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Abstract

The influence of sex has been largely described in cardiovascular diseases. Atherosclerosis is a complex process that involves many cell types such as vessel cells, immune cells and endothelial progenitor cells; however, many, if not all, studies do not report the sex of the cells. This review focuses on sex differences in human cells involved in the atherosclerotic process, emphasizing the role of sex hormones. Furthermore, we report sex differences and issues related to the processes that determine the fate of the cells such as apoptotic and autophagic mechanisms. The analysis of the data reveals that there are still many gaps in our knowledge regarding sex influences in atherosclerosis, largely for the cell types that have not been well studied, stressing the urgent need for a clear definition of experimental conditions and the inclusion of both sexes in preclinical studies.

Keywords: sex differences; vessel wall cells; immune cells; atherosclerosis; humans

1. Introduction

Atherosclerosis starts very early in life and becomes clinically relevant only after many years [1]. Compelling evidence links inflammation and adaptive immune responses to atherogenesis [2]. The potential major antigens involved in atherosclerosis are neo-epitopes derived by oxidation reactions of low density lipoprotein (LDL) or when cells undergo apoptotic death [3]. Six different morphological types of atherosclerotic lesions [4] and the involvement of numerous cells types have been described. The cell types include: endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and endothelial progenitor cells (EPCs), which participate in the arterial endothelial damage repairing endothelial dysfunction [5]; and haematogenous cells including monocytes/macrophages and cellular fragments [[6, 7] and quoted literature].

Even though the Institute of Medicine report "*Exploring the Biological Contribution of Sex*" affirms that sex affects all aspects of cellular function from "*womb to tomb*" [8] and that sex and gender could affect the outcome, interpretation and applicability of data [9], studies rarely consider the sex of donors [10], even when the effects of sex hormones are analysed [[11] and quoted literature]. Atherosclerosis exhibits numerous sex differences (SD) [12, 13]. Some of these differences have been attributed to oestrogens [14, 15]. Furthermore, SD have been found in the levels of numerous mRNAs in samples obtained from the aortas of women and men with or without atherosclerotic lesions; for example, peroxisome proliferator-activated receptor (PPAR)ϒ is more highly expressed in samples from healthy men than in samples from healthy women [16].

Although still at an early stage, some findings show that cells freshly prepared from blood or from blood vessels can be used to study sexual dimorphism [11, 17, 18]. This is relevant because there is no ideal animal model as each has its own advantages and limitations with respect to manipulation of the atherogenic process and modelling human atherosclerosis [19].

Therefore, this review focuses on sex differences in human cells in primary culture, as "*sex memory*" may be lost during culturing. For example, it has been shown that differences in the expression levels of oestrogen receptors (ER) disappear after 14 passages post-isolation [20] and GPR30 expression declines in VSMCs maintained in primary culture (vs. expression in freshly isolated tissue) [21]. The review analyses, for each cell type, the main aspects that are important in the atherosclerotic process, starting from immune cell count and function and including the influence of the menstrual cycle, menopause, and pregnancy when the donor is a woman. The effects of sex hormones in immune cells including the influence of the menstrual cycle on immune cell function have recently been reviewed, [22-25] including the effect of ageing on the influence of sex in immune cells [26]. Therefore, these specific aspects are only summarized here in relation to human cells. Moreover, differences in the expression levels and activities of sex hormone receptors have been reported, as less is known about whether they present sexual disparity at least in human cells [27]. Furthermore, our attention is focused on cell fate, namely apoptosis and autophagy. Apoptosis, a highly regulated cell death process, plays an important role in numerous pathologic conditions involving cardiovascular diseases (CVDs) [28]. Studies have documented apoptosis of VSMCs in atherectomy specimens from atherosclerotic and restenotic lesions, and apoptosis of VSMCs appears to contribute to plaque rupture [29]. Apoptosis has also been observed in macrophages and T cells within atherosclerotic lesions [30]. Increased EC apoptosis may initiate atherosclerosis, as the inducers of EC apoptosis are known risk factors for atherosclerosis [31]. Therefore, it is relevant to know whether apoptosis is a sex-divergent process. On the other hand, autophagy is a process of self-cannibalization of organelles, aggregates, proteins and toxic molecules in the lysosomes. In immune cells, autophagy is also a regulator of inflammation promoting the end of the inflammatory response [32]. Autophagy preserves the health of cells and tissues by replacing outdated and damaged cellular components. In the early stage of atherosclerosis, it appears to protect plaques against stressors through the degradation of intracellular components;

however, it appears to become dysfunctional in advanced stages of atherosclerosis [33] and quoted literature]. However, the role of autophagy in atherosclerosis remains poorly understood, and even more poorly understood is the role of sex in the autophagic processes of the cells involved in atherosclerosis. On the contrary, SD in redox state have been reviewed previously [34, 35] and are not discussed here.

Finally, as a complex interplay between lifestyle, such as smoking, and genetic risk factors is present in atherosclerosis, the effect of smoking on cell count has been considered in view of the fact that increased cell counts are considered risk factors for CVDs, and the pro-atherosclerotic activity of smoking has been considered as smoking is more hazardous for women than for men [36].

2. Human ECs

Endothelial dysfunction plays an early and pivotal role in vascular inflammation and in the pathogenesis of atherosclerosis [37]. In the context of ECs, it is important to note that ECs have significant phenotypic heterogeneity depending on the location and vessel type [5], implicating that SD found in one location cannot automatically be extended to another one. For example, ERβ is more highly expressed in human umbilical artery endothelial cells (HUAECs) than in human umbilical vein endothelial cells (HUVECs), whereas ERα expression does not differ between ECs obtained from the two vessels [38].

HUVECs express genes and proteins for ERs and ARs without significant differences between the sexes [11] (Table 1). Regarding ERα and ARs, there are not univocal results because Annibalini et al. [39] reported that male and female HUVECs do not express ERα, while ERβ and AR expression is similar (Table 1). Toth and collaborators show that HUVECs of unknown sex lack ERα and PRB but express ERβ and PRA [40]. Finally, Kim-Schulze and collaborators [41] show expression of ERs in ECs of unknown sex, whereas human cerebral vascular endothelial cell (HCVECs) express ERβ and

GPER1 but there are no reports on ERα expression in HCVECs [42]. PRA and PRB are present in freshly isolated female HUVECs and human dermal ECs [43], but only PRA is found in a commercial line of HUVECs [43]. ARs have been identified in HAECs (human aortic endothelial cells) [43], and other authors found higher AR levels in male-derived ECs versus female-derived ECs [44, 45].

2.1 Function of human ECs

ECs have many functions [46], and their impairment is a risk factor for vascular diseases [47-49]. The activity of healthy endothelium depends on the release of numerous molecules (prostacyclin, endothelium-derived hyperpolarizing factor (EDHF), NO, hydrogen sulfite (H_2S), thrombomodulin, tromboxane, antithrombin, endothelin (ET), Von Willebrand's factor (VWF), fibronectin, etc.) that have regulatory functions on VSMCs, circulating white cells, erythrocytes and PLTs and on vascular permeability [46]. Globally, sex affects the function of ECs. Compared to healthy, age-matched men, healthy women tend to have better endothelial function [50]. Endothelium-dependent vasodilation is higher in healthy females than in males. Endothelium-dependent vasodilation is also higher in females than in males after CAD and is positively associated with ageing [51-53]. Furthermore, obesity and insulin resistance reduce endothelial vasodilatation in both sexes, but this effect is more marked in men [54]. Type II diabetes impairs endothelial vasodilatation above and beyond that observed with obesity alone only in women [54]. Additionally, the vasodilation induced by methacholine, an agonist of muscarinic receptors, which is a trigger of NO and EDHF release, is bigger in premenopausal healthy women than in age-matched healthy men, whereas no difference is observed in diabetic individuals [54].

The effects of sex hormones on endothelial function have been described both *in vivo* and *in vitro*. Endothelial function has been shown to be influenced by the menstrual cycle in healthy women. In particular, endothelium-dependent vasodilation is larger during the luteal phase than during the

follicular phase [55, 56]. Nevertheless, some authors state that NO production is higher during the follicular phase than during the ovulatory phase [57]. This has not been unanimously confirmed, as Jilma and collaborators [58] have indicated that NO production is not influenced by the menstrual cycle. An apparent decline in endothelial function during menopause has been reported [59, 60]. Notably, HRT increases the levels of NO in postmenopausal women [61], indicating the importance of oestrogens in regulating the production of this relevant vasodilator. In line with previous results, Forte [62] shows that the whole-body production of NO is greater in healthy premenopausal women than in men. However, Jilma and collaborators [58] indicate that the NO concentration, such as the level of exhaled NO, is higher in men than in women [63]. In primary cultures of HUVECs, Addis and collaborators [11] show that female HUVECs express significantly higher levels of both the NOS3 gene and protein compared to male HUVECs, indicating a significant sex difference in the ability to produce the potent vasodilator NO. *In vitro,* testosterone induces NO production in HAECs that is associated with activation of NOS3, which in turn is inhibited by incubation with nilutamide, an AR antagonist, or an AR siRNA [[64] and quoted literature]. However, others do not report effects of testosterone on NO production [65, 66]. In HAECs, oestrogens also activate NOS3 [[64] and quoted literature]. In fact, it is not known whether H_2S signalling is influenced by sex, but its plasma levels are higher in healthy men than in women. This is not surprising because its synthesis is under the control of androgens [67] and OCs reduce the plasma levels of its precursor, cysteine [68].

In vitro, dihydrotestosterone induces VCAM1 expression, increasing monocyte binding to the endothelium. Furthermore, dihydrotestosterone triggers the proliferation of HAECs, probably through the up-regulation of the expression of vascular endothelial growth factor A and cyclins A and D1 [69]. The stimulation of EC proliferation in the CV system by activation of AR could contribute to the repair of EC injury/damage [[64] and quoted literature].

The potent vasoconstrictor ET1 is produced by vascular ECs as well as by many other tissues, and the endothelin system is up-regulated in a number of pathological conditions associated with endothelial dysfunction including atherosclerosis [70]. The endothelin system appears to act in a sex-specific manner. Plasma ET1 levels are higher in men than in women, and pregnant women have lower levels compared with non-pregnant women [71]. HRT increases the levels of ET1 in postmenopausal women [61]. The previous data suggest a role of sex hormones in the regulation of the endothelin system. This is confirmed by the fact that oestradiol reduces basal and stimulated ET1 secretion from HAECs. Conversely, testosterone induces an increase in the number of cells that secrete ET1, up-regulating ET1 mRNA [72]. Another vasoconstrictor, thrombin, is more active in female HUVECs than in male ones in stimulating prostacyclin and prostaglandin E_2 synthesis [73].

Finally, Lorenz and collaborators [74] report different transcriptional profiles in HUVECs from male and female donors, with 70 genes being differentially expressed between the sexes. For example, genes related to immune responses (humoral, innate and inflammatory responses) and some genes involved in metabolism (for example, leptin, insulin receptors and some apolipoproteins) are higher in female than in male HUVECs, as is the viability after serum starvation and tube formation capacity. These results indicate that some SD reside in autosomal genes.

In HUVECs, 17β-oestradiol increases the expression and activity of SR-BI/CLA-1, which facilitates the cellular uptake of cholesterol from HDL [154]. Moreover, after shear stress induction, a higher number of genes are up- or down-regulated in female HUVECs than in male ones; for example, VCAM-1 expression is down-regulated almost 22 times in female HUVECs and only 3.5 times in male HUVECs [74]. Finally, unstimulated male HUVECs release more MCP-1 and IL-8 than female HUVECs [75].

2.2 Human ECs and cell fate

As mentioned above, SD in death pathways receive little attention. Recently, the use of freshly isolated cells from male and female individuals provided information on sexual dimorphism in cell fate control. In ECs, SD regard either apoptosis or autophagy. SD in the induction of cell death may, to some extent, explain the disparity between the sexes in many human diseases including atherosclerosis.

2.2.1 Apoptosis

EC apoptosis may be relevant for endothelial injury, including atherosclerosis, and it may lead to endothelial disruption. Notably, oxidized LDL (oxLDL) induces apoptosis and autophagy (see below) in HUVECs of unknown sex [76]. Intriguingly, inhibition of apoptosis occurs from an inhibition of autophagy, whereas inhibition of autophagy occurs with an increase in apoptosis [77]. Sex hormones influence apoptotic processes. For example, testosterone enhances TNFα-induced apoptosis after serum deprivation in HUVECs [78], whereas, in human coronary artery ECs, 17β-oestradiol increases apoptosis and elevates the pre-apoptotic Fas mRNA and protein expression, the expression of FasL mRNA and the secretion of FasL protein [79]. At least in experimental animals, epoxyeicosatrienoic acids, the activity of which is controlled by soluble epoxide hydrolase, are more highly expressed in male cerebral vessels compared with female cerebral vessels [80], and they seem to protect ECs from ischaemic injury [81]. Genistein, an isoflavone that is structurally similar to oestradiol, has pro-apoptotic activity in human coronary ECs obtained from middle-aged women [82]. Finally, RLIP76, a Ral effector GTPase-activating protein, significantly alters the percentage of apoptosis only in female HUVECs [83].

2.2.2 Autophagy

Autophagy, or type II programmed cell death, might also be involved in the progression of atherosclerosis [84]. In ECs from atherosclerotic plaques obtained during carotid endarterectomy in

individuals with acute ischaemic stroke and severe carotid artery stenosis, an increase in autophagy compared with normal arteries is observed [85]. In HUVECs of unknown sex, autophagy is activated by OxLDL through the LC3/beclin1 pathway causing vascular endothelial dysfunction [76]. The increase in autophagy is blocked by 3-methyladenine, an inhibitor of autophagic response, and is increased by the autophagy inducer rapamycin [76].

A recent study characterizes the phenotype of HUVECs according to the sex of the umbilical cord donor and individuates that male cells have a higher degree of constitutive autophagy than female ones as they express higher levels of Beclin-1 and have a higher LC3-II / LC3-I ratio [11]. These results have been confirmed by ultrastructural analysis showing a higher build-up of autophagic vacuoles at different stages in male HUVECs than in female ones. However, the protein expression levels of mTOR and AKT, critical regulators of autophagy, are not different between the sexes. The tendency of male HUVECs to undergo autophagy could depend, at least in part, on increased oxidative behaviour compared with female HUVECs [11].

Another important function of ECs is the secretion of VWF, which is required for PLT adhesion to the vessel wall. EC autophagosomes contain abundant VWF protein, and its secretion is inhibited *in vitro* by inhibitors of autophagy, suggesting that inhibition of autophagy can prevent thrombosis [86]. In HUVECs, angiotensin II progressively increases autophagy, apoptosis and senescence, which are reduced by valsartan. Additionally, autophagy has an early protective effect on vascular endothelial damage due to Ang II [87], and autophagy induced by hyperglycaemia [88, 89] may have a protective effect versus senescence and apoptosis. However, it is not yet known whether the protective effect of autophagy is influenced by sex.

3. Human VSMCs

The medial layer of the vascular wall is composed of VSMCs and the synthesized extracellular matrix, and it plays a key role in the maintenance of vascular structure and function [90]. Notably, VSMCs may trans-differentiate into other cell phenotypes such as macrophages, which can lead to the formation of foam cells [[91] and quoted literature].

Very little information exists on the effect of sex on human VSMCs as the majority of studies are performed using animals as cell donors. However, it is known that ERα, ERβ, and PR mRNAs and proteins (Table 1) are present in cells from different veins and arteries such as the coronary arteries, saphenous veins, aortas, mammary arteries, and iliac arteries obtained from both men and women [92, 93]. When sex is considered, it has been found that ERβ mRNA is predominantly expressed in female VSMCs obtained from the coronary artery and saphenous vein [94]. ERβ appears to be more highly expressed in women who have not undergone HRT versus users of HRT, whereas ERα levels do not differ among cohorts. Furthermore, in HRT users, only the expression of ERα declines with age [95]. In aortas obtained by autopsy, the expression of ER decreases with the level of atherosclerosis. No change is reported in men [96].

Finally, human umbilical artery smooth muscle cells from male and female neonates display sexual dimorphism in ERβ expression, with ERβ being more highly expressed in male-derived cells, while ERα is similarly expressed in both sexes (Table 1) [97]. ERα is also localized in human VSMCs starved for five days to allow for ERα up-regulation, and ERα is more highly expressed on average in VSMCs from female donors than in VSMCs from male donors [98]. In human aortic VSMCs, PRB is similar in male- and female-derived cells, but PRA is lower in male-derived cells than in female-derived cells [99]. The GPER has also been identified in cultured VSMCs [43]. PRs have been identified in VSMCs. In particular, PRB is equally expressed in men and women, while PRA is more highly expressed in vessels obtained from postmenopausal women [43]. In VSMCs prepared from human internal mammary arteries, binding and physiological studies confirm the presence of ARs

[100]. Globally, sex hormone receptors are present, but they exhibit dishomogeneous expression in the VSMCs of different arteries.

3.1 Function of human VSMCs

Considering the presence of sex hormone receptors, it is not surprising that sex hormones affect the function of human VSMCs. Oestrogens have similarly antiproliferative effects on male and female human aortic smooth muscle cells [101], acting through ERβ, which is the prevalent isoform and mediates vasodilation and VSMC relaxation [102]. Moreover, *in vitro,* female sex hormones (oestradiol and progesterone) are able to reduce collagen deposition in female aortic VSMCs much more than testosterone*,* while testosterone up-regulates gene and protein expression of matrix metalloproteinase 3 [103]. Steroid sulfatase (STS) and oestrogen sulfotransferase (EST) are involved in the metabolism of oestrogens and are both present in aortic VSMCs. In women, STS expression declines with the level of atherosclerosis, while the decline is not evident in men. EST expression is significantly higher in male aortas than in female aortas with mild atherosclerotic changes. In women, EST is more highly expressed when the aorta exhibits severe atherosclerotic damage [104].

3.2 Human VSMCs and cell fate

Atherosclerotic lesions result from a dynamic interplay involving proliferation, autophagy and apoptosis in response to injury of the ECs and VSMCs of the artery wall [84, 105]. However, it has been suggested that autophagy can contribute to attenuation of inflammation through the eradication of damaged molecules and cellular organelles, thus preventing apoptosis of VSMCs and stabilizing the lesion [106]. Excessively stimulated autophagy may also play a detrimental role in plaque formation [106]. In this context, it is fundamental to know the influence of sex to optimize the therapeutic approach in women.

3.2.1 Apoptosis

Apoptosis and cell death are frequent events in the initial formation of plaques as well as in more evolved plaques. In the thin fibrous cap of advanced lesions, apoptosis enhances plaque rupture, thereby triggering thrombosis and myocardial infarction and contributing to plaque instability and sudden coronary death [107, 108]. Importantly, cells undergoing apoptosis need to be efficiently removed from atherosclerotic lesions because their permanence is a potent inducer of the coagulant cascade [109, 110]. Recently, it has been that mithophagy has a role on human VSMC apoptosis induced by oxLDL suggesting that mitophagy is a safeguard mechanism versus apoptosis [111]. Nevertheless, the importance of apoptosis in VSMCs and the influence of sex on apoptosis of human VSMCs have still not been studied, although animal studies suggest that there are significant SD [112-114].

3.2.2 Autophagy

VSMCs have the capacity to assume different phenotypes and this phenotype flexibility needs the integration of transcriptional, metabolic, and ultra-structural programs and in doing this autophagy seems to assume the role of main coordinator [115]. For example platelet derived growth factor BB (PDGFBB) induces autophagy reducing the transition to the synthetic VSMC phenotype and elevating cell survival in condition of increased oxidative stress [115], whereas other stimuli such as angiotensin II promotes cell death [115].

It is believed that autophagy plays an important role in the aetiology of atherosclerosis. However, the molecular basis of autophagy in the pathogenesis of atherosclerosis remains poorly understood, and even less is known about how the sex influences the process. In VSMCs obtained from atherosclerotic plaques from carotid endarterectomies in individuals with acute ischaemic stroke and severe carotid

artery stenosis, an increase in autophagy compared with normal arteries was observed [85]. Some authors suggest that an increase in autophagy is relevant to maintaining plaque stability. In the atherosclerotic plaques, autophagy may play a protective role [116], and cell death induced by low doses of statins may be reduced by the autophagy inducer 7-ketocholesterol, at least in rabbit aortic VSMCs [117], suggesting that stimulation of autophagy could protect VSMCs from death. However, excessive autophagy may cause autophagic death of SMCs, which conversely results in plaque destabilization. In this context, it is relevant to consider that human umbilical artery smooth cells from male neonates have higher levels of Beclin-1 than female cells, while the LC3-II / LC3-I ratio is similar between the sexes [97, 118]. Beclin-1 has a role in the crosstalk between autophagy and cell proliferation [89]. Therefore, the sex difference observed may be of interest. When autophagy (measured as the LC3-II / LC3-I ratio) is induced by stimuli such as verapamil, starvation and rapamycin, the autophagy programme is different in male and female human umbilical artery smooth muscle cells [118]. Importantly, the SD are stimulus specific. In brief, serum starvation-induced autophagy is more pronounced in female human umbilical artery smooth muscle cells than in male cells, while 250 nM rapamycin produces autophagy only in female cells [118]. Moreover, verapamil-induced autophagy does not exhibit sex differences, even if Beclin-1 is increased in female cells. Finally, PmTor does not differ under basal conditions, but it is significantly down-regulated by starvation in female human umbilical artery smooth muscle cells and by rapamycin in both male and female cells [118]. The SD in autophagy indicate sex-based differences in the pharmacodynamic effects of verapamil and rapamycin. Autophagy in VSMCs may be considered a housekeeping process [89] that provides protection against cell death in several vascular diseases, whereas excessive autophagy would be deleterious. In particular, the defective autophagy of VSMCs may cause irreversible cellular senescence and subsequently contribute to atherosclerosis progression and other vascular disorders. Therefore, more sex studies of this specific point are urgently needed.

4. Human PLTs

These small circulating cell fragments play a pivotal role in haemostasis and endothelial repair and are also involved in atherogenesis and thrombosis [119, 120]. There is both indirect and direct evidence that sex hormones affect human PLT biology.

ERα and ERβ have been found in circulating PLTs [121, 122], with similar expression levels in the two sexes (Table 1) [122]. These receptors can exert non-genomic activity having a pro-aggregating effect through ERβ [123]. AR has been detected in human PLTs [121].

4.1 Human PLT count and morphology

Human PLT count is influenced by sex. Women have a higher PLT count than men [124-130]. The average PLT count is also influenced by age and ethnicity [127, 128, 130]. Notably, the SD observed with HRT and OC use in adults are still present in the elderly [68, 128-131]. It is possible that the higher haematocrit values observed in men versus women, with relatively less plasma and greater *in vitro* dilution due to the addition of anticoagulant solutions, lead to the erroneous measurement of lower platelet counts in men compared with women [132, 133]. In pregnant women, there is a light thrombocytopenia due to increased platelet consumption caused by the increase in the utero-placental circulation [134]. Women with acute strokes have elevated PLT counts, although PLT reactivity is lower in women than in men [125].

In addition, SD are also detected in PLT morphology, with the mean PLT volume being higher in women than in men [126, 135].

4.2 Human PLT count and smoking

Studies do not provide univocal results regarding smoking and PLT count [128, 136-138]. It has been reported that female smokers have lower PLT counts than non-smokers, while this difference is not detected in men [128]. However, others report that smoking increases PLT count in fertile women (in the follicular phase) but not in men [136].

4.3 Function of human PLTs

Sex effects on the functions of PLTs are present, and they have recently been reviewed [[132, 139] and quoted literature]. Briefly, women have more receptors for fibrinogen [132], bind more fibrinogen after adenosine diphosphate (ADP) and present more spontaneous aggregation than men [[132, 139] and quoted literature]. Furthermore, PLT degranulation measured as CD62P expression and activation of GPIIb/IIIa measured as PAC-1 binding do not exhibit sexual dimorphism. However, when PLTs are exposed to ADP, female PLTs appear to be more activated and undergo major morphological alterations. The PLTs obtained from women produce a significant increase in phosphorylated protein kinase substrates such as RhoA and phosphorylated myosin light chain [140]. Lawrence and collaborators [133] also observed more spreading and adherence in men than in women.

Furthermore, in women without CAD, PLTs are more reactive to ADP and to thrombin receptor agonist than in men [141, 142]. At rest, PLTs of women with CAD respond better to serotonin and epinephrine compared with those of men [[132, 139] and quoted literature]. Also protease-activated receptor-mediated PLT reactivity is increased in females [143]. Finally, following mental stress, women have higher collagen-stimulated PLT aggregation responses than men [144]. SD are also measured with a test that measures both PLT adhesion and aggregation (primary haemostasis). A Korean study of healthy men and women reported that women have a significantly higher closure time than men [145]; however, this is not an univocal result [146].

SD taking into account drug response have previously described and were recently reviewed [[132, 147] and quoted literature]. The Genetic Study of Aspirin Responsiveness performed in healthy men and women revealed higher PLT reactivity in women than in men for numerous agonists [[132] and quoted literature], and this higher reactivity in women is still present after the administration of low doses of aspirin when collagen or ADP are used as agonists [[132] and quoted literature]. In contrast, SD disappear in subjects treated with clopidogrel or with both aspirin and clopidogrel [[132] and quoted literature].

Recombinant human erythropoietin (rhEPO) increases PLT aggregation induced by ADP with some SD, especially when collagen is the agonist. In fact, rhEPO increases and decreases collagen aggregation in males and females, respectively. In addition, rhEPO increases the PLT membrane glycoprotein complex α2bβ3 in both sexes suggesting that homotypic (PLTs-PLTs) aggregation occurs without significant SD. Conversely, membrane P-selectin, an index of heterotypic aggregation (PLTs-leukocytes), is increased by rhEPO only in women. These results indicate that the prothrombotic risk observed with rhEPO occurs in a sex-specific manner, with females being more sensitive [148].

Activated PLTs participate in inflammatory responses that further amplify their response and endothelial activation during plaque rupture. Activated PLTs release numerous molecules with inflammatory and mitogenic activities into the local microenvironment [149], enforcing the recruitment of monocytes and their differentiation into macrophages [150]. The formation of leukocyte-PLT aggregates in patients undergoing angioplasty and stenting is more pronounced in females than in males, whereas the expression of P-selectin and GPIIb/IIIa does not differ significantly between the sexes [139]. In this context, a special role is played by TLRs, which appear to be more highly expressed in women than in men and which interact with the immune system engaging the neutrophil population [[132] and quoted literature] and favouring PLT-leukocyte aggregates. Indeed, in women, TLR expression is associated with soluble P selectin [151]. In contrast, TLR expression in men is more

frequently associated with soluble TNFα receptor-1 and ICAM1 [151]. Notably, the expression of TLR1, TLR3, TLR6, and TLR7 is associated with body mass index in women. However, in men, the expression of TLR5, TLR7, and TLR10 is associated with the total cholesterol to high-density lipoprotein ratio [151]. Brain-derived neurotrophic factor (BDNF), a nerve growth factor, is also stored in human PLTs [152] and plays a critical role in the CV system [153]. Interestingly, PLT BDNF levels are lower in healthy women than in men [154]. The opposite is observed in individuals with stable coronary artery diseases [155].

Some SD depend on sex hormones, however the effect of the female menstrual cycle on platelet activity is not clear. Some studies show that PLTs bind more fibrinogen during the luteal phase than during the follicular phase [142, 156], whereas PLTs closure time is higher during the follicular phase than during the luteal phase [157], and others show that PLTs adhesion has a biphasic peak during the menstrual cycle [158]. However, other studies find no evidence of any associations among PLTs activity and menstrual cycle phase [159-161]. OCs are able to activate PLTs [162], and some of the effects of OCs depend on the androgenic property of progestin [157]. Furthermore, OCs elevate factor VII levels, PLTs activity, and levels of fibrinogen and plasminogen activator inhibitor-1 [163]. However others do not find any influences of OCs [159-161]. HRT leads to an increase in circulating and activated PLTs in users than in non-users [164], leaving the mean PLTs volume and aggregation rates unchanged [165]. During the third trimester, pregnancy elevates PLT aggregation and the concentration of coagulation factors and decreases fibrinolytic capacity [166]. Pregnancy also elevates the activity of calcium adenosine triphosphatase, beta-thromboglobulin and platelet factor-4, suggesting increased activation of PLTs [167].

Very recently, a more direct role of PLTs in the atherosclerotic process has been reported. Specifically, PLTs can internalize lipids and promote the formation of foam cells [168, 169]. It is not known whether these processes are sex dependent.

Generally, PLT reactivity is enhanced in women; however, this is not an univocal result. Additionally, SD also depend on the stimulus applied. Unfortunately, the paucity of data does not permit us to individuate the clinical relevance of these SD in view of the fact that the sex effect may also be dependent on hormonal status.

4.4 Human PLsT apoptosis and autophagy

PLTs have the ability to undergo apoptosis, which can be induced by a multitude of stimuli. Increased apoptosis results in thrombocytopenia, bleeding disorders and the induction of microparticles [170]. PLTs also have autophagy machinery. Constitutive autophagy is present in resting PLTs and is elevated in response to PLT activation. Inhibition of autophagy during megakaryocyte differentiation reduces PLT formation, and affect PLT function, whereas inhibition of autophagy in mature megakaryocytes induces abnormal PLT activation [171]. It seems that basal autophagy playing an important role in normal PLTs activation [172]. Indeed in diabetic PLTs the induction of mitophagy plays a beneficial role versus oxidative stress, and its absence increases thrombosis [172]. However, the role of constitutive autophagy and mitophagy is not yet clearly understood [173]. In fact, it is not known whether apoptosis and autophagy are influenced by sex.

5. Human EPCs

Endothelial dysfunction is the first step of atherogenesis, and it has been proposed that the lack of or small number of EPCs is a limitation that can lead to endothelial dysfunction [174]. Circulating male and female EPCs express classical ERα and ERβ [17], with similar levels of the receptor protein in male and female EPCs (Table 1). However, the literature is not univocal because some reports indicate that cultured human male EPCs express ERα but not ERβ [175], while a significant sex difference in mRNA levels for ERα and ERβ is reported by Fadini et al. [18], with men having the highest levels.

The receptor expression is influenced by hormonal fluctuation. In particular, the expression of ERβ is higher in premenopausal women versus men (Table 1). Furthermore, ERβ expression is higher during the ovulatory phase in comparison with the follicular and luteal phases, as well as in comparison with postmenopausal women. Meanwhile, ERα is expressed similarly in both sexes, and, in women, ERα is not influenced by variation in sex hormones [176]. The presence of ARs has been demonstrated by immunohistochemistry and by the measurement of AR mRNA and protein expression [177], but it is not known if AR is influenced by sex.

5.1 Human EPC counts

EPCs are estimated to comprise 0.02-0.2% of blood cells [178, 179]. It has been reported that the number of EPCs is higher in young fertile and pre-hypertensive women than in healthy and pre-hypertensive age-matched men [18, 180-182]. However, this is not an univocal result because one study [183] does not report SD. Additionally, some authors do not observe a significant difference in the number of EPCs between middle-aged women and men [18, 180, 184, 185], indicating that SD could be age dependent. The number of EPCs depends on numerous factors such as rhEPO, growth hormones, and some medications (statins and angiotensin converting enzyme inhibitors) [186-188]. Notably, Pelliccia et al. (2009) reported that postmenopausal women and men with CAD have the same number of EPCs [189]. The previous results suggest that the number of EPCs is regulated by sex hormones. Considering that a reduced number of EPCs is linked with the incidence of CVD [174], it would be clinically relevant to know the reference value of EPCs with respect to the hormonal phase of women.

5.2 EPC count and smoking

The effects of smoking on EPCs are complex and do not appear to be univocal. Yue et al. [190] found that circulating levels of EPCs are significantly lowered in smokers with CAD compared to controls and non-smokers with CAD. In patients with CAD, Werner et al. report a positive association between smoking and high baseline levels of EPCs [191]. Similarly, Mobarrez et al. [192] show that $CD34^+$ cells increase after smoking one cigarette. Other authors have shown that the number of EPCs is directly proportional to the number of cigarettes smoked [193]. Others found that smoking does not affect the EPC count [183]. Unfortunately, sex was not considered in the last two studies mentioned.

5.3 Function of human EPCs

The ability of EPCs to migrate and form colonies *ex vivo* is an independent marker of vascular health [194]. In basal conditions, EPCs obtained from young adult men and women do not exhibit any significant differences in the number of migrated cells [17]. However, Hoetzer et al. [194] show that EPC migration is higher in middle-aged women (58 years old) than in age-matched men, suggesting that age could influence the sex effect. Migration appears to be affected by oestrogens. However, conflicting results have been reported regarding the effect of oestradiol *in vitro*. Fadini and colleagues [18] observe that oestradiol elevates the number of colonies in males with a smaller effect on female EPCs, while others [17] found that oestradiol, at physiological concentrations, inhibits EPC migration by approximately 50% in premenopausal women but not in young men. Another compound with oestrogenic properties such as bisphenol A decreases migration only in female cells [17]. Globally, in male EPCs, migration appears to be less sensitive to oestrogenic compounds. In line with previous data, colony formation and migration are influenced by the phase of the menstrual cycle, menopause and HRT [18, 194, 195], again suggesting the involvement of oestrogens at least in females. Recently, it has been shown that patients with hypogonadism have a low number of EPCs, and testosterone replacement therapy can increase the number of circulating EPCs in men [196] indicating that

androgens could also play a role. In view of the fact that mobilization of EPCs contributes to increased neovascularization, which could play a role in the prevention of CVDs [197], the lower migratory capacity of female EPCs could help to explain the higher prevalence of endothelial dysfunction in women [198, 199]. In pre-hypertensive premenopausal women, the activity of circulating EPCs is better preserved than in pre-hypertensive men, with the number and activity of EPCs partially associated with enhanced NO production, whereas vascular endothelial growth factor or granulocyte macrophage colony-stimulating factor are not varied [182].

5.4 Human EPC and cell fate

To the best of our knowledge, there is no data regarding sex influences on autophagy and apoptosis in circulating human EPCs.

6. Human leukocytes

Leukocytes produce numerous chemokines and cytokines and express receptors for these molecules. Complex chemokine and cytokine interactions promote different pathways involved in all stages of atherosclerosis [200]. There is a paucity of research on the influence of sex on this specific point. However, a small study [201] showed that the expression of the chemokine (C-C motif) ligand 5 (CCL5) is higher in women than in men. This is similar to sCD40L [202], and people with high levels of sCD40L are considered to have high cardiovascular (CV) risk [203]. Women have higher expression levels of platelet-derived growth factor (PDGF)-AA and PDGF-BB than men [201]. Ageing modifies the expression of chemokines and cytokines, and this appears to occur in a sex-specific way: macrophage-derived chemokine is, for example, age-dependent in women but not in men [202]. Indeed, the most common polymorphisms of the fractalkine receptor (CX3CR1) gene, T280M and V249I, increase waist circumference in a sex-specific manner [204]. Women with two copies of the

T280M and V249I alleles have higher waist circumferences than women with one copy of the T280M and V249I alleles, while men with one copy of T280M exhibit a higher waist circumference [204].

The human X chromosome encodes many immune-regulatory genes such as TLRs 7 and 8. Fifteen percent of X-linked genes escape silencing, resulting in the increased expression of certain gene products in females as compared to males [26]. In addition, the X chromosome is rich in micro-RNAs (miRNAs) [26]. This is particularly relevant to SD.

6.1 Human leukocyte count

The total leukocyte count is lower in men than in women [205]. In particular, in women, the count varies with the menstrual cycle; it is higher around the 8th to 10th days of the menstrual period than around the 22nd to 24th days [206]. The global leukocyte count is considered an inflammatory marker and a risk factor for CV events [207]; it is associated with CV mortality in both sexes and with non-CV mortality in women [207]. Longevity is also associated with a lower, yet still normal, count, and this correlation is more pronounced in men than in women [208].

Therefore, it is necessary to be aware of the menstrual phase and to build up reference values for the single menstrual phase before performing leukocyte counts. The availability of these data would improve the comparison between men and women and would help to ameliorate the evaluation of CV risk in women.

6.2 Human leukocyte count and the smoking effect

Smoking affects the leukocytes count. In particular, the leukocyte count is higher in current smokers than in non-smokers [136, 205, 209]. Indeed, it is still not clear if there are significant SD because the leukocyte count has been found to be equal between sexes or higher or lower either in men or in

women [124, 209-215]. Notably, smoking increases the total leukocyte count after 20 weeks of pregnancy [216]. The above results highlight the mandatory and urgent need to conduct sex and gender research, at least for CV risk factors, in order to develop evidence-based CV prevention strategies.

7. Human neutrophils

Neutrophils comprise most of the circulating leukocytes and are pivotal in the defence against microorganisms. Historically, they have received little attention in the context of atherosclerosis; however, recent findings show that, among white blood cells, neutrophils are the strongest predictors of coronary heart disease [217].

In both men and women, neutrophils express ERα and ERβ. In women, ERα and ERβ are up-regulated in the ovulatory phase of the menstrual cycle (Table 1), and, *in vitro*, both ERs are up-regulated by 17β-oestradiol in neutrophils obtained from premenopausal women [218]. In neutrophils obtained from men, only ERα is up-regulated by incubation with 17β-oestradiol [218]. Regarding progesterone receptors (PRs), it has been described that intracellular PRs are not present in neutrophils (Table 1) [27]. Finally, the androgen receptor (AR) is expressed in neutrophil lineages from the proliferative precursors to mature neutrophils with no significant SD (Table 1) [219].

7.1 Human neutrophil count

In humans, increases in leukocyte blood counts, most notably increases in neutrophils, have been directly related to the endothelial dysfunction associated with ageing and a growing susceptibility to CVDs [220]. In line with previous results, positive correlations among neutrophil blood counts and CVDs, CV mortality, and all-cause mortality are described [221-224]. Girls have higher neutrophil counts than boys [225]. However, in adults, there are ambiguous results regarding neutrophil counts. The majority of authors have found that they are higher in women than in men [205, 226, 227], while some authors have found that they are similar between the sexes [203]. The discrepancy could arise

from the fact that this last study included only women in the follicular phase [136]. This discrepancy could be due to the relevant increase in neutrophil count observed in the luteal phase as compared to the follicular phase of the normal ovarian cycle [228]. Pregnancy modifies the granulocyte count [229]. Neutrophilia and a delay in apoptosis are observed in at-term pregnancies [230-232]. The above data suggest that the neutrophil count is dependent on sex hormones. However, the effect of exogenous hormones (oral contraceptives, OCs) is not clear, as OCs may or may not increase granulocyte numbers, depending on the type of OCs used [68, 228].

7.2 Human neutrophil count and smoking

Smoking affects the number of neutrophils [205]. The neutrophil counts of current smokers are higher than those of individuals who never smoked especially in Caucasian and Mexican-American smokers [205]. Moreover, a recent study suggests that this occurs only in men, at least in Caucasian smokers [136].

7.3 Human neutrophil function

The role of neutrophils in atherosclerosis was reported in a recent review [[233] and quoted literature]. Importantly, neutrophils are present at the sites of plaque erosion or rupture [234, 235]. Notably, there are fewer neutrophils in plaques in women than in men [236]; however, women with acute stroke have higher neutrophil activity [125].

Although the influence of sex was not considered in studies involving the activation of neutrophils, some SD emerged. In particular, the production of cytokines is higher in male cells versus female ones [237-241]. Notably, SD can depend on the stimulus and can vary according to the considered cytokine [242].

The cascade of arachidonic acid is pivotal in inflammation, and leukotrienes (LTs) are formed in atherosclerotic lesions. In human neutrophils and monocytes, the key enzyme in the synthesis of LTs, 5-lipoxygenase (5-LO), is regulated by androgens, resulting in the sex-specific formation of LTs [243]. It is now becoming clear that LTs are formed more predominantly in the whole blood or in neutrophils isolated from women than in those from men. This appears to be linked to extracellular signal-regulated kinases (ERKs), which are regulated by androgens [244], indicating that LT-induced recruitment and activation of immune cells,the induced proliferation of VSMCs and endothelial dysfunction [245] may be influenced by sex.

The neutrophils of men and women express nitric oxide synthase 1 (NOS1), which is lower in neutrophils from males than in neutrophils from females [246]. In women, the expression of NOS1 is higher during the ovulatory phase versus that measured during the first 2 days of the follicular phase in the very same donor [246]. Hormone replacement therapy (HRT) increases NOS1 protein levels and greatly induces the production of nitric oxide (NO) [246]. In neutrophils from males, *in vitro* exposure to oestrogens increases NOS1 expression, and this increase is inhibited by tamoxifen (an antiestrogenic drug, widely used for the treatment of ERα-positive breast cancer) and ICI 182780 (an ER antagonist with no agonist effects which and downregulates cellular levels of both the ERs and PRs). The increased expression of the NOS1 protein is associated with a reduction in the adhesion activity of these cells**,** which is blocked by NO inhibitors [246].

Neutrophils from females have more β-adrenergic receptors than neutrophils from males. In line with these results, the non-selective agonist of β-adrenergic receptors, isoprenaline, elevates the chemokinesis and the release of a chemotactic factor from neutrophils from females, while it is inactive in neutrophils from males [247]. On the contrary, isoprenaline inhibits IL8-induced chemotaxis, and, again, the effect of isoprenaline is sex-dependent [247]. These results suggest that stressors could have different sex effects.

Finally, CD11b expression and redox activity are similar at baseline between neutrophils from males and neutrophils from females [248]. CD11b expression is not significantly altered by physiological concentrations of oestradiol and progesterone in neutrophils from women [248]. The differences in redox state have already been discussed by Malorni and collaborators [34].

7.4 Human neutrophils and cell fate

The lifespan of neutrophils is tightly regulated, and programmed cell death participates to efficiently resolve inflammation [249]. In neutrophils, autophagy can also play a role in infectious and inflammatory diseases [250], participating in the elimination of endogenous materials and cytosol-colonizing microbes (xenophagy) [251]. Therefore, there is an urgent and mandatory need to determine the influence of sex on cell fate.

7.4.1 Human neutrophils and apoptosis

In these cells, constitutive apoptosis is reduced in women versus men [248]. Indeed, physiologic concentrations of oestradiol and progesterone delay constitutive apoptosis in both sexes but do not change Fas antibody–induced apoptosis, which is essential for the elimination of activated immune cells from the peripheral circulation. However, serum sFas (inhibitor of apoptosis) is significantly higher in men than in women, while sFasL (stimulator of apoptosis) and cytochrome c levels (released from cells during apoptosis) are lower in men than in women [252]. Notably, androgens have no effect [248]. The paucity of data on the effect of sex indicates that it is imperative to study SD in constitutive and induced apoptosis to clarify the impact of these processes on atherosclerotic diseases.

7.4.2 Human neutrophils and autophagy

Some data suggest that autophagy in white blood cells is associated with coronary artery disease (CAD). The LC3 (marker gene for autophagy) and LC3-II (a membrane marker for autophagosomes and autophagolysosomes) genes are significantly reduced in the white blood cells of patients with CAD versus controls; however, a multivariate analysis indicates that only reduction in gene expression is linked with CAD [253]. LAMP-2 gene (lysosomal membrane marker gene) expression in leukocytes is increased in patients with CAD versus controls. Unfortunately, these studies did not conduct an analysis of sex differences.

8. Human monocytes and macrophages

Monocytes represent 5-10% of the peripheral blood mononuclear cells and are progenitors of macrophages and DCs [25]. The contribution of monocytes and macrophages to atherosclerotic progression and the maintenance of vessel-wall inflammation is well established [254].

The data regarding ER expression on monocytes is controversial. Phiel and colleagues [255] found that monocytes express from low to undetectable levels of ERs; whereas Pelekanou and colleagues [256] showed that expression of α 36-kDa splice variant and G-protein coupled receptor 30/G-protein ER occurs in a sex-divergent manner. ERβ appears to be the main receptor in monocytes [43]. Intracellular PRs are not present in resting monocytes (Table 1) [27]. AR expression on human monocytes (Table 1) is higher in young men than in young women [257], but this SD in expression disappears with age [43]. Human macrophages (Table 1) express both ERα and ERβ [68, 258, 259]. Notably, the ER profile is not modified during differentiation into macrophages or DCs, and it is shared by macrophages present in the atherosclerotic plaque [256]. Some authors have shown that ERα is the predominant receptor on macrophages; whereas, ERβ is the predominant receptor on monocytes, and it is up-regulated by oestrogen only in macrophages [43]. Human macrophages also express GPER and PRs [43, 260]. The levels of ERs are higher in monocyte-derived macrophages (MDMs) from women taking OCs versus

non-users. In both OC users and non-users, ERα is inactive [68], while ERβ is more highly expressed and more active in untreated women.

In MDMs obtained from premenopausal women, the AR mRNA levels are lower than in men; MDMs from postmenopausal women have AR mRNA levels lower than both men and premenopausal women [257]. In both cases, receptor expression is lower than that in male-derived cells [43]. Indeed, in MDMs obtained from young individuals, AR protein expression does not exhibit sexual dimorphism, and, in MDMs obtained from women, the use of OCs does not influence AR expression [68].

8.1 Human monocyte count

The increase in monocyte count is associated with an increase in the risk for heart disease [261], and it is also an independent predictor of future plaque formation [262]. In non-smoking men, the presence of carotid atherosclerosis is associated with significant increases in the counts of many leukocytes subtypes. On the contrary, in non-smoking women, this correlation is absent. These results are compatible with recently recognized SD in the mechanism and pathophysiology of atherosclerosis. It is evident that monocyte counts are higher in men than in women [136, 263]. Interestingly, the monocyte count is influenced by sex hormones, at least in women, for whom sex hormones are higher in the luteal phase than in the follicular phase or during pregnancy [228, 264]. HRT in menopause reduces the monocyte count [228], while the monocyte count is not influenced by OC use [68]. Globally, these findings suggest that female sex hormones decrease monocyte numbers probably because the hormones induce mitotic arrest and apoptosis [228]. It is also important to note that some subsets of circulating monocytes such as $CD14^{++}16^{++}$ are higher in men than in women. The percentage of monocytes expressing $CD99^{++}$, an index of adhesion and diapedesis, is lower in women than in men [265]. The adipokine leptin, a satiety signal that stimulates the immune system [266], is associated with monocyte

count, especially in men [267]. Finally, ageing influences the monocyte count, which is primarily increased in women [202].

8.2 Human monocyte count and smoking

Interestingly, smoking elevates the monocyte counts only in women [136]. Transcriptomic studies show that, in humans, smoking produces perturbations of gene expression in blood cells and alterations in blood parameters [136, 268-271], suggesting that many other parameters may be influenced by smoking in a sex-specific manner.

8.3 Human monocyte and macrophage functions

Numerous SD have been described in inflammation and immunity, and they involve different pathways [22-24]. One of the most important functions of monocytes is the production of cytokines and chemokines, and some SD regarding this point have been observed. However, some of these SD have been found to be controversial *in vitro* [25]. In particular, the basal release of TNFα in men is more than twice that in women, whereas LPS-induced TNFα release is typically greater in women than in men [[25] and quoted literature]. Moreover, TNFα release from monocytes obtained from women with CAD is lower in comparison with male patients [272]. Basal IL1β, IL6 and IL8 production by monocytes and in response to LPS is similar or less in women compared to men [[25] and quoted literature]. Interestingly, the basal and LPS-induced IL1β release is higher in the luteal phase compared to the follicular phase [25]. LPS-induced IL12 release is greater in men than in women, and it is not affected by the menstrual cycle [25].

Human monocytes also produce LTs, and female-derived monocytes have a higher capacity (1.8-fold) to produce pro-inflammatory LTs than male-derived monocytes [273]. *In vitro*, the exposure to dihydrotestosterone greatly reduces LT synthesis in female-derived monocytes, while oestradiol and

progesterone have practically no effect. Notably, pregnancy elevates 5-LO by-products partly because pregnancy elevates neutrophil and monocyte counts [274]. Progesterone reduces the production of 5-LO metabolites in human primary monocytes and is more active in female cells than in male cells. The progesterone effect is rapid and reversible [243]. Phospholipase D activity and diacylglycerol formation are 1.4- to 1.8-fold lower in male-derived monocytes than in female-derived monocytes, and this is linked to increased phosphorylation of ERKs [273]. Therefore, the arachidonic acid appears to be more active in women than in men.

Male- and female-derived monocytes respond differently to leptin, an adipokine involved in regulating food intake, body weight and immunity. In fact, recombinant leptin induces CD16 expression only in cells isolated from men [267]. *In vitro*, this leptin effect is inhibited by oestrogens [275]. Considering the pro-atherotic role of leptin in human monocytes [276], the SD just described could, at least in part, explain the SD in atherosclerosis.

It is hard to investigate SD in Toll-like receptors (TLRs), especially TLR4 responsiveness (LPS is an agonist of TLR4). TLR4 is present on monocytes, macrophages, DCs and other cells that are involved in atherosclerosis by mediating monocyte/macrophage infiltration and foam cell formation [277]. Therefore, understanding the influence of sex on this system is of special interest. Unfortunately, there is no clear evidence of a sex difference in TLR4 expression on monocytes [[25] and quoted literature], although reduced TLR4 responsiveness to LPS has been observed in monocytes/macrophages derived from women *in vitro* [[25] and quoted literature].

Monocytes obtained from women with CAD have a higher expression of PPARϒ protein compared with male patients [272]. Diurnal expression of PPARα and brain and muscle aryl hydrocarbon receptor nuclear translocator like-1 (BMAL1) mRNA profiles in monocytes obtained from women in the fed state differ qualitatively and quantitatively from those of men. Diurnal PPARα and BMAL1 profiles of fasted women resembled those of men in the fed and fasted states [278]. In other words,

women change their diurnal expression profiles of PPARα and BMAL1 when switching from the fed to the fasted state, whereas men do not. This could explain why PPARα agonists such as fenofibrate significantly reduce the total bile acid concentration only in men [279] and why the combination therapy of statin and fibrate is active in diabetic men but not in diabetic women [280]. SD have also been observed with statins alone. In particular, *in vitro* exposure to simvastatin (SMV) and atorvastatin (ATV) inhibits spontaneous and LPS-induced chemotaxis especially in female-derived monocytes [281]. However, an *in vivo* study in type 2 diabetic patients did not confirm the sex-specific effect of statins. In fact, these patients, independently of sex, have the same release of TNFα, IL1β, IL6, and MCP1, which are decreased after treatment with SMV, fenofibrate, and SMV+fenofibrate [282].

In MDMs, the basal release of TNFα is influenced by OCs. Specifically, the basal release of TNFα is higher in women treated with OCs [68], while the release of TNFα induced by LPS is lower in OC users than in non-users [68]. The basal release of TNFα in MDMs is elevated in non-smoking men versus non-smoking women, but this sex difference is not present in MDMs obtained from smoking men and women [136]. The exposure to LPS increases the release of TNFα in non-smoking women and decreases it in non-smoking men, whereas no significant difference between sexes has been found in MDMs obtained from smoking people [136].

It is important to recall that 24-h LPS exposure modifies the expression and activity of ERα and ERβ in MDMs in a sex-dependent manner. In particular, LPS up-regulates the ERα level especially in male cells. On the other hand, the ERβ level is down-regulated only in female MDMs [283]. In addition, LPS increases ERα activation without any significant effect on ERβ activation status [283]. The ability of TLR agonists, including LPS, to increase ERα phosphorylation has also been reported and is associated with an increase in the inflammatory response of mesangial cells [284]. Together, these data confirm the pivotal role played by ERα in the LPS-induced inflammatory response.

Macrophages can be subdivided according to their polarization into classically activated cells (M1) or non-classically activated cells (M2) [285]. M1 enhance and sustain inflammatory responses. Conversely, M2 secrete anti-inflammatory cytokines and promote the resolution of inflammation clearing of apoptotic cells, the dampening of immune responses, and tissue repair and healing. Premenopausal and postmenopausal women have similar M1/M2 ratios [286]. Both M1 and M2 are present at different stages of human atherosclerotic plaque development [245]. M1 express ERα and ERβ, but only ERα is reduced by M1 agonists such as LPS [LPS]/interferon-γ (IFNγ) [286].

8.4 Human monocytes and macrophages and cell fate

The cellular fate of monocytes is predominantly regulated by chemokines and their receptors [287]. Granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factors, and colony-stimulating factor-1 are relevant for the differentiation of monocytes into macrophages [288]. The inhibition of apoptosis contributes to the accumulation of macrophages and the persistence of an inflammatory milieu. Autophagy in macrophages processes bulky materials, participates in inflammatory responses [289-291], contributes to cholesterol homeostasis (lipophagy) [292-294] and facilitates efferocytosis (a process in which apoptotic cells are phagocytised and removed) [295, 296]. Some studies clearly show that the atheroprotective effect of macrophage autophagy is due to increased cholesterol efflux, reduced inflammation, and improved efferocytosis [291, 295].

8.4.1 Apoptosis

Monocytes are short-lived and undergo spontaneous apoptosis, and spontaneous apoptosis facilitates the resolution of the immune response [297, 298]. The apoptosis increases with each stage of atherosclerosis, but the largest increase is observed in the vulnerable plaque [299]. In fact, the necrotic core of plaques is derived from a combination of impaired phagocytosis of apoptotic cells and

accelerated macrophage cell death [300]. Female sex hormones delay apoptosis only in peripheral blood mononuclear cells obtained from women with normal menstrual cycles but do not inhibit TNFα production [301]. The paucity of data indicates that future detailed studies on the influences of sex on apoptosis in macrophages and monocytes will be necessary.

8.4.2 Autophagy

The deregulation of autophagy in monocytes and macrophages has only recently been the focus of studies [[302] and quoted literature]. Notably, lipid droplets can also be eliminated by autophagic-dependent phenomena [293]. Peripheral blood monocytes obtained from patients with acute coronary syndrome have lower levels of autophagic markers such as beclin-1 and ATG7 [303]. Unfortunately, the influence of sex on human monocyte and macrophage autophagy is still unknown. Therefore, there is an urgent need for studies of this specific point because SD are emerging in other human cells, but the differences are cell- and parameter-specific [304].

8.5 Human macrophages and foam cells

Macrophages are the first invaders of atherosclerotic lesions and the main component of atherosclerotic plaques [305]. The adherence of monocytes to ECs and their transmigration into the sub-endothelial space to differentiate into macrophages is an early step in atherogenesis, after which they accumulate lipids to form foam cells. The recruitment of circulating monocytes to the endothelium is facilitated by cell adhesion molecules (CAMs), including intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1). The levels of soluble ICAM1 in men are better predictors of the risk of myocardial infarction than those in women [306]. Exposure to sex hormones alters cholesterol metabolism in human MDMs obtained from young men and premenopausal women [307, 308]. Exposure of human MDMs to oestrogen or progesterone reduces the accumulation of cholesteryl ester

only in female cells [309]. Therefore, oestrogen and progesterone reduce lipid accumulation in a sex-specific manner. *In vitro,* dihydrotestosterone increases monocyte-endothelial adhesion, up-regulating VCAM1 in male cells but not in female cells [309, 310].

Notably, androgens affect the expression of genes involved in lipoprotein metabolism (lysosomal acid lipase, acyl CoA:cholesterol acyl transferase and cholesteryl ester hydrolysis) in male MDMs but not in female MDMs [308]. Cholesterol transport in macrophages plays a pivotal role in atherogenesis [311]. Scavenger receptors (SRs) of LDLs are the principal contributors to cholesterol uptake in macrophages [311], and numerous types of SRs are present [311]. Cholesterol efflux occurs through ABCA1, ABCG1, and apolipoprotein E [312-314]. SD in LDL receptors and lipoprotein uptake are primarily described in animal models [315-319], whereas only few studies have been conducted using human cells. However, oestrogens regulate cholesterol transport in MDMs obtained from postmenopausal women and age-matched men. Oestrogens appear to regulate cholesterol transport by inhibiting scavenger receptor A through heat shock protein 27 [320].

In particular, oestrogens lower cholesteryl ester levels in both female and male cells, but they do not affect cholesterol efflux [321]. The reduction of cholesterol esters in MDMs should mitigate foam cell formation. Finally, oestrogens, but not androgens, have a small effect on the expression of several genes involved in cholesterol transport, but they do not affect protein expression [321]. Moreover, oestrogen-and progesterone-treated macrophages from premenopausal female donors bind a significantly greater proportion of labelled acetylated LDL [307]. In male MDMs specifically, androgens elevate cholesteryl ester content [257], but this is not univocal data [139].

9. Human DCs

DCs are constituted by different subtypes (conventional/classical DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells, and monocyte-derived CD11b+ inflammatory/migratory DCs). pDCs

express ERα and ERβ as well as CD8 T and monocyte-derived DCs (Table 1) [322], whereas intracellular PRs (Table 1) are not expressed on resting DCs [27]. AR receptors are expressed in these cells [26], but it is not clear whether there are SD.

9.1 Human DCs and smoking

A paucity of data is present on the effect of smoking on DCs, and even less data are available on the interaction between smoking and sex on DCs. In particular, the number of pDCs in the human airway is not influenced by smoking, while the number of mDCs in the blood decreases in healthy male and female smokers; however, the effect of sex was not analysed [323]. Cigarette smoke can also impair the function of human monocyte-derived DCs, enhancing IL10 secretion and prostaglandin E2 release. Furthermore, cigarette smoke extract decreases DC-mediated priming of T cells, specifically inhibiting key Th1 cytokine production and favouring the development of Th2 responses. This results in a state of arrested DC maturation and reduced DC number [324]. The above data indicate that DCs are affected by smoking, but it is still unknown whether the effect of smoking is sex specific.

9.2 Function of human DCs

Very little data are available on sex effect in DCs. It has been shown that DCs from premenopausal women respond better to TLRs than those of men, producing a large amount of IFNs in response to TLR7 (including HIV-1) and TLR9 ligands [325-327]. The difference is not present when men and postmenopausal women are compared, but HTR restores the SD versus TLR7 responses [327], indicating that oestrogens can play a role. Blocking ERs *in vitro* during pDC differentiation reduces the frequency of cytokine-producing cells in response to TLR7 stimulation, suggesting that oestrogens may act as a cell-extrinsic factor to positively regulate the TLR7 responses of pDCs in women in a cell-intrinsic manner [328]. Furthermore, the production of IFNα is primarily regulated at the transcriptional

level by the IFN regulatory factors (IRF) family, and female cells express more IRF5 (1.6 times more) than male cells [329]. Importantly, the SD in IFR5 expression are not present in $CD3^+$ T cells and in monocytes/cDCs [329], indicating a certain cell specificity. Notably, IRF5 elevates the transcription of TNFα, IL8, MIP1α, and MIP1β without SD, suggesting that sex influence is cytokine specific. Finally, IRF5 protein expression does not change with the use of OCs. In contrast, Seillet and colleagues [327] report an elevated frequency of TNFα–producing pDCs in women compared with men after stimulation with a TLR7 ligand. Finally, it is important to note that IRF5 appears to be regulated, at least in part, by ERα [329]. Cytokine specificity, *in vitro*, was confirmed by data from Segerer and collaborators [330]. They observed that progesterone and oestradiol do not affect the release of IL8, MCP1, and CCL5 from monocyte-derived DCs from healthy men and women. Finally, 17β-oestradiol induces CD40 expression through the activation of p38 and JNK, which increases minichromosome maintenance protein 6 expression, which in turn induces CD40 expression [331].

During the so called "mini-puberty" (in first six months of life, infants have circulating levels of sex hormones near the levels seen during puberty), pDCs obtained from female infants, just as those obtained from women, generate more IFNα than those obtained from male infants in response to stimulation with a stimulator of TLRs 7/8. Interestingly, androgen signalling dose-dependently down-regulates IFNα production. In the same cohorts, TNFα production in pDCs does not vary between the sexes when the DCs are exposed to a stimulator of TLRs 7/8 in a dose-dependent fashion [332].

9.3 Human DCs and cell fate

Programmed cell death is an important element of the lifespan of DCs. However, SD in apoptosis and autophagy in XX and XY cells have not been thoroughly investigated because most cell-based studies have been performed without consideration of the male or female origin of the cells.

9.3.1 Apoptosis

It has been shown that dysregulation of apoptosis in DCs is involved in sepsis-induced immunosuppression [333]. As shown in [334], in mDCs obtained from healthy donors of both sexes, apoptosis is enhanced by progesterone and oestradiol *in vitro*; however, no analysis of sex was performed in this study.

9.3.2 Autophagy

To the best of our knowledge, no SD have been reported in the autophagic process in human DCs.

10. Human lymphocytes

Approximately 30% of white blood cells are lymphocytes. They comprise three types of cells: T lymphocytes (85-90% of circulating lymphocytes) release cytokines including IL2, IL4, IL10, IFNϒ and TNFα and underlie cell-mediated adaptive immunity; B lymphocytes (5–15% of circulating lymphocytes) produce IgG and IgM antibodies and underlie antibody-driven adaptive immune responses; and natural killer cells (NKs), which are effectors of cell-mediated innate immunity. They are very well represented in atherosclerotic plaques, where T lymphocytes comprise 20% of the immune cells [14 and quoted literature]. Most of them are CD8+ cytotoxic T cells [335]. Formation of the atherosclerotic lesion and destabilization of the plaque begin with the activation of T lymphocytes and their Th2 versus the Th1 subset [245].

Human lymphocytes express both ERα and ERβ [336] (Table 1). It has also been shown that, in $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and NKs, the ERα46 isoform is the most represented [337]. Intracellular PRs are not expressed on resting lymphocytes, NKs, or DCs [27], but membrane-bound PRs are present on resting lymphocytes (Table 1) [27]. Interestingly, activated lymphocytes can up-regulate PRs [27]. Finally, the presence of ARs is still a controversial issue (Table 1) [27].

In B lymphocytes, 358 genes exhibit sexual dimorphism: compared to male-derived cells, 226 and 13 genes are found to be up- and down-regulated, respectively, in female-derived cells [338]. Furthermore, Hewagama and collaborators identified 1953 genes that exhibit sexual dimorphism in T lymphocytes [339]. Despite the genetic sex differences and the involvement of T and B lymphocytes in atherosclerotic plaques [340], the influence of sex is scarcely considered in studies of lymphocytes. Therefore, only few data are available.

10.1 Human lymphocyte counts

Men and women have similar numbers of lymphocytes [[32] and quoted literature]. This is not an univocal fact because some studies have shown higher levels of lymphocytes in male subjects [341]. However, when lymphocyte subtypes are considered, some authors report lower T lymphocyte counts in males than in females [205]. B lymphocytes are reported to be higher in females [341, 342], and studies show that women have a significantly higher percentage of $CD3^+$ and $CD4^+$ and a lower percentage of NKs than men. The lymphocyte count in women is influenced by menopausal status. The number of T and B lymphocytes and helper T cells are lower in postmenopausal women versus premenopausal women [32], but the total lymphocyte count and the subtype count do not vary throughout the menstrual cycle [228]. OCs do not affect the absolute numbers or subtype counts [228]. In contrast, HRT affects lymphocyte subtypes. The total lymphocyte counts and the percentages of T cells and Th lymphocytes are lower in HRT users than in non-users [228]. Ageing influences lymphocyte counts with T helper cells and cytotoxic T cells decreased primarily in men [202]. More older men exhibit an inverted CD4/CD8 T cell ratio compared to women [343]. In elderly women, there is an increase in the proportion of T cells that are positive for IFNϒ, IL2, IL4, and IL10 and the proportion of cells presenting IL4 or IFNϒ; whereas, in old men, a higher percentage of T cells produce

IL2, IL4 and IL13 [344]. The changes induced by ageing are more evident in males, although statistical relevance is obtained only for $CD8^+$ T cells and for effector memory cells [345].

In particular, the activation of the stress system changes the distribution of T and NKs (decreasing their ratio), independent of sex or menstrual cycle phase [346]. Globally, in women, oestrogens appear to play a role in the regulation of lymphocyte numbers.

10.2 Human lymphocyte count and the effect of smoking

Data regarding the influence of smoking on lymphocyte subsets are few and conflicting. However, one study showed that smoking status increases the lymphocyte counts only in women [136].

10.3 Human lymphocyte function

Women have more pronounced humoral and cellular immune responses to antigens than males [[32] and quoted literature]. SD in bacterial, viral and parasitic infections have been extensively reviewed by Ngo ST et al. [[32] and quoted literature]. In particular, women produce higher levels of $CD4^+$ T cells in response to immunization, with higher levels of circulating antibodies after vaccination for influenza, hepatitis B, rubella and tetanus than men [[32] and quoted literature]. Women also have higher levels of IgM than men [[32] and quoted literature].

The culture of peripheral blood mononuclear cells results in significant increases in the percentages of B cells and total T cells among females and of NKs among males [341], indicating that culture conditions have different consequences between the sexes. As previously suggested by Franconi and collaborators [347], this result indicates that sex studies require peculiar attention to experimental conditions because the very same experimental condition may affect experimental outcomes in a sex-specific manner.

Sex appears to influence lymphocyte function in an age-dependent manner. T cells isolated from older men have a lower proliferative and cytokine secretion capacity than cells from older women. The ability of monocytes to secrete a chemoattractant for activated T cells, IFN-γ-inducible protein 10 (IP-10), is reduced in a similar fashion [26]. The production of IFNϒ and IL17 in stimulated T cells from healthy older men is lower than in those from healthy young men. However, in T cells obtained from old and young women, the production of IFNϒ and IL17 is not different [348]. T regulatory cells and Th2 CD4+ helper T cells produce the anti-inflammatory cytokine IL10 [349]. The plasma levels of IL10 decline with age, with a greater decline in men than in women [350]. Interestingly, elevated levels of IL10 are associated with stroke outcomes only in women [351].

10.4 Human lymphocytes and cell fate

Apoptosis participates in the regulation of the immune response. Nevertheless, death pathways in immune XX and XY cells have not been thoroughly investigated because most cell-based studies have been performed without consideration of the male or female origin of the cells. Autophagy has been reported in T and B cells [352]. In T cells, autophagy provides substrates and modulates NFκB activation [352]. Less is known about the role of autophagy in B cells [352].

10.4.1 Apoptosis

In vitro, sex hormones delay apoptosis in B and T cells obtained from women with normal menstrual cycles [301, 353]. The activation of the stress system changes the distribution of T cells and NKs (decreasing their ratio); however, more interestingly, this is accomplished by an increase in apoptotic T helper cells independent of sex or menstrual cycle phase [346].

10.4.2 Autophagy

This cannibalistic process has been implicated in cellular survival and programmed cell death. Currently, to the best of our knowledge, it is not known whether lymphocyte autophagy is influenced by the sex of cells.

11. Human NKs

Human NKs consist of two subsets: CD56dim and CD56bright. CD56dim constitutes 90% of the total NK cell population in the peripheral blood and has high cytotoxic activity. The other 10% of NKs consist of CD56 [[354] and quoted literature]. These cells have the capacity to kill cells and to produce cytokines, and they play a central role in the innate immune response against tumours, parasites and infected cells. They also play an immunoregulatory role in the pathogenesis and progression of atherosclerosis, although it is not clear whether they are pro-atherotic or antiatherothic [355].

Both ERα and ERβ (Table 1) are expressed in human NKs [322, 336], and it has also been shown that the ERα46 isoform is the most represented [337]. Intracellular PRs (Table 1) are not expressed in NKs [27].

11.1 Human NK counts

NKs constitute approximately 5% of the leukocytes. The data on SD in NKs are scarce and discrepant. In particular, it has been reported that: a) the NK counts in males and females (postmenopausal or fertile) and OC users [[228] and quoted literature] do not differ, whereas they are higher in women with premature menopause [[32] and quoted literature]; b) the NK count varies during the menstrual cycle, increasing in the late secretory phase [[228] and quoted literature]; c) the NK count has been shown to be higher in males than in females in some studies [341], while other studies have provided evidence [356, 357] that the NK count is lower in men than in women; d) there are no SD in the subset

distributions of circulating NKs [358]. Furthermore, in older men, the proportion of positive and double-negative CD4+ cells is elevated and reduced, respectively [356]. In older individuals, a recent paper shows that there are not SD in NKs and T cells measured as a percentage of lymphocytes. However, more B cells are present in women than in men, and the ratio of CD56bright to CD56dim NKs is larger in older women than in older men [359], suggesting a sex difference in maturation of these cells. Indeed, the number of NKs increases in men and women > 60 years of age, but the increase is higher in women than in men [360]. Furthermore, during pregnancy, peripheral blood NKs are suppressed both in terms of number and activity [[228] and quoted literature], whereas mental stress increases the number of NKs in women but decreases them in men [346].

Globally, these results suggest that some of the discrepancies reported could depend on the age and hormonal status of women. The ARs and ERs are present on these cells, but sex stratification is currently missing (Table 1).

11.2 Human blood NKs and smoking

Smoking impairs the immune response and the peripheral blood leukocyte counts including the NK count, which is lower in smokers [361-363]. Moreover, an impairment in the production of IFNϒ and TNFα is reported in NKs isolated from smokers [364]. In the previous studies, stratification for sex is missing, and therefore we do not know the sex effect.

11.3 Function of human blood NKs

Data on SD of NK activity is limited. In particular, NKs in men exhibit more cytotoxic activity than those in women with regular menstrual cycles or women using OCs, who have the lowest levels of activity [[32] and quoted literature]. The menstrual cycle has no significant effect on the activity levels of NKs [365]. However, others have shown that NKs are more cytotoxic in the follicular phase than in

the luteal phase and in postmenopausal women. Males exhibit cytotoxic activity similar to that observed in the follicular phase [[32, 228] and quoted literature]. In older individuals, mature NKs obtained from women have a more cytotoxic response and produce more MIP-1β in response to a variety of stimuli [359]. These data show that sex influences NK activity in elderly individuals. The α-galactosylceramide-induced intracellular production of IFNγ, IL4, IL17 and TNF by $CD4^+$ and DN^+NKT cells is larger in women than in men [357]. Data on the *in vitro* effects of progesterone, oestrogens and androgens on human NKs are not univocal [366-368]. Finally, there are not significant SD in IL4 response, but cells obtained from men produce more IFNϒ and MIP1α than female cells [358]. Therefore, NKs may contribute to the sexual dimorphism.

11.4 Human NKs and cell fate

SD in apoptosis and autophagy in XX and XY cells have not been thoroughly investigated because most cell-based studies have been performed without consideration of the male or female origin of the cells.

11.4.1 Apoptosis

Notably, mental stress increases the apoptotic T helper cell percentage irrespective of sex or menstrual cycle phase [346]. It has been reported that NK death occurs via TNF family receptors [369], but no information is available on sex effects.

11.4.2 Autophagy

To the best of our knowledge, there is no data on the influences of sex on autophagy of human NKs.

12. Human mast cells

These cells are resident in a multitude of tissues and play a role in the defence against microorganisms [[32] and quoted literature]. Mast cells have pleiotropic roles in atherosclerosis as well as in associated complications. Indeed, the number of mast cells in humans with cardiometabolic diseases is increased compared with healthy adults [370]. Additionally, the molecules released from mast cells might contribute to the pathogenesis of CVD [370]. Mast cells contribute to plaque progression and destabilization [7]. Activated mast cells can have deleterious effects on the vessel wall, degrading the extracellular matrix, enhancing apoptosis, and recruiting inflammatory cells. The expression of ERα, ERβ, both PRs and ARs (Table 1) has been demonstrated in mast cells, and oestrogens can enhance mast cell degranulation, whereas the influence of androgens remains largely unclear [371-373]. Both oestrogen and progesterone can activate mast cells [341].

12.1 Function of human mast cells

Mast cells are implicated in inflammation during atherogenesis and plaque destabilization. Activated mast cells increase vascular leakage and the influx of leukocytes into the plaque and induce intra-plaque haemorrhage causing plaque destabilization [374]. Mast cell number is a good marker for recent lesions, and it increases in male patients, particularly in older male patients [375]. Oestrogen or progesterone induces mast cell degranulation, whereas androgens are not effective [371]. There is limited knowledge of the involvement of mast cells in SD in atherosclerosis.

13. CONCLUSIONS

Globally, an analysis of the literature shows that sex aspects have been neglected in studies of cells involved in atherosclerosis with few exceptions because the majority of *in vitro* studies do not indicate the sex of the cells used and because most of the clinical studies are comprised of a majority of men. Additionally, many studies do not consider the methodological issues of sex and gender research such

as physiological aspects (age, menstruation, pregnancy, lifestyles, etc.) and the pre-analytical conditions [347]. This could explain the great deal of variability of the results. This variability increases the difficulty in the comparison of the results of sex studies among different laboratories. The comparison is, in fact, a very complicated and hard task because investigators use cells from individuals of different ages and with different lifestyles. Furthermore, *in vitro* studies are often performed using various different methodologies such as different hormonal concentrations and different times of incubation. This leads to an increase in variability, and the obvious consequence is the presence of controversial results.

The previous observations demonstrate the need for a clear definition of experimental conditions as has been previously suggested by Franconi and collaborators [347]. In our opinion, a mandatory questionnaire should be presented to cell donors, and the very same questionnaire should be used by all researchers in order to reduce variability among laboratories. A sample questionnaire has already been proposed by Franconi and collaborators [347].

Controversial data are even present when cell counts are considered. In view of the fact that an increase in some cell numbers is a risk factor for CVD, in our opinion, it is time to establish reference values for men and women considering, at least, age, phase of life of women, and some lifestyle choices such as smoking in order to appropriately and adequately evaluate CV risk for men and women. Considering the multiplicity of cell players and their complex interactions in atherosclerosis, the analysis of the literature indicated that there is an urgent need to extend the study of sex influences not only for single cells but also for interactions among cells such as PLT-neutrophil satellitism [376]. Therefore, it appears relevant to consider gender. In order to improve the diagnosis and treatment of CAD, the research community urgently needs to understand the SD of vessel wall cells and immune cells in the specific setting of atherosclerosis. The available data suggest that cells retain memory of sex and of the

life events of the donors. For example, treatment with OCs modifies macrophage function and expression and activity of ERs [68], indicating that cells not only have a sex but also have a gender.

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Table 1. Protein expression of sex hormone receptors in human cells

Cell types	ERα	ERβ	AR	PR
ECs	M = F [11]	M = F [11]	M = F [11, 39] M > F [44, 45]	
hVSMCs	M = F [97] M < F (starved HAEC)[98]	M > F [97]	M = F [97]	Present; no gender analysis [92, 93]
PLTs	M =F [122]	M =F [122]	Present (no sex analysis) [121]	
EPCs	M = F [17, 18] = during ovulatory than follicular and luteal phases, and vs men and postmenopausal women [176]	M = F [17] > during ovulatory than follicular and luteal phases, and vs men and postmenopausal women [176]		
Neutrophils	Present (no sex	Present (no sex	M = F [219]	ND [27]

	analysis) [218]	analysis) [218]		
Monocytes	M = F [258]	M = F [258]	M > F [257]	ND [27]
Macrophages	M < F [283]	M < F [283]		
	M = F [258]	M = F [258]		
			F = F+OCs [68]	
	F < F+OCs [68]	F > F+OCs [68]		
Lymphocytes	Present (no sex analysis) [336]	Present (no sex analysis) [336]		Only membrane-bound PR [27]
NKs	Present (no sex analysis) [322]	Present (no sex analysis) [322]		ND [27]
DCs	Present (no sex analysis) [322, 327]	Present (no sex analysis); [322, 327]		ND [27]
Mast cells	Present (no sex analysis) [372, 373]	Present (no sex analysis) [372, 373]	Present (no sex analysis) [371]	Present (no sex analysis) [373]

ND: not detected