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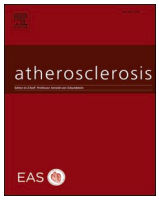

Review article

Gonadal sex *vs* genetic sex in experimental atherosclerosis

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ABSTRACT

Epidemiological data and interventional studies with hormone replacement therapy suggest that women, at least until menopause, are at decreased cardiovascular risk compared to men. Still the molecular mechanisms beyond this difference are debated and the investigation in experimental models of atherosclerosis has been pivotal to prove that the activation of the estrogen receptor is atheroprotective, despite not enough to explain the differences reported in cardiovascular disease between male and female.

This casts also for investigating the importance of the sex chromosome complement (genetic sex) beyond the contribution of sex hormones (gonadal sex) on atherosclerosis. Aim of this review is to present the dualism between gonadal sex and genetic sex with a focus on the data available from experimental models. The molecular mechanisms driving changes in lipid metabolism, immuno-inflammatory reactivity and vascular response in males and females that affect atherosclerosis progression will be discussed.

1. Introduction

The incidence of ischemic heart disease differs in relation to sex and gender; women present a lower cardiovascular disease (CVD) risk till menopause that increases thereafter [1], becoming comparable to men [2]. In concomitance, menopause comes along with a rise in risk factors such as hypertension, high circulating levels of cholesterol [3], development of carotid atherosclerotic plaque [4] and higher incidence of myocardial infarction (MI) [2]. Of note, women who experienced premature menopause had a 33% higher risk of heart failure and 9% higher risk of atrial fibrillation compared to those who did not [5]. While estrogens were identified as key players in cardiovascular protection, additional mechanisms, independent of hormones, but related to sexual chromosomes, have been proposed [6–9]. Aim of this review is to discuss factors differentially affecting atherosclerosis development in males and females as a consequence of either gonadal sex (pointing to sexual hormone differences) or of genetic sex (indicating the effect of genes selectively expressed in sexual chromosomes) with a focus on the evidence emerged from the use of experimental animal models.

2. Are experimental animal models reliable to study sex differences in atherosclerosis?

Since its introduction at the beginning of the XX century, the use of animal models has been crucial for understanding the molecular mechanisms driving atherosclerotic plaque formation and progression. However, the importance of reporting sex-differences in experimental studies of atherosclerosis has been so far underestimated [10–12], despite sex has been increasingly recognized as an independent variable of disease severity even in animal studies [13].

Indeed, the analysis of sex-related effects observed in humans through the investigation of specific molecular mechanisms in animal models is not a standard practice [14]. While experimental studies on the pathophysiology of atherosclerosis span from the use of cold vertebrate to non-human primates, few works systematically reported the results for both males and females and even less performed a statistical comparison of the differences in the two sexes [11,14].

Although rodents are the most used species for experimental research in the context of cardiovascular research, also zebrafish or larger animals like pigs or non-human primates are used (Table 1).

Zebrafish became popular in cardiovascular studies thanks to the similarity of its genome to humans [15], coupled with its rapid and large

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Table 1
Sexual dimorphism in atherosclerosis development in experimental models.

Species	Genetic modification	Intervention	Vascular bed	Sexual dymorphism in atherosclerosis development
Non-human primates	N/A	High fat high cholesterol diet	Coronary and iliac artery	M > F [21]
	N/A	High fat high cholesterol diet + Ovariectomy (F)	Coronary, carotid, iliofemoral artery	OVX F > sham F [22]
	N/A	High fat high cholesterol diet + Psychological stress (F)	Coronary artery	M < F [23–25]
Minipigs	N/A	High fat high cholesterol diet	Coronary artery	M ≤ F* [26]
	GOF PCSK9	High fat high cholesterol diet	Coronary artery, aorta (whole and arch)	M ≤ F* [27,28]
	GOF PCSK9	High fat high cholesterol diet + Orchiectomy (M)	Aorta (arch, thoracic, abdominal) and iliac artery	↑ necrotic core in orchiectomized pigs [29]
Rabbits	None, NZW, WHHL	Normal or high cholesterol diet	Aorta (arch, thoracic, abdominal)	M ≤ F* (reviewed in [14])
Mice	ApoE KO (C57BL/6 J background)	Normal or high cholesterol diet	Aorta (arch, thoracic, abdominal)	M ≤ F* (reviewed in [14])
	ApoE KO (C57BL/6 J background)	High cholesterol diet + Ovariectomy (F)	Proximal aorta and aortic sinus	OVX F > sham F [45]
	ApoE KO (C57BL/6 J background)	High cholesterol diet + Ovariectomy (F) + 17-beta-estradiol (E2) administration	Proximal aorta and aortic sinus	OVX F > OVX + E2 F [45]
	LDLR KO (C57BL/6 J background)	Normal or high cholesterol diet	Aorta (arch, whole)	M < = > F* (reviewed in [14])
	ApoE KO (C57BL/6 J background)	High cholesterol diet + E2 (M)	Proximal aorta and aortic sinus	lesion size [45]
	LDLR KO (C57BL/6 J background)	High cholesterol diet + Orchiectomy (M) + E2 or testosterone administration	Aorta (root)	ORX M > ORX M + hormones [46]
	hApoB100 and CETP Tg	High cholesterol diet + E2 administration	Aorta (arch and sinus)	↓ lesion size [44]
	ERα KO	+/− E2 administration	Aorta (whole)	↓early stage lesion size by E2 [48]
	CYP17a1/ApoE DKO	Standard and high cholesterol diet Low levels of androgens and estrogen → phenotypically F	Aorta (whole)	↑ lesion size [47]
Zebrafish	N/A	High cholesterol diet	Aorta	N/A due to the lack of sex chromosomes [18,19]

> Studies showing incresed atherosclerosis; = Studies showing no differences in atherosclerosis; < Studies showing reduced atherosclerosis.
F stands for females and M for males; OVX stands for ovariectomy and ORX for orchiectomy.
* Studies with direct statistical comparison.

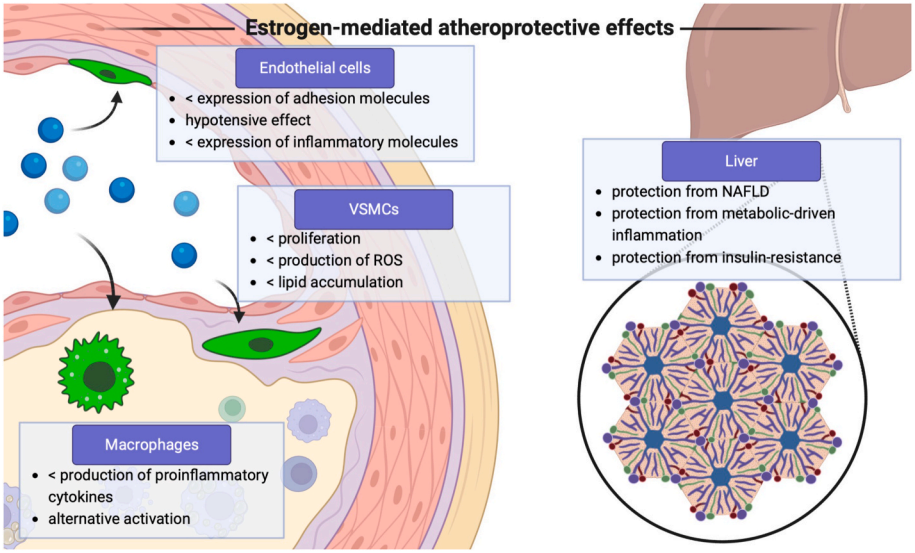

Fig. 1. Estrogen-mediated atheroprotective effects.
Estrogens exert their atheroprotective effects both locally on vascular and on immune cells but also on metabolic organs.
Specifically, in endothelial cells, estrogens promote vasodilatation and reduce the expression of adhesion and inflammatory molecules while in vascular smooth muscle cells limit lipid accumulation and proliferation. In macrophages, estrogens inhibit the release of pro-inflammatory cytokines and prevent the M1 classical activation, favoring the skewing toward an alternative phenotype (M2-like).
Systemic effects of estrogens include the reduction of non-alcoholic fatty liver disease development, hepatic inflammation, and insulin-resistance, thus dampening cardiovascular disease manifestations.

breeding. Zebrafish develops fatty streaks in the aorta upon cholesterol-rich diet feeding [16,17], but the lack of sex chromosomes limits the translation of genetic sex on disease development, despite sexual dimorphic characteristics have been reported [18,19]. Large animals, such as pigs and non-human primates, develop coronary artery disease similar to humans but practical and ethical considerations limit their use in experimental atherosclerosis [20]. Non-human primates show a sex-dependent risk of atherosclerosis similar to humans with increased coronary atherosclerosis in male compared to female, and the same is true in ovariectomized animals compared to controls [21,22]. Reduced levels of circulating estrogens as a consequence of psychological stress have been shown also to increase atherosclerosis compared to males [23–25].

In contrast, available data indicates that female minipigs develop equal or larger atherosclerotic lesions compared to males [26–28], but the literature about sex-specific difference in this species is limited, with a recent report showing that castration in hypercholesterolemic male Yucatan minipigs (which results in testosterone deficiency) does not alter the burden of dyslipidemia and atherosclerosis but increases necrotic core area in fibroatheromas [29].

Rodents remain the key species tested, with the impact of female sex frequently reported on atherosclerosis development in mice, hamsters, guinea pigs and rabbits [14,20] (Table 1). Mice are in general resistant to atherosclerosis development, although the strain C57BL6/J was shown to present an increased susceptibility to atherosclerosis when fed a cholesterol rich diet [30,31] and thus, by time, it has been genetically manipulated to exacerbate dyslipidemia and promote atherosclerotic plaque formation. The most used experimental models of dyslipidemia and atherosclerosis are mice lacking the apolipoprotein E (ApoE) or the low-density lipoprotein receptor (LDL-R). Additional experimental models include those overexpressing human ApoB, CETP or PCSK9, that is specifically induced via an adeno-associated virus vector and causes a reduced LDL-R expression in the liver [32,33], and those with the expression of a specific apoE variant (ApoE3-Leiden) (Table 1). In the following paragraphs, we will discuss how aspects related to gonadal-sex *versus* gender-sex influence atherosclerosis in murine experimental models.

3. Impact of gonadal sex on atherosclerosis

The cardiovascular protection reported in pre-menopausal women, usually associated to a less vulnerable and advanced lesion compared to males, has been directly linked to the favorable effects of estrogens and supported by the observation of a similar CVD risk between men and post-menopausal women, corresponding to a drop in estrogen levels. However, the contribution of aging itself, through a progressive degeneration of cardiovascular structures (arterial stiffening, heart hypertrophy, left ventricular wall thickening) as well as functions (endothelial dysfunction, hypertension, reduced protection from oxidative metabolism) independently of sex [34,35], should be taken into account. Nevertheless, in women, it has been reported that the higher is the age of natural menopause, the lower is the risk of mortality from coronary heart disease (CHD). Accordingly, oophorectomized young women present an increased CVD risk, further supporting the atheroprotective role of estrogens [36–39]. In spite of these findings, the atheroprotective function of estrogens has been challenged by the controversial result from different clinical studies with hormone replacement therapy (HRT) in post-menopausal women. Single versus combined administration of estrogens and progestins, might explain the different outcomes of these studies [40,41], and coupled to the observation that the earlier the HRT is started the better is the benefit [41,42]. The atheroprotective benefit of estrogens is evident in animal models and could be explained only in part by reduced plasma cholesterol [43], despite controversial results have been reported also in experimental studies [43].

Most studies on sex impact on atherosclerosis have reported an increased or at least equal atherosclerosis development in females compared to male mice [14] (Table 1). Estrogen administration in transgenic atherosclerotic mice overexpressing human ApoB100 and CETP resulted in a 2–3 times higher circulating cholesterol levels compared to other studies on dyslipidemic mice (*ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice), but still prevented atherosclerotic plaque development [44]. Ovariectomized *ApoE*$^{-/-}$ mice displayed increased atherosclerotic lesion development compared with female mice with intact ovarian function, independently of changes in plasma cholesterol levels [45]; of note this phenotype was reverted by subdermal delivery of 17-beta-estradiol (E2) at early atherosclerotic stage [45]. Estrogens have a protective role also in male: orchidectomy increases atherosclerotic lesion size in *Ldlr*$^{-/-}$ males and the administration of both E2 and testosterone limits atherosclerosis development. This protective function is lost in the presence of an inhibitor of aromatase [46], suggesting that testosterone mediated protection is guided by its conversion to estrogen. In agreement, male mice lacking the expression of CYP17A1, that is essential for the production of androgens and estrogens, are phenotypically females due to the low levels of testosterone and exhibit increased plasma cholesterol levels and atherosclerotic plaque when crossed with *ApoE*$^{-/-}$ mice [47].

A study investigating the protective effect of estrogens in early-stage atherosclerosis clearly showed that neither endogenous estrogens nor the estrogen receptor ERα are responsible for increased levels of HDL cholesterol, observed when exogenous estrogens are administrated to mice [48]. However, despite elevated levels of HDL-C are usually believed to contribute to the atheroprotection observed in pre-menopausal women, in mice HDL-C levels are not correlated with atherosclerotic plaque burden [48], suggesting that the atheroprotection of estrogens might be independent of the regulation of plasma lipid levels.

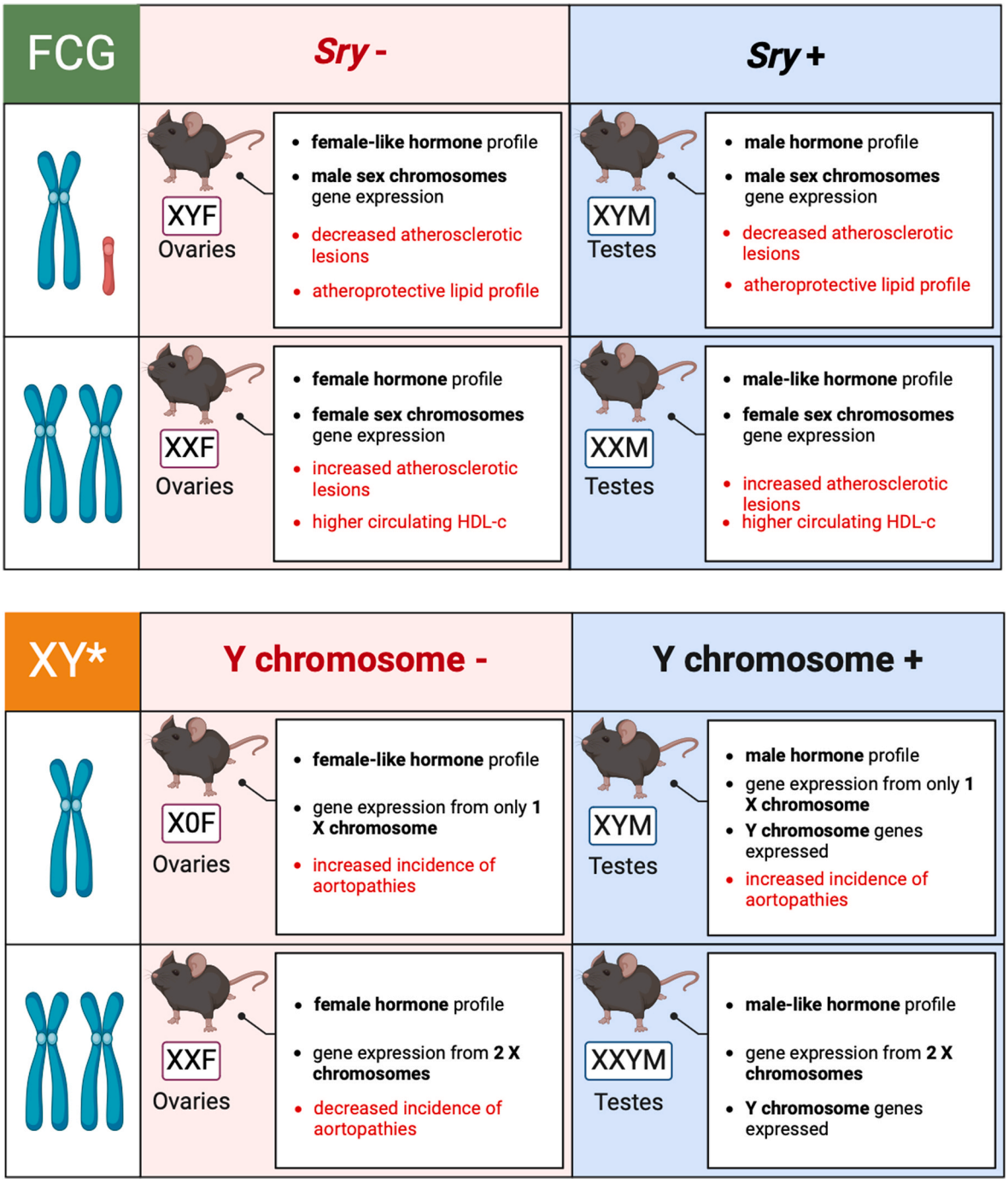

Fig. 2. Features of mouse models used to compare the impact of sex hormones or of sex chromosomes on atherosclerosis.
The four-core genotype (FCG) model can be used to understand if a sex-related characteristic is determined by sex hormones or sex chromosomes. In the FCG model, the *Sry* gene, responsible of the development of male reproductive system, is expressed on chromosome 3 instead of Y, now called Y^{-}, thus leading to mice with gonadal sex independent of the sex chromosome complement. Atherosclerosis-prone FCG mice prove the role of XX chromosomes on increasing atherosclerotic lesion size and circulating HDL cholesterol beyond the gonadal sex and circulating hormones, compared to the mice carrying the XY chromosomes that are associated with an overall atheroprotective lipid profile and decreased atherosclerotic lesion.
Further understanding on the role of sex chromosome dosage on a trait of interest can be obtained with the use of the XY* model, characterized by an improper recombination of the Y chromosome with the X chromosome, leading to X0 and XXY combination in addition to common XX and XY sex chromosomes. Thanks to this experimental model a higher increase in aortopathies has been associated with X monosomy when compared to XX mice, in line with what has been reported in Turner women.

Although important, the dimension of atherosclerotic plaque is not the only determinant of increased risk of events, and other factors such as lesion vulnerability and inflammation are to be considered. This is well established in pre-clinical studies where collagen content, smooth muscle cells and leukocyte infiltration are often evaluated as markers of disease stage, but not yet when regarding sex-driven differences. Atherosclerotic plaques of postmenopausal women are usually characterized by increased vulnerability and erosion with age, probably in concomitance with the drop in the protective effect of estrogens on the vasculature and inflammation [49,50], calling to ad hoc studies to weigh the contribution of these factor on top of aging on CV risk in women. In this issue, sex-specific effects on plaque characteristics are discussed in [51,52] and the cellular and molecular mechanisms in [53,54].

4. Estrogen receptor dependent-atheroprotective effects

In premenopausal women estradiol is released by ovaries, circulates mainly bound to the sex-hormone-binding globulin and acts on distant targets. Instead after menopause, similar to men, adipose tissue contributes almost completely to the amount of circulating estrogen through the conversion of stored or circulating androgens by aromatase, locally expressed [55,56].

The activity of estrogens is then mediated by activation of estrogen receptors (ERs) which are located in the cytosol and, upon binding with E2, dimerize and migrate to the nucleus where they interact with the estrogen responsive element, ERE, and regulate the expression of downstream genes. There are two estrogen receptors, namely type α (ERα) and type β (ERβ) which are encoded respectively by *Esr1* and *Esr2* genes [57,58]. Both receptors are present in the nervous and cardiovascular systems, but Erα in female and male reproductive organs, liver and adipose tissue, while ERβ is found mainly in the prostate, bladder, ovary, but also adipose tissue, and immune system [59]. ERα is highly expressed in endothelial cells and plays a role in mediating the effects of estrogens on the vascular endothelium, whereas ERβ stimulates the production of nitric oxide; the activation of both promotes a hypotensive effect (Fig. 1) [60]. The activation of ERα also limits the proliferation of vascular smooth muscle cells (VSMCs) in high-glucose conditions, their differentiation, the production of reactive oxygen species (ROS), and lipid accumulation (Fig. 1); in this context, the role of ERβ remains more controversial [61]. These findings have suggested that ERα activation by E2 plays an atheroprotective role on the vasculature [62], while studies on ERβ have led to divergent conclusions [63–65]. Of note, it has been shown that ERs can differently regulate gene expression in the same tissue, with ERα primarily upregulating, whereas ERβ downregulating the expression of genes related to extracellular matrix synthesis, electron transport in the mitochondria, and reactive oxygen species pathways in murine aorta [66].

Additionally, the level of both ERs in the vasculature can be regulated by sexual hormones: the analysis on endothelial cells from peripheral veins of premenopausal women showed that ERα expression is 30% lower during the early follicular phase compared to the late follicular phase [38], while increased ERα and decreased ERβ expression have been reported under long-term estrogen treatment in *in vitro* experiments [67,68]. By contrast, estrogen has been described to suppress vascular inflammation by decreasing the production of proinflammatory molecules, including cytokines and adhesion molecules [69–71] (Fig. 1). However, increased expression of ERβ over ERα has been described during aging and associated with higher oxidative stress in experimental models. This dichotomic inflammatory response is related to the different signaling pathways of ERs [72,73] and influenced by the phase of the estrous cycle in rodents [74], as well as by HRT in humans [75]. To note, the impact of HRT on inflammation is not unequivocal, and this discrepancy has been potentially associated to differences in the hormone combination or the route of administration [75,76]. In parallel, in mice levels of E2, that depend on the phase of estrous cycle - increasing in diestrus (D), and proestrus (P), while decreasing in estrus (E) and metestrus (M) over a period of 4–5 days [77,78] – have been shown to be associated to arterial stiffening [79], with higher levels of E2 resulting in a more favorable gene expression profile associated to improved cardiac and vascular function [80]. In addition to ERs, E2 can signal through the G Protein-coupled Estrogen Receptor (GPER), a receptor mainly expressed on endothelial cells and VSMCs. Its activation promotes vasodilation and inhibits VSMCs proliferation [81]. Accordingly, GPER deletion resulted in reduced vasodilation, increased inflammation and atherosclerosis [82].

Estrogens target also the liver [83]; more than 1000 genes were shown to present a sex-bias in their expression, with genes involved in triglyceride and cholesterol metabolism, or in fatty acid oxidation presenting sexual dimorphism and fluctuation during the estrous cycle [84–86]. Estrogens can mediate these effects on hepatic metabolism via both transcriptional activation or post-translational modification of ERα, ERβ and GPER [87]. Estrogens indirectly affect hepatic metabolism also by regulating growth hormone (GH) action [88]; this occurs in the central nervous system, by the regulation of pituitary GH secretion, and at the tissue levels by the activation of GH signaling [63]. Indeed, GH controls a network of transcription factors related to energetic and lipid metabolism (PPARα, CAR, FXR, SHP, SREBP, CRBP), and the absence of estrogen signaling has been associated to a metabolic-like syndrome described in GH deficiency [89].

A proper response to estrogens in the liver protects from metabolic-driven inflammation and non-alcoholic fatty liver disease (NAFLD) [88] (Fig. 1). Estrogen deficiency in ovariectomized mice, vice versa, results in hepatic insulin resistance (Fig. 1), increased fatty acid uptake but not oxidation, leading to fat accumulation in the liver and in the adipose tissue [90,91]. This phenotype was observed also in $Er\alpha^{-/-}$ mice and liver specific (LERKO) ERα knockout mice [92,93], but not in $Er\beta^{-/-}$ mice [94].

Evolutionarily, this specific metabolic response mediated by estrogens could be related to the different energetic requirements for reproduction that depend on increased metabolic flexibility of female liver [95].

Given the key role of the immunoinflammatory response during atherosclerosis, it is conceivable to speculate that the sexual dimorphism observed in the immune response might also impact the progression of CVD. It is known that rheumatoid arthritis (RA) and multiple sclerosis (MS) are more frequent in women [96], while men show a higher mortality for infectious diseases and a higher risk of developing fatal cancers [97].

In vitro, estradiol was shown to reduce the pro-inflammatory activation of both murine and human monocytes and macrophages [98,99] (Fig. 1), and their chemotaxis [100]. *In vivo*, estrogen receptor activation has been shown to modulate the production of myeloid cells, at the level of the hematopoietic niche [101]. Experimental female mice lacking the expression of ERα in hematopoietic or myeloid cells manifest obesity-induced insulin resistance and atherosclerosis [102], suggesting an immunomodulatory effect of estrogen during cardiometabolic diseases (Fig. 1). This is confirmed by the ability of estrogens to promote an alternative activation of macrophages from human PBMCs [103,104] (Fig. 1), and directly reduce metalloproteinase 12 macrophage expression in the atherosclerotic plaque [105].

5. Impact of genetic sex on atherosclerosis

When investigating the impact of sexual dimorphic characteristics, it is critical to consider not only the contribution of sexual hormones (gonadal sex) but also that of genes present in sexual chromosomes, that might present a different expression pattern in males and females (genetic sex) [106–108].

The evaluation of CVD risk in transgender people after a gender-affirming hormone-based therapy has been pivotal to evaluate the impact of gonadal versus genetic sex. Relevant to report is that transgender subjects display increased risk of myocardial infarction

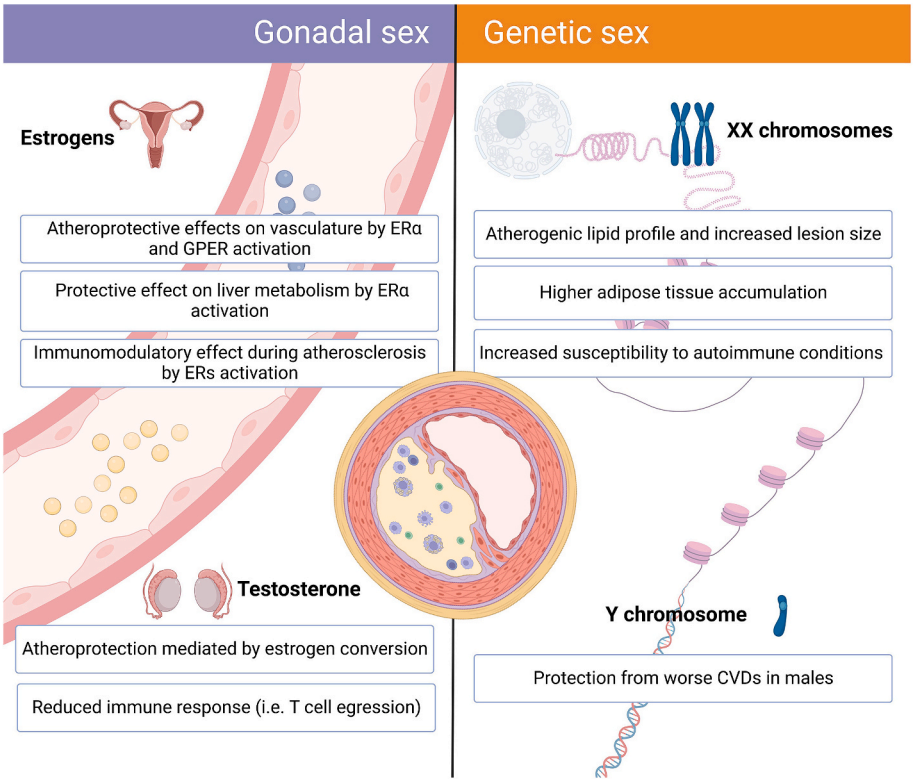

Fig. 3. Impact of gonadal sex *vs* genetic sex on atherosclerosis and atherosclerosis-related risk factors.
On the left, the impact of gonadal hormones on atherosclerosis is presented. Estrogens mediate a series of atheroprotective effects on the vasculature, on liver metabolism and on immune inflammation through the activation of nuclear and surface receptors. Testosterone directly dampens immune activation and can also be converted in estrogen thus inducing atheroprotection.
On the right, the role of genetic sex on atherogenesis is shown. The presence of two X chromosomes associates with a more atherogenic lipid profile, increased atheroma and adiposity, and increased susceptibility to autoimmune conditions. Although the Y chromosome presents a lower number of genes when compared to the X chromosome, its loss was associated to increased clonal hematopoiesis, thus predisposing to an increased risk of major cardiovascular events and worse heart failure.

compared to cisgender age-matched individuals [109,110]. In addition, a consistent change in circulating lipid profile [111,112], hemostasis and inflammation [113] are observed in people undergoing gender-affirming hormone therapy. Trans women have decreased total cholesterol, LDL-C, and triglycerides while trans men, in contrast, present an increase in these lipid parameters [111,112], with a reduction in HDL-C levels and also in HDL functionality (lower HDL cholesterol efflux capacity, HDL-CEC) [114].

The impact of genetic sex on atherosclerosis development can be investigated also in individuals with genetic conditions affecting the number of sex chromosomes, such as Klinefelter syndrome (KS), leading to male individuals with XXY chromosomes, and Turner syndrome (TS), females with X0 chromosomes. Individuals affected by any of these syndromes develop congenital cardiovascular abnormalities [115,116]. In details, KS is associated with increased cardiometabolic risk, starting from early age [117], and overall increased carotid intima-media thickness [115] and hyperlipidemia [118]. TS women display an atherogenic lipid profile [119] and intima-media thickness [120], coupled to early onset hypertension [121], and higher risk of ischemic heart disease [119].

Recently, the importance of Y chromosome has been pointed out in several studies. The loss of Y (LOY) is normally observed with aging in blood cells and, recently, has been associated with the occurrence of major cardiovascular events in patients with severe atherosclerosis, independently of the increased lesion size or plaque inflammation [122]. In addition, mosaic LOY (mLOY), a common acquired structural mutation in the leukocytes of aging men, correlates with worse cardiac fibrosis and heart failure [123], and the use of mice with mLOY, obtained through CRISPR-Cas9 technique, pointed out a role of TGFβ signaling in this phenomenon [124].

Manipulation of genetic and gonadal sex in experimental animal models offers a platform to unveil the impact of sexual chromosomes on sex-dependent mechanisms. From a biological perspective, the genetic basis of sex determination resides in the presence of XX chromosomes in females or XY in males, while the development of dimorphic sexual gonads depends on the expression of *Sry* gene (Sex determining Region Y protein) on the Y chromosome that promotes the development of a male reproductive system, while its lack, commonly due to the absence of the Y chromosome, leads to the development of female-like hormone profile and female gonads. However, epigenetic mechanisms of transcriptional repression have been identified in the regulation of female puberty. These were described as involved in the control of gonadotropin-releasing hormone (GnRH) secretion [125], thus adding further complexity to the regulation of male and female phenotype.

To discriminate genetic versus gonadal sex, one of the first models developed was the four core genotype model (FCG) [126]. This model was created by deleting the *Sry* from chromosome Y, leading to a XY^{-} mouse model presenting Y chromosome and female gonads. *Sry* insertion in the autosome 3 in the XY^{-} *Sry* mouse, results in a fertile mouse which develops male gonads. The crossing of XY^{-} *Sry* male mice with XX females leads to four possible genotypes: XX *Sry* and XY^{-} *Sry* with testis, and XY^{-} and XX with ovaries, also referred to as XXM, XYM, XYF, and XXF respectively [127] (Fig. 2).

The four-core genotype model is useful to investigate whether the sexual dimorphic characteristic of interest is caused by sex-specific gonadal hormones or the sex chromosomes per se. X chromosomes for example contain genes known to escape the X chromosome inactivation, which are thus expressed more in individuals with two X chromosomes [128]. Intriguingly, the X-inactivation escaping genes include chromatin-modifying enzymes that regulate gene expression. Indeed, sex-biased genes are enriched for proteins that are involved in deposition of epigenetic marks (such as histone modifications) and for genes that are adjacent to a subset of transcription recognition sequences, including hormone-related transcription factors, thus accounting for gene expression differences between males and females [129].

The contribution of sex chromosomes in atherosclerosis has been investigated in the FCG model crossed with $Ldlr^{-/-}$, $ApoE^{-/-}$, and WT C57BL6/J mice fed with a high-cholesterol diet (WTD). The XX chromosome complement, independently of the presence of ovaries or testes (XXF or XXM), has been associated to increased atherosclerosis,

circulating levels of cholesterol and TG, and intestinal fat absorption, while both XYM and XYF showed improved atherogenic lipid profile and lower atherosclerosis [130] (Fig. 2). Of note, this work mainly focused on lipid metabolism and lesion size as determinants of atherosclerosis development; further studies should be designed to evaluate how changes in gonadal hormones or sex chromosome complement affect plaque stability or the immuno-inflammatory response. A novel mouse model where a mutated Y chromosome (Y*) can pair with the X chromosome leading to the generation of 4 possible genotypes, comparable with XX, XY, XXY and X0 [131,132] might help understand these aspects (Fig. 2).

6. Role of genetic sex on atherosclerosis-related risk factors

6.1. Lipids and lipoprotein metabolism

In line with findings reported above, hepatic triglyceride levels under fasting show a sex dimorphism and are higher in female mice [133,134]. This was shown to strongly depend upon modifications by the epigenetic "writer" protein METTL14. The expression of this gene is higher in the presence of two X chromosomes, and liver-specific deletion of *Mettl14* from male and female mice significantly diminishes sex-specific differences in steatosis suggesting a sex-dimorphic regulation of lipid metabolism [135].

Intriguingly, also HDL-C levels are strongly related to XX chromosomes and not on gonadal hormones. XX mice present increased HDL-C levels when compared to XY mice, even after gonadectomy [136]. This difference is hypothesized to depend on the gradient of X chromosome available, as mice with two X chromosome have higher HDL-C independently of gonadal sex [136], but further studies should point out the exact mechanism underlying this phenomenon.

6.2. Vascular homeostasis

Aortopathies (i.e. aortic aneurysms, aortic dissection) which share a number of risk factors with atherosclerosis, such as inflammation, hyperlipidemia, and hypertension [137], exhibit sexual dimorphism.

In particular, angiotensin II induced abdominal aortic aneurysm (AAA) occurs 4-fold more frequently in men and in male mice compared to females [138,139]. Notably a higher frequency is observed also in women with X monosomy (Turner syndrome), suggesting that the number of X chromosomes modulate the susceptibility to aortopathies.

AlSiraj and colleagues showed that although testosterone promotes angiotensin-induced AAA [139,140], the profiling of the FCG model and XY* models highlighted a key role for sex chromosome complement. The presence of XY chromosomes in mice results in increased inflammatory gene expression pattern in the aorta which contributes to the formation of aneurysm. Interestingly, the administration of testosterone to XYF mice promotes aneurysm rupture, suggesting an influence of gonadal hormones on a phenotype already defined by sex chromosomes [141].

Furthermore, via the use of XY* model it was clarified that it is the increase in the number of X chromosomes that confers resistance to AAAs; X0F mice (which mimic the chromosome setting of the Turner syndrome) develop aortopathies at higher incidences, comparable to XY females and to XYM [142]. This has been correlated with a difference in the expression of genes escaping X-inactivation, including *Kmd5c* and *Kmd6a*, which encodes for histone-modifiers.

6.3. Immune response

While direct testing of the contribution of genetic sex on the immune response during atherogenesis has not been fully explored, it is clear that men and women present differences in the immune response that are independent of sexual hormones. The exact mechanisms explaining these differences are still elusive [143,144].

Sex-associated differences in the immune system are observed from pre-puberal age, thus it was initially hypothesized to be independent of gonadal hormones. However, a recent study using the FCG model showed how differences in T cell distribution and thymic T cell egression were correlated with levels of testosterone in the perinatal period, suggesting a regulation dependent on gonadal hormones [145]. In addition, *Sry* gene expression and male gonads development were associated with a reduced response to delayed-type hypersensitivity and the number of cells in the draining lymph nodes [146].

On the other hand, the use of the FCG model led to characterize sex-determined susceptibility to some autoimmune diseases. XX chromosomes in comparison to XY are associated with a greater susceptibility to experimental autoimmune encephalomyelitis (EAE) and lupus erythematosus [147,148]. Further studies conducted with the use of the FCG model pointed out that *Kdm6a,* a gene on the X chromosome escaping the inactivation, might be a key determinant of these differences. A lower expression of this gene, which is observed physiologically in male mice compared to female, improves the disease pathology in the $CD4^+$ T cell–mediated autoimmune EAE [149].

Immune system development and activation are therefore conditioned by both gonadal hormones and epigenetic mechanism driven by genetic sex.

7. Concluding remarks

The increasing awareness about the importance of experimental research for addressing molecular differences in atherogenesis between men and women, is improving our understanding of the role of hormones and sex chromosomes in cardiovascular risk and incidence. This information should help first to better stratify cardiovascular risk according to sex and age, second to clarify which women will benefit more from hormone replacement therapy in post-menopause, and third to explore whether gender-affirming hormone-based therapy in transgender individuals might differently affect their cardiovascular risk (Fig. 3).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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