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REVIEW

# Atherosclerosis and sex hormones: current concepts

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## ABSTRACT

CVD (cardiovascular disease) is the leading cause of death for women. Considerable progress has been made in both our understanding of the complexities governing menopausal hormone therapy and our understanding of the cellular and molecular mechanisms underlying hormone and hormone receptor function. Understanding the interplay of atherosclerosis and sex steroid hormones and their cognate receptors at the level of the vessel wall has important ramifications for clinical practice. In the present review, we discuss the epidemiology of CVD in men and women, the clinical impact of sex hormones on CVD, and summarize our current understanding of the pathogenesis of atherosclerosis with a focus on gender differences in CVD, its clinical presentation and course, and pathobiology. The critical animal and human data that pertain to the role of oestrogens, androgens and progestins on the vessel wall is also reviewed, with particular attention to the actions of sex hormones on each of the three key cell types involved in atherogenesis: the endothelium, smooth muscle cells and macrophages. Where relevant, the systemic (metabolic) effects of sex hormones that influence atherogenesis, such as those involving vascular reactivity, inflammation and lipoprotein metabolism, are discussed. In addition, four key current concepts in the field are explored: (i) total hormone exposure time and coronary heart disease risk; (ii) the importance of tissue specificity of sex steroid hormones, critical timing and the stage of atherosclerosis in hormone action; (iii) biomarkers for atherosclerosis with regard to hormone therapy; and (iv) the complex role of sex steroids in inflammation. Future studies in this field will contribute to guiding clinical treatment recommendations for women and help define research priorities.

## ATHEROSCLEROSIS

In this section, we will discuss the epidemiology of CVD (cardiovascular disease) in men and women, review the clinical impact of sex hormones on CVD, and summarize our current understanding of the pathogenesis of atherosclerosis. We will focus on gender differences in CVD, its clinical presentation and course, and pathogenesis.

**Key words:** androgen, atherosclerosis, cardiovascular disease, cell type, gender, oestrogen, progesterone.

**Abbreviations:** AHA, American Heart Association; apo, apolipoprotein; AR, androgen receptor; $AT_1$ receptor, angiotensin II type 1 receptor; BMI, body mass index; CAC, coronary artery calcium; CAD, coronary artery disease; COX-2, cyclo-oxygenase-2; CRP, C-reactive protein; CVD, cardiovascular disease; DHEA, dehydroepiandrosterone; DHT, 5$\alpha$-dihydrotestosterone; ER, oestrogen receptor; GPCR, G-protein-coupled receptor; GPER, G-protein-coupled ER; HAEC, human aortic endothelial cell; HDL, high-density lipoprotein; HRT, hormone replacement therapy; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LDL, low-density lipoprotein; LDLR, LDL receptor; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MPA, medroxyprogesterone acetate; NF-$\kappa$B, nuclear factor $\kappa$B; NOS, NO synthase; iNOS, inducible NOS; PAI-1, plasminogen activator inhibitor-1; PR, progesterone receptor; ROS, reactive oxidative species; RT–PCR, reverse transcription–PCR; SERM, selective ER modulator; TLR4, Toll-like receptor 4; TNF-$\alpha$, tumour necrosis factor-$\alpha$; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cell.

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## Epidemiology of CVD in women

The leading cause of death for men and women in the U.S.A. is CVD. However, according to statistics from the AHA (American Heart Association; www.americanheart.org), in women, mortality attributable to CVD continues to outpace that in men [1,2]. Compared with men, women have lower awareness of CVD, higher prevalence of risk factors for CVD, such as hypertension and hyperlipidaemia, and higher death rates following a stroke and myocardial infarction [3–6]. Part of the higher myocardial infarction mortality in women compared with men is due to older age and a greater number of co-morbid conditions at the time of diagnosis. However, the persistent lack of awareness in women of the threat of CVD remains a significant health-care issue [7,8]. Despite a relative increase in awareness over time, a national survey conducted by the AHA in 2006 found that only 21 % of women believe their leading health threat to be CVD, with 27 % of women continuing to cite breast cancer as their greatest health threat [3]. Subsequent surveys have indicated relatively modest gains in awareness with over half of women in the U.S.A. still unaware of CVD as their major health threat.

Women are still not benefiting equally from effective risk-prevention strategies and life-saving measures, and more widespread and routine use of existing AHA/ACC (American College of Cardiology) CVD prevention evidence-based guidelines for women is needed [9,10]. Despite the prevalence of CVD risk factors in women, the risk factors are often unrecognized, in part due to a lack of screening. Yet, two in three women have one or more of the major CVD risk factors, including hyperlipidaemia, hypertension, diabetes mellitus, physical inactivity and obesity [1,2]. Additional important considerations in the observed gender differences in CVD between men and women relate to a number of biological factors which affect the type of plaque build-up and vascular response (remodelling) to plaque [11]. Of particular interest in the present review are the influences of sex steroid hormones and their receptors on the vascular wall.

## Sex hormones and CVD

Compared with post-menopausal women or men, CVD is relatively less prevalent in pre-menopausal women. Menopause diminishes the gender protection in women and contributes to an adverse impact on cardiovascular risk variables [12,13]. Sex steroid hormones are critical determinants of the cardiovascular risk transition following menopause, attributable primarily, but not exclusively, to differences in oestrogen levels supplied to cardiovascular tissues.

In humans, the hypothesis that hormone therapy is an effective preventive measure for coronary heart disease was supported by earlier observational epidemiological studies [14]. The mechanism was attributed to oestrogen-mediated antioxidant actions, [15,16], improvements in the lipid profile [reductions in LDL (low-density lipoprotein)-cholesterol and increases in HDL (high-density lipoprotein)-cholesterol] [13,17], and direct atheroprotection of cardiovascular tissue [18–20]. Subsequent clinical trials, however, failed to confirm the benefits of combined or unopposed menopausal oestrogen therapy in women [21–23], perhaps in part due to the inclusion of post-menopausal women with antecedent subclinical CAD (coronary artery disease) in the study cohorts. In addition, the method of administration, dose and duration of use of the exogenous hormone may also be relevant.

Presently, the weight of the evidence indicates that older women, and those with known subclinical or overt coronary heart disease, should not take hormone therapy. However, age and timing of the initiation of hormone therapy in relation to the onset of menopause also appears to be an important mitigating factor in that earlier initiation of hormone therapy, timed with the onset of menopause or closely following menopause, may lead to atheroprotection and reduce coronary risk over time [24]. Thus the overall clinical utility of hormone therapy for atheroprevention in women remains controversial and complex. In addition, changes in the expression of ERs (oestrogen receptors) accompany atherogenesis. For example, normal coronary arteries of pre-menopausal women demonstrate normal ER expression, whereas in atherosclerotic vessels of post-menopausal women ERs are down-regulated [25]. Thus the direction and magnitude of the association between hormone, hormone receptor level, atherogenesis and vasculoprotection needs clarification.

One additional aspect of the vascular complexity of hormone therapy in clinical trials relates to the action of oestrogen in vascular inflammation. The role of oestrogen in inflammation is multifaceted and dependent on a number of factors, including immune status, stage of atherosclerosis, oestrogen timing/concentration/metabolism and ER status [26,27]. Oestrogen has been reported to have both an anti- and pro-inflammatory actions; the mechanisms are detailed below in the 'Current concepts' section of the present review. For example, oestradiol alone or with progestin has neutral or anti-inflammatory effects on cytokines, whereas conjugated equine oestrogen with/without progestin has pro-inflammatory potential. In addition, oral but not transdermal oestrogen therapy leads to an increase in CRP (C-reactive protein), possibly reflecting hepatic conversion of oestradiol into downstream pro-inflammatory metabolites. However, increased levels of CRP may not reflect inflammation. The CRP findings suggesting HRT (hormone replacement therapy) is pro-inflammatory is controversial in that some studies show an increase in CRP with HRT and some do not [28,29]. Overall, support for oestrogen as an anti-inflammatory

agent in the context of atherosclerosis and the vessel wall is mounting. Thus, until additional results become available, a balanced view is needed.

## Overview of the pathogenesis of atherosclerosis and gender differences in CAD

Atherosclerosis is a result of a chronic inflammatory condition of the vessel wall leading to vascular narrowing or obstruction, and is accompanied by vascular dysfunction. The disease develops over decades and remains asymptomatic for most of this time. In order to best understand the atherosclerotic process it is important to understand the key players. The vessel wall architecture consists of three layers: (i) an inner intima, a single cell layer of vascular endothelium, (ii) the vascular media composed primarily of smooth muscle cells separated from the endothelium by the internal elastic lamina, and (iii) the outer adventitial surface consisting of the vasa vasorum, pericytes and nerve endings. The inciting event(s) in the atherosclerotic process is endothelial injury and/or excess circulating lipids [30,31], leading to the development of the earliest atherosclerotic lesion, a fatty streak, representing subendothelial accumulation and deposits of lipids and LDL in the vessel wall. The subsequent vascular response is characterized by inflammation involving a number of cells including blood-borne monocytes [32]. Monocyte recruitment is promoted by a several factors, including cellular adhesion molecules, such as VCAM-1 (vascular cell adhesion molecule-1). Monocyte diapedesis into the subendothelial space, with subsequent phenotypic differentiation into macrophages which ingest oxidized lipids in an uncontrolled and unregulated fashion, result in the formation of 'foam cells', which represent engorged lipid-filled macrophages [33].

Atherosclerotic plaque progression and growth is aided by smooth muscle proliferation and migration from the media to the intima, through the internal elastic lamina, a process driven in part by endothelial-derived cytokines and chemoattractants. With time, the media develops an abnormal matrix capable of further promoting abnormal cellular proliferation and entrapment of modified lipids in the vessel wall, such that the lesion may continue to grow in the vessel wall, become more advanced and encroach on the lumen. Adaptive vascular responses to atherosclerotic plaque build-up in the vessel wall include intramural calcification and vascular dilatation. Plaque build-up can also result in development of a fibrous cap that may be prone to fissure, rupture or erosion leading to the clinical manifestations of CAD, including angina, myocardial infarction or sudden death. Plaque rupture is typically accompanied by platelet adhesion leading to formation of a vascular thrombus that can result in partial or complete vessel occlusion [34]. However, most severe clinical events (angina, unstable angina and other acute coronary syndromes, such as myocardial infarction) do not occur at plaques that produce high-grade stenoses, but rather at sites characterized by more immature disease prone to plaque instability. Figure 1 shows the development of atherosclerosis from an early fatty streak lesion to a more advanced plaque.

Findings of the landmark WISE (Women's Ischemic Syndrome Evaluation) study, an NHLBI (National Heart, Lung and Blood Institute)-sponsored multi-site study of 935 women (mean age, 59 years) with chest pain undergoing cardiac catheterization [35] demonstrate that not all women with chest pain syndromes consistent with angina will have obstructive CAD. In fact, no CAD was observed in 34 % of women, minimal (non-obstructive) CAD in 23 %, and significant CAD (>50 % stenosis of one or more coronary arteries, or multivessel CAD) was present in only 43 % of women. Gender differences in acute coronary syndromes with 'normal' coronary arteries have also been described [36,37], and are more common in women (10–25 %), and in non-white women, compared with men (6–10 %). Plaque erosion, compared with the classic plaque fissure and rupture, as the aetiology of coronary thrombosis occurs at a higher frequency in women than in men (37 compared with 18 % respectively) [36,37].

Therefore, in women, atherosclerosis can occur with seemingly normal coronary arteries. The operative mechanisms have been ascribed to gender differences in coronary remodelling that occur in the vessel wall in response to plaque build-up. The differences in remodelling have been termed positive remodelling (predominant in women) and negative remodelling (predominant in men), and have been reviewed previously [11]. In positive remodelling, plaque and narrowing can occur without focal obstruction(s) because the disease is more diffuse with less segmental stenoses. In response to diffuse plaque build-up, the internal elastic lamina thickens as the vessel dilates to accommodate the plaque (asymptomatic), such that once women present with symptoms, plaque burden is greater as the vessel has already compensated. In comparison, in negative remodelling, there is more focal obstructive CAD and segmental stenosis of the vessel wall, without diffuse narrowing. Thus, in women, gender differences in vascular remodelling in response to the atherosclerotic process can lead to ischaemia due to diffuse CAD in the absence of focal obstruction. Additional contributors to non-obstructive CAD in women include endothelial dysfunction, microvascular disease and other factors. All of these mechanisms of angina with normal coronary arteries have indeed been shown to predominate in women [38]. Lastly, the prognosis in women with myocardial ischaemia without obstructive angiography is not as benign as previously thought, with relatively

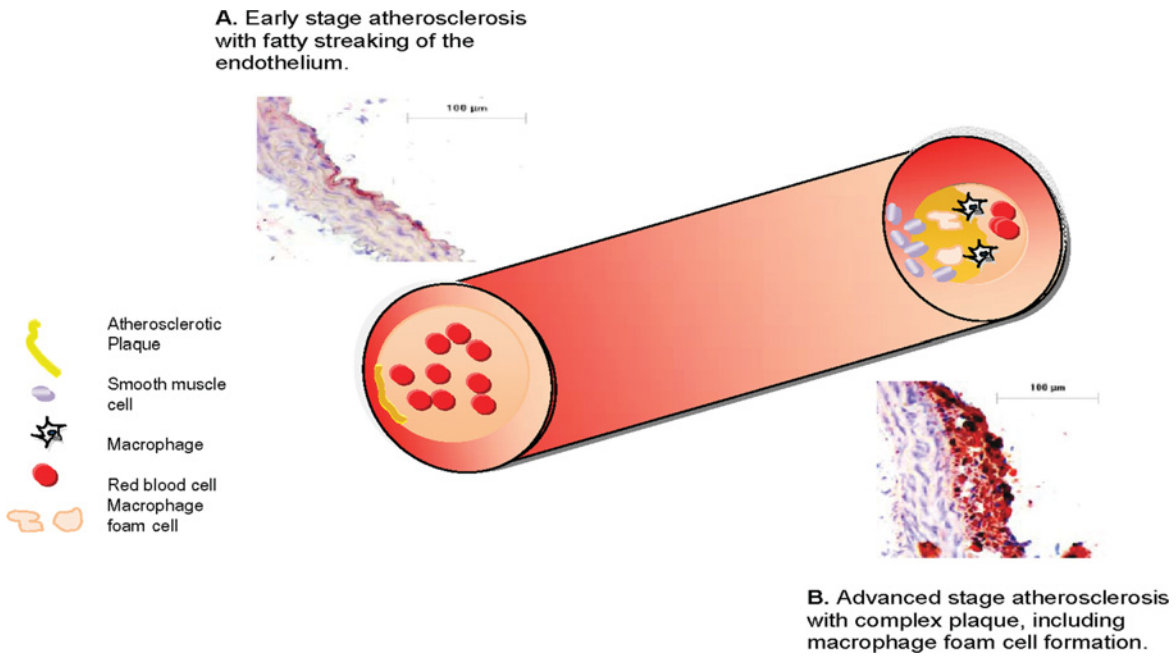


**Figure 1 Development of atherosclerosis**

The development of atherosclerosis, from an early fatty streak lesion (left side of vessel lumen) to a more advanced plaque (right side of vessel lumen), is depicted diagrammatically. Histochemistry of examples of representative cross-sections (10 $\mu$m) of lesions from the proximal aorta of cholesterol-fed mice are also shown. The vessels have been stained for lipid with Oil Red O and counterstained with Gill's haematoxylin to define the architecture. Cross-sections are orientated with the lumen to the right and the adventitial surface to the left of each image. The representative images ($\times$20 magnification) demonstrate red staining subintimal lipid deposits and fatty streaks (A), and advanced plaque with extensive subintimal lipid deposition, smooth muscle cell proliferation and foam cell formation (B).

high rates of progression to obstructive CAD, and higher short- and long-term rates of cardiac events in this population [39,40].

## SEX HORMONES AND THEIR RECEPTORS

In this section, we will direct our attention to the critical animal and human results that pertains to the role of oestrogens, androgens and progestins on the vessel wall. We will pay particular attention to the actions of sex hormones on each of the three key cell types involved in atherogenesis: the endothelium, smooth muscle cells and macrophages.

Much of the gender bias observed in atherogenesis is probably due to pleiotropic effects of the sex steroids (oestrogens, androgens and progestins) on all cell types involved in the disease process. These hormones and their receptors act through ligand-dependent, ligand-independent, genomic and non-genomic mechanisms. The most widely studied of these mechanisms is the canonical pathway in which a steroid binds its intracellular receptor, leading to dimerization of ligand-bound receptors and binding of the dimer complex to a regulatory element in the promoter regions of specific genes. Thus the receptors act as ligand-activated transcription factors. Classically, this pathway was thought to result in gene transcription; however, this pathway can also mediate inhibition of gene transcription through competition for shared co-activators or through inhibition of DNA binding by other transcription factors. Such inhibitory effects have been well-documented for ER–NF-$\kappa$B (nuclear factor $\kappa$B) interactions [41,42]. Furthermore, ligand-dependent rapid non-genomic actions can occur through classical receptors on the cell membrane which have been identified for oestrogens, androgens and progestins [43]. GPCRs (G-protein-coupled receptors) for oestrogen and progesterone have been identified on the cell surface, but remain a subject of controversy.

A receptor for oestrogen, GPR30 (GPCR receptor 30), now known as GPER (G-protein-coupled ER), has been identified intracellularly [44] and on the plasma membrane [45]. Unlike the classical ERs, GPER acts through a rapid non-genomic mechanism involving activation of the cAMP signalling pathway (reviewed in [46]). Three isoforms of a G-protein-coupled PR (progesterone receptor) have been identified in humans, but there is, as yet, no evidence that any are expressed in blood vessels [47]. Non-genomic modes of ER action, in concert with the ER-mediated regulation of transcription by multiple post-translational modifications, have also been

**Table 1 Hormone receptor expression in the vasculature**

+ = present.

| | Hormone receptors and subtypes | | | | | |
|---|---|---|---|---|---|---|
| Vascular cell type | ERs ($\alpha$ and $\beta$) | References | ARs | References | PRs (A and B) | References |
| Endothelial cells | + | [56,58,120,121] | + | [65–67,120] | + | [63,122] |
| Smooth muscle cells | + | [83,84,121] | + | [105,120] | + | [86–88,122] |
| Macrophage or monocyte | + | [88,123,124] | + | [111,113–115] | + (macrophage only) | [107,110,122] |

described. These include reversible lysine acetylation and other histone modifications (reviewed in [48]). Furthermore, spliced variants of the ER are known to exist in humans and rodents, including variants for ER$\beta$ [49,50]. Thus there is great diversity in cellular mechanisms for oestrogen action.

## Overview of hormone receptors

Two cytoplasmic ERs, ER$\alpha$ (*ESR1*)and ER$\beta$ (*ESR2*), exist in all mammals studied and have been characterized (reviewed in [51]) and both are also found on the cell surface [43]. The two receptors are products of different genes (*ESR1* and *ESR2*), but share a high degree of homology. For example, ER$\alpha$ increases and ER$\beta$ decreases apo (apolipoprotein) E expression in the hippocampus [52]. However, in the mouse aorta, the two receptors appear to have largely unique gene targets [53]. The PR exists as two isoforms, PR-A and PR-B, encoded by the same gene. The PR-B isoform mediates strong hormone-dependent transactivation in all cell types that have been examined, whereas PR-A transcriptional activity is cell- and reporter-specific, and the two isoforms regulate distinct genes in a tissue-specific manner (reviewed in [54]). To date, a single AR (androgen receptor) has been identified, transcribed from a gene located on the X chromosome [55].

Herein, we will examine the sex steroid receptor profiles and actions for the major vascular cell types important to atherosclerosis (summarized in Table 1). We will also review the systemic actions of these hormones, mediated primarily through the liver, that influence the onset and progression of atherosclerosis. In addition, results regarding vascular expression of two enzymes, cytochrome P450 aromatase (responsible for conversion of androgens into oestrogens) and 5$\alpha$-reductase [responsible for conversion of testosterone into DHT (5$\alpha$-dihydrotestosterone], will be discussed.

## Endothelial cells

ER$\alpha$ appears to be the dominant receptor in endothelium as shown in pig aortae [56] and in HUVECs (human umbilical vein endothelial cells), a widely used *in vitro* model of endothelium [57], although only ER$\beta$ was identified in HUVECs by Toth et al. [58]. Careful analysis of rat cardiac and cerebral endothelium revealed that ER$\beta$ localizes predominantly to the nucleus, whereas ER$\alpha$ is found in both the cytoplasm and the nucleus [59]. Furthermore, oestradiol up-regulates ER$\alpha$, but dramatically down-regulates ER$\beta$, in pulmonary endothelium [60]. Using a unique mouse model in which GPER drives a reporter gene, GPER has been reported to be expressed in the endothelium of small peripheral arteries but not in large arteries that are important for atherosclerotic vascular disease (aorta, carotid and renal) [61]. Experiments with a GPER-specific agonist, G-1, have revealed a role for GPER in vasodilation and the reduction in blood pressure [62]. This is, most likely, an endothelial response because GPER has not been found in vascular smooth muscle in mice.

Both isoforms of the PR have been demonstrated in freshly isolated female HUVECs by RT–PCR (reverse transcription–PCR) [63], but only PR-A was detected by immunohistochemistry in commercial HUVECs [58]. Both PR-A and PR-B were demonstrated in human dermal endothelial cells by Western blotting [64]. ARs have been identified by RT–PCR in HAECs (human aortic endothelial cells) [65] and by functional studies, Western blotting and immunohistochemistry in HUVECs [57,66]. The AR may be expressed to a higher degree in male endothelial cells than in female cells [67]. Furthermore, aromatase has been identified by RT–PCR in HUVECs [68], but not in HAECs [65], suggesting that in some, but not all, endothelial cell androgen effects may be the result of conversion into oestrogen. mRNA for the low-affinity type 1 5$\alpha$-reductase is present in HAECs, indicating that testosterone might be converted into the highly potent DHT within the endothelial cells [65].

Direct rapid effects of oestrogen on vasomotor tone have been studied extensively and are thought to be mediated by cell-surface ERs [69]. More germane to atherogenesis are oestrogen effects on endothelial proliferation, migration and inflammatory mediators. In the LDLR (LDL receptor)-deficient mouse model, selective elimination of ER$\alpha$ in endothelium abolishes the anti-atherosclerotic effects of oestrogen, suggesting the critical importance of oestrogen effects on endothelium [70]. Oestrogen promotes endothelial proliferation and increases vascular incorporation of bone-marrow-derived endothelial progenitor cells, both of which contribute to enhanced re-endothelialization

following arterial injury. The former appears to be ERα-dependent, whereas the latter requires both ERα and ERβ [71,72]. Endothelial studies have uncovered a host of anti-inflammatory actions of oestrogen that influence atherosclerosis.

Oestrogen inhibits inflammatory expression of the adhesion molecules VCAM-1 and ICAM-1 (intercellular adhesion molecule-1) in endothelium [73,74] and inhibition of VCAM-1 may occur through ERβ [75]. The cytokine chemoattractant MCP-1 (monocyte chemotactic protein-1) is unaffected by oestrogen in HUVECs, down-regulated by oestrogen in coronary artery endothelium [76], and up-regulated in splenic and dermal endothelium [77]. Thus there is tissue specificity of oestrogen action in endothelium of different vascular beds. The two ERs exert opposing effects on the promoter activity of the PAI-1 (plasminogen activator inhibitor-1) gene in endothelial cells, such that ERα induces and ERβ suppresses transcription, adding another level of complexity to the interactions between oestrogen and endothelium [78]. Finally, oestrogenic compounds vary in their effects on endothelium. One example is the 400-fold increase in potency of the oestrogen metabolite 17-epioestriol in down-regulating VCAM-1 expression compared with 17β-oestradiol [75].

Progestins also differ in their actions and potencies in endothelial cells. For example, progesterone, albeit at supraphysiological concentrations, inhibits endothelial expression of VCAM-1, whereas MPA (medroxyprogesterone acetate) does not [79]. When tested in female HUVECs, using physiological concentrations of progestins, progesterone had no effect on ICAM-1 or VCAM-1 expression, but MPA and two other progestins increased the cell-surface expression of both adhesion molecules [63]. More informative are studies of progestins in combination with oestrogen. Progesterone augments the oestrogen-mediated inhibition of ICAM-1 and VCAM-1 in human iliac artery endothelial cells, although concentrations of oestrogen in these studies were supraphysiological [80]. Proliferation of human dermal and coronary endothelial cells is inhibited by progesterone in the absence of oestrogen [64]. Furthermore, progesterone inhibits re-endothelialization of injured aortae from wild-type, but not PR-deficient, mice [64]. The effects of progestins on oestrogen-mediated proliferation of endothelium remain to be clarified in non-reproductive vascular beds.

Androgens demonstrate sex-specific endothelial responses. For example, testosterone induces proliferation of male, but not female, rat lung endothelial cells [81]. Further evidence of was provided by Celermajer and co-workers. [67,82], who demonstrated that DHT up-regulated IL (interleukin)-1β-induced cell-surface expression of VCAM-1 in cells derived from male, but not female, HUVECs. The VCAM-1 induction was AR-dependent and DHT treatment decreased the level of the NF-κB inhibitory protein. Conversely, Norata et al. [66] have shown that DHT inhibits the TNF-α (tumour necrosis factor-α)- and LPS (lipopolysaccharide)-mediated induction of mRNA for VCAM-1, ICAM-1, IL-6, MCP-1, GRO (growth-related oncogene), GM-CSF (granulocyte/macrophage colony-stimulating factor), PAI-1 and COX-2 (cyclo-oxygenase-2) in HUVECs, and that the inhibition was partially AR-dependent [66]. Transfection studies revealed that the DHT-mediated inhibition occurred through blocking of NF-κB action [66]. Testosterone also inhibits VCAM-1 expression in an AR-dependent manner by blocking NF-κB binding to DNA in HAECs [65]. Finally, Mukherjee et al. [68] have demonstrated that testosterone inhibition of VCAM-1 expression in HUVECs can be reversed by an aromatase inhibitor, suggesting that the androgen effect occurred through conversion of testosterone into oestrogen. It is difficult to reconcile the disparate results of these studies. DHT cannot be converted into oestrogen, thus the inhibitory effects of DHT on inflammatory cytokine expression cannot be explained by conversion into oestrogen. Furthermore, the observation that AR expression differs quantitatively between male- and female-derived HUVECs suggests that using mixed-gender HUVEC preparations may complicate data interpretation regarding androgen actions.

## Smooth muscle cells

ERα and ERβ have been identified in VSMCs (vascular smooth muscle cells) in the mouse [83] and in humans [63]. Interestingly, the two ERs are expressed in different ratios in VSMCs of different arteries and this is reflected in differential oestrogen-mediated expression of iNOS [inducible NOS (NO synthase)]: ERβ enhanced and ERα inhibited iNOS [84]. The GPER has been identified in cultured VSMCs [85], but not in VSMCs *in situ* in mice [61]. PRs have been detected in human, rat and mouse aortic VSMCs [86–88]. Although PR-B was equally represented in VSMCs from males in pre- and post-menopausal women, PR-A was expressed to a significantly greater extent in VSMCs, with the highest expression found in post-menopausal women [88]. ARs and 5α-reductase have been demonstrated in rat aortic VSMCs, suggesting that testosterone may be converted locally into DHT [89]. Finally, aromatase has been identified in mouse aortic VSMCs, raising the possibility that androgens may affect VSMC metabolism following conversion into oestrogen [90].

The most profound influence of oestrogen on VSMCs in the context of atherosclerosis is the inhibition of VSMC proliferation following vascular injury. This inhibitory action of oestrogen occurs in ERα-deficient, ERβ-deficient and ERα/ERβ-double-knockout mice [91–93] giving rise to the intriguing possibility that a third receptor may be in play. However, following

vascular injury there is an increase in ERβ expression in VSMCs, most dramatically in the neointima [83], suggesting a role for ERβ in vascular healing that must be independent of the anti-proliferative effects of oestrogen. The divergence of the oestrogen effects on proliferation in endothelial cells (increased proliferation) compared with smooth muscle cells (inhibited proliferation) may depend upon divergent transcriptional regulation of the proliferative mediators IGF-1 (insulin-like growth factor-1) and COX-2 in the two cell types [94]. ET-1 (endothelin-1) also inhibits VSMC proliferation, but only in females and only in the presence of oestrogen [95]. Oestrogen also reduces oxidant stress in cultured rat aortic VSMCs as measured by the production of ROS (reactive oxygen species). This antioxidant action derives, in part, from inhibition of NADPH subunit expression and, in part, from up-regulation of antioxidant enzymes, specifically ecSOD and MnSOD (extracellular and manganese superoxide dismutase) [96,97].

A consistent *in vitro* finding across species is the inhibition of VSMC proliferation by progesterone and progestogenic compounds. This decrease in proliferation is accompanied by decreases in DNA synthesis, cyclin A and E mRNA, and MAPK (mitogen-activated protein kinase) activity, and is blocked by RU486, a PR antagonist [86,93,98]. Surprisingly, Karas et al. [99] found that progesterone worsened the vascular response to injury in mice. This finding suggests that the inhibition of endothelial proliferation by progesterone overrides the progesterone-mediated inhibition of VSMC proliferation in the *in vivo* setting. In ovariectomized rabbits, progesterone reversed the oestrogen-mediated inhibition of VSMC proliferation accompanying atherogenesis [100]. This effect of progesterone on VSMC proliferation may also be secondary to the effects of progestin on the endothelium. Progesterone has been shown to up-regulate the $AT_1$ receptor (angiotensin II type 1 receptor), whereas oestrogen reduces receptor expression [101]. Thus the role of the $AT_1$ receptor in cell proliferation may contribute to the actions of progesterone *in vivo*. Progesterone also promotes the production of ROS in vascular VSMCs and antagonizes the oestrogen-mediated antioxidant protection of VSMCs [97]. In summary, although progesterone appears to be protective in cultured VSMCs, *in vivo* studies suggest that progesterone opposes the protective effects of oestrogen on VSMCs.

Although oestrogens and progestins inhibit VSMC proliferation, most studies indicate that androgens promote VSMC proliferation, and DHT induces proliferation to a much greater extent than testosterone [89,102]. However, testosterone has been shown in one system, coronary smooth muscle cells from castrated pigs, to be anti-proliferative through a mechanism independent of the AR [103]. By contrast, the adrenal androgen DHEA (dehydroepiandrosterone) inhibits VSMC proliferation and this action does not involve the ERs or AR [102,104]. The AR is expressed to a greater extent in male rat aortic VSMCs than in female VSMCs, and male VSMCs are more responsive than female VSMCs to androgens, as measured by $TXA_2$ (thromboxane $A_2$) receptor density [105].

## Macrophages

Both human monocytes and macrophages express ERα and ERβ. ERα is the predominant receptor in macrophages and is up-regulated by oestrogen in macrophages but not monocytes, whereas ERβ is the predominant receptor in monocytes and is unaltered by oestrogen in either cell type [106]. A functional splice variant of ERα is expressed prominently in human macrophages as well [106]. In mice, only ERα appears to be expressed in macrophages [107]. PRs are not present in circulating human monocytes under any conditions [108]. GPER is highly expressed in human macrophages [109]; however, both human and mouse macrophages express PRs [107,110]. AR expression in human monocytes is higher in young males than females [111], but this sex-specific difference in expression disappears with age, suggesting that the menopause increases AR expression [112]. However, AR levels do not differ between differentiated human macrophages from pre-menopausal or post-menopausal women and, in both cases, receptor expression is lower than that in male-derived cells [113]. ARs were not found by gene analysis in bone-marrow-derived mouse macrophages [107]; however, functional studies and immunohistochemical analyses suggest the presence of ARs in mouse macrophages [114,115].

Two components of macrophage metabolism are especially critical in the context of atherosclerosis: their inflammatory/immune actions and their ability to form 'foam cells' by engorging with lipid. Steroid hormones affect both the functions and the roles of the sex steroids in macrophage inflammation are clearly complex, as detailed below. As in other vascular cell types, oestrogen has anti-inflammatory effects on macrophages. Oestrogen inhibits the LPS-induced mouse homologue of MCP-1 (JE) in peritoneal macrophages [116] as well as IL-1, IL-6 and TNF-α in splenic macrophages [117]. These anti-inflammatory actions are thought to occur through oestrogen-mediated disruption of the NF-κB signalling pathway [118]. Recent evidence suggests that activation of GPER participates in the down-regulation of TNF-α and IL-6 in human macrophages [109].The effects of progestins on inflammatory mediators are less clear. Huang et al. [119] demonstrated a receptor-mediated opposing effect of progesterone on TNF-α, IL-1β and JE/MCP-1 in mouse macrophages, but did not use the steroids in combination. However, two groups have shown that testosterone up-regulates TNF-α in macrophages from male mice [114,115]. Using ARKO (AR-deficient) mice, Lai et al. [115]

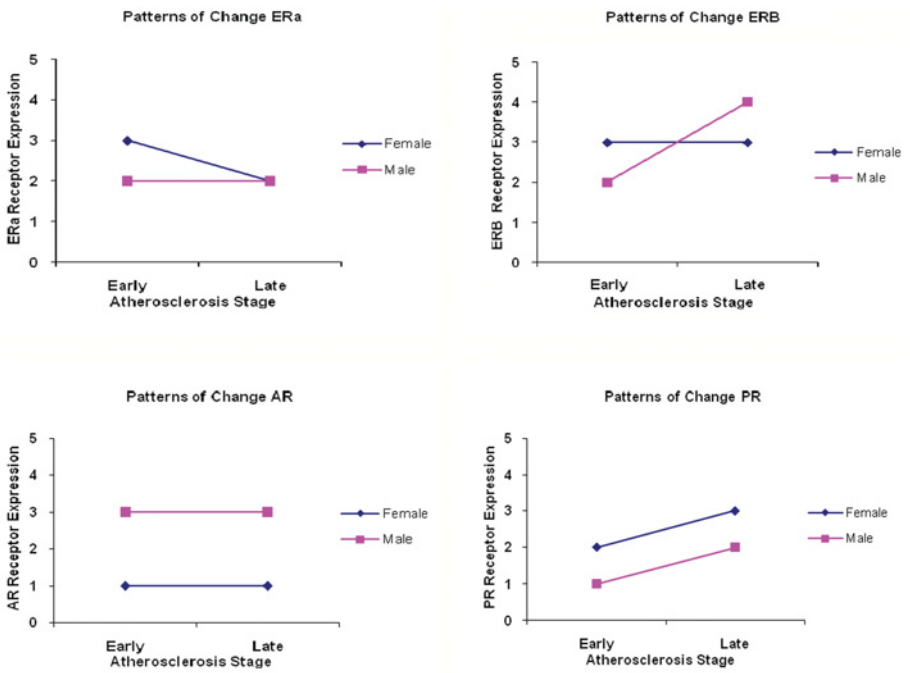


**Figure 2 Pattern of change in hormone receptor expression with development of atherosclerosis in male and female animals**

The pattern of change for the ER$\alpha$, ER$\beta$, ARs and PRs are demonstrated for mouse models of early- and late-stage atherosclerosis in male and female animals (sources of the results are as listed in Table 2).

demonstrated that androgens increase the inflammatory monocyte population, increase monocyte chemotaxis [through enhanced CCR2 (CC chemokine receptor 2) expression] and increase macrophage TNF-$\alpha$ expression [115]. Owing to sex-specific differences in macrophage AR expression, it remains of interest to examine the effects of androgens on inflammatory mediators in female macrophages.

Oestrogen has been shown to increase and testosterone to decrease TLR4 (Toll-like receptor 4) expression on mouse macrophages [125,126]. TLR4 is a cell-surface receptor that triggers two different signalling pathways, one of which activates NF-$\kappa$B, thereby inducing inflammatory genes. Thus one would expect oestrogen, which inhibits NF-$\kappa$B-dependent inflammatory mediators, to down-regulate TLR4 and testosterone to do the opposite.

The sex steroids also have significant effects on macrophage lipid metabolism. Progesterone inhibits cholesterol esterification in many cell types, in part by blocking transport of cholesterol to ACAT (acyl-CoA:cholesterol acyltransferase), the enzyme responsible for esterification [127]. Oestrogen also appears to reduce cholesterol esterification through different mechanisms: oestrogen decreases the expression of CD36, one of the receptors responsible for LDL uptake [128], and enhances HDL-mediated cholesterol efflux from macrophages [129]. In human macrophages, oestrogen- and progesterone-mediated inhibition of cholesterol esterification and, by extension, foam cell formation, occurs only in female-derived macrophages [82]. By contrast, androgens enhance cholesterol esterification only in male-derived macrophages (reviewed in [130]).

In considering the anti-inflammatory and anti-atherogenic effects of oestrogen, questions arise as to the potential protective actions of post-menopausal oestrogen replacement. It has been suggested that oestrogen may fail to protect in older women due to decreased ERs in the vessel wall. Furthermore, as atherosclerosis develops and the endothelium is disrupted, endothelial ERs are no longer present in the denuded atherosclerotic area. The findings in Figure 2 demonstrate the pattern of sex steroid receptor expression in atherosclerotic lesions in a mouse model. However, in the case of the ER, inflammation itself may also diminish ER-mediated actions due to reciprocal antagonism between NF-$\kappa$B and the ER [131,132].

## CURRENT CONCEPTS

This section will explore several key current concepts in the field, including (i) total hormone exposure time and CAD risk; (ii) the importance of tissue specificity of sex steroid hormones, critical timing and the stage of

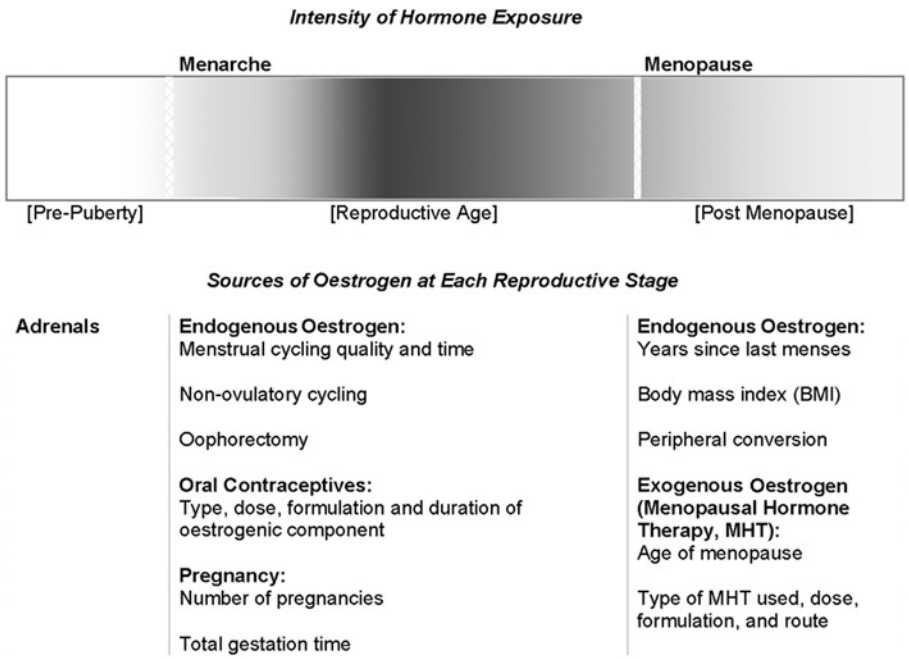


**Figure 3 Total oestrogen exposure time**

The intensity of hormone exposure from pre-puberty to reproductive age to post-menopause is shown schematically in the upper portion of the Figure. The corresponding potential sources of hormones and hormone level modulators at each reproductive stage are listed in the lower portion of the Figure.

atherosclerosis; (iii) biomarkers for atherosclerosis as they relate to hormone therapy; and (iv) sex steroids and inflammation.

## Total hormone exposure time

During her lifespan, a woman is exposed to varying types and levels of hormones. An emerging concept in the field links total hormone exposure time to the development of CAD [133]. In the pre-pubertal period, oestrogen is derived primarily from adrenal production and thus sex steroid production and levels are very low until the time of menarche. Subsequent to that, during the reproductive years, a woman may be exposed to hormones from a number of sources, including endogenous ovarian oestrogens, contraceptive hormones and hormonal fluctuations associated with pregnancy. During the menopause, transition hormone levels decline and, once in menopause, endogenous oestrogen levels fall dramatically. Variations in menopausal oestrogen levels can be observed and are due to residual ovarian oestrogenic activity as well as peripheral conversion; the latter can be affected by BMI (body mass index). Exogenous oestrogen use during menopause will also contribute to oestrogen exposure in the post-menopausal period. Thus total oestrogen exposure across the lifespan will vary from woman to woman and be dependent on the factors that contribute to hormone exposure from pre-menarche to the post-menopausal period. The continuum of hormone exposure is summarized in Figure 3.

The impact of oestrogen exposure time on the development of CAD is not yet well defined, and oestrogen atheroprotection in women is clearly more complex than time of vascular exposure to oestrogen alone. Specifically, when considering hormone exposure time up to and including reproductive age, the higher the number of years of sustained oestrogen exposure, the lower the prevalence/severity of obstructive CAD [133]. However, when considering reproductive age and post-menopausal hormone exposure time together, no anti-atherosclerotic association (angiographic CAD and adverse cardiovascular events) is observed with total oestrogen exposure duration [133]. This suggests that other factors (e.g. ER status, vascular disease stage etc.) are at play and contributing to the clinical complexity observed in the relationship of hormone exposure time and CAD.

## Hormone therapy: impact of tissue specificity, stage of atherosclerosis and critical timing

It is becoming increasingly clear that the effects of oestrogens on the blood vessel wall depend upon the extent and complexity of atherosclerotic disease present at the time hormone therapy is initiated. Potential mechanisms for atheroprotection include: (i) oestrogen, specifically 17$\beta$-oestradiol, itself; (ii) the ER; and/or (iii) serum lipids and inflammatory biomarkers. As atherosclerosis

**Table 2 Hormone receptor expression in early- compared with late-stage atherosclerosis in males and females**
Expression levels: low (+) and high (++++)

| | Receptor expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Early-stage atherosclerosis | | | | Late-stage atherosclerosis | | | |
| Receptor subtype | Female | Reference(s) | Male | Reference(s) | Female | Reference(s) | Male | Reference(s) |
| ER$\alpha$ | +++ | [121,145] | ++ | [121] | ++ | [121] | ++ | [120,145] |
| ER$\beta$ | +++ | [121,145] | ++ | [121] | +++ | [121] | ++++ | [120,145] |
| AR | + | [113] | +++ | [113] | + | [113] | +++ | [100,113,120] |
| PR | ++ | [88,147] | + | [88,147] | +++ | [88,147] | ++ | [88,147] |

evolves, the early vascular protective mechanisms of oestrogen, detailed above, recede and are replaced by oestrogen responses that are thought to be deleterious [134–136] (e.g. decreases in ER function/expression, less vasodilation, greater inflammatory activation and enhanced plaque instability).

ER$\alpha$ has been demonstrated to be a major mediator of the atheroprotective effects of oestrogen in transgenic murine models of spontaneous advanced atherosclerosis having targeted inactivation of apoE, LDLR and both the apoE and ER$\alpha$ genes [137–140]. Atheroprotection in these models occurs via reductions in lesion plaque size and lesion maturation. However, the inhibitory effects of oestrogen on atherogenesis appear to be lost once atherosclerotic lesions are established [141]. Interestingly, in a model of early-stage atherosclerosis, oestradiol inhibits and delays the development of early atherosclerotic lesions, fatty streaks, by ER$\alpha$-independent mechanisms [142].

Furthermore, in early-stage disease, both endogenous and exogenous oestrogen is effective in attenuating aortic lesion development and progression in a concentration-dependent manner [142]. ER$\beta$ does not appear to be involved in the development of early-stage atherosclerosis [143]. However, HSP27 (heat-shock protein 27), an ER$\beta$-associated protein, was demonstrated to have attenuated expression in coronary atherosclerosis and to modulate oestrogen signalling in minimally diseased arteries [144]. In pre- and post-menopausal women, ER$\beta$ is correlated with coronary calcification and thus more advanced atherosclerosis [145], suggesting a role for this ER subtype in more advanced vascular disease. Lastly, ER$\alpha$- or ER$\beta$-independent inhibition of cell growth has been demonstrated for catechol oestrogens [146] (oestrogens are converted into catechol oestrogens via an oxidation step), indicating that the ERs may not be necessary for all oestrogen actions. Thus it is becoming increasingly clear that the pleiotropic effects of hormone therapy on the vascular system and cells differ, depending not only on hormone and lipid status, but also, importantly, on the stage of atherosclerosis in the underlying blood vessel.

A summary of vascular hormone expression, and how it is modified by early- and late-stage atherosclerosis in males and female animal models, is provided in Table 2 and Figure 2. ER$\alpha$ and ER$\beta$ expression levels are higher in females compared with males, but in females ER$\alpha$ expression declines, whereas ER$\beta$ expression is unchanged, with advancing atherosclerosis. AR levels are lower, and PR levels higher, in females compared with males. With advancing atherosclerosis, in both males and females there is no change in AR expression, whereas there is an increase in PR expression.

The studies described above contribute to a better understanding of ER$\alpha$-dependent and -independent mechanisms of oestrogen atheroprotection, linked to an emerging concept coupling oestrogen action to the timing of its initiation in relationship to critical periods in the stages of atherogenesis. The so-called critical timing hypothesis [148] postulates that there is a critical time in the development of CAD when the introduction of oestrogen is atheroprotective, following which there is greater harm than good due to nascent underlying clinical or subclinical CAD. This hypothesis has been proposed as a unifying hypothesis for explaining the discrepant findings of the observational, clinical, animal and human findings regarding menopausal hormone therapy. This knowledge is potentially very important, as one of the most critical areas of new focus in this field is to understand the molecular and phenotypic interface of oestrogens with their receptors in the vascular wall with regard to the stage(s) of atherosclerosis. A better understanding of this system may lead to a better understanding of how to optimize, and when to target, hormone therapies for CVD protection in women.

## Biomarkers for atherosclerosis: microparticles and implications for hormone therapy

A considerable number of subjects develop atherosclerosis even if they have normal levels of lipids and lipoprotein concentrations, suggesting that other as yet unknown factors contribute in the disease process. Therefore a

challenge for the research and clinical communities is to determine, define and validate the use of a specific independent indicator for early asymptomatic CVD. Because atherosclerosis is a progressive disease that involves multiple cell types and biochemical processes (hypercholesterolaemia, lipid peroxidation, damage to the endothelial lining, accumulation of activated inflammatory cells and platelets in the vascular wall, and formation of fatty streaks), cell-specific markers of activation may be useful in the identification of early pathophysiological processes.

In clinical practice, biomarkers are used in the diagnosis of suspected disease, prognosis, management of established disease and assessment of effectiveness of therapy. The term 'biomarker' is used to define a specific measurable indicator of normal or pathophysiological processes. A variety of assessment methods are used to identify biomarkers, and some common biophysical and anatomical methods used in cardiovascular medicine include the ECG, measurement of vascular anatomy by ultrasound imaging of carotid intima-medial thickness, and coronary arterial plaque volume by intravascular ultrasound, quantitative angiography or computer tomography. However, soluble plasma proteins, lipids or other soluble molecules associated with particular cellular processes, such as activation or apoptosis, have been evaluated as biomarkers of cardiovascular risk, disease progression and associated cardiovascular events [149–159]. Regardless of the method of assessment of the signal (physical measurement or biochemical composition or activity), to be useful a biomarker must be specific, i.e. it must accurately assess one condition or process to the exclusion of others. Furthermore, to be useful in a clinical setting, the test to assess the biomarker should also be sensitive and specific, reproducible, cost-effective, generally available and easy to perform.

Soluble blood-borne molecules or substances evaluated as biomarkers of CVD risk and progression fall into four general categories, as detailed in Table 3. Of these, cytokines associated with inflammation and proteins associated with the coagulation cascade fail the specificity test for a biomarker as concentrations of these substances can change with infection, trauma, cancer, thrombosis and other diseases which may be related either indirectly or directly to CVD [160,161]. Furthermore, use of most soluble protein biomarkers is limited because their clearance rates and source cannot be identified.

During cellular activation, cell–cell interactions and the process of programmed cell death termed apoptosis (that can be induced by oxidative damage, inflammatory cytokines and chemokines, and shear stress) result in the shedding of sealed plasma-membrane vesicles of $<1\ \mu$m in diameter into the circulation. These sealed membrane vesicles have been called 'microparticles' or 'microvesicles'. The term 'microvesicle' more accurately describes the membrane-sealed vesicles because the term

**Table 3 Biomarkers of cardiovascular disease**

| Category of biomarkers | Examples |
|---|---|
| Lipids and lipoproteins | Lipids: total cholesterol, triacylglycerols (triglycerides), LDL and HDL, and oxidized LDL |
| | Lp-$PLA_2$ (lipoprotein-associated phospholipase $A_2$) |
| | ApoB/ApoA1 ratio |
| Inflammation-associated secretory cytokines and proteins | Interleukins: IL-1, IL-3, IL-6 and IL-18 |
| | TNF-$\alpha$ |
| | MCP-1 |
| | hsCRP (high-sensitive CRP) |
| | ICAM-1 |
| | VCAM-1 |
| Proteins associated with coagulation/thrombosis | Fibrinogen |
| | Thrombin–antithrombin complex |
| | Von Willebrand factor |
| | D-dimer |
| Activated cell-derived peptide/proteins and enzymes | BNP (brain-type natriuretic peptide) |
| | Troponin |
| | Myeloperoxidase |

'microparticles' also can be applied to aggregates of proteins/lipids and inorganic molecules or manufactured synthetic materials, such as albumin-coated polystyrene spheres [162–165]. Microvesicles are biochemically active and potentially important in the pathophysiology of CVD, coronary artery syndromes, aortic aneurysm, arterial and venous thrombosis, pulmonary embolism, systemic lupus erythematosus and antiphospholipid syndrome [166–174]. Microvesicles also participate in the transport of specific cellular signalling molecules from the parent cell to other cells which, in turn, alter the biological activity of recipient cells initiating inflammation and vascular dysfunction. For example, platelet-derived microvesicles transfer the chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) and platelet-specific immunoreactive antigens to the surface of endothelial cells [175], and endothelial progenitor cell-derived microvesicles transfer mRNA content into endothelial cells [176]. Furthermore, exposure of isolated arteries *in vitro* to microvesicles from patients diagnosed with acute coronary syndrome, end-stage renal failure or pre-eclampsia induces endothelial dysfunction compared with microvesicles from healthy subjects [177–179].

Development and progression of atherosclerosis are also associated with increased apoptosis of cells present in the lesion [180,181]. The composition of microvesicles released from apoptotic cells was different

from microvesicles shed from activated cells [182,183]. Apoptotic microvesicles have also been identified in atherosclerotic plaques by electron microscopy [184]. Upon plaque rupture, apoptotic microvesicles released into the circulation could promote atherothrombosis by providing procoagulant surface phosphatidylserine and tissue factor [185]. Indeed, the concentration of microvesicles was found to be 200-fold higher in atherosclerotic plaques than in plasma [186].

However, because the composition of microvesicles and their counts in the circulation depend upon their cell of origin and the processes that trigger their production, populations of circulating microvesicles may reflect early as well as late CVD processes. In other words, they may provide more sensitive and specific biomarkers to diagnose asymptomatic CVD processes. For example, in asymptomatic newly menopausal women whose Framingham risk scores were low (less than the 10th percentile for 10-year event risk), counts of circulating endothelium- and platelet-derived microvesicles and their thrombogenic capacity were significantly greater in women who had CAC (coronary artery calcium) scores of 93–315 Agatston units compared with age-matched women with negative and low CAC (Agatston scores 0 and <35) [187]. Of note, women with high CAC could not be distinguished from the other groups based on traditional risk factors for CVD, including BMI, blood cholesterol, lipoproteins (LDL and HDL), triacylglycerols (triglycerides) and glucose [187]. Therefore these results suggest that defined populations of microvesicles may represent potential mechanisms to identify women with vascular lesions, in this case CAC, who would not be identified by traditional screening tools. Furthermore, they suggest that factors other than elevated blood lipids may contribute to accelerated arterial calcification in some women.

The contribution of sex steroids, in particular oestrogen, to cellular interactions leading to the formation of microvesicles remains to be explored in detail as total counts of circulating microvesicles, those derived from platelets, monocytes and vascular endothelium, and those positive for phosphatidylserine and tissue factor are significantly greater in newly menopausal women with circulating oestrogen below 20 pg/ml, compared with age-matched women with oestrogen >40 pg/ml [188]. These results demonstrate that the procoagulant characteristics of the microvesicle pool increased with a decrease in endogenous oestradiol. Whether numbers and characteristics of microvesicles would change with hormone treatment in these women remains to be determined, but could represent yet another mechanism by which oestrogenic treatments could reduce progression of atherosclerosis. Recent studies also suggest that loss of ER$\beta$ may be associated with increased thrombogenicity of platelets, as the numbers of thrombogenic microvesicles are greater in ER$\beta$-knockout mice than in age-matched wild-type female mice [189].

Since atherosclerosis progresses over the lifespan, age- and sex-specific biomarkers of vascular pathophysiological processes are warranted. New biomarkers should be identified in asymptomatic male and female populations for early disease. If microvesicles are to be utilized as such, then the range in number and cellular origin needs to be established for males and females by decade of life, in health and disease, and from childhood to centenarians. Any new biomarker for an early CVD process should be specific, sensitive, cost-effective, stable, reliable, validated and easily interpreted by medical professionals. Blood-borne microvesicles could serve as sensitive and specific independent biomarkers to diagnose early (asymptomatic/subclinical disease) and late (symptomatic disease) cardiovascular pathology and provide prognostic assessment and management of individuals with suspected cardiovascular syndromes, disease and/or events. However, standardized, validated and cost-effective methodology must also be developed in order for this set of biomarkers to reach clinical utility.

As indicated by studies of inflammatory mediators in the endothelium and oestrogen itself, there is tissue specificity in oestrogen actions. Further complexity from both a metabolic and a therapeutic perspective derives from the fact that not all oestrogens are equivalent in their actions, in part because of differing affinities for ER$\alpha$ and ER$\beta$. This concept is important in the context of development of SERMs (selective ER modulators) for clinical use. SERMS (e.g. tamoxifen, raloxifene, droloxifene and the phyto-oestrogens) represent an important class of non-hormonal agents. Their activity on lipid metabolism and vascular tissue, coupled with their general oestrogen antagonist effects on breast and uterus, have been described [190–192] and reviewed [193]. However, although they are attractive candidates for clinical use and study, SERMs and phyto-oestrogens are not presently approved for clinical use in CVD management or prevention. Members of the progestin and androgen classes also differ in their actions. Furthermore, as observed in all cell types, there are sex-specific differences in response to sex steroids. Finally, there is cross-talk between the sex steroid receptor pathways and other signalling pathways, such as NF-$\kappa$B. Each of these factors must be considered when designing and analysing studies of sex steroids and atherosclerosis.

## Sex steroids and inflammation

Sex steroids have immunomodulating actions; however, their role in inflammation is complex. Current concepts relate to steroid-mediated cell- and receptor-specific pro-inflammatory and pro-oxidant vascular pathways (e.g. endothelial NO in leucocyte–endothelial interactions and oxidative reactions), and differential effects of key steroid metabolites.

Vascular inflammation resulting from mechanical or biochemical tissue injury, accompanied by ROS and/or invading pathogens, are initial events leading to the development and progression of CVD. Sex differences in the occurrence of inflammatory immunological diseases, such as rheumatoid arthritis and systemic lupus erythematosus, and the modulation of their symptoms associated with puberty, pregnancy and menopause suggest that oestrogen may be pro-inflammatory or modulate B-cell and T-cell immunity [27,194,195]. However, experimental evidence to support the concept that oestrogen itself is pro-inflammatory is controversial.

Results of studies designed to investigate the direct inflammatory nature of oestrogen suggest that physiological levels of oestrogen, and oestrogen levels attained during pregnancy and with hormone therapy inhibit secretion of pro-inflammatory cytokines [TNF-$\alpha$, IL-1$\beta$, IL-6, MCP-1, iNOS and MMPs (metalloproteinases)] and stimulate synthesis and secretion of anti-inflammatory cytokines [IL-4, IL-10, TGF-$\beta$ (transforming growth factor-$\beta$), TIMP (tissue inhibitor of metalloproteinases) and osteoprotegerin]. These effects are mediated predominantly through ER$\alpha$ signalling mechanisms in otherwise unstimulated cells [27,196]. In contrast, and in further support of the anti-inflammatory effects of oestrogen, are findings demonstrating that, with decreases in concentrations of oestrogens at menopause, secretion of pro-inflammatory cytokines increases and there is increased expression of cellular surface adhesion molecules (E-selectin, P-selectin, VCAM-1 and ICAM-1). These responses are reversed when oestrogen levels are restored to those of pre-menopause [69,197].

Oestradiol also inhibits cellular responses to LPS-induced inflammation via ER$\alpha$-mediated inhibition of NF-$\kappa$B translocation [198–201]. However, and in contrast, chronic administration of oestradiol (80 $\mu$g $\cdot$ kg$^{-1}$ of body weight $\cdot$ day$^{-1}$ for 60 days *in vivo* to ovariectomized mice) stimulates secretion of pro-inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6 and IL-12p40) and increases expression of iNOS through ER$\alpha$ [202]. Oestradiol has also been demonstrated to augment secretion of pro-inflammatory cytokine secretion from peritoneal macrophages activated by LPS through ER$\alpha$-mediated down-regulation of PI3K (phosphoinositide 3-kinase) activity and Akt phosphorylation [202]. These conflicting results point to the need for a better understanding of how oestrogen directly and indirectly modulates the production of cytokines in response to various immunologically related stimuli, as well as differences between acute and chronic responses to oestrogen (and other sex steroids). Furthermore, general statements as to whether oestrogen is pro- or anti-inflammatory must be evaluated with caution as consideration needs to be given to sex and age, the concentration and route of administration of the hormone, the cell type studied, and the magnitude of direct stimulation of other inflammatory pathways (such as TLR4) that might also amplify immune responses.

There is general agreement that oestrogen increases the synthesis and activity of NOS through genomic and non-genomic actions respectively [197]. However, there is no consensus as to how oestrogen modulates other forms of NOS in the presence of pro-inflammatory molecules. Furthermore, clarification is needed as to the contribution of the specific subtype of ERs in these processes. Metabolites of oestrogen also participate in pro- and anti-inflammatory processes. For example, 2-methoxyoestradiol can attenuate inflammation, whereas 4-hydroxyoestradiol stimulates pro-inflammatory processes by inducing ROS and DNA damage [27,69]. Oestrogen also may affect the oxidative status of cells through modulation of mitochondrial enzymes that scavenge oxygen-derived free radicals [69]. Secretion of mitochondrial damage-associated molecular products also induce an inflammatory response to injury [203], although the mechanism(s) whereby these products are modulated by oestrogen remains to be clarified.

Rapid non-genomic signalling of oestrogen is now established as a mechanism by which oestrogen affects cell function. These effects may be mediated either through membrane-associated full-length ER$\alpha$ or the recently identified GPER. Although several studies have identified the expression and signalling mechanism of GPER in cell proliferation, apoptosis, glucose haemostasis, immune responses, and in the neurological, reproductive and cardiovascular systems, a number of studies have described fundamental controversies regarding GPER expression and its specific cellular function, oestrogen binding and signalling in the same cell types [204–208]. Not addressed in this section are findings with GPER in cardiovascular pathophysiology, as they are included in our earlier discussion of the role of classical ERs (ER$\alpha$ and $\beta$), as well as PRs and ARs.

## CONCLUSION AND FUTURE DIRECTIONS

Over the past decade, landmark clinical trials have generated important results that have informed current guidelines regarding menopausal hormone therapy, thereby dramatically affecting current patterns of hormone use for millions of women. At the same time, controversies have arisen regarding the findings of such studies, and new conceptual frameworks postulated for providing unifying explanations designed to integrate all the available data. Considerable progress has been made in both our understanding of the complexities governing menopausal hormone therapy, and our understanding of cellular and molecular mechanisms underlying hormone and hormone receptor function. In addition, we now recognize that hormone receptor expression changes according to the stage of vascular

**Table 4 Future directions in atherosclerosis and sex hormone research**

| Area | |
|---|---|
| Hormone therapy | Validate critical timing hypothesis |
| | Identify ideal steroids for treatment |
| | Identify optimal routes of administration, dose and formulation |
| Hormones/receptors and inflammation | Understand mechanisms of cytokine production |
| | Discern acute and chronic responses |
| | Validate inflammatory markers as hormone-responsive biomarkers of cardiovascular risk |
| | Identify therapeutic targets for GPER |
| | Understand contribution of ER subtypes to inflammation |
| Microvesicle biomarkers | Validate specific microvesicles as biomarkers |
| | Determine influence of exogenous oestrogen and progestin |
| | Determine age- and sex-specific profiles |

disease. Importantly, in early-stage atherosclerosis, the mechanism of atheroprotection is oestradiol-mediated and independent of the extent of hyperlipidaemia, a reduction in serum lipids, or the presence or absence of the ER$\alpha$, whereas this receptor is integral to atheroprotection in advanced atherosclerotic lesions. Recognition of the importance of receptor status and the stage of atherosclerosis with regard to hormone action has guided the design of new ongoing clinical and animal studies and will in turn be critically important to consider in the interpretation of such studies.

Given the prescient biological importance of understanding CVD and its interaction with hormonal influences, clinicians and basic scientists should stay 'tuned' for new developments in this field, particularly as they relate to current concepts regarding total hormone exposure time, critical timing of menopausal hormone therapy, the characterization and testing of emerging biomarkers for CVD and hormone responsiveness, and anti- and pro-inflammatory actions of oestrogen and other hormones.

Future studies are needed to help guide the evolution of treatment recommendations for women, and to contribute to setting research priorities for clinical, translational and basic scientists in the area of hormone therapy, hormones and receptors, microvesicles and biomarkers, and hormones and inflammation, as summarized in Table 4 and below:

This includes, but is not limited to, further research into: (i) the basic mechanisms by which inflammation and hormones interact in vascular disease; (ii) mechanisms underlying direct and indirect oestrogen-mediated cytokine production in response to immunological stimuli; (iii) explanations for differences between acute and chronic responses to oestrogen and other sex steroids; (iv) our understanding of the association/causal relationship of inflammatory markers and risk factors as markers in disease, and how they may change with changes in hormonal status; (v) the feasibility of transitioning known oestrogen-responsive cardiovascular risk markers from the laboratory to the clinical setting; (vi) creating personalized risk profiles with greater sensitivity and/or specificity for CVD than currently possible; and (vii) additional randomized clinical trials to create consensus and clarify strategies for post-menopausal hormone therapy. Furthermore, the discovery of GPER as an additional ER and development of a highly specific GPER agonist, G-1, adds to the arsenal of promising potential therapeutic targets and compounds to delineate further vascular hormone–receptor interactions in the vasculature, and will provide a fruitful area for future investigation.

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