

RESEARCH ARTICLE

The clinical impact of estrogen loss on cardiovascular disease in menopausal females

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Abstract:

According to the CDC (2017), more women than men have died from heart disease over the last 20-25 years. On the contrary, premenopausal women are protected against heart and cardiovascular disease (CVD) compared to men. Following menopause, there is sharp rise in CVD mortality and morbidity in women compared to men indicating that women lose protection against CVD during menopause. This loss of CVD protection in women drives the CDC statistics. Life expectancy of women has now reached 82 (almost 35 years longer than at the turn of the 20th century). Yet, women typically undergo menopause at 50-60 years of age, which means that women spend over 40% of their life in menopause. Therefore, menopausal women, and associated CVD risk, must be considered as distinct from an aging or senescent woman. Despite longstanding knowledge that premenopausal women are protected from CVD, our fundamental understanding regarding the shift in CVD risk with menopause remains inadequate and impedes our ability to develop sex-specific therapeutic strategies to combat menopausal susceptibility to CVD. This review provides a critical overview of clinical trials attempting to address CVD susceptibility postmenopausal using hormone replacement therapy. Next, we outline key deficiencies in pre-clinical menopause models and introduce an alternative to overcome these deficiencies. Finally, we discuss a novel connection between AMPK and estrogen-dependent pathways that may serve as a potential solution to increased CVD susceptibility in menopausal women.

Keywords: cardiovascular disease, menopause, perimenopause, estrogen, estrogen receptors, hormone replacement therapy, AMP-activated protein kinase, ovariectomy, timing hypothesis

1.1. Outlook of Cardiovascular Disease in Women

Cardiovascular disease (CVD) remains the leading cause of death claiming about 600,000 (Center for Disease Control and Prevention [CDC], 2017) lives per year in both men and women¹⁻³. The most common cause of CVD is directly or indirectly related to coronary heart disease (50%)⁵ and can progress to heart failure; approximately 5.7 million people have heart failure due to CVD and roughly half of this population is women¹⁻³. Moreover, *post*-menopausal women account for 2 million of these patients⁶. In the United States, the life expectancy of women has now reached 82, almost 35 years longer than at the turn of the 20th century. Thus, a greater portion of a woman's lifespan is spent in menopause, leading to some staggering statistics; more women than men have died from CVD over the last 20-25 years (CDC, 2017). Yet, **only 54%** of women are aware of their CVD risk despite concerted efforts to educate women about CVD⁴. Compared to males, premenopausal females are protected against developing CVD⁷. After transition to menopause, protection against CVD and other CVD-dependent complications is lost and risk increases dramatically^{8, 9}. Despite the longstanding knowledge that premenopausal women are protected from developing CVD, the fundamental mechanisms underlying the shift in CVD risk that occurs with menopause remain unknown. This impedes our ability to develop therapeutic strategies to combat menopausal cardiac remodeling and its complications.

Considering the progressive loss of estrogen during menopause, a prime candidate responsible for protection against CVD in premenopausal females is estrogen. Estrogen is a naturally occurring steroid hormone that is positioned to play a unique role in cardioprotection. However, estrogen

signaling is complex and multiple molecular, genetic and cellular mechanisms have been suggested to underlie protection against CVD^{10, 11}. Estrogen is positioned to play a unique role since it can respond to environmental, genetic and non-genetic cues to impact genetic expression and cellular signaling¹¹. Investigations into the cardioprotective effect of estrogen are complicated by findings in human studies when compared to rodents. Generally, rodent models of CVD consistently demonstrate worsening pathology following surgical removal of the ovaries, ovariectomy (OVX), which can be reversed by estrogen replacement, typically in the form of 17 β -estradiol (E2)¹²⁻²¹. Unfortunately, OVX accounts for approximately 10% of the female population lacking estrogen and does not accurately reflect the majority of non-cycling, menopausal women. Despite the limitations with the OVX model, these studies provide important insight as to the role of estrogen and estrogen loss in disease etiology. In this review, we will provide an overview of key clinical studies addressing the impact of menopause and estrogen, typically through hormone replacement therapy (HRT) on CVD. We will also introduce a novel cellular and molecular mechanism mediating CVD protection in females that may provide a foundation for sex-specific therapeutic strategies.

1.2. Clinical Status of Hormone Replacement Therapy and the Timing Hypothesis

1.2.1. WISDOM and WHI Trials; Estrogen alone, progesterone alone, estrogen plus progesterone, placebo:

Despite the promise of rodent studies, the prospective Women's Health Initiative (WHI) and Women's International Study of long Duration Estrogen after Menopause (WISDOM) studies showed an increased

CVD and stroke risk with estrogen replacement in menopausal women^{22, 23}. As a result of these findings, the American Heart Association and the US Food and Drug Administration guidelines state that estrogen replacement therapy “should not be used for the prevention of heart attack or stroke”.

The Women’s International Study of long Duration Estrogen after Menopause (WISDOM) is one of the first HRT studies. In this study, approximately 6000 women were randomized to four groups and administered either estrogen alone (conjugated equine estrogens), progesterone alone, progesterone and estrogen (conjugated equine estrogen plus medroxyprogesterone), or placebo therapy and followed for over 6 years²⁴. The mean age was 62 years and the majority of the women in the study were already in menopause for 15 years²⁴. Although the study was planned to span 10 years, it was stopped prematurely due to emerging evidence from the Woman’s Health Initiative (WHI). The WHI, using a similar trial design and similar parameters, demonstrated that combined estrogen (CEE) and medroxyprogesterone (MPA) therapy in non-hysterectomized women, at an average age of 64, augmented cardiovascular risks instead of diminishing them as predicted²³.

In the WISDOM trial, estrogen-only therapy was hypothesized to decrease the risk of coronary heart disease, breast cancer, diabetes, and osteoporotic fractures, when implemented during early menopause²⁴. However, in the WISDOM trial HRT reduced fracture risk, yet increased thromboembolic, breast cancer, and cerebrovascular risk in asymptomatic postmenopausal women²⁴. Similarly, a second WHI trial with estrogen (CEE) alone versus placebo, in hysterectomized women, demonstrated an increased risk of

stroke and thromboembolisms²⁵. Consequently, results from WISDOM and WHI’s trials prompted a massive decline of HRT use during the early 2000s.

Recently (2017) WHI published results from an 18-year-follow-up to their first, estrogen (CEE) plus progesterone (MPA) versus placebo, 5.6 year-long WHI study (12.5 year post-study surveillance), and their second, CEE alone versus placebo, 7.2 year-long study (10.8 year post-study surveillance)²⁶. In this recent report, investigators analyzed all-cause mortality and cause-specific mortality during this surveillance period. Amongst the results for the all-cause mortality surveillance for each WHI trial study, one had 27.1% mortality in CEE+MPA subjects versus 27.6% mortality in placebo counterparts, while the other study had 28.3% mortality in CEE alone subjects versus 30% mortality in placebo subjects²⁶. Additionally, CVD mortality was very similar between the HRT and the placebo groups (8.9% and 9.0%, respectively), and showed no significant differences across WHI studies²⁶. Finally, there was no increased risk or mortality for CVD, stroke, or coronary artery disease (CAD), when comparing HRT groups versus placebo groups during intervention (HRT supplementation) or post-intervention (post study surveillance) of the studies. These findings suggested that WHI may have prematurely concluded that estrogen-based HRT should not be used to treat menopause-associated cardiovascular risks.

1.2.2. BEST Trials; users (estrogen, progestin, estrogen and progestin, placebo, bucindolol) vs. non-users:

During the WISDOM and WHI trials, another clinical trial, “Beta-Blocker Evaluation of Survival Trial” (BEST), was initiated and continued over a period of 3.5 years to validate three Vesnarinone (cardiotonic agent) HRT (estrogen) trials

(VesT). Contrary to the WISDOM and WHI trials, VesT results showed women undergoing estrogen-based HRT were found to have improved patient quality of life and protection against pathological remodeling of cardiac vasculature and cardiomyocytes^{27, 28}. The subjects of the study were postmenopausal women ranging from 50 to 93 years of age, with class III and IV heart failure (HF) (NYHA standards)²⁸. Women were considered for the postmenopausal group if they were already taking HRT of estrogen, progestin, or a combination of both.

BEST identified that 102 HRT users (72 using estrogen alone, 3 using progestin alone, and 27 using both) had 21% mortality, while 333 HRT non-users had a significantly higher mortality (34%; $p=0.025$)²⁸. Similarly, a higher mortality was observed in CAD HRT user subjects versus non-CAD HRT user subjects. Left ventricular ejection fraction (LVEF), a systolic measure of heart function, was measured across study groups and did not significantly differ between non-user (placebo and non-HRT), user, ischemic, or non-ischemic patients. The suggestion is that differences in observed mortality depended on CAD progression.

Overall, HRT independently improved heart function and prevented progression of CVD despite onset of CAD, which further verifies the Vesnarinone studies. However, the study also had several limitations that could be improved upon for future studies. First, the study did not monitor whether subjects were administered HRT before or continued HRT after the study. Second, the supplements used were not evenly represented in the HRT user groups; ischemic CAD patients and stage IV NYHA patients were over represented. Third, the important transitory property of menopause was not evaluated, when the study identified their patients as

postmenopausal women, ≥ 50 years of age. Identifying each group by age and associated menopause symptoms would have improved the clinics understanding of estrogen's regulatory properties in heart disease. Overall this trial supported previous evidence relating menopausal females to non-ischemic CVD, yet fell short on presenting quality echocardiographic data to describe how changes in heart function across groups explained changes in mortality.

1.2.3. HERS I and HERS II; Estrogen + progestin vs. placebo:

The discrepancies of the BEST study resulted in a 6.8 year-long Hormone and Estrogen-progestin Replacement Study (HERS I) and HERS II study. The subjects were >79 years (and ≥ 5 years after start of menopause) old women with $<$ class IV NYHA HF²⁹. Women enrolled in the study received HRT of either estrogen plus progestin, or a placebo. The trial consisted of approximately 2,700 women, of which 644 were in HF and had a history of at least one ischemic event²⁹. Out of these 644 women, 324 received estrogen and progestin, while 320 received placebo²⁹. During the first 4.1 years (HERS I trial) there were 114 deaths (18% mortality) independent of HRT. To ensure HRT did not have long-term deleterious consequences, a 2.7 year follow up trial (HERS II trial) was conducted to monitor the HERS I trial subjects, HERS II resulted in 99 more HRT- independent deaths²⁹.

The results of both trials opposed the BEST trial findings, yet showed concordance with the WHI and WISDOM trails. Therefore, a strong case can be made that HRT does not decrease mortality in HF or prevent myocardial ischemia. Unlike BEST and VesT studies, a weakness of the HERS trials is that non-ischemic HF HRT subjects were excluded. Additionally, the subjects

studied during the HERS trials were near the end of the average life span (82 years of age), where progression of ischemic HF and increased mortality are certain to penetrate the study.

1.2.4. The Timing Hypothesis: ELITE; Estrogen and progesterone vs. placebo:

The timing hypothesis states that the effectiveness of hormone replacement therapy (HRT) depends on the “timing” of estrogen delivery relative to the age of menopause onset. The hypothesis suggests that there is a window where HRT can reduce or reverse CVD during woman’s transition into menopause. Factors such as age elapsed since menopause, age alone, or combination of both influence the optimal timing of HRT to improve cardiovascular health and/or prevent CVD. More importantly these factors can instigate decrease in estrogen release or estrogen receptor sensitivity³⁰. Support for the timing hypothesis is provided in a recent (2015) clinical trial, “Early versus Late Intervention Trial with Estradiol” (ELITE), in young transitioning (into menopause) women³¹. However, another similar clinical trial, “Kronos Early Estrogen Prevention Study” (KEEPS), in young women yielded conflicting results³². Regardless of the inconclusive nature of these studies, investigators are now forced to incorporate the timing hypothesis into the proper planning of future HRT trials.

Almost a decade after the first WHI HRT study, the 7.5 year-long study (including a 2.5-year follow-up) ELITE was completed. The study consisted of 643 healthy postmenopausal women who had entered menopause in the last 6 years or less (early group) or at least 10 years previously (late group)³¹. The mean age of the early group (menopausal) was 55 years, while the mean in the late group (postmenopausal) was 65 years, and both groups contained women

who had undergone a hysterectomy³¹. Women who had hysterectomy received oral 17 β -estradiol and 4% progesterone, while the non-hysterectomy group received only 17 β -estradiol³¹. The patients were monitored for 6 years, and showed reduced carotid artery intima-media wall thickness compared to the placebo counterpart in the early group, but not the late group. This study is unique and progressive, because the phases of menopause were defined separately, and the diagnosis was based on vasculature alteration and associated post-menses symptoms. In the ELITE trial, estrogen was shown to have little effect in reversing established atherosclerosis, yet results suggested that earlier administration of estrogen-based HRT in these subjects could prevent established atherosclerosis and lower CVD associated risks³¹.

Last year, a similar study, “KRONOS Early Estrogen Prevention Study” (KEEPS), was conducted to identify if estrogen and progesterone had a greater effect on vasomotor symptoms (VMSs) in early menopausal (6-36 months post last menses; age 42-58) women versus placebo³². The study evaluated VMSs, frequency of hot flashes and sweats, self-reported insomnia, and irritability. The study concluded that HRT (both conjugated estrogen and estrogen alone) in early menopause alleviated hot flashes, night sweats, and insomnia. This four year-long study impacted the HRT community because estrogen-based HRT is currently the only solution to alleviate menopausal symptoms, other than paroxetine mesylate. Therefore, this trial brought to light the conclusion that estrogen-based HRT is not the issue, yet the protocol and/or timing for estrogen-based HRT needs to be optimized to treat patients appropriately. Additionally, their results were consistent with symptom alleviation found in the WHI and HERS trials. This trial did not look at vasculature alterations,

yet, like ELITE, demonstrated that estrogen-based HRT benefited early menopausal women.

1.2.5. FHS trial; Post menopausal women, men, and genetically modified aromatase:

The Framingham Heart Study (FHS) clinical study investigated whether the onset of menopause and lower circulating levels of estrogen were responsible for increased CVD susceptibility. The 6-year FHS (plus 6 year follow-up) was conducted in post-menopausal women and age-matched men. These individuals, 834 men and 687 women, were also in third and fourth cycle heart failure³³. The participants were screened for serum levels of estradiol, testosterone, and dehydroepiandrosterone sulfate (DHEAS). As a steroid hormone, estrogen mediates its actions through ligand-dependent interaction with two estrogen receptors (ER), ER α or ER β . The cellular response to estrogen is either rapid (non-genomic), through translocation of ER pools to the plasma membrane to stimulate second messenger signaling cascades, or delayed (genomic), by nuclear targeting to regulate gene transcription³⁴. A normal hormone level in men and postmenopausal women is characterized by moderate testosterone levels, low estradiol levels, and a low estrogen:testosterone ratio (implying high aromatase levels).

This trial focused on the variation of aromatase gene CYP19A1 and estrogen receptor isoform genes ESR1 (ER α) and ESR2 (ER β)³³. The study determined that hormone serum levels were dramatically affected and highly dependent upon CYP19A1 and ESR2 modification. This set of modifications resulted in increased susceptibility to coronary atherosclerosis in men, due to a shift in estrogen:testosterone ratios (carrier to non carrier 13% difference), estradiol levels (5%), testosterone levels (17%), and a decrease in ER β ³³. The differences in the aromatase gene expression were unique to men, while equal ESR2 gene expression levels were observed in both sexes. Additionally, the ESR1 gene seemed to display consistent hormone-level binding regardless of gene modification.

The following tables, **Table 1.1 and 1.2**, summarizes the previously discussed HRT clinical trials, and emphasizes the importance of the timing hypothesis, determining the type of hormone, and identifying key measurements of heart function. The results verify the timing hypothesis, and give a general understanding of how the clinical perspective has improved since the introduction of HRT in the early 2000s. The clinical trials tend to lack optimization, thus more preclinical data and experiments may help improve future trials.

Table 1.1. HRT Clinical Trials During Early 2000s

Clinical Trial	Estrogen Replacement and Atherosclerosis (ERA) [1996-99] [G1:79, G2:85, G3:84] ³⁵	Women's Estrogen-progestin Lipid Lowering Hormone Atherosclerosis Regression Trial (WELL-HART) [1995-2000] [G1:76, G2:76, G3:74] ³⁶	Estrogen in the Prevention of Atherosclerosis Trial (EPAT)[1999-2001][G1:111, G2:111] ³⁷	Woman's International Study of long Duration Estrogen after Menopause (WISDOM)[1999-2002][Stratum1 (S1):3721, S2:966, S3:1005] ²⁴	Beta-Blocker Evaluation of Survival Trial (BEST)[1992-2003][G1:53, G2:49, G3:168, G4:164] ²⁸	Heart and Estrogen-progestin Replacement Study (HERS) [1998-2003][G1:1380, G2:1383] ³⁸
Mean Age years	66 (≥ 5)	63.5(18)	60.3(<5 & >5)	63(15)	63(NM)	67(5)
NYHA or CAD	Asymptomatic	History of CAD	History of CAD	Asymptomatic	III (91%) , IV(8%) and CAD history	History of CAD
Length of Study (yrs)	3.2	1 (treated); 3.3 follow-up	2	6	3.5	4.1
Time Since HRT	Y	N	Y	Y	Y	Y
Intervention (mg/day)	G1: CEE (0.625) G2: CEE(0.625)+MPA(2.5) G3: PBO	G1: 17β (1)+MPA PBO(5)(12 days/month) G2: 17β (1)+MPA(5)(12 days/month) G3: PBO (6)	G1: 17β (1) G2: PBO (1) Both: Lipid lowering medication if 160mg/dL(LDL)	G1: CEE (0.625)+MPA(2.5,5,10 (varied on breakthrough bleeding)) G2: CEE (0.625)+PBO(2.5,5,10) G3: PBO(3.125)	G1: users (72 patients estrogen alone, 3 progestin alone patients, 27 estrogen+progestin) +bucindolol G2: users+PBO G3: non-users+bucindolol G4: non-users+PBO	G1: CEE (0.625)+MPA(2.5) G2: PBO(3.125)
Risk	↑Fracture, 9 CAD deaths, 19 nonfatal myocardial infarctions. No significant effects elicited HRT.	No significant ↑ or ↓ in risk for CAD in relation to HRT groups.	↑Risk of uterine cancer and diabetes mellitus. No significant effect with LDL medication.	↑Thromboembolic events, breast cancer and possible cerebrovascular events across groups.	↑Mortality (34%) in non-user versus users despite beta-blocker treatment. Also ↑deleterious events in ischemic patients.	No significant relationships between groups. 10%↑ HDL and 11%↓LDL in HRT group. ↑Thromboembolic events and gall bladder disease progression.
Benefit	Estrogen is preventative, and not intended to prevent progression of CAD or HF.	No significant benefit from HRT.	In estrogen alone group vs. PBO there was a ↓ in CIMT, atherosclerosis progression slowed and estrogen was preventative of CAD.	↓Fracture risk. Estrogen alone therapy started near menopause may ↓CHD, breast cancer, and osteoporotic fractures.	↓Mortality in user group and significant ↑ in survival rate in users without history of ischemia.	HRT had 81% adherence and PBO had 0% adherence, in first year. No ↑ in mortality, fractures, or cancer, in HRT vs. PBO groups. No HRT dependent changes in LVEF. First year see ↑CHD event, and after four years see ↓CHD event.

17β= 17-β estradiol (E2); CAD=coronary Artery Disease; CEE=conjugated equine estrogen; CHF =congestive heart failure; G#=#treatment group; CIMT=Carotid Intima-Media Thickness; HRT= hormone replacement therapy; LVEF=left ventricle ejection fraction; MPA=medroxyprogesterone acetate; MPG=micronized progesterone gel; NYHA= New York Heart Association functional classification; o-CEE=oral conjugated estrogen; PBO= placebo; PBOP= placebo patch; t-E2=transdermal estradiol

Table 1.2. HRT Clinical Trials During Late 2000s

Clinical Trial	Heart and Estrogen-progestin Replacement Study (HERSII)[2003-06][G1:1380, G2:1383] ³⁹	Early vs. Late Intervention Trial with Estradiol (ELITE)[2008-15][G1:260, G2:11, G3:272, G4:100] ³¹	Kronos Early Estrogen Prevention Study (KEEPS)[2012-16][G1:230, G2:255, G3:275] ³²	Women's Health Initiative (WHI) Estrogen-Progestin Study[1998-2017][G1:8506, G2:8102] ^{23, 26}	Women's Health Initiative (WHI) CEE alone Study[1992-2017][G1:5310, G2:5429] ^{25, 26}
Mean Age years	67(5)	Early:55(≤6) Late:65(≤10)	52(3)	63(≥1)	63.6(NM & hysterectomy)
NYHA or CAD	History of CAD	Asymptomatic	Asymptomatic	Asymptomatic	Asymptomatic
Length of Study (yrs)	4.1	7	4	3 (treated); 5.2 (follow-up); 12.5 (post-surveillance)	6.8 (treated); 5.2 (follow-up); 12.5 (post-surveillance)
Time Since HRT	Y	Y	Y	N	Y
Intervention (mg/day)	G1:CEE(0.625)+MPA(2.5) G2:PBO(3.125)	G1:17β(1)+4% micronized progesterone gel(45) G2:17β(1)+PBO(45) G3: PBO(46) G4: PBO(1)+PBO(45)	G1:o-CEE(0.45)+MPG(200 for 12days/month)+PBOP G2:t-E2(50ug)+MPG(200 for 12 days/month)+PBOP G3: PBO+PBOP	G1:CEE(0.625)+MPA(2.5) G2:PBO(3.125)	G1:CEE (0.625) G2: PBO (0.625)
Risk	1° risks are, Non-fatal (NF) MI and CHD death. 2° risks are coronary revascularization, hospitalization for unstable angina or CHF, NF ventricular arrhythmias, sudden death, stroke, ischemic attack, and peripheral arterial disease	No significant ↑ in risks in treated groups vs. PBO.	HRT treatments did not significantly affect irritability compared to PBO.	↑Risk in G1 vs. G2 for breast cancer, pulmonary embolism, stroke, death and hip fracture.	↑Risk of stroke in CEE patients versus PBO.
Benefit	HRT had 81% adherence and PBO had 0% adherence, in 4-5th year.	17β group significantly ↓ extent of CAD if initiated in early stages of menopause, because CIMT is normal.	Moderate to severe hot flashes and night sweats, all ↓ in treatment groups vs. PBO, yet no significant differences between HRT compounds. O-CEE vs. PBO ↓insomnia at 36-48 months, as did t-E2 at 48 months. Active treatment vs. PBO is uniform across race/ethnicity and BMI.	No benefit between group comparisons.	↓Risk of hip fracture and constant risk for CHD in CEE patients vs. PBO. Possible ↓ risk in breast cancer.

17β= 17-β estradiol (E2); CAD=coronary Artery Disease; CEE=conjugated equine estrogen; CHF =congestive heart failure; G#=#treatment group; CIMT=Carotid Intima-Media Thickness; HRT= hormone replacement therapy; LVEF=left ventricle ejection fraction; MPA=medroxyprogesterone acetate; MPG=micronized progesterone gel; NYHA= New York Heart Association functional classification; o-CEE=oral conjugated estrogen; PBO= placebo; PBOP= placebo patch; t-E2=transdermal estradiol

1.3. Mouse Model of Clinically Relevant Natural Menopause

One obstacle that has stalled progression of studies into sex differences in CVD onset is the lack of a rodent model of progressive ovarian failure, i.e. one that moves from perimenopause into postmenopause, similarly to humans. Most studies have used the surgical removal of ovaries (ovariectomy) as a model of menopause, however only 10% of women enter menopause surgically, as mentioned above. A more recent subset of rodent studies has utilized an ovary-intact mouse model of menopause, using the chemical 4-vinylcyclohexene diepoxide (VCD)^{40, 41}. Repeated daily dosing with VCD selectively targets the primordial follicles of the ovaries, accelerating the natural process of follicular atresia, and inducing gradual ovarian failure. This model preserves the important “perimenopause” transitional period and androgen secreting capacity of residual ovarian tissue, identical to menopausal women^{40, 42}. Using the VCD model of menopause, we demonstrated that perimenopausal, like cycling (premenopausal) females, were protected from pathological angiotensin II (Ang II)-induced hypertension while menopausal females were not, again, the same as humans⁴³. Estrogen delivered across the peri- to menopausal transition restored protection against Ang II-induced hypertension during menopause. Our novel finding that perimenopausal females remain protected, despite irregular cycling (prior to complete loss of estrogen), underscores the importance of studying the role of estrogen in CVD, across the transition from perimenopause to menopause.

The use of the novel VCD mouse model of menopause allows us to examine how increased susceptibility to the pathological process of CVD accelerates from premenopause to *perimenopause* to

menopause. By studying the transition from CVD-resistance to CVD-sensitive in menopausal females we will be able to uncover pathogenic mechanisms that contribute to menopausal susceptibility to CVD unlike previous work. Current therapies and clinical trials of CVD protection in women have focused on HRT; yet estrogen targets every organ in the body, which can lead to confounding results. We discovered that one of these pathways, the adenosine monophosphate-activated kinase (AMPK) signaling axis, is activated by estrogen through direct binding of estrogen receptors to the α -catalytic subunit of AMPK⁴⁴. In the next portion of this review, we outline a combinatorial approach to that will elucidate cellular, molecular and genetic mechanisms of menopausal susceptibility to CVD and a potentially new target.

As a steroid receptor, estrogen mediates its actions through ligand-dependent interaction with ER α or ER β . Upon binding to estrogen, ERs immediately translocate to the plasma membrane rapidly stimulating second messenger cascades (non-genomic) or to the nucleus initiating longer term changes in gene transcription (genomic)³⁴. Evidence indicates that extranuclear ERs and nuclear ERs are the same protein⁴⁵⁻⁴⁷. In many instances, extranuclear ER signaling originating at the plasma membrane is required for downstream, nuclear targeting by ERs⁴⁵⁻⁴⁷. Yet, how extranuclear and nuclear ER signaling integrate to regulate pathological remodeling in the heart is currently unknown.

AMPK is a phylogenetically conserved heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits⁴⁸. An increase in myocellular AMP, as occurs with CVD, allosterically activates AMPK and permits phosphorylation of the α catalytic subunit

at Thr¹⁷² by the upstream Liver Kinase B1 (LKB1) kinase complex⁴⁹⁻⁵¹. LKB1 acts in concert with Mo25 (mouse protein 25) and STRAD (ste-related adaptor protein) to phosphorylate AMPK potentiating its activity and promoting ATP producing pathways while inhibiting ATP consuming pathways^{50, 51}. In addition, AMPK immediately responds to ATP supply-demand imbalance inducing translocation of activated AMPK (p-AMPK^{thr172}) to the nucleus. AMPK promotes transcription through direct phosphorylation of histones (H2B) leading to its epigenetic modification; energy stress preferentially increases nuclear AMPK α 2, the predominant isoform in the heart, and not AMPK α 1^{52, 53}. The ability of estrogen to potentiate AMPK activity coupled with epigenetic modifications due to AMPK activity suggests that AMPK may permit estrogen-dependent activation of a specific gene program. Therefore, in the latter half of this review we aim to propose an interdependence of estrogen-AMPK signaling at the epigenetic level to elucidate a cardioprotective mechanism. The following will further discuss the role of sex-dependent molecular factors and their importance in disease progression and energetic status.

1.4. Molecular Determinants of Sex-driven Differences in Cardiac Disease

1.4.1. Estrogen signaling

The three main circulating estrogens in women are: estrone (E1), estradiol (E2) and estriol (E3). E2 is the most abundant sex hormone in pre-menopausal women. At the cellular level, estradiol targets a vast number of molecular pathways through interaction with its intracellular estrogen receptors (ER). ERs can act as transcription factors, regulating gene transcription in response to E2, but can also activate protein

kinase cascades through non-genomic signaling events⁵⁴.

The two classical estrogen receptors are ER α and ER β . They are members of the nuclear hormone receptor (NHR) family and are composed of several functional domains. ER α and ER β share a high degree of homology in their DNA-binding domain (~96% amino acid identity), but differ in their ligand-binding (~58% amino acid identity) and N-terminal domains (~15% amino acid identity)^{55, 56}. Both classical estrogen receptors have splice variants. ER α 36 and ER α 46 are N-terminus truncated splice variants of full length ER α 66^{57, 58}. ER β has multiple isoforms that differ in their ligand-binding domain (ER β 2, ER β 3, ER β 4 and ER β 5)⁵⁹.

In women, ovaries are the main source of circulating estradiol. However, E2 is also synthesized in extragonadal tissues, but to a much lesser extent. These include mesenchymal cells of the adipose tissue in the breast, osteoblasts and chondrocytes, aortic smooth muscle cells, vascular endothelium, and several parts of the brain⁶⁰. To fully understand the effect of hormonal changes characteristic of menopause on cardiac energetics, it's imperative to examine estrogen-dependent regulation of key energetic molecules, such as AMPK.

1.4.2. AMPK, a central regulator of cellular energetics

AMP-activated protein kinase (AMPK) is a serine-threonine kinase central to the cellular energetic homeostasis. It is a heterotrimeric complex composed of a catalytic α subunit and two regulatory β and γ subunits. AMPK is activated in response to a decrease in ATP/AMP ratio, characteristic of energetic stress, which triggers phosphorylation of its α -catalytic subunit by an upstream kinase (AMPKK).

There are at least two AMPKKs known: calcium-calmodulin dependent protein kinase kinase β (CaMKK β)⁶¹ and the tumor suppressor kinase complex LKB1^{62, 63}. The catalytic α subunit of AMPK has several phosphorylation sites. Amongst them, phosphorylation of Threonine 172 (thr¹⁷²) is usually used as an indicator of AMPK activity⁶³.

1.4.3. Link between the estrogen and AMPK pathways in the heart

AMPK signaling axis is prone to differential regulation in response to sex differences^{64, 65}. Sex hormone-AMPK signaling may, in part, be responsible for the AMPK sex dimorphism. Estradiol (E2) has been shown to activate the AMPK pathway^{66, 67}. Recently, we described a novel mechanism of AMPK activation by E2, where the α -catalytic subunit of AMPK binds to ER α and facilitates thr172 phosphorylation by the upstream kinase LKB1 in response to E2 stimulation⁴⁴. ER β also interacts with AMPK, but most likely has an inhibitory function. This is not a surprising finding, since ER β antagonism of ER α signaling has been described in other studies^{68, 69}. The suggestion is, at the very least, that regulation of AMPK activity by E2 could be fine-tuned by altering the relative expression levels of ER α and ER β in the cell. Therefore, AMPK activation would be limited by two factors: availability of E2 and ER expression.

ER expression varies between the sexes, and the magnitude of this variation is tissue specific. While cardiac ER β expression is similar in males and females, ER α 66 expression in males averages only 1% of female values in the heart. ER α 46 and ER α 36 show similar expression levels in female and male hearts⁷⁰. The overall impact of E2 signaling on AMPK activation in males is expected to be very scarce, since males have much lower circulating E2

levels compared to females⁷¹. In addition, the relative cardiac ER expression would dictate the nature of E2 effect on AMPK. One suggestion is that the decreased ER α 66 expression in male hearts, along with robust ER β expression and low E2 circulating levels, would result in a minimal impact of estradiol on cardiac AMPK activation. Further studies are needed to confirm this hypothesis. In addition, it is important to consider the effect of other sex hormones, such as testosterone.

Circulating E2 levels in postmenopausal women are very similar to those in men of the same age⁵⁸. Different tissues of the body, including uterus, kidney and cerebral cortex, compensate for the decrease in circulating E2 by increasing ER α expression. Importantly, ER α levels in the heart do not experience any significant change⁷². The lack of compensatory mechanisms, such as up-regulation of ER expression, may account for a misbalance in cardiac estrogen signaling. This can lead to alteration in downstream-targeted pathways. In fact, cardiac AMPK signaling is decreased in menopausal mice when compared to their WT counterpart^{73, 74}.

CVD is underscored by a changing metabolic and energetic landscape in cardiac cells. The dynamics of cardiac contraction and relaxation during CVD are dictated by the kinetics and energetics of the cross-bridge cycle⁷⁵. Myocardial ER α distribution is altered in heart tissue of male and female patients with dilated cardiomyopathy (DCM). In healthy hearts ER α co-localizes with β -catenin to the intercalated discs, while in cardiac tissue from DCM patients that interaction and expression pattern is lost⁷⁶. This is also accompanied by an increase in cardiac ER α expression levels in both sexes, which may represent a compensatory mechanism to this structural reorganization.

AMPK is only one example of the molecular pathways affected by impaired estrogen signaling. It is clear that the development of CVD is the combinatorial outcome of derangements in many estrogen-dependent pathways⁷⁷.

1.5. Conclusion

This review encompasses the current status of therapies for menopause and CVD. The summarized results from Table 1 conclude that the intervention of CVD during the transitional phases of menopause is not simple, and the previous HRT clinical trials have conflicting solutions. This profound variation between studies is likely due to the diverse parameters in each study, such as variation in type of administration, concentration of estrogen dose, type of HRT, population size, age of population, and length of post-treatment monitoring intervals of patients. Again, the current issue is not necessarily estrogen-based HRT, because estrogen's role in cardioprotection is still not well understood, thus it is logical to continue preclinical research continues to parse out the mechanistic insight that could improve standard procedures of estrogen based-HRT.

Our research aims to utilize the VCD menopause mouse model to navigate alternative mechanisms that may play a cardioprotective role, and elicit an anti-inflammatory response. AMPK from our studies is a key regulator in cardioprotection with respect to estrogen and estrogen receptors. Since previous studies only utilized estrogen and progesterone supplements, we hypothesize that AMPK, estrogen, and estrogen receptors are highly organized structures in relation to one another, and thus interact to restore cardioprotection. This idea is promising, yet needs to be further studied in order to translate to a clinical setting.

Eventually, we aim to find a procedure and therapy that result in high efficacy and minimal risks. Overall, menopause and CVD are still being characterized from all aspects of the field, yet current research supports a sex-dependent mechanism of disease progression in the heart.

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