescence-associated proinflammatory status in endothelial cells [180]. In a replicative endothelial cell model, miR-146a downregulation may have caused endothelial senescence by increasing the expression of its direct target, *NOX4* [181]. The relationship between miR-146a expression and estrogen has previously reported since circulating miR-146a expression was different between HRT-treated and nontreated postmenopausal genetically identical twins [121]. In addition, estradiol inhibition of miR-146a expression may be a key regulator of lipopolysaccharide-induced interferon-gamma *(IFN-γ)* expression in lymphocytes [128].

miR-21 expression is also related to estrogens and ageing; increased miR-21 levels have been found in aged heart tissues [182], in senescent vascular cells [164], and in blood samples in elderly people [183]. However, conversely, repression of miR-21 in senescent human aortic endothelial cells seems to increase the expression of antiproliferative and proapoptotic molecules [142] and may represent a diagnostic biomarker for acute myocardial infarction [184]. In cardiac fibroblasts, miR-21 action is implicated in heart hypertrophy by activating MAPK pathways [185], proinflammatory responses in endothelium under oscillatory shear stress [186], and VSMC proliferation after vascular injury [187]. However, the cardiovascular effects of estrogens attenuate these responses; indeed, estradiol downregulates miR-21 by binding to ERs in its promoter region [188] and miR-21 expression in postmenopausal women using HRT was lower than their twin sisters who did not use HRT [121]. Finally, in a murine model of pressure overload-induced cardiac fibrosis, miR-21 was among sex-specific miRNAs regulated by ERβ and was downregulated in estradiol-exposed primary

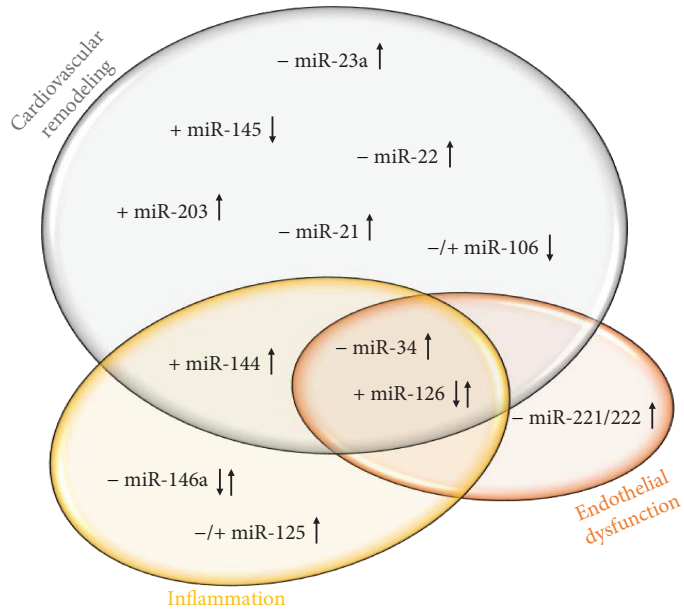

Figure 1: Estrogen-sensitive miRNAs associated with cardiovascular ageing are classified according to their role in the three main mechanisms leading to cardiovascular ageing: cardiovascular remodeling, endothelial dysfunction, and inflammation. (↓) and (↑) indicates increased or decreased expression in cardiovascular tissues during ageing, respectively. Role of estrogens on the expression of represented miRNA is also represented; (−) and (+) indicates negative or positive regulation, respectively. Controversial findings dependent on expression in different tissues/cells are indicated as (↓↑) and (−/+).

cardiac fibroblasts [135] which may explain sex-dependent differences in cardiac remodeling [189].

miR-34 is part of a well-described family of ageing-associated miRNAs related to cardiomyocyte apoptosis [190] and vascular cell senescence [191, 192]. The expression of miR-34 is elevated in old versus young aorta [173] as well as during ageing and in cardiac pathologies; however, inhibition of the miR-34 family improves heart function [193]. Moreover, miR-34 is influenced by sex and estrogens; miR-34b-3p expression is higher in male rather than female mouse hearts [143]. Furthermore, estradiol exposure decreases miR-34a expression in cultured endothelial cells [194] and is associated with SIRT1-dependent regulation of *eNOS*. Moreover, blockage of miR-34a expression was more effective in females than in males in a murine model of moderate dilated cardiomyopathy [195], highlighting the importance of studying sex-related differences in miRNA-based therapies.

miR-22 is upregulated in aged hearts [196, 197] and contributes to cardiac hypertrophy by promoting senescence and migratory activity in cardiac fibroblasts [196] and by inhibiting autophagy in cardiomyocytes [197]. Estradiol also appears to decrease miR-22 expression in primary cardiomyocytes via ERα-mediated mechanisms [198]. In addition, miR-22 downregulation increases oxidative defense by increasing expression of its direct target, Sp1 [198]: a regulatory pathway that may explain estradiol-mediated cardioprotection. A reciprocal feedback loop between ERα and miR-22 may be involved in fine-tuning the regulation of estrogen action by posttranscriptionally controlling ERα expression [199].

miR-125 is part of a family of miRNAs which are mainly associated with the activation of inflammatory cells. In macrophages exposed to lipopolysaccharide (LPS), miR-125a is upregulated [200] and miR-125b is downregulated [201]. However, more studies are needed to understand their role in activating or inhibiting inflammatory processes. The regulation of some miR-125 family members is estrogen-dependent: estradiol inhibits NF-κB activity by restoring downregulated miR-125b expression in LPS-stimulated macrophages [201] and both miR-125a and miR-125b are downregulated in lymphocytes exposed to estradiol [128]. Moreover, miR-125 activity is also associated with angiogenesis during ageing: miR-125a-5p expression is increased in endothelial cells from aged mice and is involved in impaired angiogenesis [202].

6. Conclusion

The role of sex hormones in cardiovascular physiology has been extensively studied and has been proposed as the cause of the reported sex-related differences in cardiovascular diseases. Vasodilation, inflammation inhibition, and the action of antioxidants have all been attributed to the effect that estrogens exert on the cardiovascular system. However, the effects that estrogens have on the heart and vascular tissues are themselves modified by ageing. Estrogens modify cardiovascular function by modulating gene expression, and in this sense, miRNAs have emerged as a new regulatory mechanism of both physiological and pathological processes because they regulate gene expression profiles at the posttranscriptional level (Figure 1). Studies on miRNAs

have provided insights into cardiovascular function, age-associated physiological changes, and cardiovascular pathologies. However, information about the role of miRNAs in estrogen-dependent processes in cardiovascular ageing is still scarce. Therefore, basic research to analyze sex-specific miRNA regulation can help us to understand differences in cardiovascular diseases between men and women. It may be possible to translate this new knowledge into clinical research, using miRNAs as potential tools for giving diagnoses and/or prognoses, as affordable and noninvasive biomarkers, and as a therapeutic tool for regulating (silencing or increasing) miRNA levels. Thus, future perspectives in miRNA-based therapies should consider the importance of sex-related differences in vascular ageing.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Increase and Redistribution of Sex Hormone Receptors in Premenopausal Women Are Associated with Varicose Vein Remodelling

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In chronic venous insufficiency of the lower limbs, data show that the clinical manifestation is varicose veins (VVs), and VV epidemiology suggests that sex hormones directly influence disease development through intracellular receptors. This study aimed to determine the presence and localization of oestrogen receptors (ERs), progesterone receptors (PRs), and androgen receptors (ARs) in both healthy and VV wall cells and their relationship with gender. In this study, samples from patients without a history of venous disease (CV) ($n = 18$) and with VV ($n = 40$) were used. The samples were divided by gender: CV women (CVw) = 6, CV men (CVm) = 12, VV women (VVw) = 25, and VV men (VVm) = 15. RT-qPCR and immunohistochemical techniques were performed, and increased ER and PR protein expression was found in VVw in all tunica layers. ARs were localized to the adventitial layer in the CV and were found in the neointima in VVs. mRNA expression was increased for ER and PR in VVw. AR gene expression was significantly decreased in VVm. The increase in the number of these receptors and their redistribution through the wall reinforces the role of sex hormones in varicose vein development.

1. Introduction

Varicose veins are the most common form of primary venous insufficiency, with a high prevalence of 20–60% in the Western population [1]. Various theories have attempted to explain why healthy veins become varicose (dilated and tortuous). The primary cause of varicose vein (VV) formation has not been established; however, both valvular dysfunction and venous pressure seem to play key roles in disease onset and progression [2–4]. The force of gravity and the absence of an active venous return mechanism mean that the venous wall components are subjected to intense biomechanical forces that may condition or aggravate functional failure of the venous wall [5, 6]. Macroscopic changes in the veins also occur at a microscopic level through modifications to the extracellular matrix (ECM) and cellular components

(especially of smooth muscle cells (SMCs)) [7]. The ECM is the fundamental support network of vascular walls, providing most of their structural properties. Several studies implicate variations in ECM components (collagen fibres, elastic fibres, matrix metalloproteinase (MMP), and glycosaminoglycans) and in SMCs in varicose vein pathology [7–9].

Another key point is the relationship between sex hormones and venous pathology, which primarily focuses on the increased thrombogenic risk they produce [10–12]. Female sex hormones may play a predominant role in varicose physiopathology, especially considering the influence of gender and pregnancy on varicose vein development [13]. How molecular changes occur and how hormones are involved in regulating the venous wall remain to be determined with consideration of the many variables involved, including gender differences in regulating vascular tone, differences in the stimulation or inhibition of cell populations by sex hormones, hormone actions in synthesising ECM products, and actions of hormone receptors and their activation pathways. Previous clinical and epidemiological studies have shown a predominance of varicose veins in women [12, 14, 15]; therefore, this study aimed at verifying whether steroid receptors and progesterone, oestrogen, and androgen receptors are involved in varicose vein development and whether this occurs differently in men and women. Our results show important changes in the localization of these receptors according to histological modifications when vein walls become varicose.

2. Patients and Methods

2.1. Patients and Samples. Fifty-eight saphenous vein specimens were obtained during surgery from organ donor extraction (controls with no history of venous insufficiency) or varicose vein surgery. Informed consent to participate in this study was obtained from all subjects. The project was approved by the Clinical Research Ethics Committee of the Ruber International Hospital. We divided the study population into two groups according to the sex of patients as follows.

2.2. Control Group (CV). The control group ($n = 18$) was composed of 6 vein specimens harvested from female patients (CVw) [mean age 52.4 ± 4.7, range 43–63 years] and 12 specimens from male patients (CVm) [mean age 61.2 ± 7.8, range 47–69 years].

2.3. Varicose Vein Group (VV). The varicose vein group ($n = 40$) was composed of 25 vein specimens harvested from female patients (VVw) [mean age 43.5 ± 6.6, range 46–62 years] and 15 specimens from male patients (VVm) [mean age 59.6 ± 7.3, range 53–71 years]. Segments of the saphenous vein were obtained at the time of extraction from patients with primary venous insufficiency. The Classification System for Chronic Venous Disorders (CEAP) based on clinical, aetiologic, anatomic, and pathophysiologic data was applied previously to the venous extraction [16].

Immediately after procurement, the specimens were placed in sterile minimum essential medium (MEM) and stored at 4°C and then transferred to the laboratory where they were divided into two fragments, one for microscopy (immunohistochemistry) and the other for storage at −80°C in RNAlater solution until RNA extraction and RT-PCR analysis.

2.4. Immunohistochemical Analysis. For immunohistochemical analyses, vein specimens were fixed in 4% paraformaldehyde solution, embedded in paraffin, and cut into 5 μm slices using a microtome (Microm, Barcelona, Spain). The sections were then deparaffinized, hydrated, and equilibrated in PBS (pH 7.4). We used anti-oestrogen, anti-progesterone, and anti-androgen receptor antibodies with specific secondary antibodies (Table 1). The antigen-antibody reaction was detected by peroxidase-labelled avidin-biotin procedures and avidin-alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA). The chromogenic substrate contained diaminobenzidine (DAB) for progesterone receptors (PRs) and oestrogen receptor (ER) alpha and alkaline chromogenic substrate for androgen receptors (AR). All protocols followed those of Ortega et al. [13]. After immunostaining, the tissue sections were examined under a light microscope (Zeiss, Jena, Germany). Positive cells were blind-counted under a light microscope (Zeiss) in 4 areas of 0.5 mm^2 per patient (40 high-power fields per group) by two authors. All values were expressed as the means ± SEM.

2.5. qRT-PCR. Tissue fragments of 1 cm^2 were obtained from the control and varicose veins, immersed in RNAlater solution (Ambion, Austin, TX, USA), and stored at −80°C until use. RNA was extracted through guanidine-phenol-chloroform isothiocyanate procedures using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was recovered from the aqueous phase and precipitated by adding isopropanol and incubating overnight at −20°C. RNA integrity was checked using a 1% (*w*/*v*) agarose gel and quantified by spectrophotometry. Complementary DNA was synthesised by reverse transcription using 200 ng of the total RNA with oligo (dT) primers (Amersham, Fairfield, USA) and the enzyme Moloney murine leukaemia virus reverse transcriptase (M-MLV RT, Invitrogen). cDNA was amplified by PCR using the following primers: ER sense 5′ GTG GGC GTT CCA AAT GAA AGC CAA G 3′ and antisense 5′ GAG CGC CAG ACG AGA CCA ATC ATC A 3′ at 60°C; PR sense 5′ CCC CAC GGC CAG CAG GTG CCC TAC T 3′ and antisense 5′ GAG CGC CAG ACG AGA CCA ATC ATC A 3′ at 55°C; and androgen receptor (AR) sense 5′ TAC CAG CTC ACC AAG CTC CT 3′ and antisense 5′ GTC TCA CTG GGT GTG GAA AT 3′ at 60°C. The constitutive gene glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) (primers: sense 5′ TCA CCA TCT TCC AGG GA 3′ and antisense 5′ CAC AAT GCC GAA GTG GT 3′ at 60°C) was used as a control.

The RT-qPCR mixture contained 5 μl of inverse transcription product (cDNA) diluted at 1 : 20, 10 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 1 μl (6 μM) of each primer, for a final reaction volume of 20 μl. RT-qPCR was performed in a Fast 7500

Table 1: Primary and secondary antibodies used and their dilutions.

Antigen	Species	Dilution	Provider
Oestrogen receptor (ER) alpha	Rabbit	1 : 100	RM-9101 (Neomarkers, Fremont, CA, USA)
Progesterone receptor (PR)	Rabbit	1 : 100	RM-9102 (Neomarkers, Fremont, CA, USA)
Androgen receptor (AR)	Mouse	1 : 25	Ab9474 (Abcam, Cambridge, UK)
Anti-rabbit IgG	Mouse	1 : 1000	RG-96 (Sigma-Aldrich, St. Louis, MO, USA)
Anti-mouse IgG	Goat	1 : 300	Polyclonal (Sigma-Aldrich, St. Louis, Missouri, USA)

Applied Biosystems instrument. Samples were subjected to an initial stage of 10 min at 95°C. The cDNA amplification conditions were 40 cycles of 95°C for 15 s, 60°C (the same annealing temperature for each first pair) for 30 s, and 72°C for 1 min. Fluorescence was determined at the end of each cycle. Gene expression was normalized against the expression recorded for the reference gene GAPDH.

2.6. Statistical Analysis. GraphPad Prism® 6.0 was used to perform ANOVA, Bonferroni's test (one-tail), and Student's *t*-test for unpaired data. Data are expressed as the mean ± SEM. The following p values were considered statistically significant: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Protein Analysis

3.1.1. Oestrogen Receptors (ERs). All vein samples stained positive for oestrogen receptor alpha in the vein wall. Oestrogen receptors (alpha) were localized in the cell nuclei in the three vascular wall layers (intima, media, and adventitia) in the healthy and varicose vein groups (Figure 1).

Quantifying the ER-positive cells showed that the mean was 9.03 ± 0.55 in the CVm group and 16.23 ± 1.38 in the VVm group, which was a statistically significant difference ($^{**}p = 0.005$). In the women, the mean number of positive cells was 21.13 ± 2.06 in the CVw group and 32.66 ± 1.84 in the VVw group ($^{**}p = 0.0047$). Among the control groups, the CVw group was significantly increased compared with the CVm group ($^{**}p = 0.005$) and the pathological group ($^{**}p = 0.0012$) (Figure 1(a)).

The results obtained show a clear increase in alpha oestrogen receptors in the venous walls of women with varicose veins. This increase in expression was homogenous in the three tunica layers of the varicose vein, distributed intensely throughout the wall.

3.1.2. Progesterone Receptors (PRs). In the pathological varicose veins, PR expression showed the same trend as ER expression, and this expression was more abundant than that in healthy veins, especially in women (Figure 2).

Quantitative analyses showed that the PR-positive cell mean was 11.40 ± 1.96 (mean ± SEM) in the CVm group and significantly increased to 19.16 ± 2.83 in the VVm group ($^{*}p = 0.0221$). In the CVw group, the positive cell mean was 30.71 ± 4.67, which significantly differed from the VVw group mean of 66.16 ± 2.01 ($^{***}p = 0.0006$). Significant ratios were also observed between the CV groups ($^{**}p = 0.0042$) and the VV groups ($^{**}p = 0.0021$). Overall, the pathological veins showed greater expression of the PR marker, and within groups, women showed the most PR-positive cells (Figure 2(a)).

In women with varicose veins, the presence of progesterone receptors is two times higher than that in the group without varicose veins. The expression of progesterone receptors was observed in all tunica layers of the venous wall.

3.1.3. Androgen Receptors (ARs). Androgen receptors were confined to the nuclei of a few cells in the adventitial layers of healthy veins (Figures 3(b) and 3(c)); however, in varicose veins, the positive cells were mainly located in the neointimal layer (Figures 3(d) and 3(e)).

The percentage of AR-positive cells was low in all groups (CVm = 7.48 ± 1.08, VVm = 7.24 ± 1.13, CVw = 6.02 ± 0.24, and VVw = 8.56 ± 0.79). No significant differences were found in the values among the different populations (Figure 3(a)).

3.2. Genetic Analysis

3.2.1. Oestrogen Receptor (ERs). The mean expression of ERs was 0.51 ± 0.11 RQ (relative quantity mRNA) in the CVm group, 0.88 ± 0.37 RQ in the VVm group, 1.67 ± 0.63 RQ in the CVw group, and 3.63 ± 0.46 RQ in the VVw group. Significant differences were established between the CVw and the VVw groups ($^{*}p = 0.047$) as well as between the pathology groups ($^{*}p = 0.016$) (Figure 4(a)).

3.2.2. Progesterone Receptors (PRs). Gene expression of the PRs showed a mean ± SEM of 0.51 ± 0.11 RQ in the CVm group and 0.53 ± 0.10 RQ in the VVm group, with no significant differences between the groups. For the women, the average was 0.33 ± 0.14 RQ in the CVw group and 1.01 ± 0.16 RQ in the VVw group, indicating a statistically significant increase ($^{*}p = 0.049$). A statistically significant increase was also found between the VVm and the VVw groups, $^{*}p = 0.044$ (Figure 4(b)).

3.2.3. Androgen Receptors (ARs). AR expression was detected in all patients studied. Figure 4(c) reveals that only in men was the AR mRNA expression significantly higher in the control veins than in the varicose veins ($^{**}p = 0.004$), while no significant differences were observed in AR expression between the CVw and the VVw groups. The mean AR expression was 0.53 ± 0.19 RQ in the CVm group, 0.02 ± 0.01 RQ in the VVm group, 0.28 ± 0.07 RQ in the CVw group, and 0.23 ± 0.67 RQ in the VVw group (Figure 4(c)).

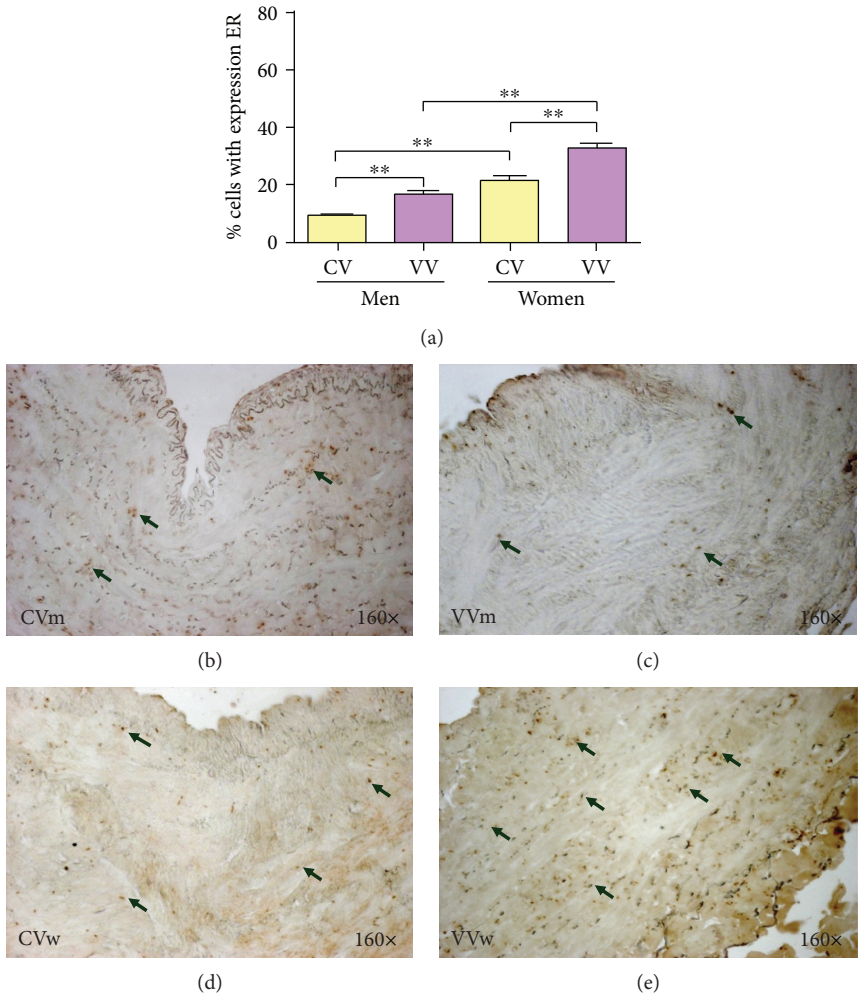

Figure 1: (a). Percentage of cells positively expressing the oestrogen receptor (ER) alpha among the study groups (CV = control vein, VV = varicose vein). (b-e). ER immunodetection images for the four analysis groups. The brown colour indicates the precipitate that correlates with ER protein expression. $^{**}p < 0.01$.

4. Discussion

In this study, we observed an increase in sex hormone receptors under varicose vein conditions in both genders. Furthermore, we want to highlight the importance of the redistribution of these receptors through the venous wall. This redistribution could be related to the pressure change in varicose veins and the histological remodelling of the vein wall.

Population age is one of the main factors affecting the presence of chronic disease. The venous system is especially affected by changes in ageing, and the effects are unequal between the genders [8]. Per the Framingham study, the annual occurrence of varicose veins is 2.6% in women and 1.9% in men [17].

This finding remains controversial because some authors consider that truncal varicose veins are equally common in men and women, while others indicate that importance lies in the reticular varicose veins and telangiectasias that are more common in women [17–19]. In women, key events are directly related to the appearance of varicose veins, such as pregnancy and menopause, which are influenced by age and hormones [19, 20]. During menopause, sex hormones play important roles, as they are involved in numerous pathologies [21]. Oestrogen receptors are present in the endothelium, SMCs, and some adventitial cells in the femoral veins [22]. Our results showed that in the varicose veins of women around menopause, there was a two-fold increase in the number of ER^+ cells and ER gene expression compared to those in the veins of healthy women. The number of ERs was about two times lower in men. These results agree with the studies of Mashiah et al. [23], who used a different methodology for the evaluation of oestrogen receptors. Several pathological anatomy studies have indicated that the human saphenous vein stains positive for progesterone receptors in both genders [24]. The staining is uniformly distributed in the SMCs of the media and subendothelium, adventitial fibroblasts, and vasa vasorum [25, 26]. Additionally, RT-qPCR has demonstrated the presence of progesterone receptor mRNA [26]. Our study showed the presence of PR in all three tunica layers (endothelium, media, and adventitia) at levels twice as high in varicose patients compared to that in healthy patients of both genders. Even in the vein walls of

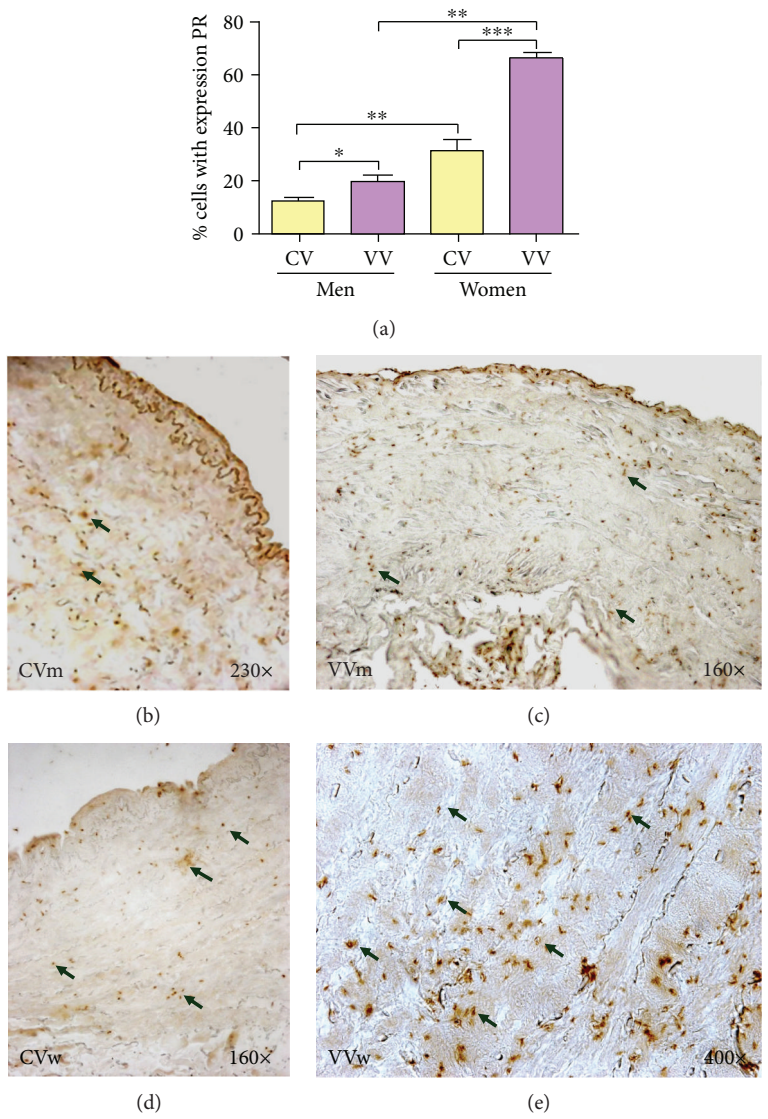

Figure 2: (a). Percentage of cells positively expressing the progesterone receptor (PR) in the study groups (CV = control vein, VV = varicose vein). (b–e). PR immunodetection images from the four analysis groups. The brown colour indicates the precipitate that correlates with PR protein expression. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

varicose veins of women, the increases in the number of PR-positive cells and PR mRNA expression were three-fold compared to those in healthy veins of women. Using the same technique, the presence of ER-delta4 isoform mRNA and protein has also been demonstrated in both mammary arterial cell and human saphenous vein cell cultures, and this expression was not located in the nucleus, which may be related to effects from nongenomic oestrogens [27]. The same author previously demonstrated ER mRNA presence in human saphenous vein SMCs, and this receptor can be activated [28].

Other authors have shown oestrogen and progesterone receptors in the human internal saphenous vein [24, 25, 28, 29]. Bergqvist et al. [25] described the positive expression of PR and ER in all samples from fertile women, but ER expression was not observed in samples from postmenopausal women or from men. Mashiah et al. [23] and Krasiński et al. [29] found positive ER expression in the ECM and SMC nuclei and PR in the nuclei of the SMC and subendothelial cells. Mashiah et al. [23] found that both receptors were expressed in the adventitial vasa vasorum cells. These authors described a nonuniform distribution of these cells, with more oestrogen and progesterone receptors (for both genders) in the varicose samples than in the controls. In the varicose samples, the numbers of oestrogen and progesterone receptors were higher in the varicose segments than in the nonvaricose segments in both men and women. The number of oestrogen and progesterone receptors in varicose veins was significantly higher in women when studied with semiquantitative methods. Our results coincide in part with those of other authors [23, 25–28] in terms of the increase in homologous receptors in varicose pathology. However, we found the presence of nuclear receptors at the adventitial level. The findings of previous authors are limited to the presence of receptors in the endothelium of the vasa vasorum. In

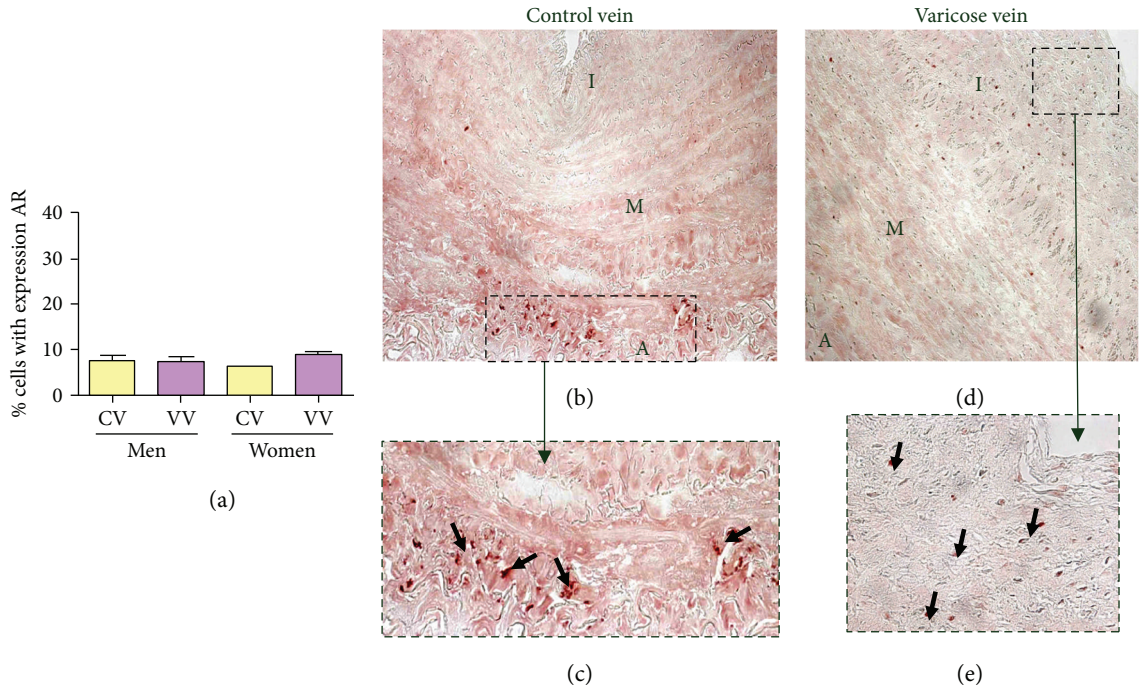

Figure 3: (a). Percentage of cells positively expressing the androgen receptor (AR) in the study groups (CV = control vein, VV = varicose vein). (b–e) AR immunodetection images in the four analysis groups. The red colour indicates the precipitate that correlates with AR protein expression.

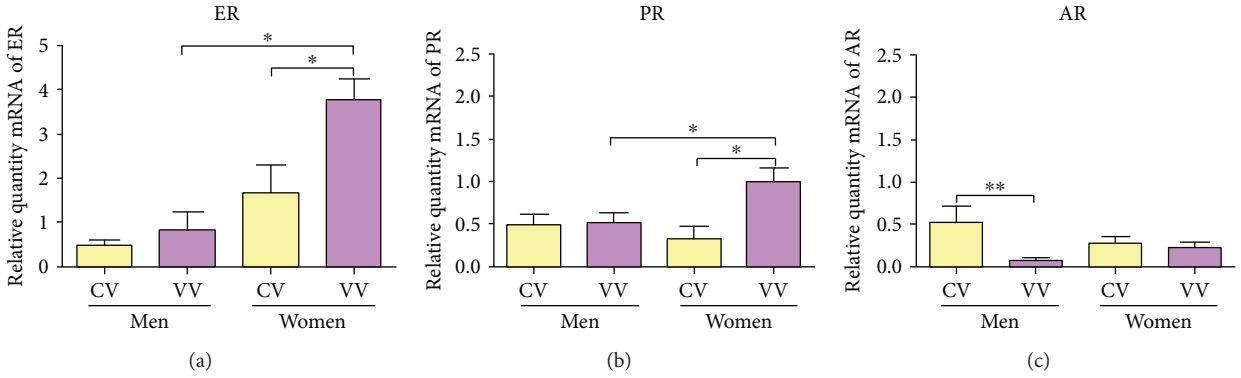

Figure 4: mRNA levels for the oestrogen receptor (ER) alpha (a), progesterone receptor (PR) (b), and androgen receptor (AR) genes (c) quantified by RT-qPCR. The results were normalized to that of the reference gene GAPDH and are provided in arbitrary units. CV = control vein and VV = varicose vein. $^{*}p < 0.05$ and $^{**}p < 0.01$.

addition, our results show protein expression and correlation with gene expression.

The direct effects of oestrogen on the human saphenous vein wall have been described in a few studies. Greater methylation of the ERα gene was found in samples from older individuals, implying lower activity [30, 31]. The authors hypothesized that the methylation associated with ERα gene inactivation could play a role in vascular tissue ageing. 17β-Oestradiol affects venous contractility, increasing the vacuum caused by ET-1 but not vasodilating it [32]. 17β-Oestradiol inhibits the Ca^{2+}-dependent vasoconstriction of saphenous veins in vitro. It also inhibits the vasoconstriction mediated by increased K^{+} as well as the contraction induced by $CaCl_2$, suggesting that E2 possibly interferes with Ca^{2+} channels [33]. These authors also described that oestradiol independently inhibits Ca^{2+}. Oestradiol dose-dependently inhibits vasoconstriction induced by phorbol dibutyrate (PDB) in media without Ca^{2+} and in SMCs depleted of Ca^{2+}, where $PGF_2\alpha$ produces contraction and oestradiol relaxes SMCs. This study suggests that part of the relaxing effect of E2 is independent of the Ca^{2+} channel blockade. Under supraphysiological conditions, 17β-oestradiol dilates the umbilical artery and vein [34]. 17β-Oestradiol does not appear to decrease α-adrenergic system activity in SMCs in vivo [35]. 17β-Oestradiol markedly dilates the femoral vein in healthy sows under physiological conditions and

in ovariectomized sows, both dependently and independently of the endothelium [22].

Other studies described a lower venous distensibility in contraction under low hormone levels (oophorectomized rats), with the response at rest equal to that of the control veins. In this model, hormone replacement partially restored the loss of compliance [36, 37]. Thus, high oestrogen levels in menopausal women have been associated with more varicose veins and greater venous distensibility [38, 39]. Endodiode-dependent vasodilation (dorsal vein of the hand) was improved in postmenopausal women with hormone replacement therapy compared with controls who received no substitution treatment [40], suggesting the relevance of these hormones.

Conversely, the study of androgen receptors in regard to its possible relationship with venous pathology has not been a research interest. Kendler et al. [41] described dysregulation of androgenic receptors in the varicose veins of men compared with healthy ulnar veins. These authors tried to relate the increase in the levels of sex hormones in the blood of varicose patients with the dysregulation of hormone receptors. Our results coincide with these findings, showing downregulated AR gene expression in men with varicose veins. Our immunohistochemical study shows, for the first time, the different locations of these receptors. In control saphenous veins (men and women), androgen receptors were found at the level of the adventitial tunica. However, in varicose veins, the location of these receptors was changed, occurring at the level of the neointima.

Some studies show that inhibition of androgen receptors can occur in the presence of high levels of progesterone [42]. Our results show changes in the location of the hormonal receptors and a greater activity of ER and PR at the adventitial level and of AR in the neointima of patients with varicose veins. This change in expression could be related to a compensatory effect via an increase in the hydrostatic and tangential pressure of the varicose veins. The increase in receptors could stimulate the proliferation of vascular cells responsible for morphological changes such as the increased diameter, thickness, and tortuosity characteristics of varicose veins, as seen by some authors in in vitro studies of endothelial cells and smooth muscle cells [43].

5. Conclusions

The above results show the effects of sex hormones on the vascular system, especially in women. Varicose veins in women are associated with increased oestrogen and progesterone receptors in all tunica layers of the vein wall. However, the location of androgenic receptors was observed in the control patients only at the level of the adventitial tunica. In varicose veins, this expression showed redistribution to the neointima. The overexpression of oestrogen and progesterone receptors and androgenic receptor redistribution in the varicose vein wall reinforce the hypothesis that hormones are involved in varicose vein pathophysiology. These studies demonstrate the necessity of developing new strategies for premenopausal women directed at preventing the development of varicose veins.

Data Availability

The data used to support the finding of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Natalio García-Honduvilla and Ángel Asúnsolo contributed equally to this work. Gemma Pascual and Julia Buján shared senior authorship in this work.

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