Heat Shock Protein 27 versus Estrogen Therapy for Post-Menopausal Atherosclerosis: Rethinking Mechanisms of Cholesterol Lowering

Running Title: *HSP27 vs. E2 Rx Post-Menopause*

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Abbreviations

AAb – anti-HSP27 (or anti-HSP25) antibodies

$ApoE^{-/-}$ - apolipoprotein E null

a.u. – absorbance units

E2 - 17β estradiol

HFD – high fat diet

HPRT - hypoxanthine-phosphoribosyltransferase

HSP27 – Heat Shock Protein 27

$HSP27^{o/e}$ – HSP27 over-expressing

LDL-C – low density lipoprotein cholesterol

LDLR – low density lipoprotein receptor

MP - menopause

mRNA – messenger RNA

NF-κB - nuclear factor kappa light chain enhancer of activated B cells

ON - overnight

OVX - ovariectomy

PBS - phosphate buffered saline

PCSK9 - Proprotein Convertase Subtilisin/Kexin type 9

rC1 – recombinant C-terminal truncation of HSP27 spanning amino acids 90-205

rHSP27 – recombinant HSP27

SDS-PAGE - sodium dodecyl sulfate and polyacrylamide gel

Abstract

The estrogen-inducible protein, Heat Shock Protein 27 (HSP27) attenuates experimental atherogenesis by reducing cholesterol levels in the blood and artery wall. Blood levels of HSP27 and natural antibodies to HSP27 are higher in healthy controls compared to patients with cardiovascular disease. Recently, we showed that vaccination of *ApoE*$^{-/-}$ mice with recombinant HSP25 (rHSP25, the murine ortholog of human HSP27) boosts anti-HSP25 levels and attenuates atherogenesis in male and female *ApoE*$^{-/-}$ mice. As estrogens and ovarian function influence HSP27 synthesis, cellular release and blood levels, we hypothesize that menopause will result in loss of HSP27 atheroprotection. Hence, we now compare the efficacy of rHSP25 vaccination *vs.* estradiol (E2) therapy in preventing the increase in atherogenesis that occurs with surgical menopause. Ovariectomized (OVX) *ApoE*$^{-/-}$ fed a high fat diet were randomized to receive 5-weekly rHSP25 vaccinations, a subcutaneous E2 slow release pellet or a combination thereof. Anti-HSP25 IgG antibodies were generated in mice vaccinated with rHSP25 or rC1, an inactive truncated form of HSP27 that acted as a control. OVX increased atherosclerotic burden by up to 65% and total plasma cholesterol levels by up to 23%. Compared to rC1/OVX (control) mice there were dramatic reductions in atherogenesis with the following treatments: -43% with rHSP25 vaccination, -52% with E2 therapy, and -82% with combined rHSP25 + E2 treatment ($p<0.0001$ for all). Relative to the rC1/OVX control mice, terminal total cholesterol levels were reduced by -22% with rHSP25 vaccination, -31% with E2 therapy and -45% with combined rHSP25 + E2 treatment ($p<0.0001$ for all). Of note, plasma PCSK9 levels were higher in E2/OVX mice than all other groups ($p<0.0001$). In contrast, OVX mice vaccinated with rHSP25 had PCSK9 levels that were -41% lower than rC1/OVX control mice ($p<0.0001$). Similarly, PCSK9 plasma protein and hepatic mRNA levels in the E2/OVX group were higher by 52% and

148% relative to rC1/OVX control mice; ($p<0.0001$ for both). Hepatic LDLR mRNA levels in the E2 treated mice were unchanged, but markedly increased with rHSP25 vaccination. In human HepG2 hepatocytes E2 increased PCSK9 promoter activity in a dose-dependent manner - an effect that could be inhibited with pan-estrogen receptor blocker ICI 182780. In contrast, the combination of [rHSP27 + PAb] decreased PCSK9 promoter activity by 64%. In conclusion, E2 therapy attenuates atherogenesis post-OVX but is accompanied by increased PCSK9 expression. In contrast, rHSP25 vaccination attenuates post-OVX atherogenesis and increases LDLR without altering PCSK9 expression. These data suggest that for the post-menopausal preventative treatment of atherosclerosis perhaps rHSP27 immunotherapy on its own, or in combination with E2 should be considered.

Keywords:

Atherosclerosis

Cholesterol

PCSK9

LDL Receptor

Estrogen

Menopause

Introduction

Our laboratory is interested in understanding why atherosclerosis is relatively silent in women compared to men – at least until menopause (MP) when estrogen levels plummet. Noting the relative abundant presence of estrogen receptors in the artery wall, we embarked on an objective discovery project looking for estrogen receptor associated proteins that might help explain important biological differences. These studies led to the discovery that Heat Shock Protein 27 (HSP27) is as an estrogen receptor beta associated protein. The relationship between HSP27 and estrogen is complex, as HSP27 acts as a repressor of estrogen-mediated transcription *in vitro*, yet its expression and extracellular release are also partially regulated by estrogens (e.g., there is an estrogen response element found in the HSP27 promoter) [1,2]. Importantly, our group [1] and four others (using objective proteomic approaches) [3-6] showed that HSP27 expression is inversely corelated with the degree of coronary artery plaque burden.

Accordingly, we then demonstrated in a series of experiments that augmenting HSP27 levels reduces atherogenesis [7-10]. Moreover, when *ApoE*$^{-/-}$ mice that over-express HSP27 are subjected to ovariectomy (OVX) HSP27 levels are lower and athero-protection is lost, unless estrogenic therapy is administered [2]. Clinically, elevated HSP27 blood levels are associated with a lower 5-year risk of myocardial infarction, stroke or cardiovascular death [8]. In women who undergo premature surgical MP because of an inherited risk of cancer, we note that HSP27 levels drop in the first post-operative year [11].

Interestingly, natural IgG auto-antibodies to HSP27 (AAbs) are detectable in human blood, and recently we demonstrated how HSP27 immune complexes (ICs) form, dock at the cell membrane

engaging with Toll-like receptor 4 (TLR4) and compete with LPS to reduce inflammatory signaling [12]. Via NF-κB pathway activation HSP27 ICs increase the expression of the low density lipoprotein receptor (LDLR) [13]. Unlike the effect of statins, the effect of HSP27 on LDLR expression is divergent from the sterol regulatory element binding protein 2-mediated dual regulation of LDLR and Proprotein Convertase Subtilisin/Kexin type 9 (PCKS9), the negative regulator of cholesterol metabolism. Furthermore, vaccination of mice with rHSP25 (murine ortholog of human HSP27), augments anti-HSP25 antibodies, reduces atherogenesis by markedly decreasing total plasma cholesterol levels and increases LDLR expression [13]. Interestingly, with rHSP25 vaccination PCSK9 levels fall, and while this may in part be due to reduced expression, it is likely due to increased PCSK9 clearance by abundant LDLRs [13].

Therefore, in this study we sought to determine if rHSP25 vaccination can combat atherogenesis post-OVX, employing a surgical model of MP in *ApoE*$^{-/-}$ mice and using estradiol (E2) as a comparator. *In vitro*, we also explore the effects of the HSP27 IC *vs*. E2 on the expression of PCSK9. Our results indicate that rHSP25 vaccination and E2 therapy are both effective in attenuating the early stages of atherogenesis. However, we were surprised to find that E2 increases PCSK9 transcription without altering that of LDLR, while HSP27 therapy lowers cholesterol (and PCSK9) by increasing LDLR expression.

Materials and Methods

Recombinant protein preparation

Plasmids encoding HIS-tagged full-length HSP27 were used to express the following recombinant proteins: i) the mouse ortholog rHSP25, or the C-terminal, biologically inactive (rC1) fragment of HSP27 (AA93-205) (**Fig. 1A**). For *in vitro* studies involving human hepatocytes, we also generated rHSP27, as previously described [14]. Using a pET-21a vector, with the plasmids were transformed into an *Escherichia coli* expression strain (DE3). Recombinant proteins were purified with a Ni-NTA resin and Q-Sepharose™ (GE Healthcare). Endotoxin was removed by Pierce High-Capacity Endotoxin Removal Resin (ThermoFisher Scientific, Waltham, MA, USA). The purity of the final recombinant proteins was determined to be more than 99% by sodium dodecyl sulfate and polyacrylamide gel (SDS-PAGE) with an endotoxin concentration lower than 2 units/mg protein measured by Limulus Amebocyte Lysate PYROGENT™ 125 Plus (Lonza). In previous experiments we demonstrated that these recombinant proteins were functionally devoid of significant endotoxin contamination, as the addition of polymyxin B had no effect on the ability of these proteins to activate NF-κB [9,15].

Murine Surgical Model of Menopause and Atherogenesis

The following animal protocol was approved by the Animal Care and Use Committee at the University of Calgary and is in compliance with the Canadian Council on Animal Care (**Fig. 1B**). Five-week-old *ApoE*$^{-/-}$ female mice were purchased from the Jackson Laboratories (JAX®, Canada; C57BL/6 genetic background, Jackson Laboratory, Bar Harbor, ME, USA; stock 002052: B6.129P2-*ApoE*^{tm1Unc}/J) and after one week of acclimatization were randomized to

bilateral OVX *vs.* a surgical sham operation (i.e., ovaries not removed). For all surgical procedures, mice were given a mixture of isoflurane/oxygen (2.5%, 1L/min respectively). Briefly, the dorsum of each mouse was incised and both ovaries were removed. Muscle and skin layers were closed with sterile synthetic bio-absorbable sutures (Ethicon, Somerville, NJ). Buprenorphine (0.05 mg/kg subcutaneously) was administered twice-daily for 3 days post-surgery for analgesia. For the mice randomized to receive E2 supplementation, after recovering for one-week post-OVX a pellet containing 0.1 mg of E2 (60-day release formulation; Innovative Research America) was implanted subcutaneously between the ear and shoulder using a trocar. This strength of E2 pellet was selected because in a pilot experiment using higher concentration E2 pellets (0.25 mg/60-day) there was a 3-fold increase in the uterine / fallopian tube weight in the E2/OVX *vs.* rC1/OVX mice. Mice were also randomized to rHSP25 or rC1 vaccination, as well as the combination of rHSP25 vaccination plus an E2 pellet. Vaccination was performed weekly with recombinant HSP25 (rHSP25; 100 µg, 75 µL) or 'rC1', a truncated HSP27 peptide consisting of the C-terminal half of the peptide, (100 µg, 75 µL) mixed with an aluminum hydroxide gel adjuvant (Alhydrogel® 2%, Al, 25 µL; Invivogen vac-alu-250, San Diego, CA). One week after the commencement of E2 and/or vaccinations, the mice began a 5-week atherogenic High Fat Diet (HFD) containing: 1.25% cholesterol and 15.8% fat, (ENVIGO Harlan Teklad diets) until euthanasia. In addition, control experiments were conducted comparing PBS *vs.* rC1 treatments in sham and OVX mice (**Supplemental Fig. 1A**).

Mice were housed (n=3 or 4 per cage) in a temperature controlled (20ºC) facility with a 12 h light/ dark cycles and had free access to autoclaved food and water. A total of 64 mice were randomized to 8 groups (8 mice/group). Plasma samples were collected weekly and animal

weight recorded prior to treatment administration. At euthanasia, mice were placed under anesthesia with isoflurane/oxygen mixture (5%, 1L/min) and adequate sedation was verified by toe and/or tail pinching to ensure absence of a withdrawal reflex response. The thoracic cavity was opened by cutting the ribs lateral to the sternum to expose the heart, and whole blood was collected by inserting a 25-gauge needle into the left ventricle. After blood collection, the right atrium pierced to permit hemorrhage, and systemic perfusion fixation with phosphate-buffered saline (PBS) thrice or until the liver turned pale in color, followed by 10% NBF. The heart and aorta were dissected, removed and immersed in NBF. The uteri and fallopian tubes were also harvested, dissected of all fat and connective tissue, photographed and weighed in order to assess the end organ effect of E2 *vs.* rHSP25 therapies.

Murine anti-HSP25 IgG antibody levels

Levels of IgG AAb were measured in murine plasma using an ELISA developed in our laboratory (**Fig. 1C**). Briefly, NUNC maxisorp plates (ThermoFisher) were coated with rHSP25 at a concentration of 500 ng/well in carbonate-bicarbonate buffer overnight (ON) at room temperature. The wells were blocked with 1% BSA/PBST, washed in phosphate buffered saline tween (PBST), and incubated with plasma at a final dilution of 1:2,000 in 1% BSA for two hrs followed by 3 more washes in PBST. A horse radish peroxidase (HRP) labeled a goat anti-mouse IgG (H&L) antibody (catalogue #115-035-062; Jackson Immunoresearch, West Grove, PA) was used as a detection antibody at a dilution of 1:5,000 and incubated for one hr at room temperature. Finally, substrate solution (3,3’,3.5’-Tetramethylbenzidine Liquid Substrate, TMB; Millipore Sigma; Oakville, ON) was added to each well and incubated for 10 mins avoiding direct light. The reaction was stopped by 2N H_2SO4 and the optical density quantified at 450 nm,

using Synergy Mx plate reader (BioTek). To establish an internal standard for the measurement of HSP25 auto-antibodies, plasma from a healthy control subject was diluted 500 times in 1% BSA/PBST and arbitrarily defined as 50 absorbance units (a.u.).

Evaluation of *en face* Atherosclerotic Lesion Area

The aorta was dissected from the ascending to the thoracic segments ending at the 4th bronchial branches and the adventitia was removed. The primary incision followed the lesser curvature of the arch opening up the aorta longitudinally. A second incision was made along the greater curvature of the arch down to the level of the left subclavian artery. The aortic lipid content was evaluated using oil red O staining (described previously) [10] and photographed using an Olympus SZ-61 stereo microscope with an Olympus UC30 camera (aortic magnification ×12; **Fig. 2A, Supplemental Fig. 1B**). The *en face* atherosclerotic aortic lesions were analyzed using Image-Pro software (Media Cybernetics) to calculate the total and atherosclerotic lesion areas. The extent of atherosclerosis was expressed as the percentage of surface area of the entire aorta covered by atherosclerotic lesions [8].

Murine Plasma Cholesterol Profiles and Lipoprotein Fractions

Total plasma cholesterol levels were determined using an enzymatic colorimetric assay kit (Wako Pure Chemical Industries, Ltd, 439-17501) (**Fig. 2C, Supplemental Fig. 1D, 2C, 2D**). The lipoprotein cholesterol distribution profile (VLDL, LDL, HDL) was evaluated in pooled plasma samples under fasting conditions after fractionation by fast protein liquid chromatography (FPLC) gel filtration on a single Superose 6 column (**Fig. 2D**).

LDLR and PCSK9 Gene Expression in Murine Liver Tissue

As previously described, RNA was isolated from mouse liver tissue and used for Real-time PCR analysis of LDLR and PCSK9 gene expression (**Fig. 3A, 4B**).[16] The specific murine primer sequence pairs, including those for the control gene, hypoxanthine-phosphoribosyltransferase (hHPRT1), are provided in **Supplemental Table 1**. RNA expression is presented as log fold change.

LDLR Protein Expression in Murine Liver Tissue

Protein was harvested from the murine livers for SDS-PAGE. Multiple Western blots were obtained for each protein assayed, and representative data are shown in **Fig. 3B, 3C**. Mouse liver tissue (200-300 mg) was placed inside a Precellys® homogenizing tube with 4 glass beads and 500 µL of RIPA lysis buffer (50 mM Tris HCL pH 7.4, 1% Triton X-100, 0.5% Sodium Deoxycholate, 150 mM NaCl, 2mM EDTA, protease inhibitor cocktail). Tissue was homogenized during two 30 s cycles at 6,000 RPM. The lysate was transferred to 2 mL tubes and further processed using a handheld sonicator at 50% amplitude for two 3 s pulses, centrifuged 45 mins at 16,000×g, and the supernatant layer below the fat and above the pellet was transferred to a new tube. Samples were centrifuged again for 10 mins at 16,000×g and the supernatants were transferred to new tubes. Protein lysates were quantified using a Bio-Rad DC Protein Assay, with equal amounts of protein (40-80 µg per well) loaded onto 10% Tris-Glycine gels for SDS-PAGE before being transferred to 0.2 µm nitrocellulose membranes ON at 4°C. Membranes were blocked in 5% Milk in TBS-Tween for 1h, washed, incubated in fresh primary antibodies with 5% bovine serum albumin (BSA) ON at 4°C, washed, and incubated in secondary antibody before ECL detection using an ImageQuant LAS4000 imager (GE Healthcare). The primary

antibodies used for Western Blots prepared using murine liver lysates were rabbit anti-LDLR (1:5,000; Abcam ab52818) and mouse anti-β-actin (1:10,000; sc-47778, Santa Cruz Biotechnology). The secondary antibodies used for WB were: anti-rabbit IgG HRP (Abcam ab97051) and anti-mouse IgG HRP (Jackson Immunoresearch 115-035-062).

PCSK9 Measurement

Plasma levels of PCSK9 (**Fig. 4A**) were measured using an ELISA kit specific to murine PCSK9 (R&D, DY3985) as per manufacturer's instructions. Samples were diluted 1:100 in 1% BSA and a PCSK9 standard curve was generated for each experiment.

PCSK9 Promoter Activity Assays

Cells from the human hepatic HepG2 cell line were grown in complete media (with 10% FBS and antibiotics) and seeded ON at 4×10^4 cells/well in a 48-well culture plates, then: i) transiently transfected with a PCSK9 promoter firefly luciferase reporter construct, incorporated in the plasmids pGL3-PCSK9-D1 [17], and ii) co-transfected with a transfection control vector (pBLSV40 plasmid constitutively expressing a *Renilla* luciferase; generously donated by Dr. David Proud University of Calgary, Calgary, AB), so that the dual luciferase activities could be measured. The stoichiometry was: 500 ng of the PCSK9-D1 promoter construct and 25 ng of pBLSV40 vector, using 1 μL of Lipofectamine®2000 Transfection Reagent (ThermoFisher). Cells were then treated with recombinant proteins (rHSP27, rC1), antibodies (unrelated IgG control, PAb), and combinations thereof for 24 hrs. PCSK9 promoter-driven firefly luciferase activity and control renilla luciferase activity were measured using the Firefly & Renilla Luciferase Single Tube Assay Kit (#30081-1; Biotium, Fremont, CA) according to the

manufacturer's protocol, with some minor modifications. Briefly, cells were washed with PBS then lysed in 110 μL of passive lysis buffer. Firefly luciferase activity was measured first by combining 30 μL of cell lysate with 50 μL of firefly working solution in a 12×75mm round-bottom tube, measuring luminescence, then adding 5 μL of *Renilla* working solution to the same tube and again measuring luminescence. Luminescence was detected using a Monolight 3010 Luminometer (BD Biosciences). Absolute firefly luciferase activity was normalized against *Renilla* luciferase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition, and 3 independent transfection assays were performed for each reporter construct. The data was log transformed in order to facilitate parametric statistical analyses (**Fig. 4C, 4D**).

Statistical analysis

For all data, continuous variables are expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed by using a one-way ANOVA (Graph Pad Prism 8, La Jolla,CA, USA) and Tukey's comparison of the groups. The direct comparison of unpaired treatment groups was performed using a Student's t-test. Quantitative PCR gene expression data were log-transformed and are reported as log-fold change in order to more normally distribute the data and permit parametric statistical analyses. A p-value < 0.05 was considered significant.

Results

a) Overview of rHSP25 Vaccination *vs*. E2 Therapy Experiment in OVX mice

To test the hypothesis that rHSP25 vaccination reduces atherogenesis in OVX mice, we subjected atherosclerosis-prone *ApoE*$^{-/-}$ mice to OVX, followed by 6 weekly subcutaneous injections with rHSP25 with the adjuvant Alum (**Fig. 1A, 1B**). Another group of mice underwent a sham surgery (the ovaries were not removed) followed by rHSP25 vaccination. For comparison with the clinical scenario of hormonal therapy for MP, a group of OVX *ApoE*$^{-/-}$ mice underwent subcutaneous implantation of a slow release E2 pellet, while another group received both E2 and rHSP25 vaccination. The inactive truncated C-terminus of HSP27, rC1 (mixed with Alum), was administered as a negative control to OVX or sham surgery mice. All mice (7-8 per group) received a HFD for 5 weeks, beginning one week after the initiation of therapies.

b) **Validation of the Vaccination response**

The rise in IgG anti-HSP25 levels were similar for all mice vaccinated with rHSP25 whether it was combined with E2 or not (**Fig. 1C**). The antibody response to vaccination with rC1 was similar to that with rHSP25, thereby indicating that rC1 remained immunogenic. E2 or PBS treated mice had very low (background) levels of AAbs.

c) **Validation of the Murine Model of Menopause and Physiological Estrogen Therapy**

We confirmed that this OVX model was appropriate for the study of MP atherogenesis by showing that OVX increased atherosclerotic burden and cholesterol levels. *ApoE*$^{-/-}$ mice subjected to OVX had more aortic atherosclerosis than sham operated mice [e.g., PBS sham *vs.* PBS OVX: 45% increase (11.8 ± 1.2 vs. 17.1 ± 1.7; respectively, p=0.03); and rC1 OVX

compared to rC1 sham: 65% increase (18.2 ± 1.9 *vs*. 11.0 ± 0.7; respectively, p=0.006)] **(Supplemental Fig. 1B, 1C)**. Similarly, total plasma cholesterol levels 1-day pre-euthanasia were higher in OVX mice compared to sham operated mice [e.g., PBS/sham compared to PBS/OVX: 23% higher (1,119 ± 32 *vs*. 1,374 ± 34; respectively, p=0.0001), and rC1/sham compared to rC1/OVX: 21% higher (1,136 ± 23 *vs*. 1,369 ± 55; respectively, p=0.002)] **(Supplemental Fig. 1D)**. As measurement of estrogen levels in mice is technically challenging, we confirmed that the estrogenic therapy in the E2 pellet (0.1 mg/60 day) provided a physiological level of hormone replacement by examining the uteri at the end of the experiment. The uterine weights of the E2 treated mice were similar to those of the sham mice, thereby confirming that the E2 dose was likely in a physiological range (**Fig. 1D, 1E**). In contrast, the rHSP25/OVX uteri were similar to those of the rC1/OVX mice showing 4.5-fold atrophy *vs*. rC1/sham mice uteri; thus, indicating that rHSP25 had no direct or indirect estrogenic properties.

d) **Validation of rC1 as a Negative Control Treatment in OVX / Atherogenesis Model**

Four control groups (PBS/sham, rC1/sham, PBS/OVX and rC1/OVX) were used to assess the validity of using rC1 as a negative control. rC1 is an excellent control treatment as it is prepared in an identical manner as rHSP25 using recombinant technology in *E. coli*. Although these recombinant proteins are treated to reduce endotoxin contaminants to very low levels, if there is residual endotoxin present in one protein it is balanced by the same risk in the other protein. The degree of atherosclerosis was similar for the sham operated mice that received PBS *vs.* rC1 (11.8 ± 1.19 *vs*. 11.03 ± 0.65 respectively; p=0.58), as well as the OVX mice that received PBS *vs*. rC1 (17.1 ± 1.67 *vs.* 18.2 ± 1.93; respectively, p=0.68) (**Supplemental Fig. 2A, 2B**). Likewise, cholesterol levels were similar amongst the PBS sham *vs.* rC1 sham mice (1,119 ± 31.64 *vs.*

1,136 ± 22.83; respectively, p=0.67) and the PBS OVX *vs*. rC1 OVX mice (1,374 ± 33.75 *vs*. 1,369 ± 54.88; respectively, p= 0.94) (**Supplemental Fig. 2C, 2D**). Therefore, for the purpose of comparing the effect of the active treatment groups (rHSP25 or E2), the rC1/OVX group was used as the reference group for the effect of OVX, as the degree of atherogenesis as well as the cholesterol levels were similar to those found in the PBS/OVX cohort.

e) **rHSP25 Vaccination and E2 Therapy Post-OVX are Comparable for Attenuation of Atherogenesis and Lowering Cholesterol**

Mice subjected to OVX alone (rC1/OVX) had 51% more aortic atherosclerosis compared to non-OVX (rC1/sham) mice (16.7 ± 1.4 *vs.* 11.0 ± 0.6; **Fig. 2A, 2B)**. The atherosclerotic burden was similar in the rHSP25/OVX and E2/OVX mice, and markedly reduced compared to rC1/OVX (p<0.0001). Interestingly, the combination of [rHSP25 + E2] in the OVX mice showed the lowest atherosclerotic burden (-82% *vs*. rC1/OVX; p<0.0001). Finally, the atherosclerotic burden was lower in the rHSP25/sham *vs.* rC1/sham mice (p=0.04).

While the baseline cholesterol levels in all mice fed a normal chow diet were similar (515 ± 82 mg/dL), by the second week of the HFD the levels peaked and then fell by varying degrees over the ensuing 3 weeks of the HFD. Terminal plasma cholesterol levels (**Fig. 2C**) were measured 1-day prior to euthanasia, with the highest levels found in the rC1/OVX group (1,393 ± 57 mg/dl), which was 21% higher (p<0.0001) than the rC1/sham mice (1,148 ± 23 mg/dl). Both the rHSP25/OVX and E2/OVX mice had similar cholesterol levels that were lower than the rC1/OVX mice (1,068 ± 8 and 964 ± 27; both p<0.0001 *vs.* rC1/OVX; respectively), while the lowest levels were found in the combined therapy [rHSP25 + E2/OVX] mice (736 ± 24;

p<0.0001 *vs.* all OVX groups). Cholesterol levels were lower in the rHSP25/sham compared to rC1/sham mice (p<0.0001). As demonstrated by FPLC, the LDL-C fraction was reduced in both the E2/OVX and rHSP25/OVX groups relative to the rC1/OVX mice (**Fig. 2D**).

f) **Unlike rHSP25 Vaccination, E2 Therapy Did not Increase LDLR Expression Post-OVX**

rHSP25 alone or in combination with E2 markedly increased hepatic LDLR mRNA expression (+219% for the rHSP25/OVX *vs.* rC1/OVX and +165% for the [rHSP25 + E2] *vs*. E2/OVX mice; p<0.0001 for both; **Fig. 3A**). LDLR protein expression also increased in the mice vaccinated with rHSP25 (50% for the rHSP25/OVX *vs*. rC1/OVX, 51% for the [E2 + rHSP25/OVX] *vs*. E2/OVX; p=0.04 and p=0.006, respectively; **Fig. 3B, 3C**). Surprisingly, E2 therapy in isolation had no effect on LDLR mRNA or protein expression.

g) **E2 but Not rHSP25 Vaccination Increases PCSK9 Gene Expression**

Plasma PCSK9 levels were much higher in E2/OVX (464 ± 13 ng/ml) compared to the other four treatment groups (*p*<0.0001) – including a 52% increase relative to rC1/OVX (**Fig. 4A**). Combining [rHSP25 + E2] therapy post-OVX resulted in a 23% reduction in PCSK9 levels (357 ± 12 ng/ml; p=0.0001) relative to E2/OVX alone. Plasma PCSK9 levels were 26% lower in the rHSP25/sham *vs.* rC1/sham mice (p=0.046). Hepatic PCSK9 mRNA expression was 148% higher in the E2/OVX compared to rC1/OVX and all other murine treatment groups (p<0.0001) (**Fig. 4B**).

The PCSK9 promoter is thought to contain two estrogen response elements (EREs), as predicted *in silico* using the MatInspector analysis tool (optimized matrix threshold = 0.9 for both;

https://www.genomatix.de/online_help/help_matinspector/matinspector_help.html). Hence, we assessed PCSK9 promoter activity in HepG2 cells treated for 24 hrs with escalating concentrations of E2 (0.1, 10, 100nM) and noted a robust dose-dependent increase in activity (+505% with 100 nM E2, p=0.0005) that was reduced by 46% (p=0.02) with the pan estrogen receptor blocker, ICI 182,780 (**Fig. 4C**). Likewise, treating HepG2 cells for 24 hrs with rHSP27 alone (100 μg) had no effect, yet there was a 64% decrease in PCSK9 promoter activity (p=0.01) when cells were treated with [rHSP27 + PAb] (**Fig. 4D**). Moreover, there was a remarkable 303% increase in PCSK9 promoter activity with E2 treatment (100 nM; p<0.0001) that shrunk by 43% (p<0.0001) with [rHSP27 + PAb] treatment.

Discussion

Using a surgical model of MP in atherosclerosis-prone *ApoE*$^{-/-}$ mice fed a HFD we demonstrate that rHSP25 vaccination, like E2 therapy, is atheroprotective. As well, we confirmed a recent observation [13] that rHSP25 vaccination promotes the expression of the LDLR – an effect that is not associated with E2 therapy. Unlike rHSP25 vaccination, E2 therapy is associated with increased PCSK9 synthesis and plasma levels (**Fig. 4A, 4B**), and showed a dose dependent increase in PCSK9 promoter activity that was partially abrogated with estrogen receptor blockade or concomitant treatment with [rHSP27 + PAb] (**Fig. 4C, 4D**).

While it was surprising to find that E2 treatment did not increase LDLR expression, perhaps it was predictable? The specific mechanisms that account for the relative protection against atherosclerotic complications in pre-MP women are poorly understood, yet there is a common belief that one of the main cardiovascular benefits of estrogens is the lowering of LDL-C due to

an increase in LDLR expression. However, on reviewing the pre-clinical and clinical literature, the effects of estrogens on LDLR vary. For example, in rats LDLR activity and expression are reduced with OVX but improve with estrogen therapy [18,19]. But, in non-OVX mice treated with (even) supra-physiological E2 concentrations, LDLR expression does not change [20,21]. As the *ApoE*$^{-/-}$ mice in our study did not show an increase in LDLR with E2, this does not seem to be due to an inherent problem with LDLR expression that is unique to this strain of mice – as it is clear that these same mice showed a robust increase in LDLR expression in response to rHSP25 vaccination. Finally, it is interesting to note that E2 treatment is even atheroprotective in *LDLR*$^{-/-}$ mice – thereby pointing to beneficial effects of estrogens that may be at a distance from LDLR modulation [22].

Similarly, the evidence that E2 modulates LDLR expression in human hepatocytes is far from convincing [23,24]. For example, there is indirect evidence of increased LDLR binding activity in liver biopsy tissue from two male patients receiving estrogen therapy for prostate cancer [25]. The doses of estrogenic compounds administered to these men were not in a physiological range, and their cholesterol levels were not lower than the normal. Given that >1,000 human hepatic genes display a sex-bias in their expression, particularly those known to participate in or regulate lipid metabolism, and that the LDLR gene is more highly expressed in the female liver, the value of these experimental results in male subjects should be interpreted with caution [26].

PCSK9 was only discovered less than 2 decades ago, and there is a lot to learn about its transcriptional regulation, including the potential role of two estrogen response elements in its promoter. Nonetheless, the estrogen mediated upregulation of PCSK9 expression noted in the

current studies does fit with clinical observations showing that: i) PCSK9 levels are higher in pre-MP women than men [27], and ii) PCSK9 levels are markedly elevated in pregnant compared to non-pregnant age-matched women (493 *vs.* 290 ng/ml) [28]. This would fit with the fact that in the third trimester of pregnancy E2 levels increase 25-50 fold and total cholesterol levels rise 50-75% [29]. Hence, we postulate that E2 drives these increases in PCSK9 and cholesterol levels in pregnancy. Whether the surges in E2 levels that occur during the normal menstrual cycle or with exogenous estrogenic therapies have the same effects on PCSK9 levels remains to be studied. One study that does not fit with our observations on the role of E2 in upregulating PCSK9 levels is the Dallas Heart Study. Specifically, these investigators found that PCSK9 levels are higher in post- *vs.* pre-MP women, and that there was no difference in PCSK9 levels between women receiving estrogenic compounds or not [27]. However, it is not clear if the higher PCSK9 levels in the older post-MP women are clearly related to loss of ovarian function, or that the estrogenic therapy that they received was physiological. Indeed, a number of other studies suggest that PCSK9 levels simply correlate with age, and this may be difficult to discount too [30-32].

Although we did not see major differences in atheroprotection with E2 *vs*. rHSP25 therapy in vivo, we have uncovered some subtle mechanistic differences that require further consideration – particularly the increase in PCSK9 with estrogens. To summarize the results of our studies, we constructed a working hypothesis incorporating the interplay between estrogens and HSP27, as well as PCSK9 and LDLR (**Fig. 5**). Briefly, E2 upregulates PCSK9 expression, yet it is also well known that E2 increases HSP27 expression and cellular release [2,33,34]. HSP27 markedly increases LDLR expression, and given the rapid recycling of the LDLR, this increase in LDLR synthesis may overpower any change in PCSK9 levels by binding PCKS9 and subjecting it to intracellular

disposal. However, in the MP scenario estrogen levels are diminished, and while PCSK9 expression is not augmented because of this, there may also be inadequate HSP27 levels to stimulate LDLR expression and the clearance of PCSK9. As a result, LDL-C and PCSK9 levels are higher compared to pre-MP. Hence, what needs to be emphasized about these data are: i) the dramatic HSP27-induced increase in LDLR (also reported elsewhere recently)[13] that not only reduces LDL-C but also PCSK9 levels, and ii) the previously unrecognized E2-mediated increase in PCSK9 expression. As long as E2 therapy is increasing HSP27 (and therefore LDLR) levels to the same extent there will be clearance of PCSK9 and a balanced lipoprotein profile. However, we do not know if this is uniformly the case for all estrogenic compounds (e.g., there may estrogen receptor specific subtleties with regards to LDLR and PCSK9 expression that have yet to be uncovered). Further study is required, particularly if PCSK9 expression may be increased by some estrogenic compounds, and therefore represent a cardiovascular or other hazard.

Currently, we are working towards understanding if this working hypothesis can be further evaluated in humans, and explore the virtues of estrogen receptor specific modulators [11,34]. As well, we are trying to better comprehend how HSP27 (or HSP25) alone or in combination with its autoantibody, modulate LDLR and PCSK9 transcription. We do know that in the presence of AAbs, HSP27 shows enhanced docking at the cell membrane, interacts with cell membrane receptors and signaling pathways, and can undergo internalization [12]. As low HSP27 and AAb levels are associated with atherosclerosis,[13] we want to develop immunotherapies that boost AAb levels in order to potentiate the biological activity of existing (low) levels of HSP27, especially in women post-MP.

Acknowledgments:

We are indebted to the Libin Cardiovascular Institute of Alberta and its community partners for supporting the O'Brien Vascular Biology Laboratory, as well as the staff in the University of Calgary Cumming School of Medicine Health Sciences Animal Resource Centre.

Author Contributions

N. Maarouf and E.R. O'Brien designed the studies. N. Maarouf performed all *in vivo* experiments. N. Maarouf, Y.-X. Chen, C. Shi, J. Deng, C. Diao, M. Rosin, V. Shrivastava performed or contributed to some of the *in vitro* and *ex vivo* research; N. Maarouf, C. Diao and J. Liu performed or contributed to the design and analysis of the promoter studies; N. Maarouf, V. Shrivastava, Z. Batulan and E.R. O'Brien analyzed all other data; N. Maarouf and E.R. O'Brien wrote the manuscript.

Conflict of Interest

EOB and YXC are inventors on US patents 8343915B2 and 8343916B2; EOB, CS and YXC are inventors on US patent application PCT/CA2016/051018; EOB is the inventor on US patent application 63/031,6402020; all intellectual property pertains to HSP27 diagnostics / therapeutics. EOB is the Scientific Co-Founder of Pemi31 Therapeutics Inc., a start-up company that controls the aforementioned intellectual property. EOB, CS and YXC have equity interests in Pemi31 Therapeutics Inc.

Figure Legends

Fig. 1. rHSP25 vaccination to prevent atherogenesis in ovariectomized *ApoE*$^{-/-}$ mice.

A) Schematic representation of rHSP25 (mouse ortholog of HSP27) and the biologically inactive truncated C-terminal of HSP27 (rC1 control) proteins. The black box denotes the alpha beta-crystallin domain that is important for protein oligomerization. The IXI box at the C-terminus is a flexible domain involved in the formation of multiple inter-subunit interactions.

B) Age matched, sexually mature female *ApoE*$^{-/-}$ mice were subjected to either OVX or sham operation (no OVX). One week later, they were randomized to the following treatments: rC1/OVX, rHSP25/OVX, E2/OVX, rHSP25+E2/OVX, rHSP25/sham and rC1/sham. A HFD was initiated one week later and continued for 5 weeks until euthanasia. There were 7-8 mice per group. See Supplemental Fig. 1 and 2 for additional control mice data.

C) Anti-HSP25 IgG levels increased in all mice vaccinated with either rHSP25 or rC1. The initiation of rHSP25 vaccination began at 7 weeks of age (one-week post-surgery) and measurements were taken every week thereafter, rising to a plateau by 3-4 weeks after the first vaccination and continuing at this level until euthanasia. Data are displayed as a.u. for OD @ 450 nm.

D) Representative photographs of uteri and fallopian tubes from each treatment groups.

Fig. 1. (continued)

E) Combined uterine and fallopian tube weights at time of euthanasia. After OVX there was marked atrophy of the uteri and fallopian tubes of mice in the rC1/OVX and rHSP25/OVX groups compared to rC1/sham mice. There was no difference in weights between the rC1/sham and the other groups (E2/OVX, [E2 + rHSP25/OVX], rHSP25/sham), thereby suggesting that the E2 supplementation was in a physiological hormonal range.

Fig. 2. Atherosclerosis and Hyperlipidemia after surgical MP

A) Photomicrographs of *en face* thoracic aortae stained with Oil Red O.

B) Quantitation of aortic atherosclerotic burden, as reflected by Oil Red O staining. Compared to the rC1 sham group there was an increase in aortic lesion area with rC1/OVX. Separately, rHSP25 and E2 reduced aortic atherosclerotic burden compared to rC1/OVX, but the largest decrease was noted with combined [E2+RHSP25] therapy. The atherosclerotic burden was lower in the rHSP25/sham *vs.* rC1/sham mice ($p=0.04$).

C) OVX resulted in an increase in total plasma cholesterol in the rC1 mice. Compared to rC1/OVX, cholesterol levels were lower post-OVX with either rHSP25 or E2 therapies, and lowest with combined [rHSP25+E2] treatment. Cholesterol levels in the rHSP25/sham *vs.* rC1/sham mice ($p<0.0001$).

D) Cholesterol measured in FPLC plasma fractions from pooled blood samples from rC1/OVX, rHSP25/OVX and E2/OVX group *ApoE*$^{-/-}$ mice (n=8 per group). Lipid sub-fractions based on size are indicated above the cholesterol graphs. Compared to the rC1/OVX mice, there are reductions in the LDL subfraction in both rHSP25/OVX and E2/OVX groups.

Fig. 3 rHSP25 and E2 differentially regulate of LDLR

A) Compared to rC1/OVX mice, hepatic LDLR mRNA increased dramatically in the rHSP25/OVX but not the E2/OVX mice. In the OVX mice, adding rHSP25 to E2 noticeably increased hepatic LDLR mRNA expression compared to the E2/OVX group. There was a marked increase in LDLR expression in the rHSP25/sham *vs.* the rC1/sham mice.

B) Hepatic LDLR protein expression in the OVX experiment involving *ApoE*$^{-/-}$ mice, assessed by Western blotting. Each LDLR protein band was normalized using β-actin as the loading control.

C) LDLR protein expression was similar in the rC1/sham, rC1/OVX and E2/OVX mice, but appreciably increased with rHSP25 vaccination or combined [rHSP25 + E2] treatment post-OVX. LDLR protein expression was similarly increased in the rHSP25/sham *vs.* rC1/sham mice.

Fig. 4 rHSP25 and E2 differentially regulate of PCSK9

A) Plasma PCSK9 protein levels increased with E2 treatment in the OVX mice, an effect that was attenuated when co-treated with rHSP25. rHS25 vaccination (alone) had no appreciable effects on plasma PCSK9 levels with or without OVX.

B) Hepatic PCSK9 mRNA expression dramatically increased with E2 treatment (alone), but did not change when co-treated with rHSP25. Hepatic PCSK9 mRNA levels were unchanged in rHSP25 vaccinated mice and were unaltered by OVX.

C) PCSK9 promoter activity in HepG2 cells was strongly upregulated by E2 in a dose dependent manner, an effect that was partially attenuated with the pan-ER blocker, ICI 182,780.

D) Compared to control, rHSP27+PAb significantly reduced PCSK9 promoter activity, while E2 markedly upregulated PCSK9 promoter activity – an effect that was partially reduced by adding treatment with rHSP27.

Fig. 5. Working Model for HSP27 IC Modulation of PCSK9 and LDL-R Pre- and Post-Menopause

A) Pre-MP estrogen upregulate both PCSK9 and HSP27 expression. However, HSP27 combines with its auto-antibody (AAb) to augment LDLR expression that then facilitates PCSK9 clearance. The HSP27 IC also has a direct effect on PCSK9 expression. The net effects are reduced LDL-C and PCSK9 levels.

B) Post-MP estrogen levels plummet and there is no increase in HSP27 to modulate LDLR and PCKS9, resulting in elevated LDL-C and PCSK9 levels.

References

1. Miller H, Poon S, Hibbert B, Rayner K, Chen YX, O'Brien ER. Modulation of estrogen signaling by the novel interaction of heat shock protein 27, a biomarker for atherosclerosis, and estrogen receptor beta. *Arteriosclerosis Thrombosis and Vascular Biology*. 2005;25:E10-E14

2. Rayner K, Sun J, Chen YX, McNulty M, Simard T, Zhao X, Wells DJ, de Belleroche J, O'Brien ER. Heat shock protein 27 protects against atherogenesis via an estrogen-dependent mechanism: Role of selective estrogen receptor beta modulation. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:1751-1756

3. Martin-Ventura JL, Duran MC, Blanco-Colio LM, Meilhac O, Leclercq A, Michel JB, Jensen ON, Hernandez-Merida S, Tunon J, Vivanco F, Egido J. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. *Circulation*. 2004;110:2216-2219

4. Park HK, Park EC, Bae SW, Park MY, Kim SW, Yoo HS, Tudev M, Ko YH, Choi YH, Kim S, Kim DI, Kim YW, Lee BB, Yoon JB, Park JE. Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. *Circulation*. 2006;114:886-893

5. Lepedda AJ, Cigliano A, Cherchi GM, Spirito R, Maggioni M, Carta F, Turrini F, Edelstein C, Scanu AM, Formato M. A proteomic approach to differentiate histologically classified stable and unstable plaques from human carotid arteries. *Atherosclerosis*. 2009;203:112-118

6. Liang W, Ward LJ, Karlsson H, Ljunggren SA, Li W, Lindahl M, Yuan XM. Distinctive proteomic profiles among different regions of human carotid plaques in men and women. *Sci Rep*. 2016;6:26231

7. Rayner K, Chen YX, McNulty M, Simard T, Zhao X, Wells DJ, de Belleroche J, O'Brien ER. Extracellular release of the atheroprotective heat shock protein 27 is mediated by estrogen and competitively inhibits acldl binding to scavenger receptor-a. *Circulation research*. 2008;103:133-141

8. Seibert TA, Hibbert B, Chen YX, Rayner K, Simard T, Hu T, Cuerrier CM, Zhao X, de Belleroche J, Chow BJ, Hawken S, Wilson KR, O'Brien ER. Serum heat shock protein 27 levels represent a potential therapeutic target for atherosclerosis: Observations from a human cohort and treatment of female mice. *J Am Coll Cardiol*. 2013;62:1446-1454

9. Raizman JE, Chen YX, Seibert T, Hibbert B, Cuerrier CM, Salari S, Zhao X, Hu T, Shi C, Ma X, Simard T, Caravaggio J, Rayner K, Bowdish D, Moore K, O'Brien ER. Heat shock protein-27 attenuates foam cell formation and atherogenesis by down-regulating scavenger receptor-a expression via nf-kappab signaling. *Biochimica et biophysica acta*. 2013;1831:1721-1728

10. Cuerrier CM, Chen YX, Tremblay D, Rayner K, McNulty M, Zhao X, Kennedy CR, de BJ, Pelling AE, O'Brien ER. Chronic over-expression of heat shock protein 27 attenuates atherogenesis and enhances plaque remodeling: A combined histological and mechanical assessment of aortic lesions. *PLoS ONE*. 2013;8:e55867

11. Batulan Z, Maarouf N, Shrivastava V, O'Brien E. Prophylactic salpingo-oophorectomy & surgical menopause for inherited risks of cancer: The need to identify biomarkers to assess the theoretical risk of premature coronary artery disease. *Women's Midlife Health*. 2018;4:7

12. Shi C, Deng J, Chiu MH, Chen Y-X, O'Brien ER. Heat shock protein 27 immune complex altered signaling and transport (icast): Novel mechanisms of attenuating inflammation. *bioRxiv*. 2020:2020.2005.2031.126581

13. Chen Y-X, Shi C, Deng J, Diao C, Maarouf N, Rosin M, Shrivastava V, Hu A, Bharadwa S, Adijiang A, Ulke-Lemee A, Gwilym BL, Hellmich A, Malozzi C, Batulan Z, Dean JLE, Ramirez FD, Liu J, Gerthoffer WT, O'Brien ER. Heat shock protein 27 immune complex upregulates ldlr expression thereby reducing plasma cholesterol and atherogenesis. *bioRxiv*. 2020:2020.2005.2021.102350

14. Chiu MH, Shi C, Rosin M, Batulan Z, O'Brien ER. Biophysical analyses and functional implications of the interaction between heat shock protein 27 and antibodies to hsp27. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 2019

15. Salari S, Seibert T, Chen YX, Hu T, Shi C, Zhao X, Cuerrier CM, Raizman JE, O'Brien ER. Extracellular hsp27 acts as a signaling molecule to activate nf-kappab in macrophages. *Cell Stress Chaperones*. 2013;18:53-63

16. Pulakazhi Venu VK, Adijiang A, Seibert T, Chen YX, Shi C, Batulan Z, O'Brien ER. Heat shock protein 27-derived atheroprotection involves reverse cholesterol transport that

is dependent on gm-csf to maintain abca1 and abcg1 expression in apoe(-/-) mice. *FASEB J*. 2017;31:2364-2379

17. Li H, Dong B, Park SW, Lee HS, Chen W, Liu J. Hepatocyte nuclear factor 1 alpha plays a critical role in pcsk9 gene transcription and regulation by the natural hypocholesterolemic compound berberine. *J Biol Chem*. 2009;284:28885-28895

18. Kovanen PT, Brown MS, Goldstein JL. Increased binding of low density lipoprotein to liver membranes from rats treated with 17 alpha-ethinyl estradiol. *J Biol Chem*. 1979;254:11367-11373

19. Ngo Sock ET, Chapados NA, Lavoie JM. Ldl receptor and pcsk9 transcripts are decreased in liver of ovariectomized rats: Effects of exercise training. *hmr*. 2014;46:3-3

20. Srivastava RAK, Baumann D, Schonfeld G. In vivo regulation of low□density lipoprotein receptors by estrogen differs at the post□transcriptional level in rat and mouse. *European Journal of Biochemistry*. 1993;216:527-538

21. van Vlijmen BJ, van 't Hof HB, Mol MJ, van der Boom H, van der Zee A, Frants RR, Hofker MH, Havekes LM. Modulation of very low density lipoprotein production and clearance contributes to age- and gender- dependent hyperlipoproteinemia in apolipoprotein e3-leiden transgenic mice. *J Clin Invest*. 1996;97:1184-1192

22. Shelton KA, Cline JM, Cann JA. 17-β estradiol reduces atherosclerosis without exacerbating lupus in ovariectomized systemic lupus erythematosus-susceptible ldlr−/− mice. *Atherosclerosis*. 2013;227:228-235

23. Semenkovich CF, Ostlund RE, Jr. Estrogens induce low-density lipoprotein receptor activity and decrease intracellular cholesterol in human hepatoma cell line hep g2. *Biochemistry*. 1987;26:4987-4992

24. Wade DP, Knight BL, Soutar AK. Hormonal regulation of low-density lipoprotein (ldl) receptor activity in human hepatoma hep g2 cells. Insulin increases ldl receptor activity and diminishes its suppression by exogenous ldl. *Eur J Biochem*. 1988;174:213-218

25. Angelin B, Olivecrona H, Reihnér E, Rudling M, Ståhlberg D, Eriksson M, Ewerth S, Henriksson P, Einarsson K. Hepatic cholesterol metabolism in estrogen-treated men. *Gastroenterology*. 1992;103:1657-1663

26. Zhang Y, Klein K, Sugathan A, Nassery N, Dombkowski A, Zanger UM, Waxman DJ. Transcriptional profiling of human liver identifies sex-biased genes associated with polygenic dyslipidemia and coronary artery disease. *PLOS ONE*. 2011;6:e23506

27. Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. Genetic and metabolic determinants of plasma pcsk9 levels. *J Clin Endocrinol Metab*. 2009;94:2537-2543

28. Peticca P, Raymond A, Gruslin A, Cousins M, Adetola E, Abujrad H, Mayne J, Ooi TC. Human serum pcsk9 is elevated at parturition in comparison to nonpregnant subjects while serum pcsk9 from umbilical cord blood is lower compared to maternal blood. *ISRN Endocrinol*. 2013;2013:341632

29. Abbassi-Ghanavati M, Greer LG, Cunningham FG. Pregnancy and laboratory studies: A reference table for clinicians. *Obstetrics & Gynecology*. 2009;114:1326-1331

30. Cui Q, Ju X, Yang T, Zhang M, Tang W, Chen Q, Hu Y, Haas JV, Troutt JS, Pickard RT, Darling R, Konrad RJ, Zhou H, Cao G. Serum pcsk9 is associated with multiple metabolic factors in a large han chinese population. *Atherosclerosis*. 2010;213:632-636

31. Ruscica M, Ferri N, Fogacci F, Rosticci M, Botta M, Marchiano S, Magni P, D'Addato S, Giovannini M, Borghi C, Cicero AFG, Brisighella Heart Study G. Circulating levels of proprotein convertase subtilisin/kexin type 9 and arterial stiffness in a large population sample: Data from the brisighella heart study. *Journal of the American Heart Association*. 2017;6:e005764

32. Jeenduang N. Circulating pcsk9 concentrations are increased in postmenopausal women with the metabolic syndrome. *Clin Chim Acta*. 2019

33. Shi C, Ulke-Lemee A, Deng J, Batulan Z, O'Brien ER. Characterization of heat shock protein 27 in extracellular vesicles: A potential anti-inflammatory therapy. *FASEB J*. 2019;33:1617-1630

34. Sun J, Ma X, Chen YX, Rayner K, Hibbert B, McNulty M, Dhaliwal B, Simard T, Ramirez D, O'Brien E. Attenuation of atherogenesis via the anti-inflammatory effects of the selective estrogen receptor beta modulator 8beta-ve2. *Journal of Cardiovascular Pharmacology*. 2011;58:399-405

Fig. 1

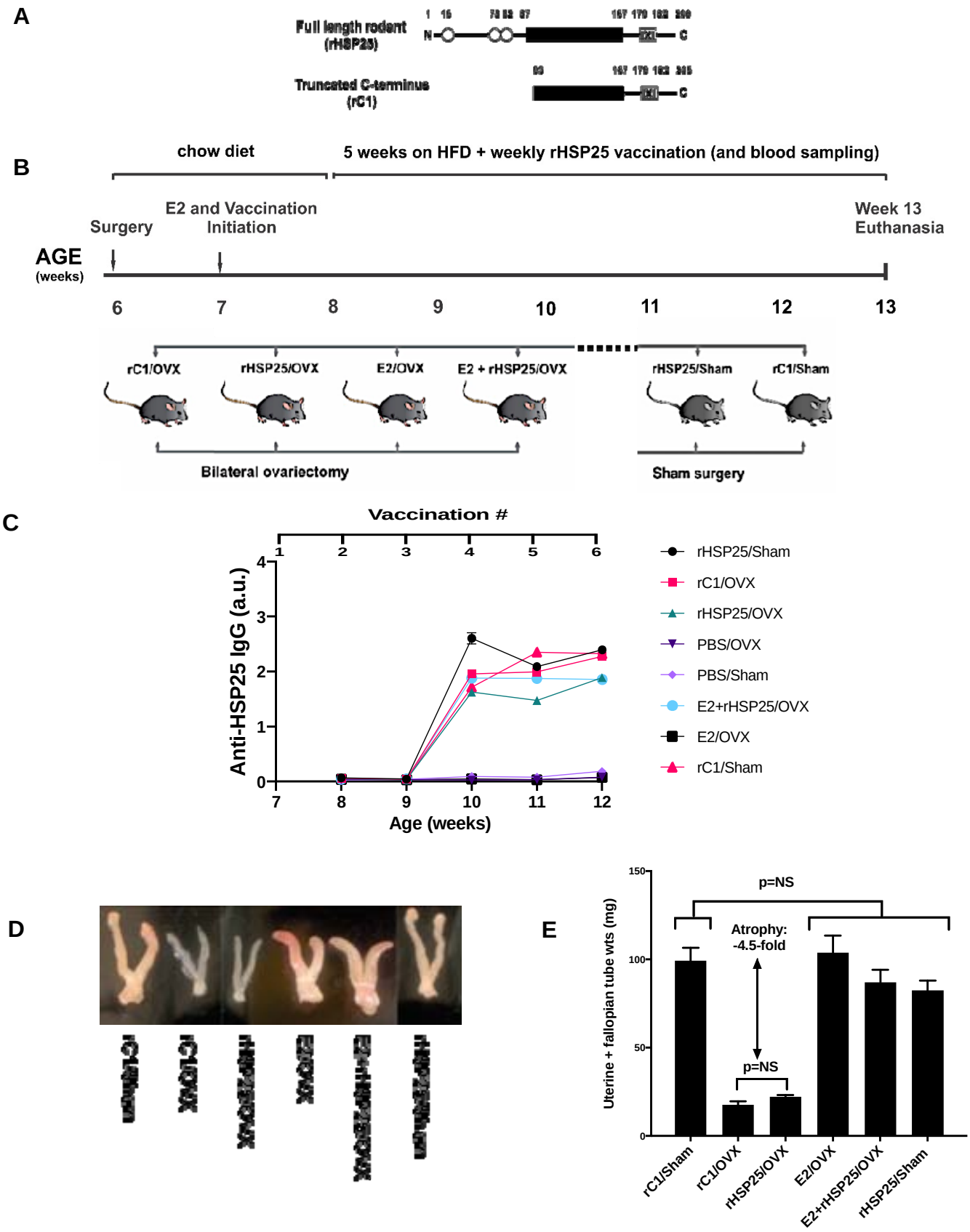
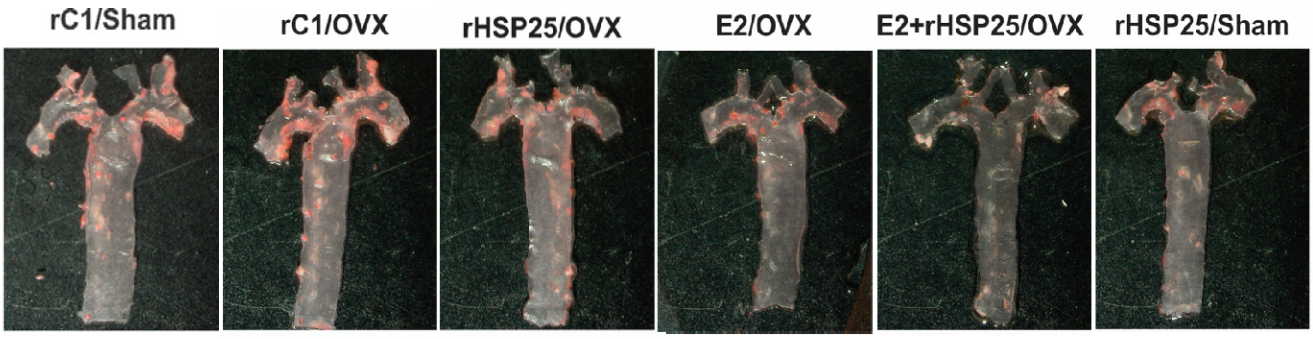

A
Full length rodent (rHSP25)
Truncated C-terminus (rC1)
B
chow diet
5 weeks on HFD + weekly rHSP25 vaccination (and blood sampling)
Surgery
E2 and Vaccination Initiation
Week 13 Euthanasia
AGE (weeks)
6
7
8
9
10
11
12
13
rC1/OVX
rHSP25/OVX
E2/OVX
E2 + rHSP25/OVX
rHSP25/Sham
rC1/Sham
Bilateral ovariectomy
Sham surgery
C
Vaccination #
Anti-HSP25 IgG (a.u.)
Age (weeks)
rHSP25/Sham
rC1/OVX
rHSP25/OVX
PBS/OVX
PBS/Sham
E2+rHSP25/OVX
E2/OVX
rC1/Sham
D
E
Uterine + fallopian tube wts (mg)
p=NS
Atrophy: -4.5-fold
p=NS
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2+rHSP25/OVX
rHSP25/Sham

Fig. 2

A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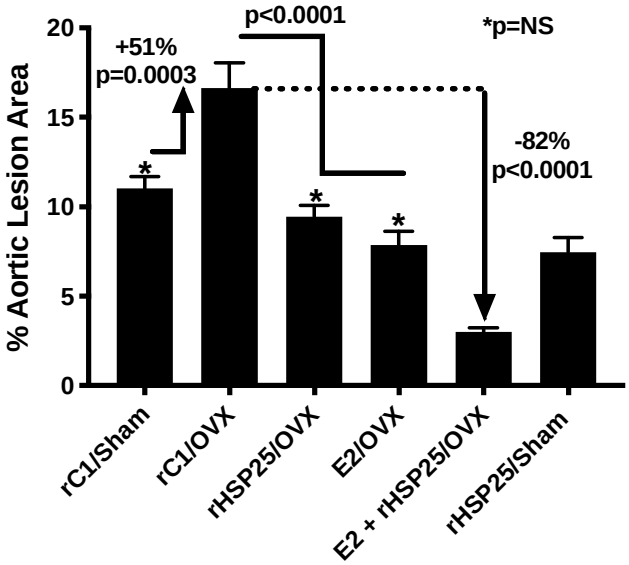
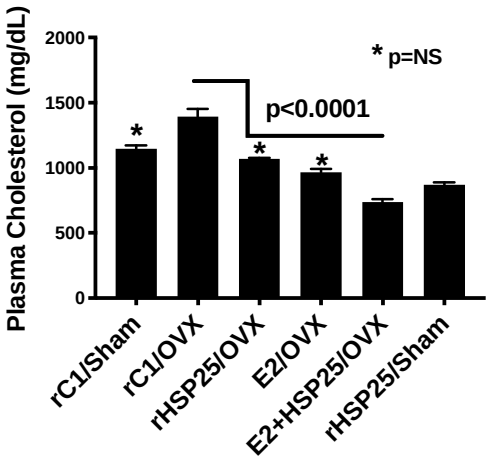
rC1/OVX
rHSP25/OVX
E2/OVX
E2+rHSP25/OVX
rHSP25/Sham

B


% Aortic Lesion Area
20
15
10
5
0
+51%
p=0.0003
p<0.0001
*p=NS
-82%
p<0.0001
*
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2 + rHSP25/OVX
rHSP25/Sham

C


Plasma Cholesterol (mg/dL)
2000
1500
1000
500
0
* p=NS
p<0.0001
*
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2+HSP25/OVX
rHSP25/Sham

D

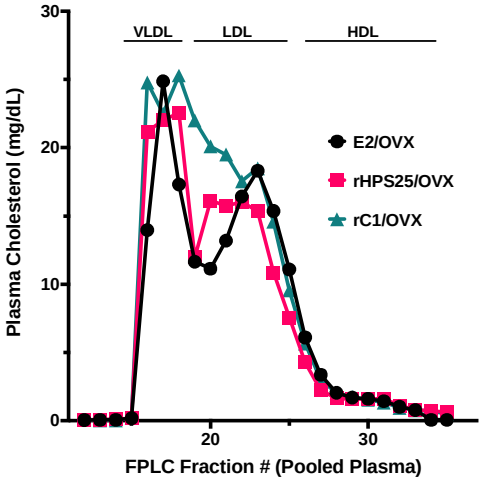
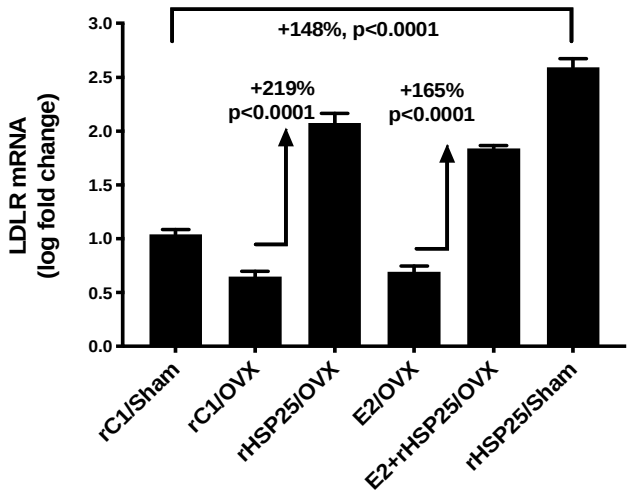

Plasma Cholesterol (mg/dL)
30
20
10
0
VLDL
LDL
HDL
E2/OVX
rHPS25/OVX
rC1/OVX
20
30
FPLC Fraction # (Pooled Plasma)

Fig. 3

A


+148%, p<0.0001
+219%
p<0.0001
+165%
p<0.0001
LDLR mRNA
(log fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2+rHSP25/OVX
rHSP25/Sham

B

LDLR
actin
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2+rHSP25/OVX
rHSP25/Sham

C

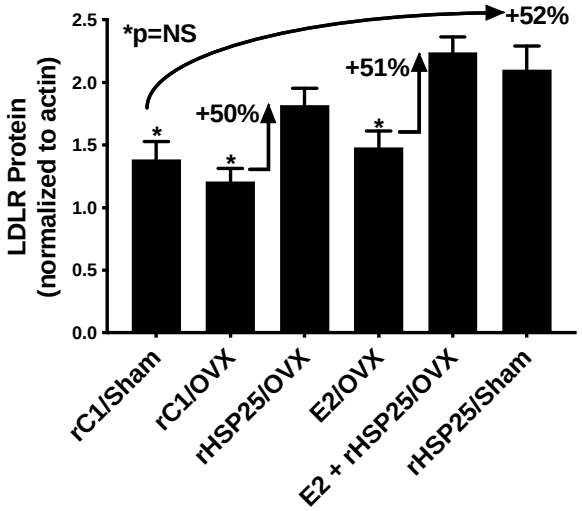

*p=NS
+52%
+51%
+50%
*
*
*
LDLR Protein
(normalized to actin)
2.5
2.0
1.5
1.0
0.5
0.0
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2 + rHSP25/OVX
rHSP25/Sham

Fig. 4

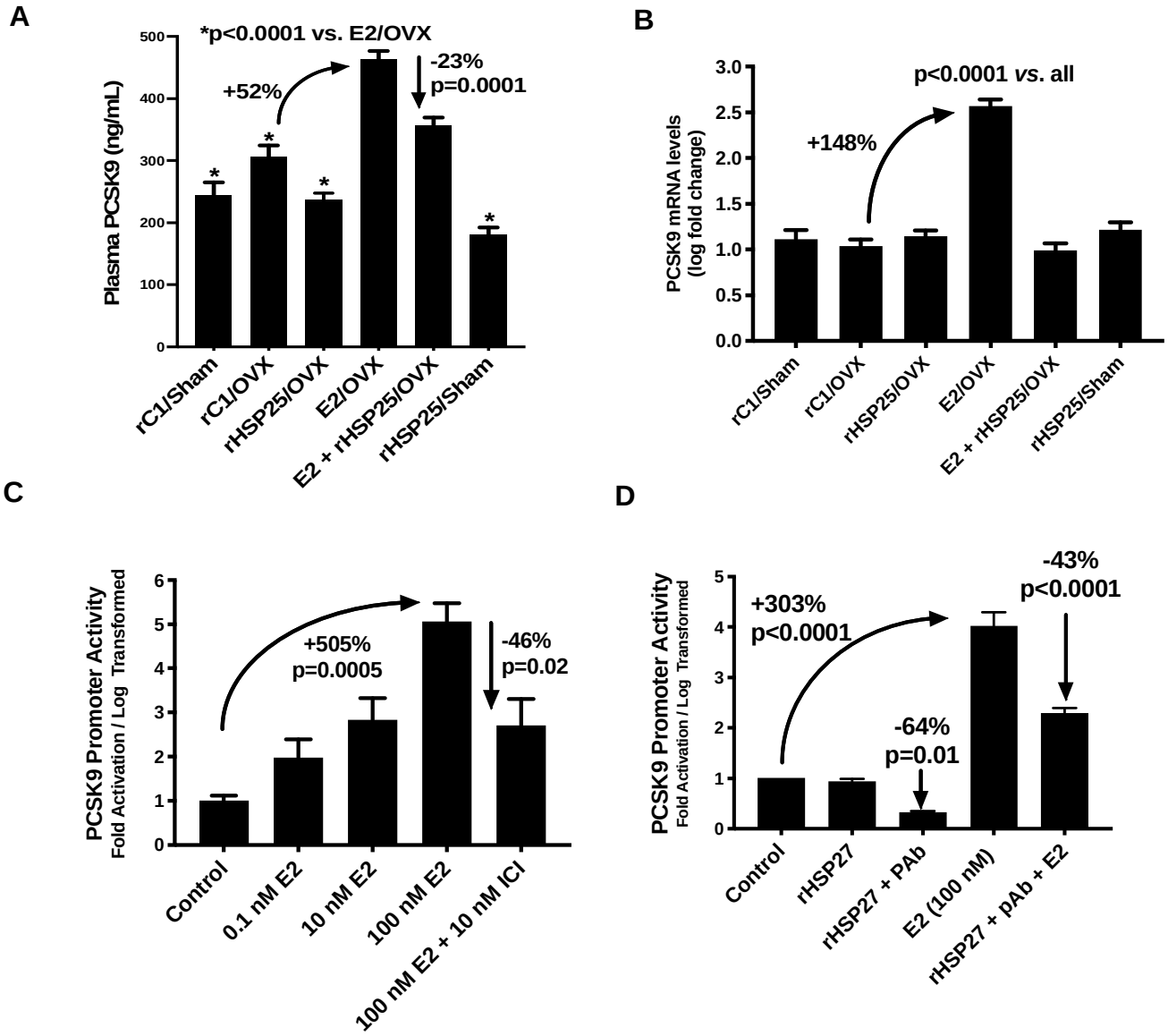
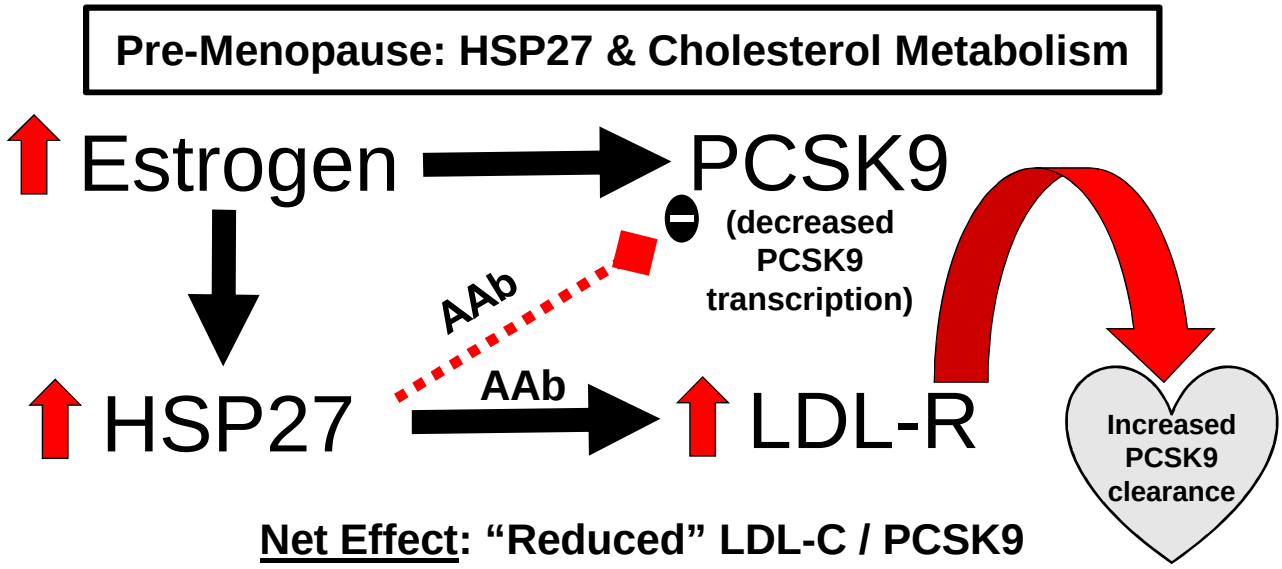
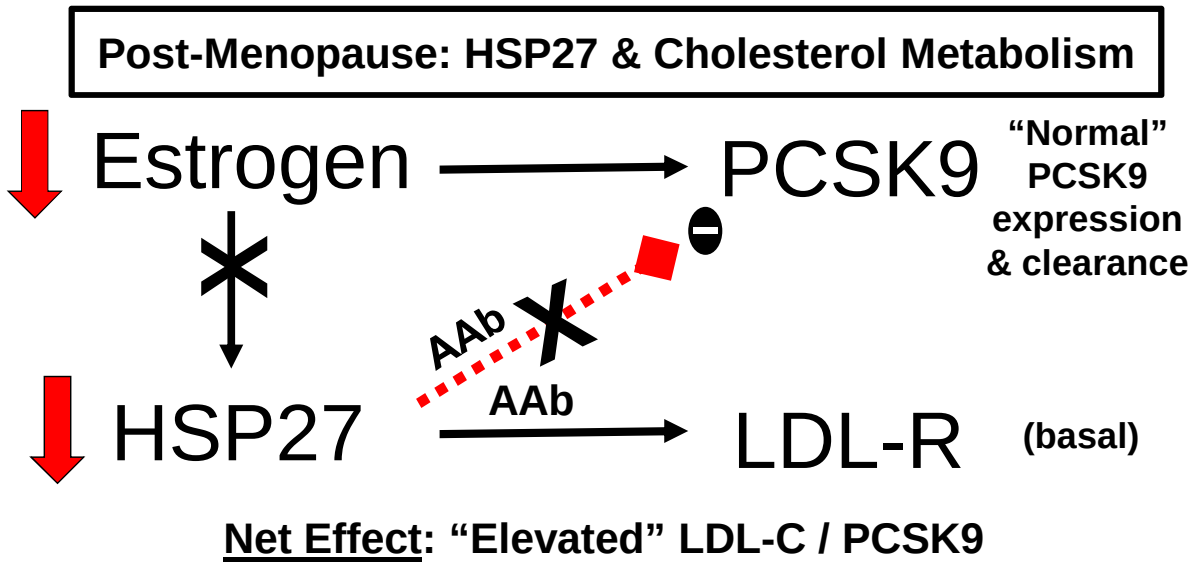

A
*p<0.0001 vs. E2/OVX
+52%
-23%
p=0.0001
Plasma PCSK9 (ng/mL)
500
400
300
200
100
0
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2 + rHSP25/OVX
rHSP25/Sham
B
p<0.0001 vs. all
+148%
PCSK9 mRNA levels
(log fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
rC1/Sham
rC1/OVX
rHSP25/OVX
E2/OVX
E2 + rHSP25/OVX
rHSP25/Sham
C
PCSK9 Promoter Activity
Fold Activation / Log Transformed
+505%
p=0.0005
-46%
p=0.02
6
5
4
3
2
1
0
Control
0.1 nM E2
10 nM E2
100 nM E2
100 nM E2 + 10 nM ICI
D
PCSK9 Promoter Activity
Fold Activation / Log Transformed
+303%
p<0.0001
-43%
p<0.0001
-64%
p=0.01
5
4
3
2
1
0
Control
rHSP27
rHSP27 + PAb
E2 (100 nM)
rHSP27 + pAb + E2

Fig. 5

A


Pre-Menopause: HSP27 & Cholesterol Metabolism
Estrogen
PCSK9
(decreased PCSK9 transcription)
AAb
AAb
HSP27
LDL-R
Increased PCSK9 clearance
Net Effect: "Reduced" LDL-C / PCSK9

B


Post-Menopause: HSP27 & Cholesterol Metabolism
Estrogen
PCSK9
"Normal" PCSK9 expression & clearance
AAb
AAb
HSP27
LDL-R
(basal)
Net Effect: "Elevated" LDL-C / PCSK9