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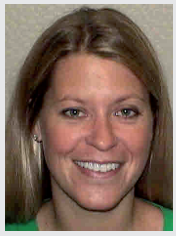

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"…there are substantial sex differences in the rate of cardiovascular events, such as myocardial infarction, stroke and venous thromboembolism, supporting the notion that sex is an important variable in cardiovascular disease risk."

The influence of sex on cardiovascular biology is gaining recognition as differences between the sexes – from the basic anatomic level to physiologic response – become apparent [1–4]. Men and women are also thought to significantly differ at the cellular and molecular levels, with sex differences reported in platelet function and coagulation factor activities [5–10]. In addition, there are substantial sex differences in the rate of cardiovascular events, such as myocardial infarction, stroke and venous thromboembolism (VTE), supporting the notion that sex is an important variable in cardiovascular disease risk [11–13].

"Considering the prevalence of thrombotic diseases, an improved understanding of how sex functions as a disease modifier is highly desirable."

Venous thromboembolism, which includes deep vein thrombosis and pulmonary embolism, affects one in every 1000 people annually, accounting for approximately 250,000 cases in the USA per year [14]. Risk factors for VTE include decreased blood flow, altered blood components and/or modifications of the vessel wall [15]. Although the existence of sex differences for incident VTE remains controversial, men are 50% more likely to suffer recurrent VTE than women [12,13,16]. In addition, several clinical risk factors for VTE differ between the sexes, such as cancer and congestive heart failure, which have strong epidemiologic sex differences. Trauma is also an established risk factor for VTE and occurs more often in males than females. Some risk factors for thrombosis are unique to women, such as pregnancy, oral contraceptive use, hormone-replacement therapy and estrogen antagonist therapies [17]. Considering the prevalence of thrombotic diseases, an improved understanding of how sex functions as a disease modifier is highly desirable. Recently, our laboratory, as well as others, have reported sex differences in thrombosis in rodent models, thus providing the opportunity for mechanistic investigation of sex-dependent cardiovascular phenotypes in these systems [18–20]. It is expected that important sexually dimorphic characteristics will continue to emerge as the field gains greater appreciation for the influence of sex on cardiovascular disease; these insights will lead to improved approaches to thrombotic disorders in patients.

## Estrogen

The precise mechanisms underlying sex differences in thrombosis remain unknown; however, differences in cardiovascular risk have been broadly attributed to the distinct hormonal profiles of men and women. The actions of estrogen within the female cardiovascular system have long been implicated in the relative protection of premenopausal women from cardiovascular events. Evidence for this protective effect is demonstrated in the increased cardiovascular risk of females during the cessation of natural estradiol production occurring at menopause. Endogenous estrogens are also suggested to have a beneficial effect on the male cardiovascular system [21]. Estrogen positively influences cardiovascular biology by decreasing vascular tone, enhancing vasodilation and vascular compliance, imparting anti-inflammatory

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and anti-oxidant properties, and improving lipid profiles [22]. By contrast, large randomized controlled clinical trials designed to determine the effect of supplemental estrogens on cardiovascular disease have shown unequivocally that oral estrogen increases the incidence of VTE and other cardiovascular events [23–26]. However, the mechanism behind this increased risk is not yet understood. Currently, oral estrogen is thought to increase VTE risk through resistance to the anticoagulant protein activated protein C (APC). This so-called 'acquired APC-resistance' may occur through changes in the levels of free protein S, protein C inhibitor and/or other inhibitors of APC [27–29]. Oral estrogens have also been found to increase levels of prothrombin fragments 1 and 2, factor VII, factor X, factor XIII, fibrinogen and other procoagulant proteins [30]. While orally administered estrogen induces a procoagulant state and increases the risk of VTE, observational studies suggest that estrogen administered transdermally has little effect on hemostasis markers and VTE risk [31,32]. Thus far, these differences in thrombosis have been attributed to first-pass metabolic effects in the liver through either increased local hepatic estrogen concentrations or the generation of an unknown active metabolite.

"The actions of estrogen ... have long been implicated in the relative protection of premenopausal women from cardiovascular events."

The contradictory effects of estrogen in cardiovascular protection and cardiovascular risk may result from estrogen's diverse biological effects and complex signaling. Estrogen signaling occurs through both genomic and nongenomic mechanisms, involving estrogen receptor (ER)α and β [33]. ERα is predominantly expressed in the uterus, mammary gland, testis, pituitary, liver, kidney, heart and muscle, while ERβ is predominantly expressed in the ovary and prostate [34]. Estrogen's effects are often dependent on the relative contributions of ERα and ERβ, thus facilitating tissue-specific responses. Selective estrogen receptor modulators (SERMs) comprise a class of drugs designed to target the estrogen receptors, having both agonist and antagonist effects at the ERs. Two SERMs, tamoxifen and raloxifene, are currently approved for the treatment of breast cancer and osteoporosis, respectively. Both drugs demonstrate antagonist properties at ERβ, but have partial agonist activity at ERα as well [35]. Both tamoxifen and raloxifene have been shown to increase the risk of VTE by approximately twofold [35–41]. Considering that the biosynthesis of procoagulant and anticoagulant effectors primarily occurs in the liver, and ERα is the predominant estrogen receptor isoform in this tissue, it is presumed that the deleterious effects of estrogen on VTE are mediated through agonist activity at liver ERα. Thus, it is plausible that selective ERβ agonists may pose less risk of thrombotic events compared with current regimens. However, further defining estrogen's mechanism of action in biological tissues of interest will aid in our understanding of estrogen's role in cardiovascular disease and allow for improved drug design.

## Growth hormone

Growth hormone (GH) is secreted in a sexually dimorphic manner and the resultant sex-specific levels of circulating GH may play an important role in thrombosis. Male secretion of GH is characterized as pulsatile, with episodic bursts occurring every 2–3 h overlying basal continuous secretion and long interpulse intervals. By contrast, the female secretory pattern is characterized by more frequent pulses and short interpulse intervals, resulting in continuous detection of GH in plasma [42,43]. These sex-specific GH secretion patterns, in turn, affect cellular signaling and gene transcription. This effect is particularly well described in the liver, where GH has been shown to induce male- and female-specific liver gene expression [44]. As previously stated, the liver is the principal organ for the biosynthesis of pro- and anticoagulant proteins, suggesting a role for GH in the establishment of sex-dependent coagulation.

"... sex-specific levels of circulating growth hormone may play an important role in thrombosis."

In support of this notion, recent findings from our laboratory demonstrate GH-dependent sex differences in thrombosis. Under normal conditions, male mice have shorter clotting times and are more susceptible to thrombosis in an *in vivo* model of pulmonary embolism (PE) compared with females. In animals with near absolute GH-deficiency, sexual dimorphism is lost, resulting in similar PE susceptibility and *in vitro* clotting times in males and females. Furthermore, administration of the male-patterned pulsatile GH to GH-deficient female mice results in a male clotting phenotype. Conversely, administration of female-patterned continuous GH to GH-deficient male mice results in prolonged clotting time that is characteristic of females [45]. While the mechanism for GH's involvement in thrombosis has yet to be defined, we have found that sex-specific GH secretion can affect the expression of genes that encode major regulators of thrombin generation, such as protein C, anti-thrombin, heparin cofactor II and protein C inhibitor. These studies demonstrate a role for GH in thrombosis and, importantly, implicate GH secretion and signaling as a mediator of sex-specific thrombotic risk.

## Effects of estrogen on GH

While estrogen and GH have many independent actions, interactions between estrogen and GH signaling and secretion have also been described. Oral estrogen increases GH levels, primarily through its effect on IGF-1 production, mediating GH resistance [46,47]. IGF-1 is the principal feedback inhibitor of GH-releasing hormone (GHRH) secretion from the hypothalamus, and attenuation of IGF-1 expression by oral estrogen releases this basal suppression, resulting in increased GH secretion from the anterior pituitary gland [47–49]. Furthermore, GH-deficient women taking supplemental estrogen require substantially higher doses of recombinant GH to achieve target IGF-1 levels [50]. Interestingly, while oral estrogen mediates GH resistance, transdermal estrogen has not

been reported to affect GH signaling [49]. The exact mechanism underlying GH resistance following orally administered estrogen remains unknown; however, recent work suggests that estrogen influences GH signaling via the liver ERα-dependent upregulation of suppressor of cytokine signaling (SOCS)2 [51,52]. SOCS2 is known to suppress GH signaling through the inhibition of the janus kinase (JAK)2 signaling protein [53,54]. Additionally, estrogen may directly inhibit JAK2 and/or its binding partner, signal transducer and activator of transcription (STAT)5 [52], both of which are important components of the GH signaling pathway. While these reported mechanisms for interaction of estrogen and GH are promising, many other pathways for crosstalk between steroid hormone receptors and cytokine receptors have been described [55]. Therefore, numerous mechanistic possibilities exist for the interaction of estrogen and GH signaling and this signaling crosstalk may be fundamental to sex-dependent thrombosis.

### Progesterone & testosterone

Other hormones, such as progesterone and testosterone, may also play a part in mediating sex differences in thrombosis, although the evidence for this is extremely limited. Progesterone has been studied as a part of oral contraceptive and hormone-replacement therapies and, in combination with estrogen, may have an effect on thrombotic risk. A recent study found that the addition of progesterone to estrogen in hormone-replacement therapy doubled the risk of VTE, although further studies – including an analysis of first-, second- and third-generation progesterones – are needed [56–58]. Testosterone has also been studied for its potential role in the increased thrombotic risk in males. However, numerous prospective cohort or nested case–control studies showed no clear association between endogenous testosterone and VTE risk [59]. At this time, whether progesterone and testosterone alone or in combination with other hormones have significant effects on thrombosis in men and women is unclear.

## Discussion

There is a growing appreciation for the influence of sex on cardiovascular disease as accumulating studies and observations substantiate the importance of sex in determining both arterial and venous thromboembolic risk; yet, we do not fully understand the precise mechanisms that underlie these sex differences. This article describes the current views of hormonal influences on thrombosis, particularly with regard to estrogen and GH; however, many important questions remain.

What are the principal mechanisms through which supplemental estrogen influences thrombosis? While estrogen increases the risk of VTE and leads to changes in the activity of coagulation-related proteins, most notably increased APC resistance, the molecular mechanism through which this occurs is not understood. Furthermore, oral and transdermal estrogens have disparate effects on VTE risk. In view of the fact that oral estrogen mediates GH resistance and increases GH secretion, while transdermal estrogen does not affect GH, we speculate that the increased VTE risk associated with oral estrogen may be due to its impact on GH signaling. Future studies are aimed at specifically determining the primary interaction(s) between these hormones and their separate and combined effects on thrombosis. This interplay between estrogen and GH holds exciting promise for the understanding of sex-specific thrombosis and the development of novel estrogen-like compounds that pose minimal risk for thrombotic disorders.

What is the effect of GH on thrombosis in humans? Our laboratory has previously reported that GH supplementation increases thrombosis in mice and this effect appears to occur through changes in the expression of multiple coagulation inhibitor genes. However, studies addressing the effects of GH in human thrombosis are sparse. A current review of the literature reveals one recent paper examining the effect of GH replacement on coagulation parameters in male and female patients with GH deficiency. No differences were found at baseline between control and GH-deficient patients, but treatment with recombinant GH led to activated partial thromboplastin time (aPTT) prolongation in males and prothrombin time (PT) prolongation in females [60]. Considering these findings, along with the increased use of supplemental GH for treatment of conditions other than hypopituitarism, an assessment of the effects of GH on coagulation is warranted.

> "Ultimately, we hope to advance the understanding of the complex biological differences between the sexes..."

What are the biological explanations for sex differences in thrombosis? Are the cardiovascular distinctions between men and women at the anatomic, physiologic, cellular and molecular levels simply stochastic, the result of distinct evolutionary pressures, or are these observed sex differences secondary to other biological processes? The stochastic model is unlikely, given that differences in hemostasis and thrombosis are found in many mammalian systems. However, whether these sex differences serve a real biological purpose remains an open question. Mouse knockout models have shown that maternal trophoblast cells lining the placental blood vessels abundantly express the coagulation-related proteins thrombomodulin, tissue factor pathway inhibitor, endothelial protein C receptor and tissue factor [61]. Thus, a delicate balance between pro- and anticoagulant proteins occurs in the placenta, presumably to regulate thrombosis and prevent fetal loss. Indeed, dysregulated thrombin generation resulting from genetic deficiency of anti-thrombin, protein C or other anticoagulant proteins has been shown to increase the risk of fetal loss and other deleterious pregnancy complications [62,63]. Furthermore, a recent report demonstrated that the loss of maternal platelets or platelet thrombin response could prevent some of the fetal loss associated with thrombomodulin deficiency [58]. Thus, there is evidence in favor of a strong biological pressure for decreased thrombin activity in females in order to protect placental blood flow. However, it is also plausible that sex differences in thrombosis are the result

of variations in hormones and/or coagulation factors that have evolved based on functions of these proteins that are unrelated to coagulation.

Ultimately, we hope to advance the understanding of the complex biological differences between the sexes and to improve the management of cardiovascular risk and treatment of disease in males and females. While this article has primarily addressed sex differences in VTE, the discussion probably extends to other thrombotic disorders, such as stroke and acute coronary syndromes. Sex differences in these diseases have been described, with similarly increased risk in men compared with women [14,64]. Moreover, the treatments for thrombotic disorders often overlap and demonstrate sex-dependence in their efficacy and associated complications. For example, sex differences in the risks and benefits of antiplatelet therapies, such as aspirin, warfarin, GPIIbIIIa inhibitors and tPA have been documented [11,65,66]. Future research aimed at understanding sex differences in risk, presentation, progression and treatment of venous and arterial thrombotic disorders will ultimately help to define novel biological mechanisms underlying these important diseases and lead to advances in sex-specific risk assessment, diagnosis or management of thrombosis-related diseases.

**Financial & competing interests disclosure**

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