Title: The stronger sex, until menopause: understanding the impact of estrogen loss on heart function

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In the first four decades of life, women are protected from cardiovascular disease (CVD) compared to men, perhaps making women the stronger sex. Over the next 5-10 years, however, ovarian follicle depletion initiates a gradual decrease in sex steroid hormones transitioning women into perimenopause. Menopause occurs in approximately the 5th decade of life and results in a near complete loss of serum estrogen (1). With menopause, the risks of coronary artery disease, peripheral artery disease, aortic calcification, and stroke substantially rise, and the decrease of circulating estrogen is hypothesized to adversely affect adipose distribution, lipid metabolism, insulin sensitivity, and blood pressure (1). In young women with either abrupt and gradual loss of estrogen due to hysterectomy, chemotherapy, and extreme psychosocial stress, risk of CVD also increases (1, 2). Multiple lines of evidence suggest that estrogen imparts a protective effect on the cardiovascular system at multiple scales, including the endothelia, arteries, and the heart itself (1, 3, 4). However, an early clinical trial that sought to diminish CVD risk in post-menopausal women by administering exogenous estrogen instead increased risk of cancer, thromboembolic events, and stroke (5). To date, the protective and detrimental roles of estrogen in the cardiovascular health of premenopausal women, postmenopausal women, and men remain poorly understood.

In 2016, the National Institute of Health began requiring that biological sex be factored into clinical and preclinical study designs to improve rigor and translation of basic science to clinical science and care (6). Despite this, recruitment of women into clinical trials remains low (7), which leaves significant knowledge gaps in the pathology and treatment of CVD in women and creates disparities in prognosis compared to men. This limited knowledge has led to delays in critical care for women presenting with myocardial infarction and increased mortality in aortic valve replacements compared to men (8, 9). In basic science research, preclinical studies persist in using predominately male animals or do not report the sex of animals within the

methods. Overall, the mechanisms by which female sex, estrogen, and age impact heart disease are critically understudied, which confounds translation from bench to bedside.

The rapid report by Joll *et al.* in this issue (10) is a welcome change from business as usual in cardiovascular research. To investigate the impact of estrogen loss on CVD in women, the authors subjected young adult female C57BL6 mice (4 months old) to bilateral ovariectomy (OVX) to induce an early menopause-like state, fed them a high cholesterol (Western) diet, and aged them to 12 months. *In vivo* echocardiogram measurements were performed at 4, 9 and 12 months, as well as bone mass density measurements using dual x-ray absorptiometry each month. At the terminal time point of 12 months, left ventricular (LV) and aortic valve (AV) tissues were harvested and stained with Mason's Trichrome and Alizarin Red S to determine collagen content and calcification, respectively. The authors found that bilateral OVX in combination with a high fat diet and aging resulted in increased LV mass, signifying LV hypertrophy and suggesting systemic hypertension. Further, no evidence of LV or AV fibrosis or calcification was found. The OVX group did have a significant decrease in bone mineral density, indicating osteoporosis development in agreement with prior rodent models (11). The stimulus for LV hypertrophy is not elucidated by Joll *et al.* but is likely related to vascular stiffening due to aging (12) and the high cholesterol diet in combination with the loss of estrogen (13). In addition, while collagen accumulation was not found, collagen type and crosslinking, which play a functional role in the stiffening of these tissues (14), could have been altered by OVX, the high-fat diet, or aging, but were not measured. Despite these limitations, the authors are to be commended for addressing the elephant in the room regarding the lack of female specific CVD research and investigating the development of CVD in aging, post-menopausal women.

Only continual action and acknowledgement of sex differences in cardiovascular health research will reduce sex-based cardiovascular health disparities. For those who take up this charge, we offer a few suggested refinements to the study design used by Joll *et al.* First,

bilateral OVX is an overly simplified model of menopause. The loss of sex steroid hormones with surgical OVX is rapid and does not mimic the gradual loss of hormones and hormone receptors in the perimenopausal to menopausal transition in human women. As an alternative, the 4-vinylcyclohexenediepoxide (VCD) mouse model of menopause simulates ovarian failure over time (15). VCD injections cause regression of small follicles and rapidly accelerate depletion of the ovarian follicle reserve. During the transition to complete ovarian depletion, the mice undergo a perimenopause phase similar to human women with corresponding hormonal changes such as decreased estrogen, increased follicle stimulating hormone, and increased luteinizing hormone. Mice receiving the VCD injection over 10-20 days begin to have extended estrous cycles that taper off into a continual diestrus anovulatory phase (15). Moreover, variable VCD dosing allows for manipulation of the perimenopausal phase and permits investigators to optimize the perimenopause phase length to the study design. Prior research combining VCD with Ang-II infusion in female C57BL6 mice showed that blood pressure increased in both the perimenopausal and menopausal phase compared to intact mice with Ang-II infusion (16). However, the use of VCD in physiological research is limited due to the carcinogenic and toxic nature of the drug to the liver and kidneys. Other off-target effects may also limit the utility of this approach for mimicking menopause in an animal model. Second, since aging is key to CVD in women, performing OVX in older rodents would better recreate the effect of hormone loss on the stiffened vasculature that likely exists in women in the 5^{th} decade of life. Third, the use of mice as a model of human disease is limited due to the robust compensatory mechanisms of the mouse in the face of injury, disease, or genetic mutations. Using the bilateral OVX with high cholesterol diet or VCD model of menopause in a larger rodent model, such as the rat, may induce more substantial LV and AV remodeling, including fibrosis, closer to the human condition. In combination with *in vitro* and *in silico* approaches, robust and physiologically relevant *in vivo* models that recapitulate the effects of female hormone loss in conjunction with aging on CVD development promise to advance equity in cardiovascular health.

In conclusion, Joll *et al.* provide a good first step in developing a mouse model that bridges the gap between sex, hormones, and age in cardiovascular health (10). This publication addresses the sorely lacking inclusion of the female sex, female sex steroid hormone effects, and lifecycle in cardiovascular research. Understanding the sex-dependent and sex steroid-dependent mechanisms of CVD development and progression are critical to diagnosis, treatment, and prognosis of women with CVD. Moreover, uncovering the ways in which estrogen protects young women's hearts, making them stronger than men's, may enable the discovery of novel therapeutics for older women and men.

Funding:

R01HL147590 and R01HL144727.

Conflicts of Interest

No conflicts of interest to disclose.

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