Cardiovascular Consequences of Estrogen Deficiency: Studies in Premenopausal Women

by

Emma O'Donnell

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Exercise Sciences
University of Toronto

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Abstract

The influence of estrogen deficiency in physically active women with functional hypothalamic amenorrhea (ExFHA) on cardiovascular regulation is unknown. Three mechanistic studies compared cardiovascular responses to exercise and orthostatic stress in ExFHA women with responses in physically active (ExOv) and sedentary (SedOv) eumenorrheic ovulatory women. Measures included calf blood flow (BF), brachial artery (BA) endothelial dependent and independent function, shear rate (SR), vascular resistance (VR), blood pressure (BP), heart rate (HR), HR variability (HRV), muscle sympathetic nervous activity (MSNA), and serum renin-angiotensin-aldosterone system (RAAS) components.

Study one examined the effects of a single bout of dynamic exercise on vascular function in ExFHA (n=12), ExOv (n=14), and SedOv (n=15) women. Pre-exercise, calf BF and BA endothelium-dependent flow-mediated vasodilation (FMD%) were lower ($p<0.05$) in ExFHA versus ovulatory women in association with higher ($p<0.05$) calf VR and lower ($p<0.05$) SR, respectively. Endothelium-independent vasodilation, assessed at baseline only, was also lower ($p<0.05$) in ExFHA. Post-exercise, calf BF was increased and VR decreased
(p<0.05) in ExFHA women, similar (p>0.05) to that observed in ovulatory women. FMD% and SR were augmented (p<0.05) post-exercise, but both remained lower (p<0.05) in ExFHA versus ovulatory women (p<0.05).

Study two investigated neurohumoral (MSNA and RAAS) BP regulation during orthostatic stress in ExFHA (n=12) and ExOv (n=17) women. Baseline systolic BP was lower (p<0.05) in ExFHA versus ExOv. Neurohumoral measures did not differ (p>0.05) between the groups at baseline. However, during hypotensive stimuli, MSNA increased to a greater extent (p<0.05), yet angiotensin II and renin were not activated in ExFHA women.

Study three examined autonomic control of HR during orthostatic stress in ExFHA (n=11), ExOv (n=17), and SedOv (n=17) women. Lower HR (p<0.05) at rest and during orthostatic stress in ExFHA was associated with markedly elevated (p<0.05) HRV due to higher (p<0.05) parasympathetic modulation. Sympathetic modulation did not differ (p>0.05) between the groups.

These studies indicate altered cardiovascular regulation in otherwise healthy ExFHA women. The influence of estrogen deficiency per se in these alterations are not clear, but in light of the etiology of amenorrhea, it is likely that complex interactions between estrogen and energy deficiency and exercise training are involved.
Acknowledgements

I would like to dedicate this thesis to my husband. My partner in life and my rock. Your love and companionship throughout this entire academic journey gave me immense strength. I could not have done this without your support and understanding.

This research could not have been possible without the many participants who gave so much of their time and effort. I enjoyed meeting each and every one of you. It was truly my pleasure to have crossed paths with so many interesting women. Thank you all.

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A heartfelt thanks also goes to my fellow students, Steve, Danielle, Marc, Ming and Sam. You are all so talented and I'm going to miss you! I look forward to our paths crossing again in the not too distant future.

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>List of Appendices</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xiv</td>
</tr>
<tr>
<td><strong>CHAPTER 1: INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Overview</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Organization of the Thesis</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Experimental Hypotheses and Purposes</td>
<td>6</td>
</tr>
<tr>
<td>1.3.1 Study One</td>
<td>7</td>
</tr>
<tr>
<td>1.3.2 Study Two</td>
<td>8</td>
</tr>
<tr>
<td>1.3.3 Study Three</td>
<td>9</td>
</tr>
<tr>
<td><strong>CHAPTER 2: REVIEW OF LITERATURE</strong></td>
<td>10</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>10</td>
</tr>
<tr>
<td>2.2 Definitions</td>
<td>11</td>
</tr>
<tr>
<td>2.3 Estrogen Synthesis, Sources, Metabolism and Receptors</td>
<td>12</td>
</tr>
<tr>
<td>2.3.1 Estrogen Biosynthetic Pathway and Sources</td>
<td>12</td>
</tr>
<tr>
<td>2.3.2 Estrogen Transport and Metabolism</td>
<td>15</td>
</tr>
<tr>
<td>2.3.3 Estrogen Receptors: Distribution, Expression and Regulation</td>
<td>16</td>
</tr>
<tr>
<td>2.4 Vascular Function</td>
<td>19</td>
</tr>
<tr>
<td>2.4.1 Endothelium and Endothelial Function</td>
<td>19</td>
</tr>
<tr>
<td>2.4.2 Endothelial Dysfunction</td>
<td>20</td>
</tr>
<tr>
<td>2.4.3 Nitric Oxide</td>
<td>20</td>
</tr>
<tr>
<td>2.5 Effects of Estrogen on the Vasculature</td>
<td>23</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Non-genomic Effects</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Genomic Effects</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Other Effects of Estrogen on Vasoregulation</td>
</tr>
<tr>
<td>2.6</td>
<td>Effects of Acute and Chronic Exercise on the Endothelium</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Shear Stress</td>
</tr>
<tr>
<td>2.6.1.1</td>
<td>Mechanotransduction of Shear Stress</td>
</tr>
<tr>
<td>2.6.1.2</td>
<td>Acute versus Chronic Shear Stress Activation: Flow Mediated Dilation and Exercise</td>
</tr>
<tr>
<td>2.6.1.3</td>
<td>Estrogen and Shear Stress</td>
</tr>
<tr>
<td>2.7</td>
<td>Vascular Effects of Estrogen and Exercise Training on Oxidative Stress, Inflammation and Lipid Profile</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Oxidative Stress</td>
</tr>
<tr>
<td>2.7.1.1</td>
<td>Oxidative Stress and Estrogen</td>
</tr>
<tr>
<td>2.7.1.2</td>
<td>Oxidative Stress and Exercise</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Vascular Inflammation</td>
</tr>
<tr>
<td>2.7.2.1</td>
<td>Vascular Inflammation and Estrogen</td>
</tr>
<tr>
<td>2.7.2.1.1</td>
<td>Anti-Inflammatory Effects of Estrogen</td>
</tr>
<tr>
<td>2.7.2.1.2</td>
<td>Pro-Inflammatory Effects of Estrogen</td>
</tr>
<tr>
<td>2.7.2.2</td>
<td>Vascular Inflammation and Exercise</td>
</tr>
<tr>
<td>2.8</td>
<td>Neurohumoral Vasoregulation: Influence of Estrogen and Exercise</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Muscle Sympathetic Nerve Activity (MSNA)</td>
</tr>
<tr>
<td>2.8.1.1</td>
<td>Muscle Sympathetic Nerve Activity and Estrogen</td>
</tr>
<tr>
<td>2.8.1.2</td>
<td>Muscle Sympathetic Nerve Activity and Exercise</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Renin-Angiotensin-Aldosterone System (RAAS)</td>
</tr>
<tr>
<td>2.8.2.1</td>
<td>Renin-Angiotensin-Aldosterone System and Estrogen</td>
</tr>
<tr>
<td>2.8.2.2</td>
<td>Renin-Angiotensin-Aldosterone System and Exercise</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Baroreflex Sensitivity and Heart Rate Variability</td>
</tr>
<tr>
<td>2.8.3.1</td>
<td>Baroreflex Sensitivity, Heart Rate Variability and Estrogen</td>
</tr>
<tr>
<td>2.8.3.2</td>
<td>Baroreflex Sensitivity, Heart Rate Variability and Exercise</td>
</tr>
<tr>
<td>2.9</td>
<td>Lipid Profile</td>
</tr>
</tbody>
</table>
List of Tables

CHAPTER 2
Table 1. Mechanisms of action and known or potential clinical consequences of altered cardiovascular function in ExFHA women.......................... 69

CHAPTER 3
Table 1: Study group demographics.......................................................... 89
Table 2: Serum measures of the study groups........................................... 90
Table 3: Hemodynamic responses at baseline and one hour post-exercise........ 91
Table 4: Brachial artery responses pre- and post-exercise across the study groups........................................................................................................... 95
Table 5: Brachial artery blood flow and shear responses pre- and post-exercise across the study groups.......................................................... 96

CHAPTER 4
Table 1: Baseline subject characteristics.................................................... 116
Table 2: Hemodynamic responses of the study groups during LBNP............ 117
Table 3: Neurohumoral responses of the study groups during LBNP............. 118

CHAPTER 5
Table 1: Demographic and anthropometric characteristics of the study groups... 137
Table 2: Blood pressure and heart rate responses of the study groups to graded LBNP........................................................................................................... 138
Table 3: Baseline HRV for the study groups.................................................. 139

APPENDIX 4
Table 1. Test re-test data, intra-class correlation and Coefficient of Variation of the main outcome variables................................................................. 249
## List of Figures

### CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Figure showing primary sources and metabolism of estrogens in premenopausal women</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Diagram showing the renin-angiotensin-aldosterone system and its effects on vascular tone and BP</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>Schema outlining the metabolic and hormonal consequences of energy deficiency, leading to estrogen deficiency, in physically active premenopausal women with FHA</td>
<td>66</td>
</tr>
</tbody>
</table>

### CHAPTER 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Endothelium-dependent and -independent responses pre-exercise for the study groups</td>
<td>97</td>
</tr>
<tr>
<td>1b</td>
<td>Percent change in brachial artery diameter pre- and post-exercise for the study groups</td>
<td>97</td>
</tr>
<tr>
<td>1c</td>
<td>Percent change in brachial artery diameter normalized for SR AUCpk pre- and post-exercise for the study groups</td>
<td>97</td>
</tr>
<tr>
<td>2a</td>
<td>Resting calf blood flow responses pre- and post-exercise for the study groups</td>
<td>98</td>
</tr>
<tr>
<td>2b</td>
<td>Peak calf blood flow responses pre- and post-exercise for the study groups</td>
<td>98</td>
</tr>
<tr>
<td>3a</td>
<td>Correlation between FMD% and SRAUCpk pre-exercise</td>
<td>99</td>
</tr>
<tr>
<td>3b</td>
<td>Correlation between FMD% and SRAUCpk post-exercise</td>
<td>99</td>
</tr>
</tbody>
</table>

### CHAPTER 4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Systolic blood pressure during LBNP</td>
<td>121</td>
</tr>
<tr>
<td>1b</td>
<td>Diastolic blood pressure during LBNP</td>
<td>121</td>
</tr>
<tr>
<td>1c</td>
<td>Mean arterial blood pressure during LBNP</td>
<td>121</td>
</tr>
<tr>
<td>1d</td>
<td>Heart rate during LBNP</td>
<td>121</td>
</tr>
<tr>
<td>2a</td>
<td>Renin responses during LBNP</td>
<td>122</td>
</tr>
</tbody>
</table>
Figure 2b. Angiotensin II responses during LBNP.................................................. 122
Figure 2c. Aldosterone responses during LBNP..................................................... 122
Figure 3a. MSNA burst frequency (bursts/minute) during LBNP.............................. 123
Figure 3b. MSNA burst incidence (bursts/100 heart beats) during LBNP.............. 123
Figure 3c. ΔMSNA bursts per 100 heart beats during LBNP................................. 123

CHAPTER 5
Figure 1a. HFlog10 during LBNP........................................................................... 140
Figure 1b. LFlog10 during LBNP......................................................................... 140
Figure 1c. Total HRV during LBNP....................................................................... 140
Figure 1d. LF/HF ratio during LBNP.................................................................... 140

CHAPTER 6
Figure 1. Summary of key findings of altered cardiovascular function in EXFHA women at baseline in response to orthostatic stress, and post-acute dynamic exercise................................................................. 156
# List of Appendices

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPENDIX 1: RECRUITMENT</td>
<td>Recruitment Poster</td>
<td>226</td>
</tr>
<tr>
<td>APPENDIX 2: QUESTIONNAIRES</td>
<td>Health, Exercise and Menstrual Cycle Questionnaire</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>Physical Activity Questionnaire</td>
<td>234</td>
</tr>
<tr>
<td>APPENDIX 3: CONSENT</td>
<td>Consent Form for study one, two and three</td>
<td>235</td>
</tr>
<tr>
<td>APPENDIX 4: RELIABILITY OF METHODS</td>
<td>Flow mediated dilation and calf blood flow</td>
<td>248</td>
</tr>
<tr>
<td>APPENDIX 5: ASSAYS</td>
<td>Serum Assay Details</td>
<td>250</td>
</tr>
<tr>
<td>APPENDIX 6: SAMPLE SIZE</td>
<td>Sample Size Calculations</td>
<td>254</td>
</tr>
<tr>
<td>APPENDIX REFERENCES</td>
<td></td>
<td>257</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-HSD</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACE2</td>
<td>Angiotensin converting enzyme 2</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropin hormone</td>
</tr>
<tr>
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<td>Angiotensin I</td>
</tr>
<tr>
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<td>Angiotensin II</td>
</tr>
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<td>AT1R</td>
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</tr>
<tr>
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<td>Angiotensin II receptor type 2</td>
</tr>
<tr>
<td>BF</td>
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</tr>
<tr>
<td>BKCa</td>
<td>Calcium-dependent potassium channel</td>
</tr>
<tr>
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<td>Blood pressure</td>
</tr>
<tr>
<td>BRS</td>
<td>Baroreflex sensitivity</td>
</tr>
<tr>
<td>C</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclo-oxygenase-1</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
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</tr>
<tr>
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<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DC</td>
<td>Deconjugation</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>Dehydroepiandrosterone sulphate</td>
</tr>
<tr>
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<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EID</td>
<td>Endothelium-independent dilation</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Estradiol-S</td>
<td>Estradiol sulphatase</td>
</tr>
<tr>
<td>Estrone-S</td>
<td>Estrone sulphatase</td>
</tr>
<tr>
<td>ET</td>
<td>Estrogen therapy</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin</td>
</tr>
<tr>
<td>ExFHA</td>
<td>Physically active women with functional hypothalamic amenorrhea</td>
</tr>
<tr>
<td>ExOv</td>
<td>Physically active women with eumenorrheic ovulatory menstrual cycles</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAI</td>
<td>Free androgen index</td>
</tr>
<tr>
<td>FHA</td>
<td>Functional hypothalamic amenorrhea</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow mediated dilation</td>
</tr>
<tr>
<td>FMD%</td>
<td>Percentage increase in brachial artery diameter induced by FMD</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>G</td>
<td>Glucuronidation</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GTN</td>
<td>Glyceryl trinitrate</td>
</tr>
<tr>
<td>GTN%</td>
<td>Percentage increase in brachial artery diameter induced by GTN</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HDLc</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary adrenal</td>
</tr>
<tr>
<td>HPO</td>
<td>Hypothalamic pituitary ovarian</td>
</tr>
<tr>
<td>HPT</td>
<td>Hypothalamic pituitary thyroid</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HRV</td>
<td>Heart rate variability</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
</tbody>
</table>
IκB  Inhibitory kappa-B
IKK  Inhibitory kappa-B kinase
iNOS Inducible nitric oxide synthase
IP3  Inositol 1,4,5-trisphosphate
IRAG Inositol 1,4,5-trisphosphate-associated cGMP kinase substrate
K+  Potassium
LBNP Lower body negative pressure
LDLc Low-density lipoprotein cholesterol
LH  Luteinizing hormone
MAP  Mean arterial blood pressure
MAPK Mitogen-activated protein kinase
MCP-1 Monocyte chemotactic protein-1
mRNA Messenger ribonucleic acid
MSNA Muscle sympathetic nerve activity
NADPH Nicotinamide adenine dinucleotide phosphate
NFκB Nuclear factor kappa-B
nNOS Neuronal nitric oxide synthase
NO  Nitric Oxide
NOS Nitric oxide synthase
NO-SGC Nitric oxide sensitive guanylyl cyclase
NOX Nicotinamide adenine dinucleotide phosphate oxidase
NTS Nucleus tractus solitarius
OxLDLc Oxidized low-density lipoprotein cholesterol
P  P450 cytochrome family
PGI2 Prostacyclin
PI3K Phosphoinositide 3-kinase
PIP3 Phosphatidylinositol (3,4,5)-triphosphate
PORH Post-occlusive reactive hyperemia
PMW Postmenopausal women

xvi
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td>Pulse pressure</td>
</tr>
<tr>
<td>PRA</td>
<td>Plasma renin activity</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin angiotensin aldosterone system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SedOv</td>
<td>Sedentary women with eumenorrheic ovulatory menstrual cycles</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex-hormone binding globulin</td>
</tr>
<tr>
<td>SNA</td>
<td>Sympathetic nerve activity</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SR</td>
<td>Shear rate</td>
</tr>
<tr>
<td>SRAUCpk</td>
<td>Peak shear rate determined by the area under the curve</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid receptor coactivator</td>
</tr>
<tr>
<td>ST</td>
<td>Sulphatases</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>Su</td>
<td>Sulphonation</td>
</tr>
<tr>
<td>T3</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TPR</td>
<td>Total peripheral resistance</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>VC</td>
<td>Vascular conductance</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cellular adhesion molecule-1</td>
</tr>
<tr>
<td>VR</td>
<td>Vascular resistance</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

1.1 Overview

Premenopausal women have a lower risk and incidence of hypertension and cardiovascular disease (CVD) compared to age-matched men (1, 2). This cardiovascular benefit is lost after surgical or natural menopause and has largely been attributed to the beneficial effects of endogenous estrogen. Evidence from human and animal studies indicate that $17\beta$-estradiol confers cardioprotection through numerous complex multifaceted mechanisms acting on the endothelial, myocardial, neurohumoral, vascular and metabolic milieu (1, 2). The proatherosclerotic and hypertensive effects of estrogen deficiency include impaired bioactivity of nitric oxide (NO), endothelial dysfunction, perturbation in autonomic function, activation of the renin-angiotensin-aldosterone system (RAAS), increased oxidative stress and changes in regulation of factors involved in inflammatory and coagulation/fibrinolytic cascades (3-6).

Estrogen deficiency is not unique to the postmenopausal period. Functional hypothalamic amenorrhea (FHA) is an important cause of reversible non-organic ovarian disruption (7). FHA is characterized by the absence of menses and profound hypoestrogenemia due to suppression of the hypothalamic–pituitary–ovarian axis (8). Three main types of FHA have been identified, all of which are commonly inter-related: stress-related amenorrhea, weight loss-related amenorrhea and exercise-associated amenorrhea (8).
Exercise-associated-FHA (ExFHA) has been observed in occupationally, recreationally and competitively physically active women. ExFHA is thought to be primarily caused by energy deficiency (insufficient energy intake without obvious caloric deficit) in response to high-energy expenditure (i.e., exercise) (9, 10). In contrast to the well known deleterious cardiovascular sequelae of FHA in individuals diagnosed with clinical eating disorders (11) or psychogenic stress (12), the cardiovascular consequences of ExFHA are less well described. However, those with ExFHA often have impaired brachial artery flow mediated dilation (FMD), lowered regional blood flow, and disordered heart rate (HR) and blood pressure (BP) regulation (13-15). While the mechanisms of action are not clear, estrogen deficiency is considered a key mitigating factor (13-15). The long-term cardiovascular consequences of these cardiovascular alterations are not yet known, but hypoestrogenemia in association with altered ovulatory cycling, including irregular menstrual cycles, is considered a contributing factor to the development and progression of premenopausal coronary artery disease (CAD) (16-18). For example, there is compelling evidence that ovarian disruption of functional hypothalamic origin, independent of other factors, is a risk factor for angiographically documented CAD during the later premenopausal years (19). Consistent with this, evidence in non-human primates also demonstrates that FHA-induced hypoestrogenemia contributes directly to the premature and accelerated progression of atherosclerosis during the reproductive years, with administration of estrogen therapy ameliorating these effects (20). Collectively, these observations support an important role for estrogen and ‘normal’ ovulatory cycling during the premenopausal years on cardiovascular health.

The following studies were undertaken to provide integrated investigations examining the mechanisms of vascular function, and the regulation of BP and HR in women with ExFHA.
The overarching purpose is twofold. Primarily, these studies are designed to clarify the influence and mechanisms of action of hypoestrogenemia on cardiovascular function during the premenopausal years. In the absence of co-morbidities, including endocrine dysfunction, chronic malnutrition or aging, women with ExFHA provide a unique model to investigate such influences. The detection of cardiovascular perturbations in this group of otherwise healthy women may identify important aspects of premenopausal estrogenic cardiovascular regulation. Secondly, these studies are intended to facilitate a detailed characterization of the cardiovascular profile of women with ExFHA. In light of the importance of normal ovulatory cycling on cardiovascular health, it is essential to understand the breadth of the potentially deleterious effects of estrogen deficiency on CV health in these women. It is anticipated that findings from the current studies will further our understanding of the role of endogenous estrogen in cardiovascular regulation, in addition to contributing to our understanding of how premenopausal estrogen deficiency might contribute to the premature development of hypertension and CAD.

1.1 Organization of the Thesis

Following a review of the pertinent literature in Chapter 2, three distinct but connected studies are presented in Chapters 3-5. In each study, exercise or orthostatic stress using lower body negative pressure (LBNP) were employed to perturbate the cardiovascular system under controlled conditions so as to elicit physiological responses that might not otherwise be apparent under resting conditions. In the first study (Chapter 3) the influence of estrogen deficiency in women with ExFHA on endothelial function was studied in two separate vascular
beds. Brachial artery (conduit vessel) flow mediated dilation (FMD) and calf (resistance vessel) blood flow were assessed both before and after 45 minutes of moderate dynamic aerobic exercise. This study was designed to extend the findings of previous studies reporting impaired percent change in resting brachial artery diameter to peak diameter (FMD%) and lowered calf blood flow (BF) in ExFHA women (14, 15, 21). It remains unclear whether these responses reflect endothelial dysfunction per se or altered vascular responsiveness and/or stimulus. Thus, in women with ExFHA, this study sought to: i) examine both conduit and resistance vessel function in response to reactive hyperemia; ii) determine whether impaired brachial artery FMD% is due at least in part to altered vessel wall stimulus, namely, shear rate, an important contributing factor to the magnitude of the FMD% response (22); and iii) investigate whether an acute bout of exercise, a recognized shear-stress mediated nitric oxide (NO) stimulus, 'restores' conduit and/or resistance vessel vasodilatory capacity to that observed in their ovulatory eumenorrheic counterpart.

The second study (Chapter 4) examined the influence of estrogen deficiency in ExFHA women on mechanisms of previously reported low resting systolic blood pressure (BP) (13, 23). While the role of estrogen deficiency in this response is unclear, older postmenopausal women (PMW) have been reported to demonstrate altered neurohumoral BP regulation in association with estrogen deficiency, including activation of the renin-angiotensin-aldosterone system (RAAS) and increased efferent sympathetic nerve activity (SNA) (2, 24). Therefore, this study sought to determine whether disordered BP regulation in young, hypoestrogenic and otherwise healthy ExFHA women is also associated with altered SNA and/or RAAS activation. To challenge BP homeostasis, SNA and RAAS were examined both at rest and in response to
arterial baroreflex-mediated changes in BP, induced by simulated orthostatic stress using lower body negative pressure (LBNP).

In the final study (Chapter 5), the influence of estrogen deficiency in women with ExFHA on autonomic control of HR was examined. This study was again prompted by findings from previous work demonstrating consistently lower resting HR in ExFHA women compared with eumenorrheic, ovulatory physically active women (13, 23, 25). Given the limited understanding of how estrogen modulates cardiac autonomic activity in humans (26, 27), this study examined autonomic modulation of HR both at rest and in response to a baroreflex-mediated cardiac autonomic regulatory challenge (again using LBNP). Specifically, the response of HR variability (HRV), which provides quantitative information on the modulation of cardiac vagal and sympathetic efferent tone (28, 29), to the application of three sequential stages of LBNP was examined.

These three integrative studies tested unique hypotheses. Additional secondary endpoints were also collected for each study to provide important ancillary information regarding related physiological concepts. These data are considered a valuable adjunct to the thesis. The individual hypotheses for each study are listed within Section 1.3 Experimental Hypotheses and Purposes (below).

In the final chapter (Chapter 6), the hypotheses are revisited in a unifying discussion that summarizes the key observations of each study, providing a global and critical interpretation of the collective findings, mechanisms, limitations and recommendations for future work.
1.3 Experimental Hypotheses and Purposes

Three studies were conducted to compare the independent effects of premenopausal estrogen deficiency on several indices of cardiovascular function. Estrogen-deplete women with ExFHA were used as an experimental model of estrogen-deficiency. Cardiovascular responses in these women were compared to those in estrogen-replete eumenorrheic ovulatory physically active women (ExOv). As a reference group, sedentary women with eumenorrheic ovulatory menstrual cycles (SedOv) were also examined. It is important to note that the studies in this thesis were originally designed to also assess amenorrheic participants both before and after 4 weeks of transdermal 17 β-estradiol (Climara 50®). This method of estrogen replacement was chosen because of its bioidentical hormone profile and the physiological level of hormone delivery, resulting in similar estrogen levels as observed during the follicular phase of the menstrual cycle. However, only 3 women elected to participate in the intervention. Thus, the thesis was not able to test a-priori hypotheses centered on examining the cardiovascular effects of estrogen replacement in ExFHA women. As such, these hypotheses have been removed from the thesis.

Primary endpoints of cardiovascular function and regulation included brachial artery and calf vascular endothelial function (conduit and resistance, respectively), lower limb muscle SNA (MSNA; using microneurography), components of the circulating RAAS (renin, angiotensin II, and aldosterone), cardiac autonomic HR modulation (using HRV), HR, and BP, all of which were measured at baseline and again following specific interventions (e.g., exercise or orthostatic stress). Baseline serum measures of ovarian and thyroid hormones, and
full lipid profile (high- and low-density lipoprotein cholesterol, total cholesterol and triglycerides) were also determined.

1.3.1 Study One

In Study one (Chapter 3), entitled ‘Impaired Vascular Function in Physically Active Estrogen Deficient Premenopausal Women is Augmented After an Acute Bout of Dynamic Exercise’, the primary study objectives were, in ExFHA women:

1. To determine the influence of estrogen deficiency on vascular function across two different vascular beds both before and one-hour after an acute bout of dynamic submaximal exercise;
2. To determine brachial artery peak shear rate (SR) and its relationship to the FMD% response; and
3. To compare responses with those observed in ExOv women.

Secondary objectives of this study included assessment of correlations between vascular responses across each of the vascular beds, and to determine meaningful correlations between vascular responses and serum measures (e.g., estradiol, triiodothyronine, lipid profile), HR and BP.

For Study One it was hypothesized that in women with ExFHA:

1. Baseline measures of i) brachial artery FMD% and endothelium-independent dilation, assessed using sublingual glycercyl trinitrate (GTN), and ii) resting and peak-ischemic calf blood flow (BF) will be lower compared with ExOV women;
2. Baseline peak shear rate (SR) assessed in the brachial artery will be lower compared with ExOV women, and will be associated with impaired FMD%; and

3. FMD%, calf BF and peak SR responses will be ‘restored’ after a single bout of submaximal dynamic exercise, such that responses are no longer different from that observed in ExOV women.

1.3.2 Study Two

In Study Two (Chapter 4) entitled ‘Discordant Efferent Sympathetic Neural and Renin Angiotensin System Responsiveness to Acute Baroreflex Stimulation during Simulated Orthostatic Stress in Estrogen-Deficient Physically Active Premenopausal Women’, the primary study objectives were, in ExFHA women:

1. To determine the influence of estrogen deficiency on neurohumoral BP regulation by assessing RAAS and MSNA at rest and in response to simulated orthostatic stress using LBNP; and

2. To compare responses with those observed in ExOV women.

Secondary objectives of this study included determination of meaningful correlations between MSNA, RAAS, systolic BP, HR and serum measures of 17 β-estradiol.

For Study Two it was hypothesized that in women with ExFHA:

1. MSNA responses will be lower compared with ExOV women;

2. RAAS responses will be lower compared with ExOV women;
3. Systolic BP and heart rate (HR) will be lower at baseline and will remain lower throughout sequential stages of LBNP compared with ExOv women.

1.3.3 Study Three

In Study Three (Chapter 5) entitled ‘Altered Autonomic Control of Heart Rate in Physically Active Women with Functional Hypothalamic Amenorrhea’, the primary study objectives were, in ExFHA women:

1. To determine the influence of estrogen deficiency on cardiac autonomic control of HR by assessing HRV at rest and in response to simulated orthostatic stress using LBNP; and

2. To compare these responses with ExOv women.

Secondary objectives of this study included determination of meaningful correlations between HRV, BP and HR responses, and serum measures of 17 β-estradiol and triiodothyronine.

For Study Three it was hypothesized that in women with ExFHA:

1. HR will be lower at baseline and will remain lower throughout sequential stages of LBNP compared with ExOv women;

2. Lower HR will be associated with higher parasympathetic modulation of HR, as determined by the high frequency component of heart rate variability (HRV), compared with ExOv women.
CHAPTER 2
Literature Review

The following literature review is republished with the permission of the Journal of Clinical Endocrinology and Metabolism, from Cardiovascular Consequences of Ovarian Disruption: A Focus on Functional Hypothalamic Amenorrhea in Physically Active Women, by O’Donnell et al., 96, 12, 2011 (25); permission conveyed through Copyright Clearance Center, Inc. The manuscript was expanded to permit inclusion of additional sections that were deemed relevant and necessary for thesis purposes.

2.1 Introduction

This chapter is organized to review the current literature pertaining to the cardiovascular effects of estrogen and estrogen deficiency in premenopausal women, with emphasis placed on physically active women with functional hypothalamic amenorrhea (ExFHA). Relevant findings in animal studies and postmenopausal women (PMW) have also been included. The first half of the review focuses on the sources, physiological actions, metabolism, and transport of estrogen, followed by endothelial and neurohumoral mechanisms of vasoregulation. The independent and combined effects of estrogen, estrogen deficiency and aerobic exercise training on vascular function are described. Literature relating to findings in physically active premenopausal women presenting with ovarian disruption in association with FHA are described exclusively in the latter half of the review. The cardiovascular consequences of
psychogenic stress (e.g., anxiety), eating disorders (e.g., anorexia nervosa), and menstrual
disturbances of organic origin (e.g., poly cystic ovarian syndrome), all of which are
independently associated with estrogen deficiency, are not reviewed here. The primary
causative factor of ExFHA is energy deficiency, and therefore consideration of the influence of
energy deficiency on cardiovascular function is also assessed in the latter section of the review.
A summary of the potential clinical cardiovascular consequences of premenopausal estrogen
deficiency associated with FHA will conclude the discussion.

2.2 Definitions

In the following review, estrogen will refer to endogenous 17 β-estradiol, and exercise training
will refer to aerobic exercise training, unless otherwise stated. Estrogen exposure will refer to
exposure to endogenous estrogens across the reproductive lifespan (25). Ovarian disruption
relates to altered hypothalamic-pituitary-ovarian function. Clinically, ovarian disruption is
characterized by symptomatic menstrual irregularities (i.e., oligomenorrhea and amenorrhea),
and sub-clinically, by asymptomatic menstrual disturbances (i.e., luteal phase defect and
anovulation in the absence of amenorrhea) (25). The most severe form of ovarian disruption,
amenorrhea, leads to chronically low circulating levels of estrogen, resulting in estrogen
deficiency (25).
2.3 Estrogen Synthesis, Sources, Metabolism and Receptors

2.3.1 Estrogen Biosynthetic Pathways and Sources

Estrogens regulate many physiological actions, including reproduction, bone metabolism, cellular growth and differentiation, lipid metabolism, energy metabolism, cognitive function, and cardiovascular function (1, 30, 31). The human body synthesizes three main estrogens: estradiol, estrone and estriol, each of which are C18 steroids derived from cholesterol, the initial precursor to steroidogenesis. Circulating cholesterol, derived from dietary fats or de novo local synthesis, is primarily transported in association with low-density-lipoproteins, which bind to specific lipoprotein receptors on the plasma membrane of steroidogenic tissue cells (32). After binding, cholesterol is taken up and stored by the steroidogenic cells, and then moved to sites of steroid synthesis (30). For cholesterol to be used in biosynthesis it must be transported to mitochondria (32). Cytosolic movement of cholesterol is facilitated by the cytoskeleton and by intracellular carrier proteins (30, 33). The subsequent translocation of cholesterol from the cytosol to the inner membrane of the mitochondria is mediated by the cholesterol transfer protein steroidogenic acute regulatory protein (StAR). StAR is an essential protein which is targeted to the outer mitochondrial membrane to facilitate the movement of cholesterol to the inner membrane where the rate-limiting enzyme of estrogen synthesis, the P450 aromatase enzyme, resides (34). Aromatization of steroids by the P450 aromatase monooxygenase enzyme complex is the final step in estrogen synthesis in the smooth endoplasmic reticulum (30). In three successive hydroxylating reactions, estradiol and estrone are formed from their precursors, testosterone and androstenedione, respectively (30).
Estrogens are produced in at least three major sites in premenopausal women: 1) the developing follicles in the ovary (primarily estradiol); 2) by aromatization of circulating androstenedione of adrenal and/or ovarian origins to estrone in peripheral tissues; and 3) by aromatization of androstenedione to estrone in estrogen-target tissues (Figure 1).

**Figure 1:** Primary sources and metabolism of estrogens in premenopausal women.

C, catechol-O-methyltransferase; DC, deconjugation; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulphate; estradiol-S, estradiol sulphatase; estrone-S, estrone sulphatase; GI, gastrointestinal; G, glucuronidation; P, P450 cytochrome family; ST, sulphatases; Su, sulphonation; 17β-HSD, 17β-hydroxysteroid dehydrogenase. Republished with permission from the Journal of Clinical Endocrinology and Metabolism [O’Donnell et al., (25)].
Estriol is produced almost exclusively from the placenta during pregnancy (31). For elaboration on its synthesis, see the following review (35). Estradiol is the most potent of the estrogens, with an activity that is 12 times greater than estrone and 80 times greater than estriol (31); thus, estradiol is considered to be the most important estrogen. The primary sources of estradiol in women are the theca and granulosa cells of the ovaries, and the luteinized derivatives of these cells (30). This ‘two-cell theory’ of estrogen biosynthesis is initiated by neuroendocrine signals from the central nervous system which stimulates the hypothalamus to release gonadotropin releasing hormone (GnRH), which in turn activates the pituitary to release the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). The appropriate secretion of the gonadotropins from the pituitary is essential for the production of ovarian estradiol and progesterone. Within the ovary, the binding of LH to LH-receptors on follicular theca cells stimulates de novo androgen production from cholesterol (32). These androgens, androstenedione and testosterone, are secreted from the theca cells and diffuse across the cellular membrane of the neighbouring follicular granulosa cells. Granulosa cells express aromatase (P450) which catalyzes androgens primarily into estradiol, but also estrone (32). Aromatase action, and thus estradiol production, in the granulosa cell is primarily regulated by FSH. Peripherally, biosynthesis of estrogen is also catalyzed by the cytochrome P450 aromatase enzyme at numerous sites, including adipose tissue, skin, adrenals, liver, breast tissue, vascular smooth muscle cells and endothelial cells (30, 36, 37). Local expression and activity of aromatase 17β-hydroxysteroid dehydrogenase and estrone sulfatases, also permits dynamic mutual conversion of estrone and estradiol (Figure 1) (30, 36, 37). Peripheral synthesis of estrogens is estimated to account for approximately 50% of all estrogens prior to
menopause (36). The level of estrogen synthesis in extragonadal tissues increases as a function of age and body weight (30).

During the menstrual cycle, estradiol production varies cyclically, with the highest serum concentrations observed in the late follicular (preovulatory) phase, followed by the mid-luteal phase, which is also accompanied by elevated progesterone concentrations. The lowest estradiol (and progesterone) levels are produced during the early follicular (menses) phase.

### 2.3.2 Estrogen Transport and Metabolism

Estradiol is secreted directly from the ovary into the blood stream. In the circulation, estradiol and other naturally occurring estrogens are bound preferentially and predominantly to sex hormone binding globulin (SHBG), but also to a lesser degree and with lower affinity to albumin (32). Only a small fraction (1-3%) of un-bound estrogen exists in the circulation (38). This fraction is considered to be bio-active and available for cellular interaction. Thus, circulating levels of SHBG, and to some extent albumin, influences both the circulating levels of estrogens and its biodisposal to target cells (31), in addition to serving as a "buffer" or reservoir, protecting estrogen from peripheral metabolism (30, 37). Estrogens are metabolized primarily in the liver by hydroxylation via enzymes of the P450 cytochrome family to form catechol estrogen metabolites, which can undergo methylation to form methoxylated estrogens (Figure 1) (30, 37). Hydroxylation of estrogens produces 2-hydroxyestrogens, 4-hydroxyestrogens, and 16α-hydroxyestrogens (catechol estrogens) (30, 37). Methylation of the 2- and 4-hydroxyestrogens by catechol O-methyltransferase produces methoxylated estrogen
metabolites (30, 37). Both the catechol estrogens and their methoxyderivatives may exert considerable estrogenic effects (37). Estrogenic metabolites may also undergo sulfonation and glucuronidation, resulting in conjugates that are eliminated via the kidneys and liver (30, 37). Hydrolysis of conjugates by the intestinal flora in the gastrointestinal tract results in deconjugation and subsequent reabsorption of the estrogens via enterohepatic circulation (30). Estrone and estradiol can also be deconjugated by sulfatases.

2.3.3 Estrogen Receptors: Distribution, Expression and Regulation

The biological effects of estrogen are mediated primarily by the estrogen receptors (ER), ERα and ERβ, both of which are classic ligand-activated transcription factors that belong to the nuclear hormone superfamily (39, 40). ERs are expressed ubiquitously throughout the body, including adipose tissue, skin, lung, brain, liver and throughout the autonomic and cardiovascular systems (1, 41-43). In the vasculature, ERs are expressed in vascular endothelium and vascular smooth muscle cells. These two cell types demonstrate distinct effects in response to estrogen exposure. In endothelial cells, estrogen promotes proliferation and inhibits apoptosis, while in vascular smooth muscle cells estrogen inhibits proliferation and migration (1, 41, 44). Experimental and animal studies show that ERα is more highly expressed in endothelial cells than ERβ (45, 46), and that ERα confers protection against vascular injury and atherosclerosis (45), with ERβ controlling for genes involved in arterial tone and blood pressure (BP) regulation (46). In the heart, myocytes from animals express both ERs (44). While their distinct biological roles currently remain unclear, studies in animals examining the sex-specific differences in the progress and development of
cardiovascular disease suggest a protective effect of ligand-activated ERs on the myocardium, including anti-hypertrophic (47) and anti-fibrotic effects (48), less severe left ventricular remodeling and better preserved function post myocardial infarction (48), and a lower incidence of tachyarrhythmia's as well as a lower prevalence of sudden cardiac death due to an ischemic event (49). However, age has been shown to be a key factor that affects gender differences in heart disease in humans. Compared to men with myocardial infarction, women less than 60 years old are at a greater risk whereas older women are at a lower risk for fatal cardiac events within 2 years after the first myocardial infarction (16).

Structurally, ERα and ERβ share a common biochemical anatomy and a high sequence homology (50). The DNA (deoxyribonucleic acid) binding domain is conserved (~97% homologous) between ERα and ERβ, as is the ligand-binding domain (60% homologous) (39, 51). However, ERα and ERβ differ in the amino terminal transcriptional control domain, AF-1, through which regulatory binding partners interact (52). Binding affinity of the various estrogens with their ligand receptors differ, with 17β-estradiol demonstrating the highest (100%) affinity for both ER-α and ER-β, followed by estrone (60 and 37%, respectively) and estriol (14 and 21%, respectively) (53).

ERα and ERβ play distinctive roles in estrogen signaling. Differences are attributed to: i) the genes encoding ERα and ERβ being differentially expressed in different tissues (41); ii) variable expression of specific co-activators (e.g., members of the p160/SRC [Steroid Receptor Coactivator] family) and co-repressors (e.g., nuclear receptor co-repressor) (reviewed by Klinge (54)) in different tissues, and iii) variability in expression of ERα relative to ERβ (31, 39, 41, 51). Thus, the relative expression of ERα and ERβ in a given cell is a major
determinant of its response to estradiol and/or synthetic, non-hormonal agonists and antagonists (41). A number of ER splice variants have also been identified, giving rise to considerable heterogeneity of ER expression (41) and subsequent altered tissue responses to estrogen (43).

Cellular levels of ERs are primarily regulated by estrogen (31, 55). There is an ER response element in many ER promoter regions, and estrogen in many cell types leads to an increase in ER mRNA (messenger ribonucleic acid) (51). In premenopausal women, studies examining the influence of cyclic variation of estrogen levels on vascular ER expression demonstrate lower (~30%) ERα expression in vascular endothelial cells during the early follicular (low estrogen) compared to the late follicular (high estrogen) phase of the menstrual cycle (56). In PMW, ERα levels in the brachial artery have also been shown to be 33% lower compared with premenopausal women during the late follicular (high estrogen) phase of the menstrual cycle (56). In contrast, estrogen binding to the ER also stimulates ubiquitination and proteosomal degradation of ERs (51). This effect of estrogen limits estrogen-induced transcriptional output and negatively regulates ER expression (51). In contrast to the variable effects of estrogens, studies consistently demonstrate that progesterone, progesterone agonists, and vitamin D3 down-regulate ER expression in breast cancer cell lines (57-59).

The signalling pathways of ER activation in endothelial cells and vascular smooth muscle cells (VSMCs) are discussed in section 2.5 Effects of Estrogen on the Vasculature.
2.4 Vascular Function

2.4.1 Endothelium and Endothelial Function

The endothelium acts to maintain vascular homeostasis through multiple complex interactions with cells in the vessel wall and lumen (60). As a multifunctional, semi-permeable barrier between circulating blood and various tissues of the body, endothelial cells participate in all aspects of vascular health. In response to both humoral and mechanical stimuli, the endothelium exerts significant autocrine, paracrine and endocrine actions that influence both physiological and pathophysiological vascular permeability, the recruitment and activity of inflammatory cells, and regulate thrombosis (61-65). The endothelium is vital for the maintenance of vascular tone. This is achieved in part by the balanced synthesis and release of vasoactive substances from the endothelial cells, which profoundly affects both the function and structure of the underlying VSMCs via complex intercellular signaling processes (65). As such, endothelial control of VSMCs plays a key role in the resulting vasoreactivity implicated in physiological or pathological circulatory processes, including VSMC proliferation and migration (65). Endothelium-derived vasodilators include nitric oxide (NO), different endothelium-derived hyperpolarizing factors and prostacyclin (PGI2) (66). Endothelium-derived vasoconstrictors include endothelin (ET-1), thromboxane A2, reactive oxygen species (ROS) and angiotensin II (Ang II) (63, 65-67). Other vasoactive substances derived from the endothelium include modulators of vascular inflammation, such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, and nuclear factor kappa-B (NF-kB) (66, 67). Haemostasis modulators include release of plasminogen activator, tissue factor inhibitor, von Willebrand factor, NO, PGI2, thromboxane A2, plasminogen-
activator inhibitor-1, and fibrinogen (66, 67). Through the balanced synthesis and release of vasoactive products endothelial haemostatic function is realized.

2.4.2 Endothelial Dysfunction

The functional integrity of the endothelium is important in the maintenance of vascular homeostasis (65). Vascular injury and/or disruption in the balance of the production of vasoactive agents can lead to endothelial dysfunction, characterized by a shift toward reduced vasodilation and a pro-inflammatory and prothrombotic state (68). Impaired production and release of endothelial nitric oxide (NO) and an increase in oxygen free radical formation are hallmark characteristics of endothelial dysfunction (61, 66, 69). Endothelial dysfunction is associated with, and involved in, many disease processes, including atherosclerosis, diabetes, hypertension, hypercholesterolemia, and inflammatory syndromes (63, 65, 68, 70-72). Importantly, endothelial dysfunction is not only a consequence of increased cardiovascular risk, but also serves as a mediator of the deleterious effects of numerous cardiovascular risk factors (73). Thus, endothelial dysfunction is considered to be a major permissive factor for the progress and development of atherosclerosis (68) and may represent an independent predictor of adverse cardiovascular events (68, 74).

2.4.3 Nitric Oxide

NO is recognized as the most important protective molecule in the vasculature, regulating vascular tone, inhibiting VSMC proliferation, inducing apoptosis, attenuating platelet
aggregation, and reducing cellular adhesion to vascular walls (69, 75-77). Multiple pathways lead to the release of vascular NO, including constitutive endothelial release due to pulsatile blood flow (i.e., shear stress), dilating factors, (e.g., estrogen, prostaglandins) and metabolites (e.g., adenosine) released from contracting skeletal muscle, sympathetic-cholinergic nerve stimulation (i.e., acetylcholine release), and NO released directly from skeletal muscle (78). NO is synthesized from L-arginine via two constitutive calcium-sensitive isoforms of nitric oxide synthase (NOS) enzymes, namely, endothelial NOS (eNOS) and neuronal NOS (nNOS), and a calcium-independent inducible isoform (iNOS) (79). iNOS is highly expressed when stimulated by inflammatory stimuli (79). nNOS and eNOS are constitutively expressed in nervous tissue and endothelial cells, respectively. Constitutive production of NO by the endothelium maintains the vasculature in a state of vasodilatation (65).

In the vasculature eNOS is quantitatively the most important source of vascular NO (69, 76, 80). Within endothelial cell caveolae (described in 2.5.1 Non-genomic Effects), eNOS is closely regulated by interaction with caveolin-1 (81). Caveolin-1 markedly attenuates eNOS activity in the resting endothelial cell by interfering with binding to the calcium (Ca\textsuperscript{2+}) regulatory protein calmodulin, thereby maintaining eNOS in an inactivated state (81, 82). In the absence of Ca\textsuperscript{2+}, calmodulin does not associate with eNOS. Acute increases in intracellular Ca\textsuperscript{2+} in response to agonists such as bradykinin, acetylcholine, estradiol, adenosine, or shear stress, result in Ca\textsuperscript{2+} binding with calmodulin, thereby displacing caveolin-1 from eNOS, facilitating enzyme activation (82-84). A highly localized caveolae-associated Ca\textsuperscript{2+} pool that independently regulates Ca\textsuperscript{2+} entry into endothelial cells has also been postulated (85). Thus, the production of NO by eNOS is Ca\textsuperscript{2+}/calmodulin dependent. The presence of the essential co-factor, tetrahydrobiopterin, is also necessary, in addition to the co-
substrates molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), and redox cofactors including enzyme bound heme, FAD (flavin adenine dinucleotide), and FMN (flavin mononucleotide) (86).

NO released from endothelial cells diffuses into adjacent VSMCs where it binds to, and activates, soluble guanylyl cyclase (SGC), leading to smooth muscle cell relaxation (87). NO sensitive SGC (NO-SGC) is an important receptor for NO signaling. Stimulation of the NO-SGC by its physiological activator NO leads to an almost 200-fold increase in the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (87). NO-SGC, similar to other nucleotide-converting enzymes, requires magnesium as a cofactor for catalysis (87). The NO-induced cGMP signal is conveyed intracellularly by the activation of several effector molecules, including cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cGMP-gated ion channels (87). Potential in vivo VSMC targets of NO-dependent activation of cGMP-dependent protein kinases include the Ca2+ activated potassium (K+) channel (BKCa) and the inositol 1,4,5-trisphosphate (IP3)-associated cGMP kinase substrate (IRAG) proteins which modulate the extracellular Ca2+ entry and intracellular Ca2+ release, respectively (82). Phosphorylation of BKCa and IRAG is believed to reduce the cytosolic Ca2+ concentration, thereby, leading to relaxation of VSMCs and favourably influencing arterial BP (82, 87).
2.5 Effects of Estrogen on the Vasculature

2.5.1 Non-genomic Effects

One of the key functions of estrogen at the level of the vasculature is the potentiation of endothelium-dependent vasodilator responses, including the rapid, non-genomic increase in production and bioavailability of NO (88, 89). The upregulation and modulation of estrogen-stimulated endothelial NO by the rapid activation of eNOS via both PI3K/Akt and MAPK signaling pathways is recognized as an important mechanism of cardioprotection. This non-nuclear signaling pathway regulates physiologically relevant processes, including stimulation of endothelial cell migration and proliferation and upregulation of NO (39). These cellular events take place via complex second-messenger signalling pathways and activation of numerous co-activators and co-repressors upon estrogen binding with its plasma-membrane receptor (90, 91). In endothelial cells, a subpopulation of non-nuclear ERs localize to plasma membrane invaginations called caveolae. These specialized cholesterol-rich plasma-membrane organelles compartmentalize signal transduction molecules on the cell surface (92), and are the preferential microdomain of ERs and ER-centred complexes (93). ERs are directly bound to caveolar proteins by scaffold proteins, such as striatin (94). Striatin facilitates ER-centred, multi-molecular complex formation, and enhances ER plasma membrane localization (93, 94). In addition to scaffold proteins, ER lipid modifications are involved in ER membrane targeting (93, 95). Specifically, ER palmitoylation is critical for plasma membrane localization and rapid estrogen–induced signalling responses (95). ERα is the predominant ER membrane-associated receptor involved in non-nuclear signalling (95, 96) and recent evidence shows an ERα splice variant, ER46, mediates estrogen-stimulated eNOS activation more efficiently than
full-length ERα, with opposite efficiency noted for genomic responses (97, 98). Within the caveolae, ERα interacts directly with caveolin-1, the major caveolar structural protein, and co-localizes with eNOS. The signalling underlying ER coupling to eNOS includes generation of the second-messengers Ca2+, cyclic AMP (cAMP), as well as activation of two G-protein isoforms (Gα, Gs), the tyrosine receptor kinase src, and the serine/threonine kinases PI3K (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase) that produces phosphatidylinositol (3,4,5)-triphosphate (PIP3), and the kinase Akt (39, 40, 98). Activated Akt directly phosphorylates eNOS on serine 1177, leading to its enzymatic activation and to the activation of ERK1 (extracellular signal-regulated kinase), which is also required to yield an increase in eNOS enzymatic activity and production of NO (1, 39, 40, 51, 96, 99, 100). Heat shock protein (HSP) 90, a chaperone molecule, independently binds to and enhances the activity of Akt by preventing its dephosphorylation (101). In addition to Akt binding, HSP90 is also associated with eNOS in the quiescent state. Upon stimulation of endothelial cells by estrogen the association between eNOS and HSP90 proteins is increased and NO promotion enhanced (102).

2.5.2 Genomic Effects

The long-term vascular effects of estrogen are produced, in part, by changes in vascular cell gene and protein expression (31). Regulation of transcriptional activity of estrogenic target genes is mediated via the ‘classic’ ligand-activated pathway (31). By passive diffusion, estrogen enters target cells and binds to intracellular, or cytosolic, receptors (1, 103). The binding of estrogen to the ligand-binding domain of the ER triggers several events, including
phosphorylation, overall conformational changes of the ER, receptor dimerization and translocation to the nucleus where the ER bind to the estrogen response element (ERE) located in the promoter region of estrogen-activated target genes (31, 39, 41, 51).

In genes that are regulated by ER but do not contain an ERE, ER-ligand complexes can bind to DNA indirectly to induce protein-protein interactions with transcription factors such as activating protein-1, SP1 (Specific Protein-1) and NF-kB, effecting 'cross-talk' transcription (51). ERs can also modulate gene transcription by ligand-independent pathways. For example, phosphorylation of ER by growth factors, or growth factor activation of coactivators, allows ER to bind to ERE or bind DNA indirectly via transcription factors (51, 104). These varied mechanisms of estrogen-regulated genomic control underscore the complexity of the long-term biologic cardiovascular actions of estrogen.

2.5.3 Other Effects of Estrogen on Vasoregulation

In addition to activation of the PI3K/Akt and MAPK signaling pathways, experimental studies demonstrate that 48hrs of estrogen exposure to pulmonary endothelial cells upregulates cyclo-oxygenase-1 (COX-1) expression (105). COX-1 is responsible for the production of prostacyclin (PGI2), a vasodilator, via metabolism of arachidonic acid (106). PGI2 activates prostacyclin receptors in VSMCs, which activates adenylate cyclase and increases the formation of cAMP (cyclic adenosine monophosphate) (107). cAMP causes VSMC relaxation by mechanisms similar to those activated by cGMP (i.e., by Ca2⁺-dependent pathways) (107). Shifting of the primary end product of endothelial COX-1 pathway away from the
vasoconstrictor prostaglandin PGH4 to the vasodilator PGI2 is also mediated by estrogen (108). Administration of progesterone with estrogen prevents the stimulatory effects of estrogen on PGI2 production in cultured human umbilical vein endothelial cells (109).

Ligand-dependent activation of ERs also influences vascular tone via the production of endothelium-derived hyperpolarizing factor (EDHF). Endothelium-dependent vasodilation after NO and PGI2 inhibition has been attributed to EDHF activity (110). Thus, enhanced EDHF activity in conditions of NO deficiency contributes to maintenance of resting and agonist-stimulated vasodilation (110). EDHF activates K+ channels and causes VSMC hyperpolarization and inhibition of Ca2+ influx via Ca2+ channels leading to VSMC relaxation (107). EDHF is prominent in small-diameter arteries and arterioles (i.e., resistance vessels), and is therefore an important regulator of regional vascular resistance, blood flow, and arterial BP (111, 112). It is postulated that the influence of estrogen on EDHF may help explain in part why premenopausal women have consistently lower BP compared with age-matched males (111, 112).

2.6 Effects of Acute and Chronic Exercise on the Endothelium

2.6.1 Shear Stress

Biomechanical forces continuously act upon blood vessels throughout the arterial tree. Specifically, pulsatile flow created by blood flow and the cardiac cycle induces: i) pressure, created by the hydrostatic forces of blood within the blood vessel; ii) circumferential stretch or tension, created as a result of distinct intercellular connections between the endothelial cells
(e.g., integrins) that exert longitudinal forces on the cell during vasomotion; and iii) shear stress, created by the frictional force of blood flow directly on the vessel wall (61, 113, 114). Collectively, these hemodynamic forces regulate fundamental vascular functions, including vascular tone, and phenotypical modulation of smooth muscle cells and endothelial cells, eliciting structural modifications of the arterial wall and modulating atherogenesis (61, 115).

Of the hemodynamic forces described, shear stress is the most potent physiological stimulus for eNOS protein activation and endothelial NO production (114, 116). Shear stress is determined by blood flow, vessel geometry and fluid viscosity (117). The nature and magnitude of shear stress can vary considerably, thereby exposing endothelial cells to diverse flow patterns (117). Constant exposure to a physiologic range of shear stress, particularly in straight sections of the arterial tree exposed to unidirectional laminar flow, facilitates the establishment of important physiologic characteristics of the artery wall that promotes an anti-inflammatory, antithrombotic, anticoagulative, profibrinolytic and antihypertrophic state (61, 113, 114, 117). Laminar flow is associated with healthy endothelial function and atheroprotection (89, 113, 114, 118, 119). Conversely, low and/or oscillatory (e.g., turbulent) blood flow, as seen preferentially at arterial branches and curves, induces a sustained activation of a number of atherogenic genes in endothelial cells, such as the monocyte chemotactic protein-1 (MCP-1), which induces monocyte infiltration into the arterial wall, thereby contributing to the pathogenesis of atherosclerosis (89, 113, 120). Retrograde blood flow, the flow of fluid in a direction other than the physiological direction, and low shear stress in association with turbulent flow are associated with endothelial dysfunction (118-120).
2.6.1.1 Mechanotransduction of Shear Stress

Shear-stress mechanotransduction activates a signaling cascade that results in production of vasodilators, including EDHF, PGI2, and NO (121). The vasodilators produced depend on the nature of the shear-stress stimulus and the endothelial phenotype (122). Endothelial and VSMCs are equipped with numerous receptors that allow them to detect and respond to shear stress (61). However, due to shear stress being the result of the parallel frictional force of blood against the vessel wall, shear stress is primarily sensed by endothelial cells (61, 116). The mechanisms by which the mechanical signal induced by shear stress is sensed by endothelial cells and transduced into second-messenger signalling that stimulates vasodilator production are complex and not yet fully understood. However, it is recognized that both force transmission, via the complex network of cytoskeletal elements (e.g., microtubules, microfilaments), and transduction of physical stimuli into biochemical signals are required for the transduction of mechanical forces (61, 114, 116). For this process to be enabled, endothelial cells must be anchored to extra-cellular matrix in order to sense and transduce signals (123). The attachment sites, called focal adhesions, are complexes of integrins, cytoskeletal and signaling proteins that mediate signal transduction (124). Activated integrins increase their association with an adaptor protein, Shc, which subsequently activates the downstream MAPKs, including ERKs and p38 MAPK (125). Mechanical stimulation of release of vasoactive substances (e.g., NO, prostaglandins) and upregulation of expression of endothelial cell genes and proteins (e.g., eNOS) that are protective against atherosclerosis help maintain normal endothelial structure and function (61, 89, 113, 114).
2.6.1.2 Acute versus Chronic Shear Stress Activation: Flow Mediated Dilation and Exercise

The acute effect of increases in shear stress on NO release underlies the principal of flow-mediated dilation (FMD) (116, 120). FMD describes the vasodilatation of an artery following an increase in luminal blood flow and internal-wall shear stress, and can be quantified as the change in vessel diameter from baseline dimensions before the application of the shear-stress stimulus (122). Shear stress is integrally involved in FMD, both as a means to normalize mechanical forces and to maintain normal endothelial function (121). Importantly, shear stress stimulus generated in the brachial artery in response to FMD is inversely related to vessel diameter (22, 126), which is also inversely associated with the FMD response (22, 126).

Immediate increases in shear, as with post-ischemic vasodilation in response to brachial artery occlusion (i.e., FMD), potently activates the eNOS protein to produce NO within seconds by acutely and transiently stimulating increases in intracellular calcium, thereby enhancing calmodulin binding to eNOS (121, 127). Long after intracellular calcium returns to baseline levels, local NO production continues by means of PI3K and Akt activated phosphorylation of eNOS, lasting as long as the increase in shear stress is imposed (121, 128).

Over several hours, prolonged increases in shear stress, as occurs with sustained exercise, causes an increase in eNOS mRNA and protein expression (129), thereby allowing endothelial cells to produce larger amounts of NO (121). This effect occurs by two distinct pathways: i) regulation of eNOS transcription, and ii) eNOS mRNA stability (129), with transcription peaking at approximately 1 hour and returning to baseline levels shortly thereafter.
Both of these pathways share a need for activation of the tyrosine kinase c-Src (118, 130). In the face of c-Src blockade, shear-induced eNOS mRNA is prevented (118). The chronic effect of shear stress-induced upregulation of eNOS and greater activation (phosphorylation) of the enzyme likely explains in part the beneficial effects of regular exercise on endothelial function (118, 121, 129). For example, in humans, chronic exercise training is associated with enhanced brachial artery endothelium dependent vasodilation (75). Similarly, exercise training in patients with established coronary artery disease (CAD) elicits improved coronary (131) and mammary (80) arterial endothelial function.

Other essential processes involved in shear induced eNOS activation include NF-kB activation. NF-kB is a heterodimer consisting of a p50 and a p65 subunit that plays a key role in regulating stress-induced, immune, and inflammatory responses (132). In unstimulated cells NF-kB is sequestered in the cytosol, bound to a class of inhibitory proteins called IkBs (129). A primary level of control for NF-kB is through interactions with an inhibitor protein called IkB. Signals that induce NF-kB activity elicit phosphorylation of IkBs is primarily mediated by IkB kinase (IKK) (132). This leads to IkB dissociation and subsequent degradation, resulting in activation of the NF-kB complex (129). The activated NF-kB complex translocates to the nucleus and induces gene expression. Shear stress induces NF-kB activity, which results in translocation of the p50 and p65 complex to the eNOS promoter region in the nucleus where they increase eNOS activity (129, 133). Although NF-kB has been implicated in the regulation of several pro-atherogenic and pro-inflammatory genes (e.g., vascular cellular adhesion molecule-1, monocyte chemoattractant protein-1), its transient activation and resulting NO production in response to shear is considered to elicit favourable anti-inflammatory and anti-
atherosclerotic properties (129). Through a negative feedback mechanism, shear-induced NO de-activates the p50 sub-unit of NF-kB, effectively inhibiting NF-kB activation (133).

2.6.1.3 Estrogen and Shear Stress

The influence of estrogen on shear stress or shear-stress mediated signalling in the vasculature *per se* is not clear. However, *in vitro* data indicate that 17β-estradiol induces β1-integrin expression in endothelial cells (134). In osteoblasts, 17β-estradiol augments shear stress responsiveness of signal transduction and gene transcription via ER–mediated increases in β1-integrin expression (135). Integrins are highly expressed in endothelial cells and are required for mechanical stretch–induced activation of extracellular signalling pathways (136). Thus, estrogen and shear stress may play synergistic roles in modulating shear stress-mediated signal transduction and gene expression, at least in these cell types.

It is noteworthy that both dynamic exercise (i.e., shear stress) and estrogen share a common but complex pathway of NO production via Akt-dependent increases in activation of eNOS (80, 137). The influence of each of these stimuli on vascular endothelial function has been shown to be independent, but not additive (14, 15, 21, 138-140). In older sedentary PMW, both acute dynamic exercise and one month of estrogen therapy (ET) independently, but not additively, restores endothelium-dependent function, with FMD responses being comparable to those observed in healthy young sedentary premenopausal women (139, 140). Whether the net effect of either stimulus is sufficient to overwhelm any effects of the other (141) or whether redundancy in the system predominates remains unknown.
2.7 Vascular Effects of Estrogen and Exercise Training on Oxidative Stress, Inflammation and Lipid Profile

2.7.1 Oxidative Stress

Oxidative stress plays a major role in the pathogenesis of atherosclerosis, vascular inflammation and endothelial dysfunction (142). It is characterized by impaired NO bioavailability and is understood to arise principally as a consequence of both the increase in production of ROS relative to antioxidant defenses and an upregulation of the enzymes involved in ROS production (142, 143). Excessive production of ROS relative to anti-oxidant defense mechanisms leads to oxidation of a number of macromolecules, including DNA, protein, carbohydrates and lipids (118). This process is commonly referred to as oxidative stress. Many ROS possess unpaired electrons and thus are free radicals, such as singlet oxygen, superoxide anion, nitric oxide, and lipid radicals (142). Other ROS, such as hydrogen peroxide, peroxynitrite, and hypochlorous acid, are not free radicals per se but have oxidizing effects that contribute to oxidative stress (144). Physiologically, ROS are fundamental to numerous physiological cellular signaling pathways in many cell types within the cardiovascular system (142). Pathophysiologically, ROS contribute to vascular dysfunction and remodeling through oxidative damage by several mechanisms, including reducing the bioavailability of NO, impairing endothelium-dependent vasodilatation, causing apoptosis, stimulating endothelial cell migration, and activating adhesion molecules and inflammatory responses, leading to endothelial cell dysfunction (144, 145).
Among the many enzymatic systems that are capable of producing ROS, NADPH oxidases (NOX), and uncoupled eNOS have been extensively studied in vascular cells. Cardiovascular risk factors increase the expression and/or activity of NOX in the vascular wall, thereby enhancing the production of ROS (69). In many types of vascular disease, NOX and eNOS are upregulated in an analogous manner (69). Their respective products, superoxide anion and NO, react rapidly to form peroxynitrite (69, 146). Peroxynitrite leads to depletion of thiol groups, damage to DNA, nitration of proteins, and oxidization of tetrahydrobiopterin, an essential co-factor of eNOS (69, 146, 147). Diminished tetrahydrobiopterin levels promote superoxide anion production by eNOS uncoupling, thereby diminishing NO bioavailability (69, 146).

Free radical concentrations can be lowered by decreasing free radical production or by increasing elimination. This can be achieved in part via: i) administration of non-enzymatic antioxidants (e.g., vitamin C), ii) by administration of agents that interfere with NOX induced superoxide formation (144, 145), or iii) stimulation of endogenous enzymatic antioxidant systems (e.g., superoxide dismutase, glutathione peroxidase), such has been shown with exercise training (148).

2.7.1.1 Oxidative Stress and Estrogen

Experimental studies demonstrate that estrogens exert both cytoprotective and antioxidant properties, potentially offering protection against the deleterious effects of ROS (149-151). Indeed, estrogens are known to be potent scavengers of free radicals, forming moderately
stable radicals from less stable radicals by donating a hydrogen atom (149, 150, 152, 153).

Structurally, the phenolic A-ring of estrogens appears to be a critical component of this antioxidant activity, as other sex hormones which lack this ring (e.g. progesterone), have no cytoprotective effects (154). Experimental studies also show that both 17 β-estradiol and 2-hydroxyestradiol, a natural metabolite of 17 β-estradiol, inhibit oxidative damage of lipids, including LDLc (152, 153, 155, 156). Oxidized LDLc (OxLDLc) plays a pivotal role in the pathophysiology of atherosclerosis, including endothelial dysfunction (157). Of interest catecholestrogens, such as 2-hydroxyestradiol, appear to afford similar or greater protection against lipid peroxidation than phenolestrogens (i.e., 17 β-estradiol, estrone and estriol) (151-153, 155, 156, 158, 159). \textit{In vitro} data also show that estrogen lowers endothelial production of superoxide anion, thereby increasing the bioavailability of NO (160).

In premenopausal women, studies demonstrate significantly lower lipid peroxide concentrations, as well as significantly higher glutathione peroxidase activity compared with PMW (161). Significant increases in endometrial glutathione peroxidase have been observed during the high compared with the low estrogen phase of the menstrual cycle (162). Conversely, hypoestrogenemia is associated with loss of the antioxidant and cytoprotective effects afforded by estrogen. For example, estrogen-deplete physically active premenopausal women with eumenorrheic but anovulatory menstrual cycles demonstrate increased basal erythrocyte superoxide dismutase (SOD) compared with ovulatory women (163). SOD is the major enzymatic antioxidant defence system against superoxide (164, 165), with elevated levels suggesting increased superoxide production (163). Consistent with this, experimental data in animals also show that ovariectomy increases and 17 β-estradiol diminishes SOD (166). Administration of 17 β-estradiol also decreases lipid peroxidation in ovariectomized rats (166).
Such data support a beneficial effect for endogenous estrogen on both intracellular antioxidant enzyme activity and free-radical scavenging abilities against lipid peroxidation.

2.7.1.2 Oxidative Stress and Exercise

Exercise increases both oxygen consumption and production of ROS including superoxide and hydrogen peroxide (148, 167). Increased shear stress also increases vascular generation of ROS via increased activation of endothelial NOX (168, 169). However, chronic exercise training results in upregulation of antioxidant defence mechanisms (148). Studies report increases in the expression of several antioxidant vascular proteins such as eNOS and extracellular SOD, and Cu/Zn-SOD (164, 165, 170, 171), and down-regulation of several pro-oxidant vascular proteins, including subunits of endothelial cell and VSMC NOX and angiotensin receptor type I, in response to exercise training (171-173).

SOD rapidly converts superoxide, resulting in hydrogen peroxide formation. Endogenous hydrogen peroxide has been shown to play an important role in the endothelial adaptation to exercise training by transiently stimulating an upregulation of eNOS via phosphorylation of Ca$^{2+}$/calmodulin-dependent kinases (165, 171, 174). In skeletal muscle, exercise training similarly stimulates activities of antioxidant enzymes, including catalase (175), and glutathione peroxidase (167), resulting in improved capacity to overcome subsequent exposure to oxidative stress. Thus, in a hormetic manner, chronic exercise training results in adaptations in vascular and skeletal muscle antioxidant capacity that results in protection against the deleterious effects of oxidants and subsequent cellular damage.
2.7.2 Vascular Inflammation

Atherosclerosis is recognized as a chronic inflammatory disorder that results from the interactions between modified lipoproteins and various components of the immune system (176, 177). The local production of pro-inflammatory cytokines in the artery wall in response to ox-LDLc promotes endothelial dysfunction, regulates macrophage and T-cell activation and proliferation, alters VSMC phenotype, induces apoptosis, and promotes plaque rupture and thrombosis (176, 178). Thus, chronic low-grade inflammation, indicated by modest (2–4-fold) elevations in circulating pro-inflammatory cytokines, contributes importantly to all stages in the development and progression of atherosclerosis (176, 179). Inflammatory variables shown to modify cardiovascular disease (CVD) risk includes the acute-phase reactant C-reactive protein (CRP), TNF-α (tumour necrosis factor-a), IL (interleukin)-6, IL-2, IL-7, IL-8, IL-18, soluble CD 40 ligand (sCD40L) and macrophage colony-stimulating factor (176, 178, 180, 181). A key event in the inflammatory response in the vasculature is the localized recruitment of various leukocyte subsets through endothelial-dependent mechanisms (182). Initially, the inflammatory activation of endothelial cells, which express adhesion molecules, such as selectins (e.g., E- and P-selectin), VCAM-1, and intracellular adhesion molecule-1 (ICAM-1), facilitates tethering and rolling of leukocytes on the inflamed endothelial surface (179, 182). Chemotactic factors, such as MCP-1, released by the vascular cells trigger cell arrest at or near cell–cell junctions, attracting the migration of leukocytes to extravascular spaces (179, 182). Stimulated immune cells then secrete and release chemical mediators, such as inflammatory cytokines (e.g., IL-1, IL-6, TNF-α, etc.), free radicals and growth factors, that induce a broad spectrum of local and systemic responses (55, 179, 183). For example, inflammatory cytokines stimulate VSMCs proliferation and migration, in addition to excessive extracellular matrix
deposition, leading to thickening of the vascular intima and arterial stiffness (179). Further, cytokine stimulated endothelial cells undergo functional alterations resulting in a prothrombotic and proinflammatory phenotype (179). Endothelial dysfunction induced by inflammation also leads to alterations in regulation of vascular tone due to imbalance between production of vasodilator and vasoconstrictor substances in the vascular wall (179).

2.7.2.1 Vascular Inflammation and Estrogen

Both pro-inflammatory and immunosuppressive effects of estrogen have been reported, with pro-inflammatory responses in humans commonly being reported in association with chronic autoimmune diseases (55). This section will focus on the anti-inflammatory effects of estrogen, followed by a brief discussion of the potential pro-inflammatory effects.

2.7.2.1.1 Anti-Inflammatory Effects of Estrogen

Epidemiological studies suggest a beneficial role for endogenous estrogen in preventing vascular inflammation (179). In premenopausal women, serum levels of soluble ICAM-1 are negatively correlated with circulating estradiol concentrations across the phases of the menstrual cycle (184). Consistent with this, PMW taking hormone replacement therapy demonstrate down-regulation of serum inflammatory markers, including ICAM-1, VCAM-1 and E-selectin (185), MCP-1 (186), TNF-α (187), and vascular endothelial growth factor (188). In animals, estrog enic inhibition of inflammation initiated by TNF-α has been consistently
demonstrated (55). Experimental data also show that 17 β-estradiol significantly inhibits basal IL-6 secretion in human umbilical vein endothelial cells (189). In endothelial cells, studies indicate that ERα mediates the inhibitory effects of estrogen of most, but not all, cytokine-induced expression of cell adhesion molecules (96, 190). However, in cultured VSMCs, ERβ has been shown to mediate the estrogenic inhibition of TNF-α-induced inflammation (191). Collectively, these data support an anti-inflammatory effect of estrogen, with hypoestrogenemia resulting in increased levels of inflammatory markers.

Postmenopausal status is associated with higher prevalence of obesity (183), and it is known that adipose tissue is a primary source of inflammatory mediators (192). The increase in adiposity in PMW is associated in part with the decline in endogenous estrogen (193) (194). The development of obesity and obesity-related disorders, such as metabolic syndrome and inflammation may therefore be favorably modulated by estrogen (195).

A further mechanism explaining estrogen’s anti-inflammatory role relates to the NF-kB pathway. There are two phases in the interaction between estrogen and NF-kB: i) an acute NF-kB activating response that mediates cellular protection, and ii) a late, chronic response to estrogen that inhibits NF-kB (132, 196). Estrogen has been shown to interact with the NF-kB pathway, both by the direct inhibition of NF-kB DNA binding, and also by altering NF-kB activation through regulation of IKK activation and IkB expression (132). Via ER-dependent mechanisms, estrogen interacts with NF-kB to modulate both its cytoplasmic and nuclear activity (197). In human coronary artery endothelial cells, rapid, non-genomic physiologic effects of estrogen have been shown to activate, and long-term genomic effects inhibit, NF-kB activation (196).
2.7.2.1.2 Pro-Inflammatory Effects of Estrogen

Under pathophysiological conditions, estrogen-induced stimulation of eNOS activity may lead to increased generation of the superoxide free radical, further reinforcing proinflammatory and proatherogenic effects (179). Further, under conditions of enhanced oxidative stress, NO production by estrogen could provide more substrate to react with superoxide, potentially resulting in the formation of the potent inflammatory molecule peroxynitrite (179, 198). Peroxynitrite, via different pathways, can also stimulate and inhibit transcription factor NF-κB (199). NF-κB is an important transcription factor involved in the regulation of genes that encode for a large number of proteins involved in inflammatory responses (146). NF-κB activation effects dual and opposing roles such that activation can lead to the expression of products that are both pro-inflammatory and anti-inflammatory, in addition to pro-apoptotic and anti-apoptotic (132). Importantly, chronic NF-κB activation is associated with numerous inflammatory pathological conditions, including diabetes, heart failure, atherosclerosis and endothelial dysfunction (200-202).

Overall, the potential negative effects of estrogen in mediating vascular oxidative stress under pathophysiological conditions can result from elevated free radical production, stimulated transcription of inflammatory factors via NF-κB, and reduced NO bioavailability (132, 179, 198, 200-202).
2.7.2.2 Vascular Inflammation and Exercise

It is widely accepted that acute and chronic exercise influence the number and function of circulating cells of the innate immune system (e.g., neutrophils and monocytes) (203). Following acute exercise, there is a transient increase in circulating levels of anti-inflammatory cytokines, whereas chronic exercise reduces basal levels of pro-inflammatory cytokines (176). For example, circulating level of IL-6, both a pro- and anti-inflammatory cytokine, is markedly increased in response to exercise. IL-6 released from working muscles increases exponentially in plasma (up to 100 fold), with the magnitude of response related to the intensity and duration of exercise, the mass of muscle recruited, and the individual endurance capacity (203, 204).

The cardiovascular significance of this response is unknown, but elevated IL-6 is linked to all-cause and CVD mortality in patient populations (176, 205), and has been suggested to directly promote atherosclerosis by enhancing endothelial expression of chemokines and adhesion molecules, increasing endothelial dysfunction and promoting a procoagulant state in obesity (176, 206). In contrast, it is thought that muscle-derived IL-6 in response to exercise may act in a hormone like manner to mobilize extracellular substrates and/or augment substrate delivery during exercise (204). Importantly, IL-6 released from skeletal muscle also stimulates post-exercise production of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist, and also exerts inhibitory effects on TNF-α (204). TNF-α is a pro-inflammatory cytokine that induces the expression of vascular adhesion molecules such as ICAM-1 and E-selectin (207, 208) and is thought to play a central role in the development of CVD (176, 178).

Chronic exercise training is generally associated with modest reductions in circulating levels of TNF-α and IL-6, and increased levels of IL-10 (209). Thus, exercise is associated with modest improvements in markers of systemic low-grade inflammation (176, 209, 210), and is thought,
in general, to confer anti-inflammatory effects (176, 178, 209). However, it is unclear whether exercise-induced changes in innate immune function influence cardiovascular disease risk or outcome (203). Evidence suggests that the cardioprotective effect of exercise may, to some extent, be ascribed to the anti-inflammatory effect of regular exercise, potentially mediated via a reduction in visceral fat mass and/or by induction of an anti-inflammatory environment with each bout of exercise (e.g., through increases in circulating anti-inflammatory cytokines, such as IL-10) (203).

2.8 Neurohumoral Vasoregulation: Influence of Estrogen and Exercise

Systemic arterial BP, flow and blood volume are closely regulated by a group of interrelated mechanisms, including neural (autonomic) and humoral (circulating or hormonal) factors. Neurohumoral effects on cardiovascular function can be divided into four categories: i) adrenal medullary effects caused by circulating catecholamines (epinephrine and norepinephrine), ii) the sympathetic stimulation of renal juxtaglomerular cells that activates the renin–angiotensin-aldosterone system (RAAS), iii) the effects of cardiac sympathetic nerves, and iv) the effects of vascular sympathetic nerves (211, 212). The net effect of these neurohumoral responses is to produce arterial and venous constriction, increase cardiac contractility, and increases in blood volume (187). Autonomic imbalance (i.e., increased sympathetic and/or decreased parasympathetic activity) contribute to the pathogenesis of several disease states, including hypertension, obesity, heart failure and diseases of the vasculature as well as myocardial
ischemia (24, 26, 213, 214). It is also associated with increased risk for sudden death and/or susceptibility to ventricular arrhythmias (215), and orthostatic intolerance (216).

Common indirect surrogate measures of neurohumoral cardiovascular activity include heart rate variability (HRV), heart rate (HR), heart rate recovery after exercise, baroreflex sensitivity (BRS), plasma catecholamine levels, and measures of circulating humoral factors, such as the RAAS (215). Direct measures of regional sympathetic activity by assessment of muscle sympathetic nerve activity (MSNA) have also been reported (215). Of these indices, the following review describes HRV, BRS, RAAS and MSNA, and how they are influenced by estrogen and exercise training.

2.8.1 Muscle Sympathetic Nerve Activity

MSNA is a direct measure of vascular vasoconstrictor nerve efferent neural activity to skeletal muscle, and can be measured at superficial nerve sites, such as the peroneal or radial nerve, using microneurographic recordings (217). MSNA is linked to arterial BP via the baroreflex (218), occurring with cardiac rhythmicity (211). Acute decreases in arterial BP cause reflex sympato-excitation and increases in arterial BP cause sympato-inhibition (218). Thus, SNA is thought to contribute importantly to the control of blood flow and BP (219), and influence cardiovascular disease manifestations, such as hypertension and heart failure (24, 220).

Importantly, aging is associated with increases in whole body sympathetic neural activity (217, 221), (222), reflected in part by increases in whole body norepinephrine spillover and increases in MSNA of $\approx$1 burst per minute per year, starting around age 30 years (217, 221).
Marked interindividual variability in the frequency of MSNA outflow is observed at
rest and in response to various stimuli (217, 221). Older individuals also tend to demonstrate
higher BP than younger individuals (222). Although evidence supports elevated SNA in
experimental and human primary (essential) hypertension (223, 224), the relationship between
MSNA and BP is not clear. The association between MSNA and mean arterial BP is reported
to be absent prior to the age of 40 years in both men and women, with MSNA and mean
arterial BP becoming modestly associated after this age, with older women demonstrating a
stronger association than older males (24). Lack of association between BP and MSNA prior
to the age of 40 years suggests that BP regulation may be less sympathetically mediated in
younger individuals, but that with increasing age, the contribution of sympathetic neural
control to BP may become increasingly significant. Importantly, SNA assessed in the lower
limb vasculature does not reflect SNA directed to other target organs, such as the kidneys or
heart. Thus, measures of nerve efferent neural activity to skeletal muscle estimates MSNA
only.

2.8.1.1 Muscle Sympathetic Nerve Activity and Estrogen

Studies in humans report that estrogen levels are inversely associated with MSNA (24, 225,
226). Premenopausal women demonstrate lower MSNA than age-matched males and older
PMW (24, 225, 226). PMW also demonstrate higher MSNA than age-matched males (24, 225,
226), with transdermal estrogen therapy shown to lower MSNA (227). However, the effects of
the menstrual cycle on MSNA are less clear. Some (228), but not all (229, 230) studies report
increased resting MSNA and plasma norepinephrine during the mid-luteal phase (high
estrogen, high progesterone) compared with the early follicular phase (low estrogen, low progesterone). Reasons for inconsistent findings across the menstrual cycle are not known, but likely reflect differences in methodologies used to perturbate BP (e.g., pharmacological versus physiological) to elicit MSNA responses, difficulties in accurately determining menstrual cycle phases, and timing of assessment. With estradiol and progesterone postulated to exert opposing sympathoexcitatory effects (228), it is particularly challenging to determine the independent effects of the ovarian hormones on SNA during the luteal (high estrogen, high progesterone) phase of the menstrual cycle.

Despite MSNA being under arterial-baroreflex control, the relationship between MSNA and arterial BP is not clear. In men and women under the age of 40 years, MSNA and arterial BP are not correlated (24). In contrast, MSNA and arterial BP become modestly associated in older (>40 years) men and women, with older women demonstrating a greater strength of relationship than age-matched males (217, 221). The stronger MSNA and BP relationship in older women parallels epidemiological data indicating a higher prevalence of hypertension in women by age 60 (231). These observations suggest that prior to the age of 40 years BP regulation is less sympathetically mediated in younger than older individuals, in whom the contribution of sympathetic neural control to BP may become increasingly significant. However, the influence of estrogen deficiency per se in MSNA and BP responses in PMW is unclear. In support of a direct modulatory effect of estrogen that is independent of aging, short-term (2 days) transdermal estrogen therapy in PMW has been shown to lower MSNA without changes in arterial BP (227). Experimental studies similarly demonstrate that acute estradiol administration to ovariectomized rats rapidly suppresses HR and renal and splanchnic SNA without changes in mean arterial BP (232). Such responses suggest direct non-genomic
effects of estrogen on central nervous autonomic centers (232) (227). In support of a genomic mechanisms of action, longer-term (8 weeks) transdermal, but not oral, estrogen therapy has been shown to lower both MSNA and diastolic BP without augmenting arterial baroreflexes in normo-tensive PMW (233). Differences in MSNA and BP responses with short- and long-term estrogen therapy, and differences in these responses with different routes of administration of estrogen therapy underscores the complexity of the effects of estrogen on sympathetic outflow.

In PMW and young men, the occurrence of sympathetic bursts in MSNA is correlated with diastolic BP variance, total peripheral resistance and cardiac output (211, 221, 234). These relationships are absent in premenopausal women. Thus, compared with men and PMW, premenopausal women with high resting MSNA do not necessarily have high total peripheral resistance or a low cardiac output, suggesting that MSNA may not be a good indicator of vasoconstrictor tone in this population (211, 221, 234, 235). Such differences allude to potential sex and age-related differences in mechanisms that regulate BP (221, 234). In premenopausal women, these differences include less autonomic and α–adrenergic receptor support of arterial BP (236, 237), enhanced β–adrenergic receptor-mediated dilatation (238), less ability to buffer increases in BP via the baroreflex (236), and lower α–adrenergic receptor sensitivity to noradrenaline (239). The influence of estrogen deficiency on these mechanisms of BP control in young and older women are not yet known.
2.8.1.2 Muscle Sympathetic Nerve Activity and Exercise

MSNA is increased during mild-intensity isometric (240) and dynamic handgrip exercise (240), during moderate-to-high intensity isometric leg extension exercise (241), and during arm cycling (242). In contrast, MSNA assessed in the contra-lateral, non-exercising limb, is lowered during moderate-intensity upright one-legged cycling (243). Conversely, dynamic one-legged cycling in the supine position does not alter MSNA outflow (243). A role for the central cardiopulmonary baroreflex in MSNA regulation during mild-moderate intensity dynamic exercise has been proposed (244). In contrast, the muscle metaboreflex is the primary-mechanism by which MSNA is stimulated during small-muscle, isometric exercise in humans (245). During dynamic exercise, muscle metaboreflex-stimulation of MSNA also occurs, but only at or above moderate, submaximal intensities (245). Similarly, low intensity isometric handgrip exercise does not activate MSNA (246).

Data pertaining to the effects of exercise training on MSNA are scarce, with no research systematically focused on women. Further, possible interactions between estrogen and exercise on MSNA have not been reported. However, studies show that exercise training decreases resting arterial BP and total peripheral resistance (247), with decreases in total peripheral resistance associated with decreases in renal (248, 249) and muscle (250, 251) sympathetic nerve activity. In light of the strong inverse association between cardiac output and MSNA (211), decreases in resting BP after 3 months of exercise training in association with increased resting cardiac output (247) likely reflects decreased sympathetic nerve activity.
2.8.2 Renin-Angiotensin-Aldosterone System (RAAS)

The RAAS is integrally involved in the regulation of blood volume and systemic vascular resistance, which together influence cardiac output and arterial BP, in addition to regulating water and electrolyte homeostasis (173, 252). Traditionally, the RAAS is considered to function as a circulating system, with the classical RAAS cascade beginning with the release of renin into the circulation. Renin is a proteolytic enzyme that is primarily synthesized, stored, and secreted by the juxtaglomerular cells of the kidney. Synthesis and release of renin is a key determinant of the activity of the RAAS, occurring in response to four major stimuli, including: i) sympathetic nerve activation, acting via β1-adrenoceptors; ii) renal artery hypotension; iii) decreased sodium delivery to the distal tubules of the kidney; and iv) negative feedback by direct actions of angiotensin II on the juxtaglomerular cells (253, 254). When renin is released into the blood, it acts upon a circulating substrate, angiotensinogen, that undergoes proteolytic cleavage to form the decapeptide angiotensin I (Ang I, or Ang I-(1-10)) (254). The enzyme angiotensin converting enzyme (ACE) cleaves two amino acids from Ang I to form the octapeptide, angiotensin II (Ang II, or Ang II-(1-8)) (254). Ang II is the primary active product of the RAAS. The effects of Ang II are primarily mediated by two distinct angiotensin receptors, AT1R and AT2R. In the classical pathway, Ang II exerts its physiological effects through activation of the AT1R (Figure 2), including: i) vasoconstriction of VSMCs and renal arterioles; ii) regulation of sodium and water balance by promoting the release of the sodium-retaining hormone aldosterone from the adrenal cortex; iii) stimulation of the sympathetic nervous system at the level of the brain stem, autonomic ganglia, and presynaptic Ang II receptors of sympathetic nerve terminal neurons; and iv) interaction with baroreflexes, resulting in withdrawal of vagal tone (254-256). The AT1R also mediates the
pathophysiological actions of Ang II, including cell growth and proliferation, inflammatory responses and oxidative stress (253). Thus, chronic activation of RAAS through AT1R is considered to contribute importantly to endothelial dysfunction and disease states such as hypertension and heart failure (253, 256). In contrast, the AT2R mediates vasodilation, exerts anti-proliferative and anti-apoptotic effects on the VSMCs, and inhibits growth and remodelling in the heart (253). While it is evident that the AT receptors exert opposing effects, the exact role and importance of AT2R are not known at this time.

As described above, an important role of Ang II is the promotion of aldosterone release (257). Aldosterone, the final endocrine signal in the RAAS, is produced in the zona glomerulosa of the adrenal cortex. This mineralcorticoid hormone targets epithelia in the kidney and colon to regulate sodium (re)absorption and potassium secretion (257, 258). As such, aldosterone is a major regulator of sodium and potassium balance, playing an important role in electrolyte balance and extracellular volume regulation, and thus, BP. Ang II and plasma potassium are the principal regulators of aldosterone production. Thus, aldosterone is under negative feedback control, responding negatively to increases in BP and decreases in potassium, and responding positively to decreases in BP and increases in potassium (258). Increased aldosterone biosynthesis has been postulated as a key independent mechanism contributing to hypertension and cardiovascular disease (257, 259).

It is now recognized that the RAAS is not only a circulating system, but also a tissue paracrine/autocrine system (253). The discovery of all RAAS components in various tissues, including the pituitary, brain, kidney, adrenal glands, adipose tissue, ovaries, testes, skin, heart, VSMCs and endothelial cells has led to the ’local’ or ‘tissue RAAS’ concept (253, 260). This
concept is based on local RAAS actions leading to tissue-based synthesis of Ang II (261). Of
importance to this synthesis is the binding of prorenin, the precursor of renin, and renin to the
cell surface in tissues (253). Physiologically, this action provides an independent mechanism
to generate Ang II locally in excess of the Ang II that is produced in the circulation (253, 261).
In the vasculature, local RAAS influences cardiovascular homeostasis through its effects on
both AT receptors, and mediates long-term effects on vascular remodeling by stimulating
proliferation of VSMCs and fibroblasts (261).

Recent studies have identified novel local RAAS components, including a new
metabolising enzyme (ACE2), smaller angiotensin peptides (e.g. Ang-(1-7) and Ang-(1-9)),
and new AT receptors, AT3R and AT4R. ACE2 is involved in the generation of alternative
angiotensin peptides, particularly through the conversion of Ang II to Ang-(1-7) and Ang I to
Ang-(1-9) (261). Ang-(1-7), derived from the cleavage of Ang-(1-9) by ACE, is reported to
modulate and antagonize the effects of Ang II (Figure 2). The putative effects of Ang-(1-7)
include vasodilation and anti-proliferation (261). These effects are thought to be mediated by a
unique receptor, the MAS receptor, that does not bind Ang II (262). The functions of AT3R
and AT4R are currently not well defined. However, these receptors are thought to influence
thrombosis by mediating the release of plasminogen activator inhibitor I through Ang I and II,
and the truncated peptides Ang III and Ang IV (261, 262). These recent discoveries, taken in
conjunction with the classical RAAS pathway, emphasize the complexity and diversity of the
physiological and pathophysiological role of the RAAS in circulatory and tissue homeostasis
(253, 256).
Figure 2. The renin-angiotensin-aldosterone system and its effects on vascular tone and BP.

ACE, angiotensin converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; AT1R, angiotensin receptor type 1; AT2R, angiotensin receptor type 2; BP, blood pressure; MAS-R, mas receptor.

2.8.2.1 Renin-Angiotensin-Aldosterone System and Estrogen

Estrogen is known to influence all components of the RAAS. The promoter region of the angiotensinogen gene is responsive to estrogen, with concentrations tending to be higher in women compared with men, and higher in premenopausal women compared with PMW (263, 264). Administration of estrogen-containing oral contraceptive formulations to premenopausal women (265) and oral ET to PMW (266) results in significantly increased plasma concentrations of angiotensinogen compared with non-users. The increased level of
angiotensinogen in women taking oral exogenous estrogen is thought to be primarily due to the ‘first-pass’ bolus effect following oral ingestion leading to transiently high hepatic exposure.

In contrast, transdermal estrogen therapy, which avoids the ‘first pass’ bolus effect, elicits mild to no stimulation of angiotensinogen production (264, 267).

Plasma renin activity (PRA), an estimation of renin in plasma by measuring the rate of formation of Ang I, is lower in premenopausal women than in age-matched males and older PMW (267). However, in PMW, the effects of exogenous estrogen on PRA and plasma renin levels have been shown to be inconsistent, suggesting either unchanged, decreased or elevated levels (266-271). In premenopausal women, plasma renin and PRA have been reported to be elevated during the luteal compared with the follicular phase of the menstrual cycle (272). In contrast, oral contraceptives are reported to increase PRA, but not plasma renin, levels (265, 273). Variable findings are thought to largely reflect complexities and variances in the choice in measure of renin in conjunction with the technological aspects of the biochemical assay (264).

Numerous studies show that oral ET in PMW increases serum Ang I and II levels, and decreases serum ACE activity without altering BP (268, 269, 274-276). Unchanged BP despite altered RAAS components has been attributed in part to the reported increase in serum levels of bradykinin, a vasodilator, in response to lowered ACE activity (268). In premenopausal women, stable arterial BP across the menstrual cycle despite elevated levels of plasma renin activity in the luteal compared with the follicular phase (272, 277, 278), has been postulated to be due to down-regulation of the tissue responsiveness to increased levels of Ang II, suggesting a blunting, rather than activation, of the RAAS system (272). This postulate is supported with
animal data showing that estrogen has direct inhibitory effects on vascular (252) and adrenal (279) AT1R expression, in addition to attenuating acute Ang II-induced aldosterone release (279). Conversely, estrogen deficiency has been shown to upregulate AT1R in ovariectomized animals (252, 280-282). Moreover, Ang-(1-7) elicits vasodilation during proestrus as compared to diestrus in rats (283). In ovariectomized animals, estrogen replacement also restores a significant dilator response to Ang-(1-7) (283), possibly via mechanisms that involve attenuation of NOX-induced ROS production (284).

Unlike other RAAS components, serum aldosterone is considered to be modulated by progesterone, and not estrogen (285). Consistent with this, studies show elevated serum aldosterone levels in the luteal (high progesterone) phase of the menstrual cycle (285), and no influence of oral ET on aldosterone levels in PMW (266, 269, 286).

In addition to decreasing AT1R expression, estrogen increases AT2R expression (287). Activation of the AT2R by the RAAS opposes the effects of the AT1R pathway by eliciting vasodilation. The classical vasopressor actions of Ang II via the AT1R pathway are thought to be offset by enhanced estrogen-dependent activation of AT2R (287). Both enhanced AT2R expression, and a greater AT2R/AT1R ratio in female compared with male rats has been reported (287). Sex differences in AT expression indicate a potential mechanism through which estrogen may confer greater vasodilator/vasoconstrictor balance in women than in men (287). Whether this contributes to the frequently observed lower BP in premenopausal women than in age-matched men remains unknown.
2.8.2.2 Renin-Angiotensin-Aldosterone System and Exercise

The influence of exercise training on the vascular RAAS is not well described (173). However, data show that aerobic exercise training in patients with coronary artery disease (80) and hypertension (288) attenuates Ang II-induced constriction, reduces NOX expression and activity, as well as blunts AT1R expression in atherosclerotic coronary arteries (172). The ACE genotype may also be an important determinant of the vascular responses to exercise training (289, 290). For example, in skeletal muscle ACE can be encoded by one of two ACE variants, type I or type II. The type II genotype has been associated with i) increased aerobic power in CAD patients in response to exercise training (289), and ii) enhanced endothelial function in endurance athletes (290). Overall, exercise appears to confer beneficial effects on various components of the RAAS. These alterations may contribute to the lower BP frequently observed in endurance athletes (291).

2.8.3 Baroreflex Sensitivity and Heart Rate Variability

Autonomic regulation of cardiac function is primarily determined by the interactions between sympathetic and vagal mechanisms on the sinus node (292). As such, HR provides an index of the net effects of autonomic tone. Autonomic control of HR can be quantitatively and non-invasively assessed by two different non-related methods: heart rate variability (HRV) and baroreflex sensitivity (BRS). Each of these methods, and how they are influenced by exercise and estrogen, are discussed below.
Baroreflex Sensitivity: BRS is an indicator of autonomic control of cardiovascular function (293), and is commonly expressed as the slope of the fitted line for the change in RR interval (ms) per change in systolic BP (mmHg) (214). The arterial baroreceptors are important regulators of the short-term stability of arterial BP. Originating in the carotid sinuses and the aortic arch, the arterial baroreceptors are mechanosensitive nerve endings that function as sensors in a negative feedback control system (294). Spontaneous variations of BP cause a conformational change in the baroreceptors leading to changes in afferent neuronal firing (293). Activation and deactivation of the arterial baroreceptors elicits reflex bradycardia and tachycardia, respectively (251). The inverse relationship that exists between HR and arterial BP is the fundamental tenet of arterial baroreflex control (251, 293).

Baroreflex mediated vascular effects are also caused through stimulation (e.g., loading) of the arterial baroreceptors, effecting sympathetic inhibition leading to reflex vasodilation (251). In contrast, the deactivation of the arterial baroreceptors (e.g., unloading), elicits sympathetic-mediated vasoconstriction (294). Thus, vascular compliance is a major determinant of the activity and magnitude of deformation of the baroreceptors, with stiffer vessels contributing to decreased BRS (293, 294). Diminished BRS is a risk factor for cardiac arrhythmias and sudden cardiac death (214). Conversely, increased BRS is an important physiological determinant of electrical stability of the heart (295).

Heart Rate Variability: Heart rate variability (HRV) describes the tonic baseline autonomic function in terms of variations of both instantaneous HR and consecutive R-R intervals (296). Non-invasive HRV analysis provides quantitative information on the modulation of cardiac vagal and sympathetic efferent activities (297). Elevated vagal or
diminished sympathetic control of HR is associated with lower resting HR and elevated HRV (27). High HRV is considered to be cardioprotective (297). Conversely, elevated sympathetic or diminished vagal control of HR is reflected as high resting HR and low HRV (297). Low HRV is suggested to be causally related to the pathobiology of coronary heart disease (298), and as a precursor to the development of hypertension (299), with studies demonstrating an inverse association between HRV and BP across a wide range of BP values (300). In women, low HRV has also been reported to be a risk factor for cardiac sudden death (27) and all cause mortality (301).

2.8.3.1 Baroreflex Sensitivity, Heart Rate Variability and Estrogen

Female reproductive hormones have been shown to influence cardiovascular autonomic regulation. Experimental studies show that in rodents, male status and withdrawal of female sex hormones through ovariectomy is associated with decreased BRS and HRV compared with intact females (302-307). Acute peripheral intravenous and central administration of 17β-estradiol increases parasympathetic and decreased parasympathetic modulation of HR in both males and ovariectomized rodents (302-304, 307, 308). Consistent with these findings, estrogen deficiency due to oophorectomy or menopause in women is associated with decreased BRS (309, 310) and HRV (225, 309-315), with ET administration eliciting favourable alterations in BRS (304, 309, 310) and HRV (225, 311-314). Studies also show higher BRS and HRV in premenopausal women compared with age-matched males and with older PMW (310, 316, 317). During the menstrual cycle, studies report increased HRV due to decreased sympathetic and/or increased parasympathetic activity in the late follicular versus the luteal
phase (318, 319). However, the influence of the menstrual cycle on BRS is less clear. Some (228, 320), but not all (321), groups report greater sympathetic BRS, assessed using the slope relating MSNA and diastolic BP, during the low estrogen compared with the high estrogen phase of the menstrual cycle. Higher cardiocagal BRS (slope relating R-R interval and systolic BP) during the luteal (high estrogen, high progesterone) compared with the follicular (low estrogen, low progesterone) phase of the menstrual cycle has also been reported by some (322), but not all (228, 323) groups. BRS assessed by the phynlephrine pressor test and Valsalva maneuver during the high estrogen (pre-ovulatory) phase of the menstrual cycle has also been shown to be significantly higher than those during the early follicular and mid luteal phases (320). Reasons for discrepancy in findings across the menstrual cycle are not clear, but are likely related to differences in techniques used to derive findings and the inherent difficulty in accurately quantifying stage of menstrual cycle. Despite these discrepancies, most of the studies described above support a favorable modulatory effect of estrogen on BRS and HRV, with estrogen deficiency associated with decreased BRS and HRV.

Cellular and molecular mechanisms involved in the estrogen-induced modulation of autonomic function are not yet fully understood. However, in the central nervous system, both ERα and ERβ are localized in significant concentrations on cell bodies, axons and terminals of autonomic regulatory nuclei throughout the neuraxis, from the spinal cord to the vasomotor centre, including the nucleus tractus solitarius (NTS), the nucleus ambiguus and the ventrolateral medulla, suggesting a role for estrogen in autonomic control (42, 324). In accordance with this, injections of exogenous estrogen into the medulla decreases arterial BP, HR, and renal sympathetic nerve activity and enhances reflex control of the HR in male rats and ovariectomized rats (307, 324, 325). Experimental evidence also supports both inhibitory
and stimulatory roles for estrogen on central GABAergic (326) and glutamatergic (327) neurotransmission, respectively. Both types of neurotransmissions are necessary for the modulation of central baroreflexes (328). In addition, estrogen has been shown to cause significant augmentation of the density of central α-2 adrenoceptors (329), which are known to facilitate baroreflex function (328). In addition to the central actions of estrogen on autonomic function, it has been shown that in female rats, estrogen modulates autonomic tone by prolonging cardiac parasympathetic responses via a potentiation of the activity of choline acetyltransferase, the enzyme responsible for the biosynthesis of acetylcholine, at the sinoatrial node (330, 65). Thus, increased cardiac parasympathetic tone in response to estrogen likely contributes importantly to favourable autonomic modulation of HRV and BRS.

2.8.3.2 Baroreflex Sensitivity, Heart Rate Variability and Exercise

Studies in both animals and humans demonstrate that exercise training is associated with decreased sympathetic autonomic drive and/or increased parasympathetic drive, reflected in part by reduced (331) or blunted (332) arterial baroreflex-mediated sympatho-excitation, lower resting HR (333-335), increased HRV (333, 334, 336, 337), and increased BRS (334, 338, 339). Mechanisms of action for increased HRV and decreased resting HR in response to endurance training are not well understood, but studies in humans and animals suggest increased vagal activity (335), alterations in cardiac pacemaker function (340), and improvements in baroreflex mediated control of HR (334, 341). The exercise training lowering effect on BRS has been proposed to be due to two possible mechanisms: i) alterations in the
baroreflex arc, and ii) altered vascular compliance. Experimental and human studies demonstrate that exercise training as well as acute exercise influences BRS through central alterations in the baroreflex arc via the nucleus ambiguus (342) and the NTS (343), both of which play a key role in baroreflex mediated control of HR. Arterial compliance is increased in response to habitual aerobic exercise training, particularly in middle-aged and older adults (344-346). Increased arterial compliance is postulated to lead to improvements in the mechanoelastic properties of the vessel wall, resulting in increased sensitivity of the baroreceptors (294). In younger individuals, in whom arterial compliance is already high, regular endurance exercise has been shown to increase BRS through alterations in the neural component of the baroreflex arc, and not through changes in vessel wall compliance (338). Whether such autonomic adaptations are due to alterations in central integration of baroreceptor input, efferent autonomic outflow, or sinoatrial node responsiveness are not yet known (338).

Research on the cardiovascular autonomic effects of exercise training has been substantially under-represented in women. This notwithstanding, evidence from observational studies show that habitual aerobic conditioning enhances cardiovagal BRS, increases HRV, and decreases resting HR in premenopausal and PMW compared to their sedentary peers (27, 333, 347). Mechanisms of action, as described above, have not yet been confirmed in women. Of interest, volume of exercise training has also been reported to influence HRV, with young women reporting high volume (>3 days/week, >1 hour/session) demonstrating greater HRV compared with women reporting low-to-moderate volume (<2 days/week, <45 minutes/session) exercise training (348, 349). In contrast, Middleton et al., (350) recently reported decreased cardiovagal BRS and HRV in endurance trained young eumenorrheic
women compared with age-matched sedentary eumenorrheic women. However, absence of control for menstrual phase, lack of detection of ovulation, and small sample size in the study by Middleton and colleagues (350) may have confounded these study findings.

2.9 Lipid Profile

Disturbances in lipid metabolism that result in unfavorable alterations in the plasma lipid profile increase the risk for CVD (351). Cholesterol, triglycerides, and high-density lipoproteins are important constituents of the lipid fraction of the human body. Low-density lipoprotein cholesterol (LDLc) is the primary cholesterol-containing atherogenic lipoprotein (60-70% cholesterol) and plays a key role in the transport of cholesterol in the blood to all tissues, primarily the liver (352). Elevated serum level of LDLc is recognized as an independent risk factor for coronary heart disease (352) and is associated with abnormal vasodilatory function in response to FMD (353). High-density lipoprotein cholesterol (HDLc) is considered to be atheroprotective (20-30% cholesterol) (354). The cardioprotection afforded by HDLc is associated with reverse cholesterol transport (355), inhibition of the atherogenic effects of oxidized LDLc (356), and increase in the half-life of eNOS (357). These beneficial effects of HDLc are associated in part with healthy endothelial function (353, 356, 357). Conversely, a low level of HDLc (<40 mg/dL) is an independent predictor for risk of cardiovascular diseases (358, 359), and is associated with endothelial dysfunction (354). Triglycerides (TG) are considered to be an independent risk factor for CAD (360), yet the direct association between elevated TG levels and CVD, and endothelial dysfunction, remains somewhat controversial. This is, in part, because TG: i) share an inverse association with
HDLc, with TG enhancing HDLc catabolism (361), ii) promote the formation of small dense LDLc particles, and facilitate their oxidation and migration into the arterial wall (362), and iii) elevated TG frequently co-exist with other CAD risk factors, such as hypertension and abdominal obesity (361). Factors known to influence the lipid profile include gender, heredity, hormone replacement therapy, sedentary lifestyle, cigarette smoking, excessive alcohol intake, overweight and obesity, diabetes, diet, and thyroid dysfunction (363, 364).

2.9.1 Lipid Profile and Estrogen

Estrogen lowers LDLc by upregulating LDL receptors in the liver and enhancing LDLc catabolism (365). Estrogen also increases HDLc by upregulating HDL receptor scavenger receptor class B type 1, thereby enhancing cholesterol efflux (366). In premenopausal women, the favorable effect of endogenous 17 β-estradiol is associated with lower LDLc levels compared to age-matched men (367). Lower LDLc, TC/ HDLc, and LDLc/HDLc ratios have also been reported during the luteal (high estrogen) compared with the follicular (low estrogen) phase of the menstrual cycle (368). Further, number of menses per annum is also significantly and inversely associated with LDLc and apolipoprotein B levels (14), suggesting that perturbations to menstrual cyclicity, and thereby estrogen exposure, can negatively influence cholesterol metabolism. In contrast, estrogen deficiency due to surgical and natural menopause is associated with evolution to a more pro-atherogenic lipid profile that includes increased LDLc, and decreased HDLc (369), with estrogen therapy shown to reduce total cholesterol (TC) levels compared to non-users, typically through lowering of LDLc, and/or an increase in HDLc (370-372).
2.9.2 Lipid Profile and Exercise

Sustained aerobic exercise training also favorably modifies much of the lipid-related atherosclerotic risk factors. Studies show that chronic exercise training increases circulating HDLc concentrations, lowers triglyceride and TC concentrations, and in some individuals, decreases LDLc concentrations (358, 373-375). The lipid-lowering mechanisms of action of exercise are not clear, but moderate- to high-exercise intensity for at least 30 minutes duration, three times per week, appears to consistently and significantly increase HDLc (376). A single acute bout of exercise can also elicit definite effects on blood lipids, including a transient increase in HDLc and reduction in serum TG (377).

2.10 Premenopausal Estrogen Deficiency: Cardiovascular Consequences

CAD is recognized as the leading cause of death in premenopausal women in the United States (16) and Canada (378), yet the pathophysiology of CAD in premenopausal women remains surprisingly under-investigated. Studies examining the potential influence of endogenous estrogen levels on CAD suggest that the timing of cardiac events, such as myocardial infarction or unstable angina, may be influenced by fluctuations of estrogen throughout the menstrual cycle, with greater number of events reported during the early follicular (low estrogen) compared with all other phases of the menstrual cycle (379, 380). Hypoestrogenemia in association with altered ovulatory cycling, including irregular menstrual cycles, is reported to be an important contributing factor to the development and progression of premenopausal
CAD (16-19). However, in women with irregular menstrual cycles of organic origin, hypoestrogenemia likely co-exists with other cardiovascular risk factors, such as hyperandrogenemia, metabolic syndrome, or hyperinsulinemia, as commonly reported in women with poly cystic ovarian syndrome (381), making it difficult to discern the independent cardiovascular effects of estrogen deficiency. This notwithstanding, the negative consequences of a history of irregular menstrual cycles on cardiovascular disease risk has been documented (17, 18). In the Nurses Health Study, the single largest cohort study of women to date, retrospective reports of a history of irregular menstrual cycles was linked with a 53% increased risk for fatal or nonfatal CAD compared with regularly cycling women (18). In a smaller study, premenopausal women under the age of 55 years with confirmed myocardial infarction retrospectively reported a higher lifelong incidence of irregular menstrual cycles compared with controls (17).

In an attempt to isolate the potential deleterious effects of hypothalamic hypoestrogenemia on the cardiovascular system, the Women’s Ischemia Syndrome Evaluation (WISE) study investigated the relationship between circulating estrogen levels and angiographically confirmed CAD in a small group (n=95) of premenopausal women (19). Independent of other risk factors, disruption of ovulatory cycling, specifically, estrogen deficiency, due to functional hypothalamic amenorrhea (FHA) associated predominantly with psychogenic stress, was identified as a risk factor for angiographically documented CAD (19). Collectively, these findings support an important role for ‘normal’ ovulatory cycling and cardiovascular health. Prospective studies designed to examine the cardiovascular disease risk of ovulatory disruption in premenopausal women are clearly warranted.
2.11 Menstrual Disturbances in Exercising Premenopausal Women

Menstrual disturbances exist across a spectrum, from subtle and asymptomatic (luteal phase defect and anovulation) to severe and symptomatic (oligomenorrhea and amenorrhea). While not all women who participate in physical activity will experience menstrual disturbances or ovarian disruption, hypothalamic menstrual aberrations across this spectrum are frequently observed among physically active women (382-385), including women with physically demanding occupations (386, 387). For example, a recent study demonstrated that across 166 menstrual cycles from 87 eumenorrheic women, 95% of sedentary women displayed ovulatory cycles compared with only 48% of physically active women (383). The estimated prevalence of the most severe menstrual disturbance, amenorrhea (i.e., cessation of menses for at least 90 consecutive days (388)), ranges between 2-46% among competitive and recreational athletes (382-385), as compared with 2-5% in non-exercising women (383, 388, 389). It is noteworthy that the prevalence of amenorrhea among competitive athletes and recreationally physically active women are reported to be similar (383, 388, 389). Duration of amenorrhea or number of amenorrheic ‘episodes’ are rarely reported.

Menstrual disturbances in weight-stable exercising women have been causally related to energy deficiency in association with a high energy expenditure (i.e., exercise) frequently combined with restrictive eating patterns without obvious caloric deficits (9, 10, 390). The interrelated spectrums of energy availability, menstrual function, and bone health comprise the medical condition of The Female Athlete Triad (391, 392). In response to energy deficiency, multiple hormonal and metabolic alterations, including decreased resting energy expenditure
(393), indicate a hypometabolic state in women with exercise-associated FHA (ExFHA). These alterations are presented in Figure 3 (below). Importantly, exercise per se has been demonstrated to not disrupt menstrual function when energy balance (i.e., a eucaloric state) is maintained (390). Furthermore, no set weight point or critical fat mass has been identified that enables menstruation (394).

2.12 Metabolic and Hormonal Alterations in Physically Active Women with Functional Hypothalamic Amenorrhea

In response to energy deficiency numerous endocrine and metabolic secretory aberrations are evident in women with ExFHA (Figure 3). Perturbations in several hypothalamic-pituitary axes occur, including ovarian, adrenal and thyroid. Reported alterations in circulating hormones include: decreased peripheral conversion of thyroxine to triiodothyronine (T3) that results in decreased circulating levels of free T3 (395), low thyroxine (396), low-to-normal thyroid stimulating hormone and increased reverse T3 (397), increased secretion of corticotrophin-releasing hormone, adrenocorticotropin hormone, cortisol and endogenous opioids (395, 398, 399), and increased ghrelin and peptide YY (23, 400, 401). Low-to-normal fat mass in response to high energy expenditure and insufficient caloric intake in women with ExFHA (23, 400) is also associated with altered adipose-derived hormone secretion, namely decreased leptin and increased adiponectin (23, 396, 400). Importantly, administration of leptin to hypothalamic amenorrheic women with low leptin levels has been demonstrated to restore
menstruation and neuroendocrine alterations (402). Thus, hypoleptinemia in association with low fat mass may be a key signal in ovarian suppression in women with ExFHA.

Low fat mass in women with ExFHA may contribute to overall estrogen deficiency via reduced synthesis of estrone; fat mass is a major source of estrone (31). Some estrogens are also produced in peripheral tissues (e.g., breast, muscles) via steroidogenic enzyme conversion of adrenal dehydroepiandrosterone (DHEA) and its sulphate ester (DHEAS), both of which are important precursors of estrogens. Estrogens augment ACTH-stimulated adrenal estrogen production by increasing DHEA(S) synthesis (403). Conversely, excessive cortisol production leads to a down-regulation of ACTH, resulting in reduced DHEA(S) synthesis (403). However, the influence of estrogen deficiency and mildly elevated cortisol production in women with ExFHA on DHEA(S) synthesis, and thereby peripheral estrogen synthesis, is unknown.

Despite energy deficiency, body weight in women with ExFHA is stable, and has been demonstrated to be similar to that observed in eumenorrheic physically active women (13, 23, 400). Compositionally, fat free mass has been shown to be both similar and higher in women with ExFHA compared with their eumenorrheic counterpart (13, 23, 400). However, resting metabolic rate adjusted for fat free mass has been demonstrated to be lower in ExFHA women than in eumenorrheic athletic women (23, 404). This seemingly paradoxical finding has been attributed to an adaptive response to conserve energy in the face of inadequate caloric intake, thereby maintaining 'normal' body weight (404).
**Figure 3.** Schema outlining the metabolic and hormonal consequences of energy deficiency in physically active premenopausal women with FHA. Republished with permission from the Journal of Clinical Endocrinology and Metabolism [O’Donnell et al., (25)].

GnRH, gonadotropin releasing hormone; CRH, corticotropin-releasing hormone; PYY, peptide YY; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; ACTH, adrenocorticotropin hormone; DHEA(S), dehydroepiandrosterone (sulphate); HPT, HPO and HPA, hypothalamic pituitary-thyroid, -ovarian and -adrenal, respectively; T3, triiodothyronine; ……. may also influence hypothalamus; ---- postulated inhibitory effect.
2.13 Cardiovascular Related Findings in Physically Active Women with Functional Hypothalamic Amenorrhea

The currently known cardiovascular alterations in women with ExFHA, complete with postulated mechanisms of action and clinical relevance, are summarized in Table 1 (page 69). The following sections of the review discuss these alterations.

2.13.1 Flow Mediated Dilation

Estrogen, caloric restriction and regular aerobic physical activity, via different mechanisms, are independently associated with enhanced synthesis and/or bioavailability of endothelial NO (1, 405, 406). However, in estrogen deficient women with ExFHA the beneficial effect of exercise training (and possibly caloric restriction) on resting endothelial function appears to be obviated. For example, brachial artery FMD, an endothelium-dependent response predominantly mediated via enhanced eNOS (122), is impaired in female athletes with ExFHA (14, 15, 21). Furthermore, resting and peak-ischemic calf blood flow in response to post-occlusion reactive hyperemia is lower, and vascular resistance higher, in women with long-term (average 271 days) ExFHA compared with physically active and sedentary ovulatory women (13). Of interest, women with short-term (90-120 days) ExFHA also demonstrate impaired resting, but not peak, calf blood flow responses compared with ovulatory women, suggesting duration of estrogen deficiency may be important, with longer duration possibly resulting in both structural and functional vascular alterations. This postulate has yet to be confirmed. This notwithstanding, impaired FMD, blunted regional blood flow and increased
regional vascular resistance in women with ExFHA suggests the presence of generalized impaired endothelial function. While this postulate and mechanisms of action have not yet been investigated, impaired FMD is believed to reflect diminished endothelial NO bioavailability due to chronic estrogen deficiency (13, 25, 407). Some (14, 408) but not all (13) studies also report vascular inflammation and increased OxLDLc potential in amenorrheic athletes. Whether these factors contribute to altered endothelial function in women in ExFHA has yet to be determined. Of interest, ghrelin and resting energy expenditure, both of which are elevated and decreased, respectively, in women with ExFHA, have also been reported to predict regional vascular resistance (13). Collectively, these studies suggest that the combined effects of metabolic, endocrine and hormonal adjustments to energy deficiency seemingly contribute to the altered vascular phenotype observed in women with ExFHA.
Table 1. Mechanisms of action and known or potential clinical consequences of altered cardiovascular function in ExFHA women.

<table>
<thead>
<tr>
<th>Physiological Outcome</th>
<th>Mechanisms of Action</th>
<th>Known or Potential Clinical Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impaired FMD</td>
<td>Estrogen deficiency leading to impaired NO bioavailability? Role of metabolic or psychogenic stress unclear.</td>
<td>Impaired FMD is a permissive factor in the development and progression of atherogenesis. Not yet known if women with ExFHA are at increased risk of premature development of CAD.</td>
</tr>
<tr>
<td>Lowered regional blood flow</td>
<td>Estrogen deficiency leading to impaired NO bioavailability? Role of metabolic or psychogenic stress unclear.</td>
<td>Cardiovascular consequences of lowered regional blood flow in women with ExFHA are unclear.</td>
</tr>
<tr>
<td>Increased regional vascular resistance</td>
<td>Counter-mechanism to help defend/ regulate BP in the face of lowered SBP? Role of estrogen deficiency, and metabolic or psychogenic stress unclear.</td>
<td>Increased peripheral resistance is associated with increased arterial stiffness. Not yet known if women with ExFHA demonstrate altered arterial stiffness.</td>
</tr>
<tr>
<td>Lowered resting SBP</td>
<td>Potentially favorable adaptation to caloric restriction? Role of estrogen deficiency or metabolic stress unclear.</td>
<td>Low 'normal' resting BP is favorable to vascular structure and function. However, hypotension may result in orthostatic intolerance. Orthostatic intolerance in women with ExFHA has not yet been investigated.</td>
</tr>
<tr>
<td>Lowered resting HR</td>
<td>Potentially favorable adaptation to caloric restriction? Role of estrogen deficiency or metabolic stress unclear.</td>
<td>Low resting HR is associated with favorable autonomic tone. Autonomic function has not yet been reported in women with ExFHA.</td>
</tr>
<tr>
<td>Increased lipid peroxidation potential</td>
<td>Decreased anti-oxidant status and/or increased pro-oxidant status in the face of estrogen deficiency? Role of metabolic stress unclear.</td>
<td>Lipid peroxidation is implicated in the pathogenesis of atherosclerotic vascular disease. Not yet known if 'potential' to lipid peroxidation contributes to vascular dysfunction in women with ExFHA.</td>
</tr>
</tbody>
</table>

Republished with permission from the Journal of Clinical Endocrinology and Metabolism [O’Donnell et al., (25)].
2.13.2 Inflammation

Studies in amenorrheic, oligomenorrheic and eumenorrheic athletes demonstrate a negative correlation between soluble VCAM-1, a serum marker of vascular inflammation, and FMD, suggesting endothelial inflammatory activation in women with endothelial dysfunction (14). In contrast, baseline serum markers of inflammation, including VCAM-1, CRP, TNF-α, IL-6, and homocysteine have been reported to be similar between amenorrheic and eumenorrheic athletes (14). This finding is inconsistent with studies reporting increased circulating inflammatory markers in hypoestrogenic PMW (183, 409). High prevalence of overweight and obesity in PMW (183, 409) likely contributes to this discrepancy due to adipose tissue in obesity being a primary source of inflammatory mediators (192). Of interest, estrogen has been shown to favorably regulate body weight and adipose metabolism (194), yet hypoestrogenic women with ExFHA demonstrate low-to-normal fat mass (13, 23, 400), unaltered levels of serum inflammatory markers (14), and a hypometabolic (395, 396, 399-401, 410, 411), suggesting a disassociation between energy metabolism and estrogen deficiency in these women. The unique neuroendocrine profile and/or high energy expenditure in women with ExFHA likely contributes to this disassociation. Indeed, exercise training and caloric restriction are both independently and in combination associated with favourable effects on adiposity and secretion of pro-inflammatory adipokines (204, 412, 413). Caloric restriction has also been shown to confer favourable anti-inflammatory effects in part via enhanced production of glucocorticoids (e.g., cortisol) and ghrelin, both of which have been experimentally demonstrated to exert anti-inflammatory effects (414, 415). Both cortisol and ghrelin are elevated in women with ExFHA (23, 399), but their influence on vascular tone remains unknown.
2.13.3 Vascular Endothelial Function: Other Potential Regulators

Endocrine and metabolic alterations in women with ExFHA (Figure 3) may have important consequences for vascular function. For example, triiodothyronine (T3), adiponectin and cortisol, all of which are altered in women with ExFHA, demonstrate vasoregulatory effects (416-419). T3 administration has been shown to enhance endothelial function in humans (416). In human cells, in vitro evidence suggests adiponectin protects against endothelial dysfunction via protecting the endothelium against damage induced by OxLDL (417), and by increasing eNOS and NO bioavailability (419). Conversely, cortisol administration has been demonstrated to decrease NO-dependent vasodilation in humans (418). Cortisol production, which is mildly elevated in women with ExFHA (395, 398, 399), also leads to a down-regulation of adrenocorticotropin hormone, resulting in reduced synthesis of DHEA and DHEAS (403). DHEA levels are essential for peripheral synthesis of estrogens. While circulating DHEA levels in women with ExFHA are not yet known, DHEA administration enhances endothelial NO-dependent vasodilation in ovariectomized rats (420). The conflicting influence of these vasoactive factors iterates the complexity of determining mechanisms of action of vascular function in women with ExFHA.

2.13.4 Restoration of Vascular Endothelial Function

Administration of exogenous estrogens (ethinyl estradiol based oral contraceptive), or recovery of endogenous estrogen (resumption of menses), restores FMD responses in women with ExFHA (21, 421, 422). Restoration of endothelial function with short-term (four weeks)
administration of folic acid without change in menstrual status has also been reported (423).
Mechanisms of action of folic acid on arterial function in women with ExFHA are not known, but the vascular benefits of folic acid have been reported to include enhancement of tetrahydrobiopterin levels, prevention of NO degradation, and improvements in circulating homocysteine levels (424).

2.13.5 Vascular Smooth Muscle Cell Function

NO, whether produced by the endothelium or administered exogenously, causes healthy arterial vascular smooth muscle cells to dilate, thereby increasing blood flow. Impaired arterial dilation in response to an exogenous source of NO (i.e., impaired endothelium-independent vasodilation) may reflect decreased smooth muscle cell responsiveness to NO and/or physiological or pathophysiological structural changes in the arterial wall (425). Some studies (21) but not all (14, 15) report impaired endothelium-independent brachial artery vasodilation in response to exogenous NO administration in women with ExFHA. Others (13) also report attenuated peak-ischemic calf blood flow in women with long-term (average 271 days), but not short-term (≤100 days) ExFHA. These observations suggest that estrogen deficiency, particularly of long duration, may influence vascular structural properties. Additional potential mechanisms of action may include increased synthesis and release of vasoconstrictor substances such as endothelin-1 or thromboxane A₂. Augmented vasoconstriction secondary to elevated sympathetic tone or mechanical abnormalities leading to reduced compliance or remodeling of the vessel wall limiting vasodilatory capacity may also be implicated.
2.13.6 Lipid Profile and Oxidized Low Density Lipoproteins

Women with ExFHA demonstrate elevations in TC, LDLc, apolipoprotein B, and TG concentrations (14, 426, 427) compared with their eumenorrheic and/or oligomenorrheic counterparts. However, values are not above recommended guidelines for cholesterol management (358). That low caloric and dietary fat intake (426, 427), combined with aerobic exercise training, does not favorably alter cholesterol levels in women with ExFHA is inconsistent with the reported decrease in LDLc in humans in response to mild to moderate caloric restriction (428) and chronic aerobic exercise training (429-431). In contrast, elevated HDLc is also reported in women with ExFHA (426). HDLc is increased with exercise training (429-431) and, depending on dietary content, may be increased or decreased with caloric restriction (428, 432). HDLc serves a number of anti-atherogenic roles, including reverse cholesterol transport. It is not known if elevated HDLc counteracts the negative effects of elevated TC and LDLc in women with ExFHA.

Plasma LDLc and apolipoprotein B in women with ExFHA have been shown to be inversely associated number of menstrual cycles per year and with brachial artery FMD (14), suggesting a role for estrogen deficiency in both lipid metabolism and FMD. Endothelial dysfunction in humans with hypercholesterolemia is associated with decreased synthesis and release of NO (433, 434), and increased inactivation of NO by superoxide anion due to increased oxidative stress (435). It is not known whether similar mechanisms of action are evident in women with ExFHA.

In amenorrheic versus eumenorrheic athletes some studies (408), but not all (436, 437), report adverse increases in post-exercise LDLc peroxidation potential as determined by greater
oxysterol formation derived from the enzymatic and non-enzymatic peroxidation of cholesterol, and decreased diene conjugation formation time (i.e., decreased ability of LDLc to resist peroxidation). Reasons for equivocal findings among studies in women with ExFHA are unclear, but may in part be due to both the diverse methods of oxidative stress assessment and the varied exercise stimuli. Other considerations include exercise intensity, duration, and the training status of the individual, all of which are recognized as important contributors to the oxidant response to acute exercise (148). Whether OxLDLc contributes to impaired FMD in women with ExFHA remains unknown.

2.13.7 Blood Pressure

Lower resting systolic BP has been observed in women with ExFHA compared with their eumenorrheic counterpart (13, 23, 438). Although mechanisms of action are unclear, it is recognized that the sympathetic nervous system plays a key role in regulating arterial BP in humans. Thus, low resting systolic BP in women with ExFHA suggests diminished sympathetic outflow, despite hypoestrogenemia. Such findings are consistent in part with studies reporting lower arterial BP in healthy humans in response to caloric restriction without malnutrition (428) and in endurance trained individuals (439). Other important regulators of BP include the RAAS. Studies describing RAAS and its role in regulating BP and/or vascular function in estrogen deficient physically active women have not yet been reported. As described earlier, estrogen influences tissue and circulating RAAS through several mechanisms, including upregulation of angiotensinogen (263, 264), variably altered renin (266-271), increased Ang I and Ang II (268, 269, 274-276), inhibitory effects on AT1R expression (252), and increased Ang-(1-7) (283). As
such, it is reasonable to speculate that estrogen deficiency in premenopausal women with ExFHA influences BP through alteration in any combination of the components of the RAAS cascade.

2.13.8 Baroreflex Sensitivity, Heart Rate and Heart Rate Variability

To date, only one study has examined BRS in women with ExFHA (440). This study reports similar cardiovagal BRS (baroreflex control of HR) as determined during i) phase II and IV of the Valsalva maneuver, and ii) an orthostatic challenge (20-minutes of 60° head-up tilt), in amenorrheic and eumenorrheic athletes (440). Thus BRS appears to be preserved in women with ExFHA. It is possible that the concurrent and competing effects of exercise training and/or caloric restriction mitigates the negative effects of hypoestrogenemia. Aerobic exercise training independently modulates the intrinsic properties of several autonomic-related nuclei in the central nervous system (441), promoting improved BRS (442). Caloric restriction has also been demonstrated to independently increase BRS in rodents (443, 444), patients with anorexia nervosa (445), and in obese humans (446). Although mechanisms of action of increased BRS with caloric restriction remain unclear, the adipokine leptin has been proposed as a strong candidate (447). The influence of hypoleptinemia, as observed in women with ExFHA (396) on BRS, or other indices of autonomic function, remains to be investigated.

Reports of HRV responses in women with ExFHA have yet to be published, but lower resting HR compared with their ovulatory eumenorrheic exercising counterpart (13, 23) suggests possible increased parasympathetic modulation of HR control in these women. Lower resting
HR in ExFHA than in similarly trained eumenorrheic women also suggests that factors other than habitual exercise training may be contributing to lower resting HR in women with ExFHA. The interplay between estrogen and endurance training in these responses is unclear, but it is known that moderate negative energy balance has a marked effect on vagal tone in animals, as evidenced by lowered resting HR and increased HRV (443, 448, 449). Thus, lower resting HR in women with ExFHA compared with their ovulatory counterpart (13, 23) may be modulated by negative energy balance. This postulate awaits investigation.

2.14 Summary and Conclusion

The known biology of estrogen supports a beneficial cardiovascular effect of this ovarian hormone. Consequently, the premenopausal years are generally considered to be cardioprotective. Despite premenopausal status and participation in regular aerobic exercise training, young hypoestrogenic women with ExFHA demonstrate cardiovascular alterations, including impaired FMD, increased LDLc and TC, and increased oxidative stress. These observations are in stark contrast with the known independent cardiovascular benefits of aerobic exercise training, and indeed moderate caloric restriction, and reflect a cardiovascular phenotype that is similar to older women who have transitioned through menopause. In contrast, potentially favourable cardiovascular adaptations in women with ExFHA include increased HDLc, decreased resting HR and BP, and low fat mass. The clinical relevance of the multiple collective and competing cardiovascular adaptations in women with shorter and longer duration of ExFHA are not yet known. However, since impaired endothelial function is a recognized permissive factor in the development and progression of atherogenesis, and preliminary data
suggest altered ovulatory cycling may be a risk factor for clinical cardiovascular disease and increased cardiovascular events (19, 379, 380), determining the breadth of the cardiovascular effects of ovarian disruption in women with ExFHA is important.

Thus, the following studies were conducted in women with ExFHA to explore mechanisms of previously documented altered cardiovascular regulation. The studies were designed to answer the following questions: i) is low resting HR associated with altered cardiac autonomic control?; ii) is low arterial BP associated with altered neurohumoral BP regulation?; iii) is impaired FMD in the conduit vessels associated with altered shear stress?; and, iv) can an acute bout of dynamic exercise restore FMD in the conduit vessels and calf blood flow in resistance vessels? Collectively, the following investigations were intended to both broaden the understanding of the short-term cardiovascular effects of premenopausal estrogen deficiency, and to further characterize the cardiovascular profile of women with ExFHA.
CHAPTER 3

Impaired Vascular Function in Physically Active Estrogen Deficient Premenopausal Women is Acutely Augmented after a Single Bout of Dynamic Exercise

3.1 Introduction

During their reproductive years, women are at lower risk of cardiovascular disease than age matched men (4). This cardiovascular protection has largely been attributed to the actions of estrogen. In the vasculature estrogen plays a key role in modulating vascular function, including enhanced production and bioavailability of endothelium-derived nitric oxide (NO). NO is a potent vasodilator that confers cardiovascular protection via anti-inflammatory, anti-coagulant and anti-atherogenic mechanisms (69). Decreased endothelial production and release of NO leads to loss of cardiovascular protection and is a recognized hallmark characteristic of endothelial dysfunction, a major permissive factor in the progress and development of atherosclerosis (69). Estrogen deficient postmenopausal women (PMW) demonstrate brachial artery endothelial dysfunction and impaired regional blood flow, with estrogen therapy augmenting vascular function in association with increased production and bioavailability of NO (450, 451).

Estrogen deficiency is not unique to the postmenopausal period. In premenopausal women, functional hypothalamic amenorrhea (FHA) is a common cause of reversible disruption to ovulatory cycling, characterized by suppression of the hypothalamic–pituitary–ovarian axis,
absence of menses and profound hypoestrogenemia. FHA is frequently reported in recreationally active women and competitive athletes (ExFHA) in association with chronic energy deficiency due to high energy expenditure that is often combined with restrictive eating patterns without obvious caloric deficits (9). To date, studies report that women with ExFHA demonstrate altered vascular function, including low brachial artery flow mediated dilation (FMD) and decreased regional blood flow (13-15, 21, 421). Impaired NO production (13-15, 21) and increased regional vascular resistance (13) in response to estrogen deficiency have been implicated. The clinical significance of altered vascular function in physically active women with FHA (ExFHA) is not yet known. However, it has recently been identified that independent of other risk factors, estrogen deficiency of functional hypothalamic origin is a risk factor for angiographically documented coronary artery disease during the later premenopausal years (19).

In addition to estrogen, shear stress also mediates increases in endothelial NO production. Immediate increases in shear stress, as with FMD, potently activates endothelial NO production within seconds (119). A positive linear relationship between the percentage increase in vessel diameter from baseline in response to FMD (FMD%) and peak (452) and total (29) hyperemic shear stress stimulus in healthy arteries has been demonstrated. Conversely, retrograde blood flow and low shear stress in association with turbulent flow are associated with endothelial dysfunction (119). The contribution of shear stress to FMD% responses in women with ExFHA has not yet been reported.

The current study sought to examine mechanisms of altered vascular function in women with ExFHA. The effects of an acute bout of dynamic exercise, a known stimulus to induce increases in endothelial NO production due to increases in blood flow and shear stress, on both
conduit and resistance arterial function was investigated. Previous studies report increased brachial artery FMD% after a single bout of dynamic exercise in healthy estrogen deficient PMW (139). Based on this, and previous (13-15, 21) findings, it was postulated that vascular function in conduit and resistance vessels would be impaired in women with ExFHA, and that a single bout of dynamic exercise would acutely restore vascular responses, similar to that observed in eumenorrheic physically active women. It was further postulated that FMD% responses would be positively associated with local shear stress both before and after exercise.

3.2 Methods

Subjects: Volunteers were recruited by posters targeting both sedentary and physically active women for a study on women’s health (Appendix 1). Screening procedures included general questions regarding exercise and eating behaviour, and menstrual and medical health (Appendix 2). Eligibility criteria for the study included, 1) age 18 to 35 yrs; 2) good health determined by a medical examination; 3) no chronic illness, including diabetes, hyperprolactinemia, poly-cystic ovarian syndrome, and thyroid disease; 4) stable menstrual status over the preceding 3 months (i.e., absence of menses or menstrual cycles between 25-35 days); 5) non-smoker; 6) not currently dieting and weight stable for the preceding 3 months; 7) no history or current clinical diagnosis of eating disorders; 8) absence of hormonal therapy for at least 6 months; and 9) no other contraindications that would preclude participation in the study. The study was approved by the institutional committee on human research by the Ethics Review Board at the University of Toronto and the University Health Network. All volunteers signed an approved informed consent document (Appendix 3).
Experimental Design: Volunteers were recruited consecutively over three years. Forty-two women completed the current study. Volunteers were grouped according to their exercise (exercising or sedentary) and menstrual and ovulatory status (eumenorrheic and ovulatory, or amenorrheic). The details of this classification scheme are outlined below.

Exercise, Menstrual and Ovulatory Status: Exercise status was defined as “sedentary” when purposeful exercise was less than 2 hours per week and “exercising” when purposeful exercise was more than 2 hours per week (13). In conjunction with the hours of exercise activity criterion, an objective criterion measure of peak aerobic capacity of $<40 \text{ ml/kg/min}$ defined sedentary status and $\geq 40 \text{ ml/kg/min}$ to reflect exercising status, was used (13).

Menstrual status was determined by self-report. Women were identified as being either eumenorrheic, defined as having regular menses (10-13 menstrual cycles per year) or amenorrheic, defined as cessation of menses for at least $>90$ days (388). Ovulatory status of eumenorrheic women was confirmed during the menstrual cycle prior to testing. Ovulation was detected using an industry recognized ovulation hormone kit (Clearblue Easy ovulation test, Unipath Diagnostics, Waltham, MA). Detection of more than 2 consecutive anovulatory menstrual cycles resulted in withdrawing the participant from the study.

Study Groups: Three groupings were established based on exercise and menstrual status: 1) sedentary women with ovulatory menstrual cycles (SedOv; $n=15$), 2) exercising women with ovulatory menstrual cycles (ExOv; $n=14$), and 3) exercising women with FHA (ExFHA; $n=12$).
**Protocol:** All testing took place in the morning, starting between 08:30-09:30 hours in a quiet ambient room (22-24 °C). Volunteers were 8 hours fasted and had abstained from exercise and caffeine for 24 hours and alcohol 12 hours prior to testing. All measures were obtained during the early follicular phase (days 2-6) in menstruating women, and on a random day for amenorrheic women. Blood sampling, using standard venipuncture techniques, were completed after 10 minutes of seated rest. After an additional 20 minutes of quiet supine rest, baseline measures of HR and BP were recorded and calf BF, brachial artery endothelium-dependent and independent function were assessed. Immediately post-baseline recordings, participants exercised (brisk treadmill walking) for 45 minutes at 60% of their peak aerobic capacity. Exercise intensity was monitored using the corresponding HR recorded at 60% during a peak oxygen consumption test as determined on a separate day (see below). To avoid sensations of hunger or hypoglycemia after exercise, subjects consumed a standard light low-fat lunch. Measures were repeated, in the supine position, 60 minutes post-exercise.

**Blood Pressure and Heart Rate:** Resting measurement of systolic (SBP), diastolic (DBP), and mean arterial BP (MAP; 1/3 pulse pressure + DBP) and HR were recorded using an automated device (Dinamap Pro 100, Critikon, USA) on the left upper-arm. Continuous BP and HR were also recorded during each testing session on a beat-to-beat basis using a photo-plethysmographic method on the middle digit of the right hand (Finapres, Ohmeda, Madison, WI).
**Anthropometric Measures:** Total body mass (recorded to the nearest 0.5 kg) and height (recorded to nearest 1.0 cm) were determined using a physician’s balance scale (Detecto, Webb City, MO). Body mass index (BMI, kg·m²) and body surface area (BSA, m²; 0.007184 x height(cm)0.725 x weight(kg)0.425) (453) were calculated.

**Body Composition:** Dual-energy x-ray absorptiometry (DXA; Prodigy, General Electric Lunar Corporation, Madison, WI, enCORE 2002 software, version 6.50.069) was utilized to determine body composition by a qualified operator. Percent body fat and lean mass were determined. A subject precision study (n=28) in premenopausal women demonstrated precision of 0.6%.

**Peak Aerobic Capacity:** Peak aerobic capacity (VO₂ peak) was measured once by a progressive treadmill test to volitional exhaustion on a separate day prior to the study day. Breath-by-breath expired gas samples were collected continuously to determine ventilation and oxygen consumption using a calibrated automated metabolic cart (Moxus Modular VO₂ System, Applied Electrochemistry Inc., Pittsburgh, PA).

**Brachial Artery Assessment:** End-diastolic, ECG-gated (R-wave), high resolution B-mode ultrasound images and pulsed-wave Doppler flow velocities of the brachial artery were acquired by an experienced operator, continuously and simultaneously during brachial artery assessment using a 10MHz linear array transducer (Probe L10-5; Vivid 7, GE Medical Systems, Milwaukee, WI). B-mode images were attained above the antecubital fossa in the longitudinal
plane at a reproducible point in the distal third of the upper arm. A probe holder was used to ensure stable transducer position throughout the studies, with Doppler angle correction of 69° employed to allow optimal perpendicular arterial wall imaging (454) and determination of Doppler flow velocities (455). Endothelium-dependent (forearm cuff-occlusion flow mediated dilation; FMD) and endothelium-independent dilation using sublingual glyceryltrinitrate (GTN; 400 µg), were determined. Images were digitally acquired on a personal computer using Virtual Dub 1.5.9 software (Copyright © 1998-2003 by Avery Lee) and stored for off-line analysis using a validated automated edge-detection software program (SpLiNeS, www.dii.unisi.it (456)), thereby eliminating subjective error (455). The FMD protocol consisted of a one-minute baseline, followed by a 5-minute occlusion of the forearm (50 mmHg above systolic BP [SBP]), just below the antecubital fossa, followed by 3 minutes of recovery. Fifteen minutes after the FMD stimulus, a recovery scan was recorded for one minute. Sublingual GTN was then administered and diameter and Doppler flow images recorded continuously for 5 minutes.

**Brachial Artery Diameter Analysis:** Baseline artery diameter (mean of 30 consecutive images), FMD% (peak % increase in arterial diameter from baseline diameter) and GTN% (% change in arterial diameter from baseline 5 minutes following GTN administration) were determined. Diameters were calculated from trailing edge to leading edge of the intima-blood interface. Mean blood velocity (MBV), expressed as the velocity-time integral (cm·s⁻¹), was calculated as the peak envelope of the blood flow velocity signal of the velocity waveform within a user-defined region of interest. Brachial artery blood flow (BBF; ml/min) was calculated as $\text{MBV} \pi (D^2/4) \text{HR}$, where $\pi$ is 3.14, D is the vessel diameter (cm), and HR is heart rate.
Shear rate (SR; cm·s⁻¹) was calculated as MBV*8/D (454); the SR average was assessed at baseline (resting shear 10 second average) and for 15 seconds immediately post-cuff release to determine peak shear (peak SR mean). Peak shear is also expressed as the sum of the area under the curve (SRAUCpk) for the 15 seconds immediately post-cuff release, which was calculated using the trapezoidal method. Normalization of FMD% to SRAUCpk was also determined (FMD%/SRAUCpk). Brachial artery diameter reproducibility using same-day measures, separated by one hour, was completed in healthy premenopausal women (n=7; 18-35 years of age). Repeated measures of baseline diameter (Appendix 4) yielded a correlation coefficient of r=0.97, an intraclass correlation coefficient (ICC) of 0.8 (95%CI 0.835, 0.994), and a coefficient of variation (CV) of 1.53%. Repeated measures of FMD% (Appendix 4) yielded a correlation coefficient of r=0.95, an ICC of 0.7 (95%CI, 0.147, 0.932), and a CV of 10.58%. These reproducibility data are consistent with others (458).

Peripheral Blood Flow: Calf blood flow (BF) was assessed in the supine position using venous occlusion strain-gauge plethysmography as previously described (459), with the calf slightly elevated above heart level. An indium-gallium strain gauge (D. E. Hokanson, Inc., Bellevue, WA) was placed around the widest girth of the calf set to approximately 10 g of tension (459). An occlusion cuff was placed around the ankle and remained inflated to 200 mmHg. A second occlusion cuff was placed above the knee and sequentially inflated to ~50 mmHg, using on-off cycles of 7 seconds, for two minutes. Peak-ischemic calf BF measures in response to post-occlusive reactive hyperemia (PORH) were attained by inflating the occlusion cuff to 50mmHg above SBP for 5 minutes, at which time it was rapidly deflated followed by sequential inflations.
to ~50mmHg for two minutes. Baseline and peak calf BF was determined from the slope of the time-leg volume curve (ml/min/100ml). Calf vascular conductance (calf VC; calf BF/mean arterial pressure [MAP]; ml/min/100ml/mmHg), and vascular resistance (calf VR; MAP/calf BF x 100; U) were calculated.

**Serum Measures:** Eight-hour fasted blood samples were collected from the ante-cubital vein using standard venipuncture techniques. Serum was analyzed using commercially available kits for 17β-estradiol, progesterone, prolactin, free testosterone, sex-hormone binding globulin (SHBG), high-density lipid cholesterol (HDLc), total cholesterol (TC), and triglycerides using standard chemical assay procedures. Low density lipid cholesterol (LDLc) was determined by the method of Friedewald (460). Serum free triiodothyronine (T3) was also assessed to provide an estimate of energy status, with low T3 levels indicating low energy status (i.e., energy deficiency) (395). All assays were run by the Core Laboratory at the Toronto General Hospital. Detailed serum collection and assay specifications are provided in Appendix 5.

Total testosterone was indexed to SHBG to calculate the free androgen index (FAI, calculated as 100 x [total testosterone/SHBG]). Increased FAI provides an estimate of increased biologically available androgens.

**Statistics:** Statistical analysis was performed after confirmation of a normal distribution. For between-group analyses, data were analyzed using one-way analysis of variance (ANOVA), and when a significant main (fixed) effect was observed, Bonferroni methods were used to
determine where the significant differences existed. Adjustment of brachial FMD% responses for artery diameter and SR were assessed using analysis of covariance (ANCOVA). For within group analyses of pre- versus post-exercise responses, repeated measures ANOVA were used. Using pooled data and separately grouped data, Pearson’s correlational analyses were used to determine significant linear independent associations between vascular function and variables of interest. Data were analyzed using packaged software (SPSS version 20; SPSS Inc., Chicago, IL). A significance level of $p<0.05$ was used to detect the differences for statistical procedures. All data are presented as mean ± SEM.

3.3 Results

Participant Characteristics: Participant characteristics are summarized in Table 1. Groups did not differ ($p>0.05$) in age, height, weight, BSA or lean mass. BMI trended ($p=0.06$) to a lower value in physically active women versus sedentary women. Average duration of amenorrhea for the ExFHA group was ~1000 days (range 153-3132 days). Exercising groups had higher ($p<0.01$; main effect) cardiorespiratory fitness and lower ($p<0.05$; main effect) percent body fat compared with sedentary women.

Serum Measures: Serum measures assessed at baseline are presented in Table 2. Comparison of chronically low estrogen levels in ExFHA women with cyclically low estrogen levels during the early follicular phase of the menstrual cycle in ovulatory women revealed lower estradiol levels ($p<0.05$) in ExFHA compared with ExOv women. Prolactin ($\mu$g/L) was also lower
(p<0.05) in ExFHA versus SedOv and ExOv women. Consistent with energy deficiency, free T3 was lowest (p<0.05) in ExFHA compared with all other groups. Serum measures of progesterone, testosterone, insulin, LDLc, HDLc, TC and TG did not statistically differ (p>0.05) between the groups (values not reported). While SHBG was lower (p<0.05) in SedOv women than in exercising women, FAI did not differ (p>0.05) between the groups.

Blood Pressure and Heart Rate: Pre- and post-exercise hemodynamic responses are summarized in Table 3. Between groups, pre-exercise baseline HR, SBP, and pulse pressure (PP) were significantly lower (p<0.05; main effect) in ExFHA versus SedOv and ExOv women. MAP and DBP did not differ between the groups (p>0.05). Post-exercise, ExFHA women demonstrated lower (p<0.05) HR, SBP, PP and MAP compared with ExOv and SedOv women. Responses did not differ (p>0.05) between ovulatory women, both before and after exercise. Within-groups, HR was increased post-exercise (p<0.05) in all groups. In ExFHA, DBP and MAP were also significantly (p<0.05) decreased, and PP increased post-exercise. In SedOv, SBP and MAP were lowered post-exercise (p<0.05). ExOv women demonstrated no changes (p>0.05) in BP measures in response to exercise.
Table 1. Study group demographics.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=15)</th>
<th>ExOv (n=14)</th>
<th>ExFHA (n=12)</th>
<th>P (main effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>23.1 ± 0.5</td>
<td>23.5 ± 1.2</td>
<td>24.8 ± 0.9</td>
<td>0.400</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.5 ± 1.2</td>
<td>164.8 ± 1.7</td>
<td>165.6 ± 1.7</td>
<td>0.604</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>59.1 ± 1.4</td>
<td>57.8 ± 1.7</td>
<td>55.8 ± 2.3</td>
<td>0.430</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.1 ± 0.5</td>
<td>21.4 ± 0.5</td>
<td>20.3 ± 0.7</td>
<td>0.062</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0.856</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>30.7 ± 1.9*</td>
<td>24.0 ± 1.8</td>
<td>21.6 ± 2.1</td>
<td>0.006</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>38.4 ± 1.2</td>
<td>41.8 ± 1.5</td>
<td>42.4 ± 2.0</td>
<td>0.147</td>
</tr>
<tr>
<td>VO₂ peak (ml/kg/min)</td>
<td>38.8 ± 0.7*</td>
<td>46.9 ± 1.2</td>
<td>46.3 ± 1.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Amenorrhea (days)</td>
<td>-</td>
<td>-</td>
<td>1012 ± 234</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are mean±SEM

BMI, body mass index; BSA, body surface area; VO₂ peak, peak oxygen uptake.

* SedOv vs. ExOv and ExFHA

a Range 153-3132
Table 2. Serum measures of the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=15)</th>
<th>ExOv (n=14)</th>
<th>ExFHA (n=12)</th>
<th>P (main effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (pmol/L)</td>
<td>130.9 ± 10.9</td>
<td>161.8 ± 24.1</td>
<td>83.8 ± 15.2\textsuperscript{a}</td>
<td>0.014</td>
</tr>
<tr>
<td>Progesterone (nmol/L)</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>0.871</td>
</tr>
<tr>
<td>Prolactin (µg/L)</td>
<td>14.0 ± 1.5</td>
<td>13.1 ± 1.3</td>
<td>9.0 ± 0.6\textsuperscript{b}</td>
<td>0.017</td>
</tr>
<tr>
<td>Free T3 (pmol/L)</td>
<td>4.3 ± 0.1</td>
<td>4.4 ± 0.2</td>
<td>3.6 ± 0.5\textsuperscript{b}</td>
<td>0.001</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>2.4 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>0.298</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>40.5 ± 2.7\textsuperscript{c}</td>
<td>51.2 ± 2.6</td>
<td>51.1 ± 4.1</td>
<td>0.040</td>
</tr>
<tr>
<td>FAI (au)</td>
<td>6.3 ± 0.7</td>
<td>4.6 ± 0.4</td>
<td>4.9 ± 0.7</td>
<td>0.157</td>
</tr>
<tr>
<td>LDLc (mmol/L)</td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>0.185</td>
</tr>
<tr>
<td>HDLc (mmol/L)</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>0.260</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>3.9 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>4.4 ± 0.3</td>
<td>0.277</td>
</tr>
</tbody>
</table>

Values are mean±SEM

Au, arbitrary units; FAI, free androgen index; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; SHBG, sex hormone binding globulin; TC, total cholesterol; T3, triiodothyronine.

\textsuperscript{a} ExFHA vs. ExOv

\textsuperscript{b} ExFHA vs. SedOv and ExOv

\textsuperscript{c} SedOv vs. ExOv and ExFHA
**Table 3.** Hemodynamic responses at baseline and one hour post-exercise.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=15)</th>
<th></th>
<th>ExOv (n=14)</th>
<th></th>
<th>ExFHA (n=12)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PreEx</td>
<td>PostEx</td>
<td>PreEx</td>
<td>PostEx</td>
<td>PreEx</td>
<td>PostEx</td>
</tr>
<tr>
<td>HR (b/min)</td>
<td>59±2</td>
<td>71±2*</td>
<td>55±2</td>
<td>68±2*</td>
<td>50±3*a</td>
<td>60±2*b</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>101±2</td>
<td>98±2**</td>
<td>102±2</td>
<td>101±2</td>
<td>92±2*a</td>
<td>90±2*b</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>57±2</td>
<td>55±1</td>
<td>57±2</td>
<td>54±2</td>
<td>56±2</td>
<td>52±1*</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>72±1</td>
<td>69±1**</td>
<td>72±2</td>
<td>70±1</td>
<td>68±2</td>
<td>65±1*b</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>44±2</td>
<td>43±2</td>
<td>45±1</td>
<td>46±1</td>
<td>36±2*a</td>
<td>38±2*b</td>
</tr>
</tbody>
</table>

Values are mean±SEM

b/min, beats per minute; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; PP, pulse pressure; SBP, systolic blood pressure.

* Pre vs. post-exercise within groups, $p<0.01$

** Pre vs. post-exercise within groups, $p<0.05$

*a* ExFHA vs. SedOv and ExOv pre exercise, $p<0.01$

*b* ExFHA vs. SedOv and ExOv post exercise, $p<0.01$
Brachial Artery Responses: Pre-Exercise: Pre-exercise brachial artery responses are summarized in Table 4. Between groups, baseline brachial artery diameter, peak FMD diameter and time to peak FMD diameter did not differ \((p>0.05)\) between groups pre-exercise. FMD\% was significantly lower \((p<0.01;\) main effect) in ExFHA versus ExOv and SedOv pre-exercise (Figure 1a). FMD\% responses did not differ between ovulatory women. Using ANCOVA, FMD\% remained significantly lower in ExFHA after independently controlling for brachial artery diameter, \(F(2,38)=26.67, p<0.01,\) and SRAUCpk, \(F(2,37)=23.34, p<0.01,\) and after controlling for these covariates in combination, \(F(2,36)=22.81, p<0.01.\) FMD\% normalized for SRAUCpk (FMD\%/SRAUCpk) also did not alter \((p<0.01)\) the study findings. GTN\% was lower \((p<0.05;\) main effect) in ExFHA versus ExOv and SedOv women (Figure 1a), in whom responses did not differ \((p>0.05)\). Post-Exercise: Between-group analyses demonstrated similar \((p>0.05)\) post-exercise baseline diameters and similar peak FMD\% diameters (Table 3). Time to FMD peak diameter was not different \((p>0.05)\) between ovulatory women but was higher \((p<0.05)\) in ExFHA women. FMD\% remained significantly lower in ExFHA women \((p<0.05)\) than in SedOv and ExOv women, in whom responses did not differ \((p>0.05)\) (Figure 1b). Using ANCOVA, FMD\% post-exercise remained significantly lower in ExFHA after independently controlling for brachial artery diameter \(F(2,38)=8.460, p<0.01,\) and SRAUCpk, \(F(2,35)=6.11, p<0.01,\) and after controlling for these covariates in combination \(F(2,34)=6.23, p<0.01.\) In contrast, FMD\%/SRAUCpk did not differ \((p=0.66;\) main effect) between groups (Figure 1c). Within-groups, pre- versus post-exercise baseline brachial artery diameter and time to peak FMD did not differ in any group \((p>0.05).\) Conversely, FMD peak diameter was increased \((p<0.05)\) within all groups. FMD\% post-exercise was significantly increased from pre-exercise \((p<0.01)\) in ExFHA women (Figure 1b), but was not altered in ovulatory women.
Brachial Artery Blood Flow and Shear Rate: Pre-Exercise: Brachial artery BF (BBF) responses are summarized in Table 5. Baseline BBF, mean SR and SRAUCpk were significantly lower ($p<0.05$) in ExFHA than in SedOv and ExOv women. Post-Exercise: Between groups, baseline and peak BBF were lower ($p<0.05$) in ExFHA versus ExOv women only. In contrast, baseline SR mean and SRAUCpk were lower ($p<0.05$) in ExFHA than in both ovulatory groups, in whom responses did not differ ($p>0.05$). Within-groups, baseline and peak BBF and baseline SR mean were increased ($p>0.05$) pre- versus post-exercise in SedOv and ExOv women only. ExFHA women trended toward increases for baseline and peak BBF ($p=0.06$ and $p=0.07$, respectively). SR peak was not altered within- or between-groups post-exercise ($p>0.05$). Conversely, SRAUCpk was increased ($p<0.05$) within all groups.

Calf Blood Flow Responses: Pre-Exercise: Calf BF responses pre-exercise are presented in Figure 2. Baseline and peak calf BF were significantly lower ($p<0.05$) in ExFHA compared with SedOv and ExOv women. Baseline and peak calf VC (ml/min/100ml/mmHg) were also lower ($p=0.025$ and $p=0.033$, respectively; main effect) in ExFHA (2.2±0.2 and 30.3±2.3) versus SedOv (3.2±0.2 and 40.4±2.5) and ExOv (3.2±0.4 and 40.1±3.9). Conversely, baseline calf VR (arbitrary units) pre-exercise was higher ($p<0.01$; main effect) in ExFHA (47.7±3.1) versus SedOv only (31.9±1.6). Peak calf VR was also significantly higher ($p=0.02$; main effect) in ExFHA (3.6±0.3) than in SedOv (2.6±0.2) and ExOv (2.7±0.2). Post-exercise: Between groups, baseline and peak calf BF (Figure 2), VR and VC did not differ ($p>0.05$). Within groups, exercise significantly ($p<0.05$) increased baseline calf BF and VC, and decreased
baseline VR in all groups. Peak calf BF and VC were increased ($p<0.05$) and peak VR decreased ($p<0.05$) in ExFHA women only.
Table 4. Brachial artery responses pre- and post-exercise across the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=15)</th>
<th>ExOv (n=14)</th>
<th>ExAmen (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PreEx</td>
<td>PostEx</td>
<td>PreEx</td>
</tr>
<tr>
<td>BA Diameter (mm)</td>
<td>3.05 ± 0.09</td>
<td>3.08 ± 0.08</td>
<td>3.12 ± 0.07</td>
</tr>
<tr>
<td>FMD Peak Diameter (mm)</td>
<td>3.28 ± 0.09</td>
<td>3.35 ± 0.08*</td>
<td>3.40 ± 0.06</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>8.02 ± 0.53</td>
<td>8.90 ± 0.58</td>
<td>8.77 ± 0.73</td>
</tr>
<tr>
<td>FMD Peak time (s)</td>
<td>47.2 ± 2.3</td>
<td>51.6 ± 3.8</td>
<td>43.6 ± 3.5</td>
</tr>
<tr>
<td>Pre GTN Diameter (mm)§</td>
<td>3.09 ± 0.14</td>
<td>-</td>
<td>3.11 ± 0.08</td>
</tr>
<tr>
<td>GTN Diameter (mm)§</td>
<td>3.64 ± 0.15</td>
<td>-</td>
<td>3.63 ± 0.05</td>
</tr>
<tr>
<td>GTN (%)§</td>
<td>17.13 ± 1.81</td>
<td>-</td>
<td>16.72 ± 1.27</td>
</tr>
</tbody>
</table>

Values are mean±SEM

BA, brachial artery diameter; FMD, flow mediated dilation; GTN, glyceryl trinitrate; s, seconds.

* Pre- versus post-exercise within groups (p<0.05); a ExFHA vs. SedOv and ExOv pre exercise, p<0.01; b ExFHA vs. SedOv and ExOv post exercise, p<0.01; c ExFHA vs. SedOv and ExOv pre exercise, p<0.05; § GTN responses in subset only (SedOv n=10, ExOv n=11, ExFHA n=7).
Table 5. Brachial artery blood flow and shear responses pre- and post-exercise across the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=15)</th>
<th>ExOv (n=14)</th>
<th>ExFHA (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PreEx</td>
<td>PostEx</td>
<td>PreEx</td>
</tr>
<tr>
<td>Resting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Flow (ml/min)</td>
<td>97.2±11.9</td>
<td>140.2±16.4*</td>
<td>166.8±17.4</td>
</tr>
<tr>
<td>Shear Rate Mean (cm/s)</td>
<td>73.1±6.9</td>
<td>87.3±6.8*</td>
<td>75.1±6.9</td>
</tr>
<tr>
<td>Peak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Flow (ml/min)</td>
<td>308.4±19.7</td>
<td>390.2±35.7*</td>
<td>315.5±19.9</td>
</tr>
<tr>
<td>Shear Rate Mean (cm/s)</td>
<td>245.8±12.2</td>
<td>251.9±17.2</td>
<td>220.2±15.5</td>
</tr>
<tr>
<td>Shear Rate AUC (a.u.)</td>
<td>2312±149</td>
<td>2764±233*</td>
<td>2062±163</td>
</tr>
</tbody>
</table>

Values are mean±SEM

* Pre- versus post-exercise within groups, \( p<0.05 \)

\( ^a \) ExFHA vs. ExOv and SedOv within condition, \( p<0.05 \)

\( ^b \) ExFHA vs. ExOv within condition, \( p<0.05 \)
Figure 1. **A**: Endothelium-dependent (FMD) and -independent (GTN) responses for the study groups pre-exercise; **B**: Percent change in brachial artery diameter pre- and post-exercise for the study groups; **C**: Percent change in brachial artery diameter normalized for SRAUCpk pre-and post-exercise for the study groups. * ExFHA vs. SedOv and ExOv within conditions, \( p<0.05 \); ** Pre- vs. post-exercise within groups, \( p<0.05 \).
**Figure 2.** Resting (A) and peak (B) calf blood flow responses pre- and post-exercise (closed and open bars, respectively) for the study groups.

* ExFHA vs. SedOv and ExOv pre-exercise, $p<0.05$;

** Pre-exercise vs. post-exercise, within groups, $p<0.05$
Figure 3. Scatterplots showing the correlation between FMD% and SRAUCpk pre-exercise (A) and post-exercise (B).
FMD% and GTN% Correlates:  

**Pre-Exercise:** Using pooled data, pre-exercise FMD% was positively correlated with estradiol ($r=0.57$, $p<0.01$), PP ($r=0.57$, $p<0.01$), SRAUCpk ($r=0.54$, $p<0.01$), and SR mean peak ($r=0.40$, $p=0.01$); see Figure 3. Pre-exercise, both FMD% and GTN% were inversely associated with brachial artery diameter ($r= -0.37$, $p<0.05$ and $r= -0.39$, $p<0.05$, respectively).  

**Post-Exercise:** FMD% was positively correlated with post-exercise measures of SRAUCpk ($r=0.45$, $p=0.01$), PP ($r=0.31$, $p=0.04$), and SR mean peak ($r=0.35$, $p=0.03$), and inversely associated with brachial artery diameter ($r= -0.33$, $p=0.03$).

Sub-group analyses showed that ExFHA and ExOv women did not independently demonstrate significant ($p>0.05$) FMD% correlates pre-exercise. In contrast, SedOv women demonstrated strong positive correlations between pre-exercise FMD% and estradiol ($r=0.50$, $p<0.05$), mean SR peak ($r=0.61$, $p<0.05$) and SRAUCpk ($r=0.62$, $p<0.01$). Post-exercise, FMD% was not significantly associated with any measure in any group.

Calf Blood Flow Correlates: Baseline hormonal and lipid markers were not associated ($p>0.05$) with pre-exercise measures of calf BF, VC or VR.

3.4 Discussion

The novel findings of the current study are twofold. Firstly, decreased endothelium-dependent dilation in ExFHA women is partly, but not fully, explained by lower SRAUCpk. Exercise acutely increased both FMD% and SRAUCpk in ExFHA women, but remained lower than that
observed in sedentary and ovulatory eumenorrheic women. However, normalization of post-exercise FMD% for SRAUCpk negated differences between the groups. Secondly, blunted calf BF responses in ExFHA women are explained solely by increased calf VR, with exercise eliciting increases in both resting and peak-ischemic calf BF and decreases in calf VR, resulting in responses that do not differ from those observed in eumenorrheic women. Endothelium-independent dilation in the brachial artery was also blunted in ExFHA women pre-exercise. Mechanisms of action remain unknown. Collectively, these findings support that mechanisms of altered basal vascular function in hypoestrogenic ExFHA women are likely multifactorial, and may include increased regional vascular tone and/or decreased hemodynamic load (i.e., low shear stress) 'seen' by the vessel wall. That dynamic exercise acutely restores both endothelium-dependent resistance vessel function and conduit vessel responses when normalized for peak shear stress stimulus suggests vascular responsiveness is preserved in ExFHA women. The clinical relevance of these findings remain to be determined.

**Pre-Exercise Vascular Responses:** The findings of low baseline brachial artery FMD%, low baseline and peak calf BF, and increased calf VR in women with ExFHA are in agreement with previous studies (13-15, 21). Altered vascular responses in women with ExFHA have been postulated to be a consequence of impaired endothelial NO synthesis and/or increased degradation due to estrogen deficiency (13, 14, 21). 17 β-estradiol rapidly activates endothelial NO synthase (eNOS) through the phosphatidylinositol-3-kinase (PI3K)/AKT pathway (107). eNOS is quantitatively the most important source of vascular NO, which, once formed, diffuses into the adjacent smooth muscle cells and induces vasorelaxation (107).
Through the same PI3K/AKT pathway, but via different mechanisms, constant exposure of the endothelium to a physiologic range of shear stress stimulates eNOS activation (119). Under specific conditions, the acute effect of increases in shear stress on NO release underlies the principal of FMD (116). Importantly, the shear stress stimulus generated in the brachial artery in response to FMD is inversely related to vessel diameter, which is also inversely associated with the FMD response (22, 126). It is also known that the magnitude of FMD is related to the magnitude of the imposed stimulus (22, 126). Thus, altered endothelium-dependent function in women with ExFHA may reflect lower shear stress-mediated NO stimulus rather than impaired vascular function per se. In agreement with this, the data demonstrate similar baseline brachial artery diameter among the groups, but lower SRAUCpk in ExFHA versus ExOv and SedOv women. However, adjustment of FMD% for both SRAUCpk and brachial artery diameter, both independently and in combination, did not alter the findings, suggesting other factors are contributing to basal endothelium-dependent responses in ExFHA women.

Impaired brachial artery endothelial-dependent and -independent vasodilatory responses combined with decreased calf BF and increased calf VR in ExFHA women suggests possible increased sympathetic vasoconstrictor tone in multiple vascular beds. While the exact influence of estrogen deficiency on these responses is unclear, NO is known to contribute to vascular tone not only by eliciting vasodilation but also by modulating sympathetic vasoconstrictor tone (461). In young premenopausal eumenorrheic women, vasoconstrictor responses to sympathetic stimuli are reported to be lower compared with age-matched men (462). Furthermore, estrogen therapy in PMW decreases vascular efferent sympathetic nerve traffic to the lower limb vascular bed (233), and also reduces vasoconstrictor responses to
norepinephrine (43). Thus, in addition to enhanced NO bioavailability, estrogen may also influence vasodilator capacity through reduced sympathetic activity and attenuated responsiveness to sympathetic-mediated vasoconstriction (i.e., enhanced functional sympatholysis). The latter may be mediated through increased β2-adrenergic receptor and decreased α–adrenergic receptor sensitivity (234, 239, 462). Whether estrogen deficiency in ExFHA women does indeed influence vasoconstrictor tone, possibly via alteration to any one or combination of these mechanisms, remains to be determined.

Lower endothelium-independent dilation in response to GTN in ExFHA than in ovulatory women is in accord with some (21), but not all (14, 15) studies in amenorrheic athletes. Causes of blunted GTN% in women with ExFHA are unknown, but is suggestive of altered smooth muscle cell responsiveness to NO and/or an altered ability of the smooth muscle cells to relax due to reduced compliance or remodelling of the vessel wall. Vascular remodeling and endothelial dysfunction has been experimentally shown to be inversely related (463). While it is not yet known whether blunted GTN%, or indeed FMD%, in physically active women with FHA is due to structural modifications of the vessel wall, estrogen has been reported to protect against vascular remodelling (464). Mechanisms of action include scavenging of reactive oxygen species and inhibition of smooth muscle cell proliferation (464).

Post-Exercise Vascular Responses: For the first time, the current study reports that following an acute bout of dynamic exercise, women with ExFHA demonstrate partially restored FMD% in the brachial artery and fully restored calf BF responses when compared to values observed in eumenorrheic women. Importantly, FMD% remained lower in ExFHA women than in both
ovulatory groups, in whom exercise did not influence FMD%, or peak calf BF, measures. Similar pre- and post-exercise conduit and resistance artery vascular responsiveness in trained and untrained ovulatory women suggests that: i) there is no additive effect of regular moderate exercise training on endothelial function, possibly due to estrogen and exercise sharing a common NO (PI3K/Akt) pathway; and/or ii) vascular responsiveness is already operating at an optimal functional 'ceiling' in healthy young women, beyond which it is physiologically unnecessary and/or potentially deleterious.

The finding of partial, but not fully restored post-exercise FMD% in ExFHA women is in contrast with a previous study reporting fully restored post-exercise brachial artery FMD% in healthy estrogen deficient PMW, with FMD% no longer differing from that observed in healthy eumenorrheic premenopausal women (140). Since this (140) and the current study utilized an identical exercise intervention protocol, differences in models of estrogen deficiency (older PMW versus young premenopausal with FHA) and/or FMD methodology (upper arm versus forearm cuff occlusion) likely explain the discrepancy in findings. Increased, but not fully restored, FMD% in ExFHA women may also be partially explained by differences in magnitude of the post-ischemic shear stress stimulus to NO synthesis. Brachial artery SRAUCpk was increased in all groups post-exercise but remained lowest in ExFHA women. Thus, as with pre-exercise FMD% responses, the magnitude of the imposed stimulus may be contributing in part to this finding. However, the data show that normalization of brachial artery FMD% for SRAUCpk post-exercise negated differences between the groups. Therefore, post-exercise FMD% in ExFHA women may be considered to be fully restored when adjusted for the hemodynamic stimulus 'seen' by the artery. While useful for comparing responses between subjects, the standardization of 'how' to normalize the FMD response, and
the clinical utility of using the normalized FMD response (FMD%/SR AUC), has yet to be established (465).

Resting calf BF was significantly increased post-exercise in all groups. Elevated post-exercise calf BF is a well documented phenomenon, but causes remain unknown (466). This notwithstanding, increases in resting calf BF in the current study were associated with decreases in calf VR in all three groups. This observation is in agreement with the documented reduction in systemic VR both during and after large-muscle dynamic exercise (467). Mechanisms of action include decreased sympathetic outflow and related sympatholysis, and increased release of vasodilator substances such as NO (468). In contrast with resting calf BF post-exercise, peak calf BF post-exercise was increased and calf VR decreased in ExFHA women, but were unaltered in both ovulatory groups. Increased regional BF and decreased regional VR in ExFHA women post-exercise suggests altered sympathetic outflow to the lower limb vascular bed. This notion is consistent with studies reporting decreased efferent muscle sympathetic nerve activity in normotensive (467) and hypertensive (469) individuals following exercise. In contrast, similar pre- versus post-exercise peak calf BF and FMD% responses in estrogen replete sedentary and physically active women in the current study suggests sympathetic outflow is not altered by chronic exercise training or acute exercise, possibly due to operating at a lower basal level than ExFHA women. This latter postulate is consistent with the reported sympatho-inhibitory effects of estrogen (24, 233, 324).

**Limitations:** In the current study, only SRAUCpk was able to be assessed. It is currently recommended that SR AUC be assessed until peak vasodilation (122). However, peak SR
continues to be commonly reported and used as a determinate of the FMD% stimulus. Shear stress is determined in part by fluid viscosity, which was not quantified in this study.

Retrograde shear is associated with impaired FMD% (119). In the current study only antegrade flow was assessed. It is unclear whether retrograde shear contributes to impaired FMD% in ExFHA women. Attenuated post-exercise FMD% in ExFHA women may be related in part to altered smooth muscle cell responsiveness to endothelium-derived NO. Post-exercise GTN% would have helped confirm this postulate, but due to post-exercise hypotension and concerns regarding safety of the research subjects, this measure was not collected. Energy deficiency elicits numerous endocrine and metabolic secretory aberrations. The influence of these aberrations on vascular function was not able to be considered in the current study. Finally, the use of small sample sizes in the study groups may have hindered the ability to detect differences when in fact they were present.

Conclusions: Women with ExFHA demonstrate altered conduit vessel endothelial-dependent and -independent reactivity and decreased calf BF, suggesting that hypoestrogenemia may partially obviate the recognized vascular benefits of both regular exercise and premenopausal status. Pre-exercise, low FMD% in ExFHA women is partially explained by low peak SR. Conversely, low calf BF is solely explained by elevated calf VR. Compared with estrogen replete women, dynamic exercise acutely restores calf BF in ExFHA women by decreasing VR. In contrast, FMD% is only partially restored by dynamic exercise. Normalization of FMD% for peak SR negated post-exercise, but not pre-exercise, differences between the groups. However, interpretation of post-exercise vascular responses is difficult due to the hemodyanamic and sympathetic changes associated with acute exercise. This notwithstanding,
altered vascular function in ExFHA appears to be due in part to increased regional
vasoconstrictor tone, decreased shear stress-mediated NO vasodilation, and/or impaired
vascular responsiveness. The clinical relevance of these findings have yet to be determined.
CHAPTER 4

Discordant Efferent Sympathetic Neural and Renin Angiotensin System Responsiveness to Acute Baroreflex Stimulation during Simulated Orthostatic Stress in Estrogen-Deficient Physically Active Premenopausal Women

4.1 Introduction

Premenopausal women are at lower risk for hypertension and cardiovascular disease compared with age-matched men and postmenopausal women (PMW) (4). This advantage is associated in part, with the well documented favorable vasoregulatory and sympatho-inhibitory effects of ovarian hormones, particularly estrogen (2, 24, 450). Conversely, estrogen deficiency after menopause is associated with increased sympathetic nervous system activity (SNA), increased vascular tone, and altered renin-angiotensin-aldosterone system (RAAS) activity (2, 24, 450). Collectively, these neurohumoral perturbations are thought to contribute to the higher incidence of hypertension and the marked acceleration in the development and progression of atherosclerosis reported in PMW compared with age-matched men and younger premenopausal women (2, 231).

Estrogen deficiency is not unique to the postmenopausal period. In premenopausal women, functional hypothalamic amenorrhea (FHA) is a common and reversible cause of non-organic disruption of ovulatory cycling that is characterized by marked hypoestrogenemia (7), similar to that observed in postmenopausal women (25). Greater prevalence of FHA is reported
among recreationally and competitively physically active women (383, 388) compared with non-exercising women (388). It has been previously shown that exercising, weight-stable women with long-term estrogen deficiency (ExFHA) have impaired regional blood flow, and increased regional vascular resistance compared with both exercise trained and sedentary ovulatory women (13, 23). Others also report impaired flow mediated dilation in the brachial artery (14, 15, 21, 422). These findings suggest an elevated risk for hypertension in this cohort. However, paradoxically, ExFHA demonstrate lower, not higher, systolic BP than their eumenorrheic counterpart (13, 23, 438), suggesting altered BP regulation in amenorrheic athletes. Neurohumoral mechanisms of BP regulation in ExFHA women have not yet been reported.

Therefore, in estrogen deficient women with ExFHA, the purpose of this study was to examine patterns of sympatho-excitation, RAAS activation and arterial baroreflex mediated increases in HR in response to orthostatically induced changes in arterial BP during graded lower body negative pressure (LBNP). In keeping with the previously observed lower basal HR and BP in ExFHA women (13, 23, 438), it was hypothesized that hemodynamic responses during LBNP would also be lower in ExFHA women compared with eumenorrheic physically active women. Consistent with the reported sympatho-inhibitory effects of estrogen (24, 233, 324), it was also hypothesized that efferent muscle SNA (MSNA) responses would be higher and serum RAAS components elevated in ExFHA versus estrogen replete eumenorrheic physically active women.
4.2 Methods

Subjects: Physically active eumenorrheic and amenorrheic women were recruited for this study. Subject recruitment methods and study eligibility criteria are as previously described in detail on page 80.

Experimental Design and Study Groupings: Measures of BP, HR, lower limb MSNA and serum RAAS were obtained at rest and during three graded stages of LBNP. Volunteers were grouped according to their menstrual status (ovulatory [ExOv; n=17] or amenorrheic [ExFHA; n=12]). The details of this classification scheme are as previously outlined on page 81.

Exercise status: As described in detail previously on page 81, ‘exercising’ status was defined as i) >2 hours of structured physical activity per week, and ii) a peak aerobic capacity of ≥40 ml/kg/min (13).

Procedures: LBNP was applied using a custom-built chamber allowing for simultaneous application of simulated orthostatic stress and recording MSNA from the right peroneal nerve while the participant lay supine with the body sealed at level of the iliac crest. The right leg was held in position by form-fitting supports. HR, BP, respiratory excursions and MSNA were recorded simultaneously at baseline and during each stage of LBNP. Blood sampling was completed at baseline and during the last minute of each stage of LBNP.
Protocol: All testing occurred in the morning, starting between 0930-1030 in a quiet ambient room (22-24 °C). Volunteers were at least 2 hours fasted and had abstained from exercise and caffeine for 24 hours and alcohol 12 hours prior to testing. All measures were obtained during the early follicular phase (days 2-6) in eumenorrheic subjects, and on a random day for amenorrheic subjects. Once MSNA was detected, an 8-minute baseline recording was completed followed by sequentially graded application of LBNP at -10mmHg, -20mmHg and -40mmHg. These levels of LBNP were chosen to enable gradual unloading of cardiopulmonary and arterial baroreceptors and to permit comparison of results with previous studies examining the effects of estrogen on arterial BP regulation during LBNP (286, 470). Each level was sustained for 8 minutes, followed by a 5-minute recovery stage (i.e., no LBNP). BP, HR, and MSNA were recorded continuously. The LBNP was terminated if presyncopal evidence was detected, including a drop in systolic BP below 80mmHg, pallor, and subjective feelings of nausea, dizziness and/or light-headedness.

Body Composition and Anthropometric Measures: Height, weight, lean mass and fat mass were assessed as previously described in detail on page 83.

Peak Aerobic Capacity: Methods of assessment are as described previously on page 83.

Muscle Sympathetic Nerve Activity: Multi-unit recordings of post-ganglionic MSNA were obtained with a unipolar tungsten electrode inserted selectively into a muscle-nerve fascicle of
the right fibular (peroneal) nerve using previously described techniques (471). The nerve signals were amplified, filtered (bandwidth 700 to 2000 Hz), rectified, and integrated to obtain a mean voltage display of sympathetic nervous activity. A recording of MSNA was considered acceptable when the following criteria were met: i) spontaneous bursts of neural discharge synchronous with the heart rate; ii) no response to arousal stimuli or skin stroking; iii) an increase in nerve burst frequency with apnoea; iv) a signal to noise ratio of 3:1 (472). With the subject lying quietly, recordings were acquired at baseline and during each stage of LBNP. Signals were digitized (LabVIEW, National Instruments Corporation, Austin, TX, USA) for off-line analysis using customized software. Variables of interest included burst incidence (bursts per 100 heart beats) and burst frequency (bursts per minute).

**Blood Pressure and Heart Rate:** Measurements of systolic (SBP), diastolic DBP), mean arterial BP (MAP; 1/3 pulse pressure + DBP) and HR were recorded on the left upper-arm using an automated device (Dinamap Pro 100, Critikon, USA). Brachial BP and HR were assessed at one minute intervals at baseline (three consecutive measures) and throughout each stage of LBNP using an automated upper arm cuff (Dinamap Pro 100, Critikon, USA). Continuous recordings of BP and HR were also acquired. HR was recorded using lead II of an electrocardiogram, and BP was recorded on the index finger using a photoplethysmographic device with orthostatic correction (Portapres Model-2, Finapres Medical Systems BV, USA). Brachial BP and HR recorded during the last three minutes of each LBNP stage were averaged to acquire a mean value for each LBNP stage.
Serum Measures: A polyethylene catheter was inserted into the right cubital vein by a medically trained nurse to facilitate multiple blood draws over time. Baseline samples were analyzed for 17 β-estradiol, progesterone, free triiodothyronine (T3), and components of the RAAS (renin, Angiotensin II [Ang II], and aldosterone). Angiotensinogen was not assessed in the current study as others have previously reported no effect of simulated orthostatic stress on this RAAS component in healthy pre- and post-menopausal women (286). Blood samples for components of the RAAS were also collected during the last minute of each sub-atmospheric pressure stage (-10, -20 and -40 mmHg). All assays were run by the Core Laboratory at the Toronto General Hospital. Detailed serum collection and assay specifications are provided in Appendix 5.

Statistics: Data screening, conducted prior to statistical analysis included outlier detection, and examination of variable distributions within each of the two groups for normality. No outliers were detected. All data sets were normally distributed. Group differences at baseline were detected using one-way analysis of variance (ANOVA). Within- and between-group analyses of responses to LBNP, including change (∆) in measures compared to baseline, were determined using repeated measures ANOVA. When assumptions of sphericity were violated, Greenhouse-Geisser correction was used. Using pooled data, Pearson’s correlational analyses were used to determine significant linear independent associations between BP and neurohumoral variables of interest. Data were analyzed using packaged software (SPSS version 20; SPSS Inc., Chicago, IL). A significance level of $p < 0.05$ was used to detect the differences for statistical procedures. All data are presented as the mean ± SEM.
4.3 Results

Subject Characteristics: Characteristics of the study groups are presented in Table 1. Duration of amenorrhea was ~1000 days in women with ExFHA. The two study groups did not differ \((p>0.05)\) in age, height, weight, calculated body mass index (BMI), body composition or cardio-respiratory fitness level. Serum measures of progesterone were similar \((p>0.05)\) between groups. In contrast, estrogen and free T3 were significantly lower \((p<0.05)\) in women with ExFHA than in ExOv women.

Baseline Hemodynamics and RAAS: Baseline arterial BP and HR are shown in Table 2. Systolic BP (SBP), pulse pressure (PP), mean arterial BP (MAP), and HR were lower \((p<0.05)\) in ExFHA women compared with ExOv women. Baseline diastolic BP (DBP) did not differ between the groups \((p=0.25)\).

Blood samples for RAAS components was successfully collected in 20 subjects (ExOv, \(n=11\); ExFHA, \(n=9\)). Of the 9 subjects that were not tested for RAAS, 7 were not able to be successfully catheterized and 2 expressed preference to not be catheterized. Baseline serum measures of renin, Ang II and aldosterone are presented in Table 3. No between-group differences for baseline serum components of the RAAS \((p>0.05)\) were detected.

Baseline MSNA: Acceptable microelectrode recordings of MSNA were obtained in 22 women (ExOv, \(n=12\); ExFHA, \(n=10\)). Table 3 reports MSNA bursts/minute (burst frequency) and
bursts/100 heart beats (burst incidence). No differences in baseline MSNA were detected
($p>0.05$) between groups.
Table 1. Baseline subject characteristics.

<table>
<thead>
<tr>
<th></th>
<th>ExOv (n=17)</th>
<th>ExFHA (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.7 ± 1.2</td>
<td>25.4 ± 1.1</td>
<td>0.308</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.7 ± 1.5</td>
<td>167.8 ± 1.4</td>
<td>0.316</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>57.5 ± 1.9</td>
<td>58.4 ± 2.1</td>
<td>0.761</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>20.9 ± 0.5</td>
<td>20.7 ± 0.7</td>
<td>0.828</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>23.5 ± 1.8</td>
<td>21.8 ± 2.1</td>
<td>0.592</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>41.9 ± 1.6</td>
<td>44.2 ± 1.7</td>
<td>0.377</td>
</tr>
<tr>
<td>VO(_2) peak (ml/kg/min)</td>
<td>47.1 ± 1.3</td>
<td>46.3 ± 1.9</td>
<td>0.666</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>154.8 ± 24.5</td>
<td>87.0 ± 13.9*</td>
<td>0.045</td>
</tr>
<tr>
<td>Progesterone (nmol/L)</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>0.634</td>
</tr>
<tr>
<td>Free T3 (pmol/L)</td>
<td>4.2 ± 0.2</td>
<td>3.6 ± 0.1*</td>
<td>0.017</td>
</tr>
<tr>
<td>Amenorrhea (days)(^a)</td>
<td></td>
<td>1061 ± 319</td>
<td>-</td>
</tr>
</tbody>
</table>

Values mean±SEM.

BMI, body mass index; VO\(_2\) peak, peak oxygen consumption; T3, triiodothyronine.

* ExFHA vs. ExOv

\(^a\) Range 150-3132 days
Table 2. Hemodynamic responses of the groups during LBNP.

<table>
<thead>
<tr>
<th></th>
<th>Ba</th>
<th>-10mmHg</th>
<th>-20mmHg</th>
<th>-40mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ExOv</td>
<td>ExFHA</td>
<td>ExOv</td>
<td>ExFHA</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>105±2</td>
<td>94±2***</td>
<td>101±2§</td>
<td>93±2*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>60±1</td>
<td>58±2</td>
<td>56±2§</td>
<td>56±2</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>75±1</td>
<td>70±2**</td>
<td>71±2§</td>
<td>68±2</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>45±2</td>
<td>36±2***</td>
<td>45±2</td>
<td>37±2*</td>
</tr>
<tr>
<td>HR (b/min)</td>
<td>56±2</td>
<td>47±2***</td>
<td>58±2</td>
<td>48±2*</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

* Significantly different between-groups for given LBNP, \(p<0.05\).

** ExFHA versus ExOv at baseline, \(p<0.05\).

§ Significantly different from baseline within-groups, \(p<0.05\).
Table 3. Neurohumoral measures during graded LBNP.

<table>
<thead>
<tr>
<th></th>
<th>Ba</th>
<th>-10mmHg</th>
<th>-20mmHg</th>
<th>-40mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ExOv</td>
<td>ExFHA</td>
<td>ExOv</td>
<td>ExFHA</td>
</tr>
<tr>
<td>Renin (pmol/L)†</td>
<td>8.0 ± 2.0</td>
<td>4.0 ± 0.8</td>
<td>7.4 ± 1.8</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>Ang II (pmol/L)†</td>
<td>3.9 ± 0.8</td>
<td>2.3 ± 0.3</td>
<td>4.1 ± 0.9</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>Aldosterone (pmol/L)†</td>
<td>88 ± 16</td>
<td>132 ± 32</td>
<td>74 ± 8</td>
<td>117 ± 32</td>
</tr>
<tr>
<td>MSNA (bursts/min)‡</td>
<td>8.4 ± 1.6</td>
<td>9.8 ± 2.2</td>
<td>10.4 ± 2.0</td>
<td>13.1 ± 2.7</td>
</tr>
<tr>
<td>MSNA (bursts/100 beats)‡</td>
<td>15.1 ± 3.0</td>
<td>21.9 ± 4.8</td>
<td>18.3 ± 3.6</td>
<td>27.9 ± 5.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

† ExOv n=11; ExFHA n=10.
‡ ExOv, n=12; ExFHA, n=10

* Significantly different between-groups for given LBNP, p<0.05.

§ Significantly different from baseline within-groups, p<0.05.
Hemodynamic and RAAS Responses to LBNP: LBNP elicited similar ($p > 0.05$) within-group decreases in SBP, MAP and DBP ($p < 0.001$ for all measures), and increases in HR ($p < 0.001$) (Figure 1). In contrast, LBNP did not elicit within-group changes in PP ($p = 0.15$). Significant between-group responses to graded LBNP were observed for SBP ($F(1,27) = 13.60, p < 0.01$), PP ($F(1,27) = 11.56, p < 0.01$) and HR ($F(1,27) = 26.02, p < 0.01$), with ExFHA women demonstrating consistently lower values than ExOv women. While between-group DBP responses to LBNP did not differ ($F(1,27) = 0.62, p = 0.45$), MAP trended toward different responses between groups ($F(2.38, 64.34) = 29.21, p = 0.06$). ∆HR and ∆BP responses to LBNP did not differ ($p > 0.05$; all measures) between the groups.

RAAS responses to LBNP are shown in Figure 2. Within groups, LBNP elicited significant changes in renin ($F(1.27, 19.09) = 10.04, p < 0.01$), and Ang II ($F(1.67, 25.28) = 5.62, p = 0.01$), but not aldosterone ($p > 0.05$). Significant between-group interactions in response to LBNP were also observed for renin ($F(1.27, 19.09) = 5.32, p = 0.03$), Ang II ($F(1.68, 25.28) = 7.86, p < 0.01$), and aldosterone ($F(1.54, 26.17) = 4.48, p = 0.03$). Specifically, ExFHA women demonstrated no activation of either renin or Ang II compared with ExOv women (Figure 2a and 2b), who demonstrated the anticipated reflex activation of these RAAS components in response to LBNP. LBNP lowered aldosterone in ExFHA women, but increased aldosterone in ExOv women (Figure 2c). Within- and between-group analyses using ∆ measures of each RAAS component did not alter ($p > 0.05$; all measures) the findings.

MSNA Responses to LBNP: Within-groups, LBNP caused MSNA bursts/minute ($p < 0.001$) and bursts/100 beats ($p < 0.001$) to increase similarly ($p > 0.05$) in both groups (Figure 3 and 3b). In
contrast, between-groups, MSNA bursts/100 heart beats \((F(1,20)=4.95; p=0.04)\), but not bursts/minute \((p>0.05)\), was significantly higher in ExFHA women versus ExOv women at -20 and -40 mmHg. Similarly, between-group measures of ΔMSNA bursts/100 heart beats, but not ΔMSNA bursts/minute, was significantly greater \((F(1,20)=4.42, p<0.05)\) in ExFHA versus ExOv women at -20 and -40 mmHg (Figure 3c).

**Correlations:** Using pooled data, MSNA responses at baseline and during LBNP were not correlated \((p>0.05)\) with serum measures of RAAS components, estradiol, progesterone, free T3, or measures of arterial BP. Using data from ExFHA women only, baseline MSNA bursts/minute was positively correlated with HR \((r=0.84, p<0.01)\). MSNA responses during LBNP were not correlated with any measure of BP or RAAS. In ExOv women, MSNA responses at baseline and during LBNP were not correlated \((p>0.05)\) with measures of BP or RAAS.
Figure 1. Systolic BP (A), diastolic BP (B), mean arterial BP (C), and HR (D) for the study groups during graded LBNP. L × G, LBNP × Group interaction. A significant main effect (p < 0.001) for LBNP was observed for all measures. * Significantly different between groups within condition, p < 0.05.
Figure 2. Serum renin (A), angiotensin II (B), and aldosterone (C) during the last minute of each stage of LBNP. * Significantly different between groups within condition, p<0.05.
Figure 3. MSNA bursts per minute (A) and per 100 heart beats (B), and change (Δ) in MSNA bursts per 100 heart beats from baseline (C) during graded LBNP. * Significantly different between groups within condition, $p<0.05$. 
4.4 Discussion

This is the first study to report hemodynamic, efferent SNA and RAAS responsiveness during orthostatic stress in women with ExFHA. The novel findings indicate that compared to BMI-, age- and fitness-matched estrogen replete women, estrogen deficient women with ExFHA demonstrate: i) similar basal efferent sympathetic outflow to the lower limb and similar plasma Ang II and renin levels, yet lower resting HR and SBP; ii) greater increases in MSNA burst incidence during orthostatic stress yet lower BP and HR responses; and iii) an absence of reflex activation of the RAAS, despite decreases in BP during orthostatic stress. While the exact role of estrogen deficiency in these responses is unclear, these findings suggest that neurohumoral control of arterial BP during orthostatic stress is shifted towards increased sympathetic and decreased humoral influences in ExFHA women.

A key finding of the study was that similar basal MSNA and serum RAAS levels exist in ExFHA and ExOv women despite significantly lower measures of resting MAP and SBP in women with ExFHA. While the influence of estrogen deficiency per se on these findings is not clear, these data suggest that lower resting supine arterial BP in ExFHA women, a consistent finding with previous studies (13, 23), is not associated with efferent MSNA or circulating RAAS. In contrast, challenging BP homeostasis using simulated orthostatic stress revealed a shift in neurohumoral arterial BP control in the estrogen deficient compared with estrogen replete women. Graded LBNP elicited increases in MSNA and HR, and decreases in SBP, in both amenorrheic and eumenorrheic women. However, SBP and HR responses were consistently lower, and MSNA burst incidence (bursts/100 heart beats), but not MSNA burst frequency (bursts/min), higher in physically active women with FHA. Importantly, ΔHR and
∆SBP did not differ between the groups, but ∆MSNA burst incidence remained higher in ExFHA than ExOv women. Thus, ExFHA women demonstrate greater reliance on sympathetic activation in defending arterial BP during hypotensive stress compared with ExOv women, suggesting a role for estradiol in the modulation of SNA patterns of sympatho-excitation. This notion is consistent with studies reporting a sympatho-inhibitory effect of estrogen (233, 324), including lower autonomic and alpha-adrenergic control of resting arterial BP (236, 239), enhanced β-adrenergic receptor-mediated dilatation (238), and a reduction in vasoconstrictor responses to sympathetic stimuli (462, 473) in young women versus men.

In contrast to the well documented sympatho-inhibitory effects of estrogen at rest (24, 233, 324), the effects of estrogen on vasomotor sympathetic outflow in response to simulated orthostatic stress are less clear. Studies report lower (474) and similar (475) MSNA responses to head-up tilt in young women versus men. In premenopausal women, total MSNA in response to graded LBNP (470) and head-up tilt (323) is augmented when both estrogen and progesterone levels are elevated (mid-luteal phase) compared with the low hormone (early follicular) phase of the menstrual cycle. In contrast, studies also report no effect of 4 weeks of estrogen therapy on efferent MSNA in healthy PMW during LBNP (476). Reasons for discordance between these studies are not clear, but use of different orthostatic challenges and the opposing sympatho-excitatory effects of estradiol and progesterone (228) are likely of importance.

Of interest, the finding of similar ∆HR increases and ∆SBP decreases during simulated orthostatic stress in ExOv and ExFHA women suggests arterial baroreflex mediated increases in HR in response to LBNP is not influenced by hypoestrogenemia. Although not directly assessed in the present study, this observation concurs with reports of preserved baroreflex sensitivity.
using the Valsalva manoeuvre during head-up tilt in amenorrheic athletes (440). However, these (440) and the current findings are in contrast with studies demonstrating favorable modulatory effects of estrogen therapy on baroreflex sensitivity in PMW (309, 310). The differences in arterial compliance, and hence arterial baroreceptor functioning in younger versus older women may contribute to these divergent findings.

Another key finding of the study was that unlike their eumenorrheic counterparts, women with ExFHA do not demonstrate reflex activation of the RAAS in response to hypotensive stimuli. This observation is consistent with findings in estrogen deficient PMW during graded LBNP (286). Taken together, these findings suggest that estrogen deficiency may inhibit RAAS activation during orthostatic stress. The influence of estrogen deficiency on the RAAS has not yet been elucidated, but numerous studies report that estrogen modulates the RAAS. For example, estrogen therapy in PMW increases plasma renin activity, serum Ang I and II levels and decreases serum angiotensin converting enzyme-1 activity (268, 269, 274-276). Of interest, despite RAAS activation, BP is unaltered in PMW taking oral estrogen therapy (268, 269, 274-276). Similarly, arterial BP is well regulated throughout the menstrual cycle despite significant fluctuations in plasma renin activity (272, 278). The discordance between RAAS activation and arterial BP control in premenopausal women is thought to be due in part to ovarian hormone-mediated altered tissue sensitivity to circulating hormones of the RAAS (272). In PMW taking oral estrogen therapy, the absence of the vasopressor actions of Ang II acting on the Ang II type 1 receptor pathway may also be secondary in part, to enhanced estrogen-dependent activation of novel RAAS vasodilator pathways (477). These include bradykinin (268), Ang 1-7 (283), ACE2 (478), and activation of the Ang II type 2 receptor.
Whether novel RAAS pathways or tissue sensitivity to RAAS are altered in women with ExFHA remains to be determined.

Limitations: Several study limitations are acknowledged. Hydration status, or the sodium or protein status of the participants was not assessed or controlled. Altered hydration status can influence hemoconcentration levels of plasma markers, and sodium depletion and protein overfeeding can result in increased activity of the RAAS (480). Since mild negative energy balance without impaired micronutrient status is linked to FHA in physically active women (25), its role cannot be isolated from that of estrogen deficiency. Mild caloric deficit is associated with favourable alterations to both arterial BP (432) and efferent SNA (481) in animals and humans. Circulating catecholamines, vascular tone or cardiac output, all of which play a central role in the regulation of arterial BP, were also not assessed. It is also acknowledged that measures of circulating RAAS does not reflect tissue RAAS levels, and that SNA recorded in the lower limb cannot be extrapolated to other organs including the kidney and heart. Finally, the group sample size may have limited the ability to detect true differences in certain endpoints.

Conclusion: ExFHA and eumenorrheic women demonstrate similar neurohumoral regulation of arterial BP at supine rest. In contrast, marked alterations in both MSNA and RAAS responses to simulated orthostatic stress are evident in estrogen deplete versus estrogen replete women. Notably, women with ExFHA demonstrate greater increases in MSNA burst incidence and absence of activation of the RAAS during graded LBNP. The mechanisms underlying a shift
towards greater efferent SNA and lower humoral support of BP during orthostatic stress are unclear, as is the role of estrogen *per se*. However, these data support that neurohumoral regulation of arterial BP is altered during hypotensive stimuli in hypoestrogenic women with ExFHA. The clinical implications of increased efferent sympathetic outflow to the lower limb skeletal muscle vascular bed in ExFHA women on cardiovascular risk remains to be determined.
CHAPTER 5
Altered Autonomic Control of Heart Rate in Physically Active Women with Functional Hypothalamic Amenorrhea

5.1 Introduction

Hypoestrogenemia in association with altered ovulatory cycling is an important contributing factor to the development and progression of premenopausal coronary artery disease (CAD) (16-19). Retrospective studies indicate that a history of irregular menstrual cycles is associated with increased risk for fatal and nonfatal CAD compared with regularly cycling women (18), with altered exposure to estrogen across the reproductive life span considered to be a key factor (20). Independent of other risk factors, disruption of ovulatory cycling due to functional hypothalamic amenorrhea (FHA) has also been recently identified as a risk factor for angiographically documented CAD in premenopausal women (19). FHA is a reversible cause of ovarian disruption in premenopausal women that is characterized by absence of menses and marked hypoestrogenemia (7). The prevalence of FHA is notably higher among weight-stable physically active women (~3-44%) compared with sedentary women (~2-5%) in association with energy deficiency (i.e., caloric deficit) (383, 388).

Cardiovascular protection by estrogen is mediated through numerous mechanisms, including favorable modulation of the autonomic nervous system (1, 225). The autonomic nervous system plays an important role in cardiovascular regulation under physiological and pathophysiological conditions (26). Heart rate variability (HRV), which reflects the modulation
of cardiac vagal and sympathetic efferent activities (28), is linked to cardiovascular disease risk. Diminished HRV is associated with increased cardiac arrhythmias and sudden cardiac death (28), and is linked to the pathobiology of CAD (298). In postmenopausal women (PMW), estrogen deficiency is associated with decreased HRV (311, 314). Conversely, estrogen therapy in PMW is associated with increased HRV (225, 482). HRV has not yet been reported in estrogen deficient pre-menopausal physically active women with FHA (ExFHA). However, studies report lower resting heart rate (HR) in ExFHA women compared with eumenorrheic physically active women (13, 23). This paradoxical observation suggests that despite estrogen deficiency, HRV is increased in ExFHA women. While it is unclear whether HR is modulated by the independent or combined effects of energy deficiency, estrogen deficiency and exercise training, the characterization of autonomic control of HR in ExFHA women remains to be determined. Therefore, this study sought to examine cardiac autonomic balance in ExFHA women at rest and during orthostatic stress using graded lower body negative pressure (LBNP). These responses in ExFHA women were compared with responses in cardiorespiratory-matched physically active estrogen replete women with ovulatory eumenorrheic menstrual cycles. In light of the previously reported lower resting HR in ExFHA women (13, 23), it was hypothesized that HRV would be higher than in their eumenorrheic ovulatory counterpart.

5.2 Methods

Subjects: Physically active eumenorrheic and amenorrheic women were recruited for this study. As a reference group, sedentary eumenorrheic women were also recruited. Subject recruitment and study eligibility criteria have been previously described in detail on page 80.
**Experimental Design and Study Groupings:** Measures of BP and HR were obtained at rest and during three graded stages of LBNP for the purposes of determining HRV. Three groupings (exercising and ovulatory, ExOv, n=17; sedentary and ovulatory, SedOv, n=17; exercising and amenorrheic, ExFHA, n=12) were identified according to their ovulatory status and exercising status. The details of these classification schemes are as previously described on page 81.

**Procedure and Protocol:** The procedure and protocol are as described previously on pages 110-111.

**Blood Pressure and Heart Rate:** Automated brachial BP and HR, and continuous plethysmographic BP and HR measures, were collected as described previously on page 112.

**Respiration Rate:** Respiration rate was continuously recorded at baseline and during each 8-minute stage of LBNP using a pneumobelt placed around the lower abdomen and connected to a transducer. Respiration rate was averaged for each 8-minute recording to obtain mean responses.
Heart Rate Variability: Signal output was to a Gould Viper recorder (Gould Instrument Systems, Madison, WI, USA) where they were digitized (LabVIEW, National Instruments Corporation, Austin, TX, USA) for off-line beat-to-beat analysis of HR, BP, and spontaneous breathing frequency using customized software. Signals were sampled at a frequency of 200 Hz, with the exception of the electrocardiogram, which was at 1000 Hz. Individual HRV data were averaged over 256 beat sequences (483). Ectopic beats were manually deleted and replaced via linear interpolation from adjacent cardiac cycles. Only tracings with <5% ectopy-corrected beats were accepted for analysis.

Analyses of HR intervals (R-R) in the frequency domain was performed by fast Fourier transform. Power spectral components were obtained at very low (VLF: 0.001–0.05 Hz), low (LF: 0.05 to 0.15 Hz) and high (HF: 0.15 to 0.5 Hz) frequencies in absolute units (au; ms²). Due to the known skewed nature of HRV data (483), LF and HF raw data were also normalized (%) and log transformed (log10). Total power was calculated as VLF+LF+HF ms². Normalized units (nu) were calculated as: LFnu=LF power/(total power-VLF power) x 100, and HFnu=HF power/(total power-VLF power) x 100. Although the LF band is modulated by both the sympathetic and the parasympathetic nervous systems (483), LF was determined to reflect sympathetic efferent modulation. HF was interpreted as vagal efferent modulation. The LF/HF ratio (ms²) was calculated to determine cardiac sympatho-vagal balance (483).

Body Composition and Anthropometric Measures: Height, body weight, fat mass and lean mass were assessed as previously described on page 83.
Peak Aerobic Capacity: Methods of assessment are as described previously on page 83.

Serum Measures: Non-fasted serum samples were analyzed for 17 β-estradiol and progesterone on the day of testing. Samples were collected at least 20 minutes before data collection. During a separate study visit, overnight 8-hour fasted serum samples were collected during days 2-6 of the preceding menstrual cycle for ovulatory women, or on a random day for amenorrheic women. These fasted samples were analyzed for 17 β-estradiol, progesterone, testosterone, sex-hormone binding globulin, prolactin, insulin and glucose. Measures of 17 β-estradiol and progesterone were not statistically different between menstrual cycles for ovulatory women, or between days for ExFHA women. Thus, average serum values of ovarian hormones over two menstrual cycles, and one time measures of all other serum markers are reported here. Serum free triiodothyronine (T3) was also assessed to provide an estimate of energy status, with low T3 levels indicating low energy status (i.e., energy deficiency) (395). All assays were run by the Core Laboratory at the Toronto General Hospital. Detailed serum collection and assay specifications are provided in Appendix 5.

Statistics: All variables are expressed as mean ± standard error of the mean (SEM). HRV raw data (ms2) demonstrated positively skewed distribution for all frequencies. Consequently, variables LF and HF were log-transformed (log10), and normalized (as described in Heart Rate Variability in Methods), to minimize the variance and normalize the distribution. One-way
ANOVA was used to detect baseline differences in HRV outcome measures between groups. When significant differences were detected, Bonferroni methods were used to determine where the significant differences existed. Within- and between-group analyses of responses to LBNP, including delta responses from baseline, were determined using repeated measures ANOVA. When assumptions of sphericity were violated, Greenhouse-Geisser correction was used. Using pooled data, Pearson’s correlational analyses were used to determine significant linear associations between HRV, HR, BP and serum measures. A significance level of \( P<0.05 \) was used to detect the differences for all statistical procedures. All analyses were run on SPSS version 21.0; SPSS Inc., Chicago, IL.

5.3 Results

Subject Characteristics: Subject characteristics are presented in Table 1. Groups did not differ \( (p>0.05) \) in age, height, weight, or body composition. Sedentary women were significantly less aerobically conditioned \( (p<0.001) \) compared with exercising women. Serum measures of 17 \( \beta \)-estradiol (pmol/L) did not differ \( (p>0.05) \) between ovulatory groups. In contrast, 17 \( \beta \)-estradiol was lower \( (p<0.05) \) in ExFHA \( (87.8\pm14.7) \) compared with ExOv only \( (148.7\pm19.2) \).

Progesterone was similar \( (p>0.05) \) between groups \( (1.8 \pm 0.7 \text{ nmol/L}; \text{ pooled mean}) \). Free T3 (pmol/L) was significantly lower \( (p<0.05) \) in ExFHA \( (3.6\pm0.6) \) compared with ExOv \( (4.3\pm0.1) \) and SedOv \( (4.2\pm0.2) \) women.
Blood Pressure and Heart Rate: Baseline BP and HR measures are shown in Table 2. Between groups, baseline HR and SBP were lower \((p<0.05)\) in ExFHA women than in SedOv and ExOv women, in whom HR and SBP did not differ \((p>0.05)\). Baseline DBP was similar \((p>0.05)\) between the groups. Baseline MAP was lower \((p<0.05)\) in ExFHA than in ExOv women only. Within groups, graded LBNP elicited a significant increase \((p<0.05)\) in HR, and a decrease \((p<0.05)\) in SBP, DBP and MAP. No LBNP x group interactions were observed for BP and HR measures \((p>0.05)\). Between-groups, analyses showed that HR and SBP responses to LBNP remained significantly lower \((p<0.05)\) in ExFHA compared with SedOv and ExOv women. DBP and MAP responses to LBNP did not differ \((p>0.05, \text{main effect})\) between the groups. No between-group differences for BP and HR were detected during graded LBNP for SedOv and ExOv women \((p>0.05)\). Delta responses (data not shown) for all HR and BP responses did not differ \((p>0.05)\) between the groups.

Heart Rate Variability: Baseline HRV is presented in Table 3. HRV data was unavailable in one SedOv woman as the study was terminated because of presyncopal symptoms during orthostatic stress testing at -20 mmHg. At baseline, groups did not differ \((p>0.05)\) in VLF, LF, LF/HF ratio, LFnu, HFnu, and LFlog10. ExFHA women demonstrated higher \((p<0.05; \text{main effect})\) baseline HF (ms2), Total HRV, and HFlog10 compared with SedOv and ExOv women. LF/HF ratio trended lower \((p=0.09)\) in ExFHA women than ovulatory women. Baseline measures of HRV did not differ \((p>0.05)\) between ExOv and SedOv women. HRV responses to graded LBNP are presented in Figure 1. Significant within-group reductions in Total HRV \((F(1.92, 82.66)=7.18, p<0.01; \text{main effect})\), HFnu \((F(2.31, 99.29)=46.54, p<0.001)\), and
HFlog10 ($F(1.63, 70.18)=72.411, p<0.001$) were observed at -20mmHg and -40mmHg compared with baseline. Conversely, LBNP elicited significant increases in LF/HF ratio ($F(1.42, 61.07)=30.156, p<0.001$), and LFnu ($F(2.29, 102.92)=44.239, p<0.001$) at -40mmHg compared with baseline. Measures of VLF ($p=0.74$; main effect), LF ($p=0.36$; main effect), and LFlog10 ($p=0.26$; main effect) were unaltered by LBNP. No LBNP x Group interactions ($p>0.05$) were detected. Between-group differences were observed, with women with ExFHA demonstrating significantly higher Total HRV ($p=0.001$; main effect), HFnu ($p<0.001$; main effect) and HFlog10 ($p<0.001$; main effect) compared with SedOv and ExOv women. LF/HF ratio was significantly lower ($p=0.02$; main effect) at -20mmHg and -40mmHg LBNP in ExFHA versus SedOv women only. LBNP measures of HRV did not differ ($p>0.05$) between SedOv and ExOv women. Delta HRV responses also did not differ ($p>0.05$) between the groups.
Table 1. Demographic and anthropometric characteristics of the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv n=17</th>
<th>ExOv n=17</th>
<th>ExFHA n=11</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.5 ± 0.6</td>
<td>23.5 ± 1.2</td>
<td>25.4 ± 1.1</td>
<td>0.312</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.1 ± 1.5</td>
<td>166.2 ± 1.5</td>
<td>167.2 ± 1.5</td>
<td>0.400</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.6 ± 1.5</td>
<td>58.0 ± 1.9</td>
<td>58.7 ± 2.3</td>
<td>0.953</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>21.7 ± 0.5</td>
<td>20.9 ± 0.5</td>
<td>20.8 ± 0.8</td>
<td>0.438</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>28.7 ± 2.3</td>
<td>24.7 ± 2.0</td>
<td>21.8 ± 2.2</td>
<td>0.118</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>38.8 ± 1.4</td>
<td>42.0 ± 1.6</td>
<td>44.1 ± 1.3</td>
<td>0.092</td>
</tr>
<tr>
<td>VO$_2$ peak (ml/kg/min)</td>
<td>38.9 ± 0.8*</td>
<td>46.6 ± 1.3</td>
<td>47.8 ± 2.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

BMI, body mass index; VO$_2$ peak, peak oxygen consumption.

* SedOv vs ExOv and ExFHA.
Table 2. Blood pressure and heart rate responses of the study groups to graded LBNP.

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>SBP</th>
<th>DBP</th>
<th>MAP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SedOv†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ba</td>
<td>61 ± 2</td>
<td>102 ± 2</td>
<td>59 ± 1</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>-10 mmHg</td>
<td>61 ± 2</td>
<td>98 ± 2§</td>
<td>55 ± 1§</td>
<td>69 ± 1§</td>
</tr>
<tr>
<td>-20 mmHg</td>
<td>65 ± 3</td>
<td>96 ± 1§</td>
<td>53 ± 1§</td>
<td>67 ± 2§</td>
</tr>
<tr>
<td>-40 mmHg</td>
<td>80 ± 3§</td>
<td>94 ± 2§</td>
<td>51 ± 2§</td>
<td>66 ± 2§</td>
</tr>
<tr>
<td><strong>ExOv†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ba</td>
<td>57 ± 2</td>
<td>105 ± 2</td>
<td>61 ± 1</td>
<td>76 ± 1</td>
</tr>
<tr>
<td>-10 mmHg</td>
<td>58 ± 2</td>
<td>102 ± 2§</td>
<td>57 ± 2§</td>
<td>72 ± 2§</td>
</tr>
<tr>
<td>-20 mmHg</td>
<td>62 ± 3§</td>
<td>100 ± 2§</td>
<td>54 ± 2§</td>
<td>70 ± 2§</td>
</tr>
<tr>
<td>-40 mmHg</td>
<td>72 ± 3§</td>
<td>98 ± 1§</td>
<td>53 ± 2§</td>
<td>68 ± 2§</td>
</tr>
<tr>
<td><strong>ExFHA†</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ba</td>
<td>47 ± 2*</td>
<td>94 ± 2*</td>
<td>58 ± 2</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>-10 mmHg</td>
<td>48 ± 2*</td>
<td>93 ± 2*</td>
<td>56 ± 2</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>-20 mmHg</td>
<td>49 ± 2*</td>
<td>92 ± 2*</td>
<td>53 ± 2§</td>
<td>66 ± 2§</td>
</tr>
<tr>
<td>-40 mmHg</td>
<td>60 ± 2*§</td>
<td>89 ± 2*§</td>
<td>51 ± 2§</td>
<td>64 ± 2§</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

Ba, baseline; DBP, diastolic BP; HR, heart rate; MAP, mean arterial BP; SBP, systolic BP.

† LBNP main effect for each measure within each group, p<0.001.

* ExFHA versus SedOv and ExOv within condition.

§ Significantly different from baseline within groups.
### Table 3. Baseline HRV for the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv n=17</th>
<th>ExOv n=17</th>
<th>ExFHA n=12</th>
<th>P (main effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absolute (ms²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>686 ± 135</td>
<td>1024 ± 265</td>
<td>1023 ± 165</td>
<td>0.398</td>
</tr>
<tr>
<td>HF</td>
<td>837 ± 122</td>
<td>948 ± 130</td>
<td>2364 ± 757*</td>
<td>0.012</td>
</tr>
<tr>
<td>VLF</td>
<td>818 ± 115</td>
<td>1160 ± 179</td>
<td>1705 ± 549</td>
<td>0.114</td>
</tr>
<tr>
<td>LF+HF</td>
<td>1524 ± 220</td>
<td>1971 ± 341</td>
<td>3386 ± 894**</td>
<td>0.034</td>
</tr>
<tr>
<td>Total (LF+HF+VLF)</td>
<td>2347 ± 253</td>
<td>3137 ± 349</td>
<td>5092 ± 1310*</td>
<td>0.021</td>
</tr>
<tr>
<td>LF/HF</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± .01</td>
<td>0.095</td>
</tr>
<tr>
<td><strong>Normalized (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF (LF/[LF+HF]*100)</td>
<td>44 ± 3</td>
<td>46 ± 5</td>
<td>34 ± 4</td>
<td>0.129</td>
</tr>
<tr>
<td>HF (HF/[LF+HF]*100)</td>
<td>56 ± 3</td>
<td>54 ± 5</td>
<td>66 ± 4</td>
<td>0.129</td>
</tr>
<tr>
<td><strong>Transformed (log10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>2.7 ± 0.1</td>
<td>2.8 ± .01</td>
<td>2.9 ± 0.1</td>
<td>0.516</td>
</tr>
<tr>
<td>HF</td>
<td>2.8 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>3.2 ± 0.1*</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

* ExFHA vs SedOv and ExOv

** ExFHA vs SedOv
Figure 1. HF log10 (A), LF log10 (B), Total HRV (C) and LF/HF ratio (D) responses during graded LBNP. * ExFHA versus SedOv and ExOv women, p<0.05.
**Respiration Rate:** Respiration rate did not differ ($p>0.05$; main effect) between groups at baseline (14.2±0.4 breaths/minute; overall baseline mean ±SEM), nor was it altered by LBNP ($p>0.05$, main effect).

**Correlates of HR and HRV:** Resting HR was strongly and positively associated with estradiol ($r=0.52$, $p=0.001$) and T3 ($r=0.61$, $p<0.001$). In contrast, baseline measures of HRV were not associated ($p>0.05$) with serum measures of estradiol or T3. HFlog10, LF+HF and Total HRV were strongly associated ($p<0.05$) with HR at rest and in response to LBNP.

**5.4 Discussion**

This is the first study showing that moderately-trained women with FHA demonstrate altered neural contributions to the modulation of HR when compared with eumenorrheic and ovulatory trained and untrained women. Overall, the data indicate: i) elevated parasympathetic neural contribution to HR control; ii) unaltered cardiac sympathetic modulation; iii) unaltered cardiac autonomic sympatho-vagal balance; and iii) increased Total HRV that is solely due to increased cardiac parasympathetic modulation. While the influence of estrogen deficiency *per se* on these findings is not clear, associations between serum markers of energy status (i.e., triiodothyronine) and HR imply a potential role for energy deficiency in these observations.
**Estrogen and Cardiac Autonomic Control:** In the current study, the show that low resting HR in estrogen deficient physically active women is associated with greater cardiac parasympathetic modulation. Higher cardiac vagal tone in ExFHA women persisted during simulated orthostatic stress. In contrast, cardiac sympathetic modulation and sympatho-vagal balance (i.e., LF/HF ratio) does not differ from that observed in estrogen replete women, both exercise trained and untrained. The finding of marked increased cardiac vagal tone in hypoestrogenic women is in contrast with human studies of surgical (oophorectomized) and natural menopause reporting low resting cardiac parasympathetic and high sympathetic modulation of HR (225, 311, 314, 315). Conversely, estrogen therapy restores cardiac sympatho-vagal balance to pre-surgery levels in oophorectomized women (311), and improves sympatho-vagal balance in naturally postmenopausal women (225, 311, 314, 315). Cellular and molecular mechanisms of estrogenic action on cardiac autonomic control remain unknown. However, the localization of a significant population of both estrogen receptor-alpha and beta on cell bodies, axons and terminals of autonomic regulatory nuclei throughout the neuraxis, implicate a key role for estrogen in autonomic control (42, 183, 324). In light of this, it seems likely that the paradoxical effects of estrogen deficiency on cardiac autonomic modulation of HR in the young otherwise healthy ExFHA women is influenced by other factors that may be associated with ovarian disruption. Two such factors include energy deficiency (i.e., caloric deficit) and aerobic exercise training, both of which have been independently shown to alter autonomic control of HR (27, 333, 443, 481).
Energy Deficiency and Cardiac Autonomic Control: FHA in weight-stable physically active women is causally linked to mild negative energy balance in association with increased energy expenditure that is frequently combined with subclinical restrictive eating patterns (9, 10, 484). In animals, moderate negative energy balance is associated with decreased sympatho-excitation (481). Consistent with this, caloric restriction without nutrient deficit in humans is associated with favorable decreases in resting HR and SBP (432). Exact mechanisms of action are not known, but are thought to include decreased oxidative stress and inflammation (485, 486), and modifications in the actions of the hypothalamic-pituitary neuroendocrine pathways (487). In response to energy deficiency, numerous endocrine and metabolic secretory aberrations can occur. In ExFHA women such perturbations include decreased circulating levels of T3 and leptin and elevated levels of cortisol, adiponectin and ghrelin (23, 400, 411). The influence of these neuroendocrine alterations on autonomic activity in ExFHA women are not yet known. This notwithstanding, the current study reports resting bradycardia (HR <50 beats/min) in association with increased cardiac vagal modulation of HR, and low SBP in ExFHA women compared with both sedentary and exercise trained ovulatory women. These observations, although less marked, are consistent in part with findings in anorexia nervosa patients (488). However, vagal modulation of HR in anorexia nervosa is variably reported to be increased (488), unaltered (489) or decreased (490) compared with healthy controls (488). Mechanisms of action are not yet known, but of interest, cardiac autonomic disturbances in anorexia nervosa are reported to be unaltered with short and long-term restoration of weight (491). Although ExFHA women are weight-stable and not malnourished, it remains to be determined whether restoring energy balance reverses cardiac hypervagal tone in these women.
Exercise Training and Cardiac Autonomic Function: Aerobic conditioning in humans increases parasympathetic and decreases sympathetic cardiac modulation (27, 333). These neural adaptations produce bradycardia and increased HRV, decreased arterial baroreflex-mediated sympathoexcitation, and improvements in baroreflex control of HR (27, 333, 334, 341). While the mechanisms of action remain unclear, alterations in the intrinsic SA node pacemaker (340), and neurotransmission at the level of the nucleus tractus solitarius (343) have been implicated. In the current study, the data show similar cardio-respiratory fitness in both of the exercising groups, but lower HR and higher cardiac vagal tone in ExFHA than in ExOv women. Consistent with this, cardiac vagal tone has been similarly reported to be elevated in aerobically exercise trained ovariectomized animals (492, 493). The mechanisms accounting for greater cardiac vagal tone in exercise trained hypoestrogenic versus estrogen replete animals and humans are not clear, but findings suggest that: i) hyper vagal cardiac tone is likely elicited via non-exercise training mediated pathways, and/or ii) estrogen may play an important and independent role in autonomic adaptations induced by exercise training (493). Further studies are warranted to confirm these postulates.

Limitations: HRV responses reflect cardiac autonomic tone only, and cannot be extrapolated to sympatho-vagal balance to other target organs such as kidney or skeletal muscle. Respiratory sinus arrhythmia, or the HF (vagal) component of HRV, is influenced by both the depth and frequency of respiration (494). Despite having similar breathing frequencies (~14 breaths per minute) among the study groups, it is possible that women with ExFHA may have different tidal volumes, which were not recorded. Although similar cardio-respiratory fitness was observed
between the exercise trained groups, it is possible that HRV was influenced by differences in exercise training volume and intensity (27, 333, 349). Finally, the small sample size of the study groups may have limited the ability to detect true differences and associations.

**Conclusion:** Estrogen deficient women with ExFHA demonstrate altered neural contributions to the modulation of HR compared with ovulatory eumenorrheic physically active and sedentary women. Specifically, ExFHA women demonstrate lower resting HR in association with increased cardiac vagal modulation yet demonstrate preserved cardiac sympathetic tone. These perturbations persist during simulated orthostatic stress. The influence of estrogen deficiency *per se* and the clinical relevance of these findings remain unclear and warrant further study. However, in light of the etiology of FHA in physically active women, it is likely that these findings are the result of complex interplay between estrogen deficiency, energy deficiency and exercise training. It is not yet known whether enhanced cardiac vagal modulation in women with ExFHA confers cardioprotection or alternatively reflects systemic autonomic dysfunction.
CHAPTER 6
Summary, Unifying Discussion and Conclusions

6.1 Summary

Using physiological mechanistic studies, the hypotheses tested in this dissertation provided a framework for examining the short-term cardiovascular effects of premenopausal estrogen deficiency in women with ExFHA. These studies also further characterized the cardiovascular profile of women with this condition. The three experiments that formed the basis of this thesis are summarized below.

The first study (Chapter 3) compared the influence of estrogen deficiency in women with ExFHA on endothelial function across two different vascular beds: conduit vessels (brachial artery) and resistance vessels (calf). Using forearm (brachial artery) and thigh and ankle (calf BF) cuff occlusion methods, changes in brachial artery vessel diameter (FMD%) and calf blood flow were investigated both before and one-hour after an acute bout of dynamic exercise. Endothelium-independent dilation (GTN%) of the brachial artery was also assessed at baseline only. Responses in ExFHA women were compared with that in age-, cardiorespiratory fitness- and BMI-matched estrogen replete physically active eumenorrheic ovulatory (ExOV) women. For reference purposes, sedentary eumenorrheic ovulatory (SedOv) women were also assessed. In eumenorrheic women (SedOv and ExOv), all measures were determined during the early follicular (low estrogen and low progesterone) phase of the menstrual cycle.
The **first hypothesis** stated: baseline measures of brachial artery FMD% and GTN%, and resting and peak-ischemic calf BF, will be lower in ExFHA women compared with ExOV women. **Key Findings:** In support of this hypothesis, baseline brachial artery diameter did not differ \((p>0.05)\) between the groups. Baseline FMD%, GTN%, and resting and peak regional calf BF responses were lower \((p<0.05)\) in women with ExFHA compared with ExOV women.

The **second hypothesis** stated: Pre-exercise peak SR (determined by the area under the curve; SRAUCpk) assessed in the brachial artery would be lower in ExFHA women compared with ExOV women, and would be associated with impaired FMD%.

**Key Findings:** In support of this hypothesis, SRAUCpk was lower \((p<0.05)\) in ExFHA women than in ExOV women pre-exercise. Using pooled data, SRAUCpk was strongly and positively associated with FMD% \((r=0.54, p<0.001)\). However, pre-exercise FMD% adjusted for SRAUCpk remained lower \((p<0.05)\) in ExFHA compared with ExOV women.

The **third hypothesis** stated: FMD%, calf BF and brachial artery peak SR responses will be ‘restored’ in ExFHA women after a single bout of submaximal dynamic exercise, such that responses will no longer be different from that observed in ExOV women. **Key Findings:** This hypothesis was rejected as exercise significantly \((p<0.05)\) increased SRAUCpk and FMD% in ExFHA women, but did not restore FMD% to levels observed in ovulatory women \((p<0.05)\). Of note, adjustment of post-exercise FMD% for SRAUCpk removed significant differences between the groups. Resting calf BF was increased \((p<0.05)\) to a similar \((p>0.05)\) level in all groups. In contrast, peak calf BF was increased \((p<0.05)\) in ExFHA women only, similar \((p>0.05)\) to the level observed in ExOV women, in whom pre- versus post-exercise
peak calf BF did not differ ($p>0.05$). For all vascular responses, ExOv and SedOv women did not differ ($p>0.05$).

**In summary, this study demonstrates altered conduit and resistance vessel function in ExFHA women compared with ExOV women. Dynamic exercise augments, but does not fully restore, conduit vessel function in ExFHA women. In contrast, resistance vessel function is acutely restored post-exercise.**

Study two (Chapter 4) sought to investigate neurohumoral mechanisms of BP control in ExFHA women. Efferent muscle SNA and serum RAAS components were assessed at baseline and during graded LBNP. Responses in ExFHA women were compared with that in estrogen replete ExOv women during the early follicular phase of the menstrual cycle. The **first hypothesis** stated: Systolic BP and HR responses to LBNP will be lower in ExFHA women compared with ExOv and women. **Key Findings:** Consistent with the findings in study one, women with ExFHA demonstrated lower ($p<0.05$) resting HR and systolic BP compared to ExOv women. Orthostatic stress elicited the anticipated increases ($p<0.05$) in HR and decreases ($p<0.05$) in systolic BP in both groups, but ExFHA women demonstrated consistently lower ($p<0.05$) HR and systolic BP at all stages of LBNP, thereby supporting this hypothesis.

The **second hypothesis** for study two stated: In women with ExFHA, MSNA responses at rest and during orthostatic stress will be higher compared with ExOv women. **Key Findings:** The results were contradictory, offering both support and refutation of this hypothesis. While similar ($p>0.05$) baseline MSNA bursts/100 heart beats and bursts/min were observed amongst groups (counter to the hypothesis), MSNA bursts/100 heart beats, but
not bursts/min, were higher \( (p<0.05) \) in ExFHA women compared with ExOv women during LBNP, thereby supporting the hypothesis.

The **third hypothesis** for study two stated: **RAAS responses to LBNP will differ in ExFHA women compared with ExOv women.** **Key Findings:** Baseline measures of renin and Ang II did not differ \( (p>0.05) \) between ExOv and ExFHA women. However, graded LBNP elicited expected increases \( (p<0.05) \) in renin and Ang II in ExOv women, but did not alter \( (p>0.05) \) these RAAS components in ExFHA women, thereby supporting this hypothesis.

**In summary, this study demonstrates altered neurohumoral BP regulation during hypotensive stress in ExFHA compared with ExOv women, with ExFHA women demonstrating a greater reliance on sympathetic nerve activity and less reliance on the RAAS to defend arterial BP during orthostatic stress.**

Study Three (Chapter 5) sought to examine mechanisms of low resting HR in ExFHA women. Autonomic control of HR in women with ExFHA was assessed at baseline and during simulated orthostatic stress elicited by graded LBNP. Responses in ExFHA women were compared with estrogen replete ExOv and SedOv women during the early follicular phase of the menstrual cycle. The following hypothesis was tested: **Autonomic regulation of HR, as determined by HRV, will be different in women with ExFHA than in ExOv and SedOv women.** **Key Findings:** Results of the study support acceptance of this hypothesis. The data show markedly elevated \( (p<0.05) \) vagal (i.e., HFlog10) modulation of HR both at rest and during simulated orthostatic stress in ExFHA compared with ovulatory women. In contrast, sympathetic modulation of resting HR (i.e., LFlog10) and sympatho-vagal balance (LF/HF ratio) did not differ \( (p>0.05) \) between the groups at any time point. In all groups, graded
LBNP elicited increases \( (p>0.05) \) in LF/HF ratio, and decreases \( (p<0.05) \) in Total HRV and HFlog10. LBNP but did not alter \( (p>0.05) \) LFlog10 in any group.

**In summary, this study reports altered neural modulation of HR both at rest and in response to hypotensive stimuli in ExFHA compared with ExOv women, with ExFHA women demonstrating markedly elevated parasympathetic cardiac tone.**

### 6.2 Unifying Discussion

The overarching objective of this thesis was to answer two fundamental questions: i) How does hypoestrogenemia during the premenopausal years influence vascular function, BP control and HR regulation, and ii) what are the mechanisms involved? Evidence from human and animal studies indicate that 17\(\beta\)-estradiol confers cardioprotection through numerous multifaceted mechanisms including endothelial, myocardial and neurohumoral effects \( (1, 2) \). In stark contrast, recently published primary and secondary prevention randomized controlled trials of postmenopausal estrogen therapy have produced neutral or negative results \( (495-498) \). Reasons for discordance between the known biology of estrogen and the results of clinical trials of postmenopausal HRT \( (495-498) \) remain clear. However, in PMW, the use of varied hormonal regimens that are derived from non-bioidentical hormones, the influence of aging, and increasing likelihood of one or more co-morbidities likely play a role. In the absence of aging, organic endocrine abnormalities or co-morbidities, otherwise healthy weight-stable ExFHA women provide a unique model to explore the cardiovascular effects of estrogen deficiency. The three studies presented in this thesis were designed to explore mechanisms of
altered vascular function and BP and HR regulation in hypoestrogenic ExFHA women. To date, such studies have not been reported.

6.2.1 Mechanisms of Altered Vascular Function in ExFHA Women

Study One identified that low calf BF can be attributed solely to increased calf VR. In contrast, basal FMD% in ExFHA women was partially explained by (i.e., correlated with) several factors, including SRAUCpk and baseline vessel diameter. However, these factors were not able to fully explain low basal FMD% in ExFHA women. Within the limits of this thesis, evidence from the first and second study suggest that similar to calf BF, basal FMD% responses in ExFHA women may also be blunted due to increased vasoconstrictor tone. Evidence to support this postulate includes decreased endothelium-independent function (GTN%) and higher efferent MSNA during hypotensive stimuli in ExFHA women compared with ovulatory women. Although MSNA was not statistically different at baseline between eumenorrheic and amenorrheic women, it is not known whether MSNA responses are similarly transduced in estrogen replete and estrogen deplete physically active women. It is tempting to speculate that in hypoestrogenic ExFHA women the vasoconstricting effects of MSNA are offset to a lesser extent compared with estrogen replete women. Along these lines, it is possible that a higher basal vasoconstricting tone prevails in ExFHA women. These adaptations may serve to blunt the vasodilatory capacity of the vessels in order to help maintain perfusion pressure in the face of borderline hypotension in ExFHA women. Although this requires confirmation, the data also support transient suppression of sympathetic tone in estrogen deficient, but not replete, women following exercise (i.e., decreased calf VR,
significantly augmented FMD%). These findings suggest that basal sympathetic outflow to the periphery may be augmented in ExFHA women. The testing of GTN% post-exercise would further elucidate the role of vasoconstrictor tone in conduit function, but given the presence of post-exercise hypotension in many subjects, this measure was not able to be determined. Notwithstanding, the data suggest a role for increased vasoconstrictor tone in blunting both conduit and resistance vessel responses in ExFHA women.

### 6.2.2 Mechanisms of Altered Blood Pressure Regulation in ExFHA Women

Despite a lower 'set-point' for HR and arterial BP in ExFHA women, orthostatically challenged BP appears to be well defended in ExFHA women. Similar \( \Delta \)HR and \( \Delta \)BP responses and absence of syncopal symptoms during graded orthostatic stress in both groups of women suggests unaltered arterial baroreflex control of BP in ExFHA women. However, \( \Delta \)MSNA burst incidence, which is also baroreceptor mediated, was increased to a significantly greater extent at higher levels of orthostatic stress in ExFHA compared with ExOv women. At these higher levels of orthostatic stress (-20 & -40 mmHg), arterial baroreceptors are activated (499). In contrast, the lower level of orthostatic stress (i.e., -10 mmHg), activates the cardio-pulmonary baroreceptors which contribute minimally to reflex sympathetic activation in human skeletal muscle during orthostatic stress (499). Consistent with this, the data identify little to no activation of MSNA or the RAAS at the lower level of LBNP in both ExOv and amenorrheic women. At higher levels of LBNP (-20 and -40mmHg) MSNA was significantly
increased from baseline in both groups, although more markedly so in amenorrheic women, with the RAAS being activated in ExOv women only at these levels of LBNP. These observations identify that during an orthostatic challenge: i) both the RAAS and MSNA contribute to arterial BP control in ExOv women; yet, ii) MSNA, but not RAAS, contributes to arterial BP control in ExFHA women. Collectively, these findings illustrate the redundancy in the systems that regulate BP, and suggest that MSNA is the primary regulator of arterial BP in ExFHA women, while humoral control is diminished.

6.2.3 Mechanisms of Altered Heart Rate Regulation in ExFHA Women

Study Three identified lower resting HR, and lower resting HR responses during LBNP in ExFHA women compared with ExOv women of similar cardiorespiratory fitness (VO2 peak ~46 ml/kg/min) and SedOv women. That both exercising groups were similarly trained suggests that the lower HR (~47 beats per minute) in ExFHA women was not exercise training induced. The finding of a strong positive association between HR, HRV indices of parasympathetic tone and serum T3 (a marker of energy status) suggests an important role for energy deficiency in the observed responses in ExFHA women, in whom T3 levels were lowest. In contrast, serum T3 was not associated with indices of sympathetic modulation of HR or cardiac sympathovagal balance, measures of which did not differ between amenorrheic and ovulatory women at any time point. The finding of greater parasympathetic and unaltered sympathetic modulation of HR during LBNP in ExFHA women is consistent with data from Study Two, reporting unaltered sympathetic outflow to the kidneys (as suggested by non-activation of the RAAS during LBNP), yet is in contrast with the finding of greater
sympathetic outflow to the lower limb vascular bed during orthostatic stress. These observations suggest that elevated efferent SNA in ExFHA women is not systemic, rather it is selectively increased in the vasculature, possibly to help maintain cardiac output in the face of low resting HR.

6.2.4 Energy Deficiency versus Estrogen Deficiency

Evidence from this thesis supports that the cardiovascular alterations observed in ExFHA women collectively reflect compensatory adaptations to maintain cardiovascular homeostasis in the face of multiple competing factors, including the independent and combined effects of estrogen and energy deficiency and exercise training (see Figure 1). However, the similar cardiovascular and hormonal responses for all outcome variables in sedentary and exercising ovulatory women, suggests that moderate exercise training has minimal effect on cardiovascular regulation in healthy young women. This observation also suggests a potential lack of effect for exercise training on the diverse cardiovascular findings between the similarly aerobically conditioned ExOv and ExFHA women. Moreover, the presence of impaired vascular function in ExFHA women despite similar cardiorespiratory fitness, suggests that the positive vascular effects of regular exercise training are obviated. While it is not yet known whether impaired vascular function in ExFHA women is a harbinger of future atherosclerosis, such findings suggest a potentially profound effect of estrogen deficiency on vascular function in these otherwise healthy women. This postulate is consistent with the greater reported frequency and clustering of many traditional vascular disease risk conditions (e.g.,
hypertension, diabetes, obesity) in PMW versus age-matched men (500), and a more rapid
decline in FMD% in PMW with aging compared with men (501). These observations, taken
together with the data from this thesis, suggest a key role for the ovarian hormones in healthy
vascular function in women. Other cardiovascular perturbations in ExFHA women that are
consistent with a modulatory effect of estrogen deficiency include increased MSNA and non-
activation of the RAAS during hypotensive stimuli. Conversely, data from this thesis also
supports a role for energy deficiency in the cardiovascular perturbations observed in ExFHA
women. Serum T3 was associated with both HR and HFlog10, and evidence of energy
conserving mechanisms in ExFHA women was observed, such as a lower 'set point' for both
HR and arterial BP and increased cardiac parasympathetic tone. Similar energy conserving
mechanisms are observed in both hibernating animals and calorically restricted humans (502).
However, despite the seeming independent cardiovascular effects of estrogen and energy
deficiency, and the apparent absence of effect of exercise training, it is likely that the observed
cardiovascular perturbations in ExFHA women are the consequence of complex interactions
between these three factors.
Figure 1. Summary of key findings of altered cardiovascular function in ExFHA women at baseline, in response to orthostatic stress and post-acute dynamic exercise.

↑ increased; ↓ decreased; ↔ unaltered; ---, potential role; CBF, calf blood flow; CVR, calf vascular resistance; HF, high frequency HRV; HRV, heart rate variability; ?, unknown.
6.3 Cardiovascular Phenotype of ExFHA Women

The cardiovascular phenotype observed in ExFHA women has characteristics of anorexia nervosa patients, older PMW, and young males. The vascular phenotype is consistent with estrogen deficient PMW (450, 451), in addition to estrogen and energy deficient anorexia nervosa patients (11, 503). The cardiac autonomic phenotype of ExFHA women (low resting HR, elevated parasympathetic cardiac tone) is analogous to that observed in anorexia nervosa patients (11, 488), but not PMW, who demonstrate elevated sympathetic and/or decreased parasympathetic control of HR (333), suggesting energy deficiency may play a key role in this response. Finally, the neurohumoral responses in ExFHA are very similar to those observed in PMW (286), and, to some extent, are also parallel to findings in: i) young healthy sedentary males (also “estrogen deficient”), who demonstrate higher MSNA compared with age-matched females (24, 225, 226), and ii) endurance-trained young males, who demonstrate higher MSNA compared with their sedentary peers (504). Collectively, these observations, taken with the findings in this thesis, suggest a primary role for estrogen in vascular and peripheral neurohumoral regulation, but that energy deficiency may be more influential in regulating cardiac autonomic tone. The effects of exercise training are not clear.

6.4 Study Limitations

Assessment of FMD% remains a research tool, due in part to both the technical and biological variability associated with this measurement. Standardized guidelines have been proposed to minimize technical variability (122, 505). Currently, adjusting FMD% for shear stress, and if
required, brachial artery diameter, is considered the best approach to minimize biological variability (122). However, several limitations of this approach are recognized. Firstly, the clinical utility of using the 'normalized' FMD% response (FMD%/SR AUC) has yet to be established (465). Secondly, statistical concerns arise when using ANCOVA methods to adjust the FMD% response, due to the frequent violation of assumptions necessary for reliable use of ratio-normalization, including regression slopes that have y-intercepts greater than zero, nonlinear and unstable relations, skewed data distributions, and heteroscedastic variance (506). Thirdly, normalizing the FMD% also does not take into account the compliance of the artery, which affects both the shear rate calculation and the stimulus for FMD% (507).

It is conceivable that the observed partially restored FMD% versus fully restored peak calf BF post-exercise in ExFHA women is a consequence of the specificity of the chosen exercise stimulus (brisk walking) itself. However, studies report mild to moderate dynamic lower-limb exercise elicits marked (3-4 fold) increases in blood flow and shear stress in the non-working brachial artery during exercise (508). Thus, it seems unlikely that the exercise stimulus in Study One would not be sufficient to elicit increases in blood flow and shear stress in the brachial artery of ExFHA women.

In the absence of post-exercise GTN%, the cause of low pre exercise GTN% in ExFHA women remains unclear. Assessment of GTN% after exercise would have helped clarify whether vascular function (e.g., vasodilator capacity or arterial compliance) and/or vascular structure are altered in ExFHA women, as supported by the finding of low GTN% pre-exercise in these women.
While it is difficult to determine the independent cardiovascular effects of energy and estrogen deficiency in ExFHA women, the administration of exogenous estrogen would have helped delineate the cardiovascular effects of estrogen deficiency. The effects of different routes of estrogen administration may be of interest since studies in PMW report differences in the cardiovascular effects of transdermal versus oral estrogen therapy (233, 268, 286, 509, 510). Increasing energy intake in ExFHA women with a goal to resume menses would permit investigation of the cardiovascular effects of energy deficiency. It is reasonable to expect that the cardiovascular effects of estrogen administration (i.e., estrogen replete, energy deficient) versus resumption of menses (i.e., estrogen replete, energy replete) would differ. To date, no study has examined this postulate.

6.5 Future Studies

Although impaired NO production and bioavailability in response to estrogen deficiency remains the primary hypothesis of impaired FMD% in ExFHA women, the role of endothelium-derived NO has not yet been confirmed. Examination of the potential role of other vasoactive substances that are also modulated by estrogen, such as EDHF, thromboxane, prostacyclin, and free radicals (e.g., superoxide anion) (105, 108, 110, 151), have similarly not yet been reported in ExFHA women. Studies designed to assess arterial compliance and vascular structure, both of which are influenced by estrogen (464, 511), are also lacking. The vascular effects of neuroendocrine alterations in response to energy deficiency in ExFHA women, such as elevated circulating cortisol, ghrelin and adiponectin (23, 395, 398-401), also warrant investigation.
Energy deficiency is also associated with altered cardiac structure and function. Anorexia nervosa patients demonstrate decreased cardiac muscle mass, cardiac output, bradycardia, increased total peripheral resistance and hypotension (11). Decreased plasma levels of atrial natrietic peptide and norepinephrine have also been reported (512, 513). Although not starving, this thesis reports that energy deficient ExFHA women similarly demonstrate low resting HR (~50 beats/min) and arterial BP (~92/57 mmHg), and increased regional VR. It remains to be determined whether cardiac dimensions, cardiac output, systemic peripheral resistance or circulating sympathetic humoral factors are also altered in ExFHA women.

This thesis reports absence of activation of the circulating RAAS during hypotensive stimuli in ExFHA women. Future studies that examine vascular tissue RAAS, including novel RAAS components in ExFHA women would be of interest. Novel RAAS components are known to elicit vasodilation (261), and could potentially contribute to the lower arterial BP consistently observed in ExFHA women.

It is unclear whether increased parasympathetic cardiac modulation in ExFHA women reflects autonomic dysregulation or is cardioprotective. Studies designed to investigate arterial baroreflex sensitivity will provide important information regarding the functioning of the autonomic nervous system in ExFHA women.
6.6 Conclusions

Data from this thesis identify disruption in cardiovascular regulation in ExFHA women compared with physically active eumenorrheic women (summarized in Figure 1). The observed cardiovascular perturbations likely reflect compensatory adaptations to maintain homeostatic cardiovascular stability in the face of estrogen and energy deficiency. For example, estrogen deficiency appears to be the prime candidate in the disruption of vascular function and neurohumoral regulation of BP in ExFHA women, possibly a consequence of strategies combined to maintain cardiac output and defend arterial BP. In contrast, energy deficiency may play a role in elevated cardiac vagal tone in ExFHA women, possibly as a mechanism to conserve energy. However, the role of exercise training in these responses remains unclear. This notwithstanding, altered vascular function in otherwise healthy 

recreationally active young women with FHA suggests that the cardio-protective benefits associated with both premenopausal status and participation in regular physical activity are mitigated in these women. Further studies are required to determine if diminished estrogen exposure over the reproductive lifespan in physically active women with FHA increases long-term cardiovascular health risk.
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APPENDIX 1

YOUNG WOMEN WANTED for Research Study

Researchers at the Clinical Cardiovascular Physiology Laboratory (Toronto General Hospital) and at the Cardiovascular Regulation Laboratory (University of Toronto) are currently recruiting healthy young physically active AND non-active premenopausal women to participate in a study looking at THE EFFECTS OF ESTROGEN AND EXERCISE ON CARDIOVASCULAR HEALTH.

Study Criteria
1. Aged between 18-35 years
2. Physically active >3hrs/week or <2hrs/week
3. Regularly menstruating or not menstruating at all
4. Not on oral contraceptives, not pregnant or postpartum
5. Have no known cardiovascular disease, diabetes mellitus, high blood pressure, or any other chronic diseases
6. A non-smoker and otherwise healthy

Measures
1. Blood vessel and heart health assessment, both before and after exercise, using a number of tests, including: arterial stiffness, calf blood flow, blood vessel and blood pressure regulation
2. Body composition, fitness test and some blood draws

Time Commitment
1. Regularly menstruating women: 4 visits to the laboratory across one to two menstrual cycles
2. Women that have not menstruated for at least 3 consecutive months: 4 visits to the laboratory across a 30-day period

Contact Information
If you are interested and would like more information, please contact Dr Paula Harvey (Primary Investigator), or Emma O'Donnell (PhD Research Student) at: 416-340-4755 or email: emma.odonnell@utoronto.ca

RENUMERATION PROVIDED
APPENDIX 2

Health, Exercise and Menstrual Cycle Screening Questions
MEDICAL AND MENSTRUAL CYCLE HEALTH QUESTIONS

Directions: Please answer the following questions regarding your medical and menstrual cycle history. If you have any questions, ask one of the investigators.

Study Code___________________________            Date:_______________________________

1. Weight:_______(pounds/kilograms)

2. Height:_______(feet, inches)

3. Age:_______(years)

4. Gender:  F

5. Date of Birth:       /          /19

6. Ethnic Category:________________________________________________

7. Racial Category: (please check ONLY one)
   _____ Aboriginal/First Nations/Inuit
   _____ African-Canadian/ Black/Caribbean
   _____ Asian
   _____ Latin American
   _____ Middle Eastern
   _____ South-Asian
   _____ White/Caucasian/European
   _____ Other

8. What was the (approximate) date of your last your last medical check-up?
   ______________________________________________________________________

9. If you have had a medical check-up in the last 12 months, were you considered to be in good health?
   _____ Yes
   _____ No

If you answered NO, please explain _______________________________________________
10. Please describe any current illness or conditions:
_____________________________________________________________________
_____________________________________________________________________
_____________________________________________________________________

Please answer the following 7 questions (#11 to #17) regarding your weight history as an ADULT (from age 18 and up):

11. Highest past weight (not counting pregnancy, if applicable):_______ (pounds/kilograms)

12. Age at highest past weight:_______ years of age

13. How long were you at this weight? _______months

14. Lowest past weight:_______ (pounds/kilograms)

15. Age at lowest past weight: _______years of age

16. How long were you at this weight? _______months

17. Would you say that you are weight stable (do not fluctuate more than ± 5 lbs in a given week) or that your weight fluctuates frequently (> ± 5 lbs on a regular basis)?
   _______Yes, I am weight stable (my weight does not fluctuate > ± 5 lbs)
   _______No, I am not weight stable (my weight fluctuates > ± 5 lbs)
18. How old were you when you first menstruated?
   
   \[I \text{ was } ______ \text{ years old when I first menstruated.}\]

19. Have you given birth in the last 12 months?
   
   ______ No
   
   ______ Yes. \(I \text{ gave birth } _____ \text{ (number) months ago.}\)

20. Have you gone for any length of time without menstruating (e.g., no period for >90 days)?
   
   ______ Yes
   
   ______ No (skip to # 25)

21. If the answer to #20 is yes, are you currently not having menstrual cycles (e.g., amenorrhea)?
   
   ______ Yes (go to question #22)
   
   ______ No (go to question #23)

22. If you answered YES to question #21, please state how many months it has been since your last period. ________________________________

23. If you answered NO to question #21, please state your age when you experienced absence of menstrual cycles for >90 days ________________________________

24. When you experienced absence of menstrual cycles for >90 days, do you recall if you were exercising excessively, dieting, or under a lot of psychological stress (i.e., exams, high work load) during this time?
   
   ______ Yes, \(I \text{ was } \) ________________________________\( (\text{describe} \) at that time
   
   ______ No, I don’t recall

25. Currently, what is the average length of your menstrual cycle (from the beginning of menstrual flow [menses] to the beginning of the next menstrual flow [menses])? The average cycle length is 28 days.
   
   \[My \text{ average cycle length is } ______ \text{ days}\]
   
   ______ I am not currently having menstrual cycles. However, before I began to have amenorrhea (no menstrual cycle for >90 days), my average cycle length was _______ days.
26. Currently, for how many days do you typically experience menstrual flow each cycle? Please circle the correct response.

0 days   1 day   2 days   3 days   4 days   5 day   5+ days

27. In the past 12 months, estimate how many menstrual cycles you have had?

I have had _______cycles in the past 12 months

28. Are you currently taking oral or any other kind of hormonal contraceptives (i.e., Nuvaring)?

_______Yes

_______No (skip to # 31)

29. If the answer to #28 is YES, what kind of hormonal contraceptive are you using?

____________________________________________________________________

30. If the answer to #28 is YES, how long have you been taking hormonal contraceptives?

____ < 3 months  ____ 3-6 months  ____ 6-12 months  ____ 1-1.5 years  ____ > 1.5 years

31. Have you ever taken hormonal contraceptives OR other hormones for amenorrhea (absence of menstrual cycles) or irregular periods.

_______ Yes

_______ No

If you answered YES, please explain: __________________________________________

_________________________________________________________________________

_________________________________________________________________________

32. Have you ever had any of the following surgical procedures (check all that apply):

_______ Hysterectomy

_______ Oophorectomy (removal of one or both ovaries)

_______ Other gynecological procedure(s) (please list)

_________________________________________________________________________
33. Have you ever been diagnosed with any of the following (check all that apply)
   _____ Endometriosis
   _____ Fibroids
   _____ Poly cystic ovarian syndrome
   _____ Any cancer (please describe)__________________________
   _____ Thrombosis (blood clot), whether personal or familial
   _____ Gall bladder disease
   _____ Any tumours (please describe)__________________________
   _____ Heart disease
   _____ Familial heart disease or high blood pressure

34. Do you currently smoke? _____ No _____ Yes
   If yes, how many cigarettes do you smoke per day? ______

35. Did you smoke in the past? _____ No _____ Yes
   If yes, for how long? ______

36. Have you ever been diagnosed with an eating disorder?
   _____ Yes (please answer #37, 38, 39 and 40)
   _____ No (skip to question #41)

37. Please check the correct diagnosis:
   _____ Anorexia
   _____ Bulimia
   _____ Obsessive/Compulsive binge eating
   _____ Bulimarexia
   _____ Other, please list:______________________________________

38. Have you ever received professional counseling for eating problems?
   _____ Yes (please answer #34 and #35)
   _____ No (go to #36)

39. Please specify length of counseling:
   __________________ Began
   __________________ Ended

40. Are you currently in counseling for food related problems?
   _____ Yes
   _____ No
41. Do you currently have, or have you ever had, a stress fracture in the lower limb (e.g., leg, foot, ankle, pelvis)?
   ______ Yes (please answer #42)
   ______ No

42. If you answered yes to #36, please explain.

___________________________________________________________________________
_________________________________________________________________________
### CURRENT AND PAST PHYSICAL ACTIVITY

1. With respect to your physical activity or exercise training in the **PAST 2 YEARS**, state the following:

<table>
<thead>
<tr>
<th>Type</th>
<th>Frequency (d/wk)</th>
<th>Duration per session (min/day)</th>
<th>Years at that Dose of Exercise</th>
</tr>
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2. With respect to your physical activity or exercise training in the **PAST 6 months** state the following:

<table>
<thead>
<tr>
<th>Type</th>
<th>Frequency (d/wk)</th>
<th>Duration per session (min/day)</th>
<th>Years at that Dose of Exercise</th>
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<tr>
<td><strong>Same as above?</strong></td>
<td>Please just place a check mark here</td>
<td>______________________________</td>
<td></td>
</tr>
</tbody>
</table>

3. At what age did you start engaging in physical activity/exercise training regularly?

_______________________________________________________________________
_______________________________________________________________________
Consent Form for Participation in a Research Study

TITLE: Cardiovascular consequences of estrogen deficiency: studies in premenopausal women.

PRINCIPAL INVESTIGATOR:
Dr. P. J. Harvey, BMBS, PhD, FRACP  UHN, Toronto General Hospital  (416) 340-4755

CO-INVESTIGATOR:
Dr J. M. Goodman, PhD  University of Toronto  (416) 978-0762

You are being asked to take part in a research study. Before agreeing to participate in this study, it is important that you read and understand the following explanation of the proposed study procedures. The following information describes the purpose, procedures, benefits, discomforts, risks and precautions associated with this study. It also describes your right to refuse to participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about its risks and benefits to be able to make an informed decision. This is known as the informed consent process. Please ask the study doctor or study staff to explain any words you don’t understand before signing this consent form. Make sure all your questions have been answered to your satisfaction before signing this document.
Background
Hypertension (high blood pressure) and cardiovascular disease (disease of the heart and blood vessels) affect the lives of thousands of Canadians. Women are at lower risk of these two diseases than men of the same age until they reach menopause. Studies in animals and humans suggest that estrogen may protect women against high blood pressure and disease of the heart and blood vessels before they reach menopause. However, it is now known that low estrogen levels are not restricted to postmenopausal women. For example, over recent decades there has been an increase in the number of women participating in regular exercise for fun, work and sporting competition. This is usually thought to be a good thing, because a physically active life-style is a healthy lifestyle, helping to protect against cardiovascular disease and high blood pressure. However, when young women are very physically active often they develop changes in their menstrual cycle. Sometimes, these changes are associated with lower levels of estrogen. Amenorrhea, the most severe change in menstrual cycle health, results in the complete lack of menstruation and very low estrogen levels. Importantly, these estrogen levels are similar to that seen in much older postmenopausal women. The effects of such low estrogen levels on blood vessel and heart health in physically active premenopausal women that have stopped menstruating are largely unknown.

Purpose
This study was designed to assess the effects of estrogen levels and exercise on factors which contribute to the control of blood pressure and the health of the heart and blood vessels in premenopausal women. To conduct a thorough study of these effects we require 3 groups of premenopausal women. We plan to look at heart and blood vessel health in young (18-35 yrs) healthy women who are physically active, and are either regularly menstruating (10-13 cycles per year; group 1), or have not menstruated for at least the last three consecutive months (i.e., they have amenorrhea; group 2). For the purposes of comparing physically active women with women that do not exercise regularly, we will also study a group of physically inactive (less than 2 hours of structured physical activity per week) premenopausal women who are menstruating regularly (group 3).

*Menstruating women* will be tested once on all measures. *Women that do not menstruate* (i.e., they have amenorrhea) will be tested before and after 4 weeks of estrogen replacement (i.e., they will be tested twice on all measures). The estrogen replacement will be given in the form of a patch that is applied to the skin weekly. The type and dose of estrogen given in this patch is the same as that found in menstruating women. Testing women that do not menstruate both before and after using the estrogen patch will allow us to look more closely at the effects of estrogen on heart and blood vessel health.
We would like you to read through this summary to familiarize yourself with the study requirements (i.e., number of visits, time needed to complete the visits), the benefits and the potential risks of this study.

**Procedures**

*Regularly Menstruating Women:* For women that are menstruating regularly, it is expected that the study period will involve monitoring across 2 menstrual cycles in total: one pre-study cycle (consisting of keeping a menstrual and exercise log; we will provide you with these), and one study cycle, where, in addition to completing an additional menstrual and exercise log, you will complete four study visits so we can run a number of tests on you (these tests are detailed below in Study Visit 1, 2, 3 and 4). If, for any reason, you are not able to complete all the tests during this study period, any outstanding tests will be completed during an additional (second) study period. Before initial testing, you will be given an ovulation hormone test kit (Clearblue Easy ovulation test), for the purposes of identifying whether you ovulated or not. You will be given instruction as to how and when you will use the kit. It is important that we know your ovulatory status such that we can reasonably predict your estrogen and progesterone profile across the menstrual cycle. If you ovulate, we will go ahead and arrange testing sessions to take place during days 1-6 of your next menstrual cycle, day 1 being the first day of bleeding. If you did not ovulate (i.e., you are anovulatory), and you would still like to remain in the study, we will need to wait until your next menstrual cycle to re-assess your ovulatory status. It is not uncommon for menstrual cycles to vary between ovulatory and anovulatory month over month in physically active women.

*Women that do not Menstruate at all:* For women that have not menstruated for at least 90 days, the study period will be 60 days total, made up of two 30-day study periods. During one study period you will receive 4 weeks of estrogen replacement via a skin patch, followed by 10 days of progestagen tablets. The progestagen is very similar to the natural hormone progesterone, and is a standard and important part of any course of hormone replacement given to women who have a uterus (i.e., womb). During the second study period you will not take any estrogen or progestagen. The order of the study periods (i.e., estrogen and no estrogen) will be assigned to you randomly. Overall, you will complete one ‘initial visit’ (described below) plus four ‘Study Visits’ during one 30-day study period (Study Visits 1, 2, 3 and 4; described below), and three ‘Study Visits’ during a second 30-day study period (Study Visits 2, 3, and 4). Thus, most measurements will be performed twice, for a total of 7 ‘Study Visits’ across two 30-day periods. Body composition testing and aerobic fitness testing however, will only be measured once (as described in Study Visit 1). All study visits will be on separate days and completed during the fourth week of each 30-day study period.
Initial Visit
Assessment of your suitability to be included in the study will require a minimum of one visit to the laboratory (Clinical Cardiovascular Physiology Laboratory, Room 413, 6th floor, Eaton Wing South, Toronto General Hospital). It is expected that this visit will take about 1.5 hours. You will be asked questions about your personal information (such as name, telephone number, address, etc.), general medical history, as well as your menstrual cycle history and exercise history. Provided you meet all aspects of the study entry requirements, and you agree to participate in this study by giving written consent (at the end of this document), we will measure your height and weight, you will have a urine test to confirm that you are not pregnant, and you will receive further information about the study and the study requirements.

Study Visits
Study Visit 1 will be completed before all other Study Visits. Study Visits 2, 3 and 4 (described below) will be done in a randomized order, meaning the order of the visits will be decided by chance, so they may not happen in the order currently described in this consent. Once this order has been determined, you will be told what the order of Study Visits will be. The Study Visits are as follows:

Study Visit 1
Aerobic Fitness Testing
Study Visit 1 will take about 45 mins to complete and will look at your aerobic fitness level. First, we will measure your blood pressure in the usual manner. Then we will fit you with a heart rate monitor around your wrist. You will then perform an exercise test on a standard treadmill. You may choose the speed of the treadmill. We will increase the incline of the treadmill gradually until you reach your maximum effort. This test normally lasts about 8-12 minutes. You may stop the test at any time. You will be asked to go without alcohol and caffeine 24 hours before your appointment.

Study Visit 2
Blood Vessel Responses, High Intensity Exercise, and Body Composition
Study Visit 2 will take about 3 - 3.5 hours to complete and will look at your Blood Vessel Responses before and after a short bout (approximately 10 mins) of high intensity exercise. Your Body Composition will also be measured. You will be asked to go without alcohol and caffeine 24 hours before your appointment. The following tables describe the measures and procedures for these tests:
**Blood Vessel Responses**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-test</td>
<td>You will rest for 10 minutes lying down before we take any measures</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>We will fit you with a heart rate monitor which you will wear on your wrist like a watch</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>A small cuff will be placed around your right middle finger and a standard cuff will also be placed around your upper arm</td>
</tr>
<tr>
<td>Blood Vessel Responses</td>
<td>A small pencil-like probe will be pressed on the skin surface over a blood vessel. The probe does not penetrate your skin. The blood vessel sites we are interested in are in your wrist, your neck, your upper leg (near the groin), and in your foot.</td>
</tr>
<tr>
<td>Blood Draw</td>
<td>A small amount of blood will be drawn from your arm in the usual manner</td>
</tr>
</tbody>
</table>

**High Intensity Exercise**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise</td>
<td>You will perform an exercise test on a standard treadmill. You may choose the speed of the treadmill. We will increase the incline of the treadmill gradually until you reach your maximum effort. This test normally lasts about 8-12 minutes. You may stop the test at any time</td>
</tr>
<tr>
<td>Recovery</td>
<td>Once you have finished the exercise test you will lie down for 60 minutes. During this time we will measure your blood pressure and heart rate as described above in ‘Blood Vessel Responses’.</td>
</tr>
<tr>
<td>Blood Draw</td>
<td>A small amount of blood will be drawn from your arm in the usual manner</td>
</tr>
<tr>
<td>Blood Vessel Responses</td>
<td>After 60 minutes of recovery we will repeat the measures of ‘Blood Vessel Responses’ using a small pencil-like probe (as described above)</td>
</tr>
</tbody>
</table>

**Body Composition**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body composition</td>
<td>This test will measure how much of your body weight is fat or muscle (body composition). This test is done by DEXA</td>
</tr>
</tbody>
</table>
Study Visit 3

Blood Vessel Responses to Moderate Intensity Exercise

This visit will take approximately 4.5 hours and will look at your Blood Vessel Responses before and after Moderate Intensity Exercise. You will be asked to come to the laboratory having eaten no breakfast and having done no exercise. The following tables describe the measures and procedures for these tests:

<table>
<thead>
<tr>
<th>Measure</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-test</td>
<td>You will rest for 10 minutes lying down before we take any measures.</td>
</tr>
<tr>
<td>Blood Draw</td>
<td>A catheter (a small tube) will be inserted into a vein in your arm. This is common practice for collecting blood samples during a study session.</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>We will measure your heart rate by applying standard electrodes to your chest.</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>A small cuff will be placed around your right middle finger and a standard cuff will also be placed around your upper arm.</td>
</tr>
<tr>
<td>Cardiac Output</td>
<td>We will measure how much blood your heart pumps per minute (cardiac output), by taking a photograph-like picture using ultrasound techniques. We will place a hand-held probe on your skin near the top of your chest bone.</td>
</tr>
<tr>
<td>Blood Vessel Responses (1): Arm</td>
<td>Similar to cardiac output, an ultrasound picture of the blood vessels in your arm will be taken to look at your blood vessel health. This test will be done before and after inflating an upper arm blood pressure cuff for 4.5 minutes.</td>
</tr>
<tr>
<td>Blood Vessel Responses (2): Skin</td>
<td>To look at the differences in responses between different types of blood vessels, we will also look at the blood vessels in your skin in your forearm. We will tape a small round disc to your forearm and take measures of skin blood flow before and after inflating an arm blood</td>
</tr>
</tbody>
</table>
Blood Vessel Responses (3):
Arm and skin

<table>
<thead>
<tr>
<th>Measure</th>
<th>Procedure</th>
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</thead>
<tbody>
<tr>
<td>Pressure cuff</td>
<td>for 4.5 minutes.</td>
</tr>
<tr>
<td>Blood vessel measurements will then be repeated after taking a spray of nitroglycerin under the tongue. Nitroglycerin is commonly used in studies to help the vessels relax.</td>
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</tr>
<tr>
<td>Blood Vessel Responses: Calf</td>
<td>To look at blood vessel responses in your calf, we will wrap a blood pressure cuff around your knee and one above your ankle. A special thin elastic tube will then be wrapped around the calf to measure calf blood flow. This test will be done before and after inflating the blood pressure cuffs, together, for 4.5 minutes.</td>
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**Moderate Exercise**

<table>
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<tr>
<th>Measure</th>
<th>Procedure</th>
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<tbody>
<tr>
<td>Exercise</td>
<td>You will exercise on a standard treadmill for a maximum of 45 minutes at a moderate exercise intensity (60% of your maximum). Typically, this exercise involves walking briskly or jogging slowly, depending on what is comfortable for you.</td>
</tr>
<tr>
<td>Recovery</td>
<td>Once you have finished the exercise you will lie down for 60 minutes. During this time we will measure your blood pressure and heart rate as described above. We will also draw a small amount of blood from your arm.</td>
</tr>
<tr>
<td>Blood Vessel Responses: Arm and Skin</td>
<td>After 60 minutes of recovery we will repeat the measures of ‘Blood Vessel Responses’ for the arm (1), skin (2), and arm and skin (3), as described above.</td>
</tr>
<tr>
<td>Cardiac Output</td>
<td>This measure will be repeated as described above.</td>
</tr>
</tbody>
</table>

**Study Visit 4**

*Blood Pressure Responses and Regulation*

Testing for this study visit will take about 4.5 hours and will look at your Blood Pressure Responses and Regulation. You will be asked to come to the laboratory having not exercised and having not eaten breakfast. The following tables describe the measures and procedures for these tests:
## Blood Pressure Responses and Regulation

<table>
<thead>
<tr>
<th>Measure</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-test</td>
<td>You will rest for 10 minutes lying down before we take any measures.</td>
</tr>
<tr>
<td>Blood Draw</td>
<td>We will draw blood samples as described in Study Visit 3.</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>We will measure your heart rate as described in Study Visit 3.</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>We will measure your blood pressure as described in Study Visit 3.</td>
</tr>
<tr>
<td>Blood Pressure Regulation: Microneurography</td>
<td>We will measure nerve activity in your lower leg, just below the knee on the outer side of your leg, using a method called ‘microneurography’. First, to find the nerve we use a pen-shaped electrical probe to cause a muscle twitch or tingling. These sensations are annoying, but not painful, and disappear when the probe is removed. After we have found the nerve we will put a tiny wire through the skin into the nerve. When the wire enters the nerve you will again experience twitching or tingling. We leave the pin in the nerve to record its activity during the experiment. Since the pin is very thin, we do not need to give any anaesthetic. In most people, a good recording takes 20-30 minutes. If a good recording cannot be made after an hour the microneurography testing may be stopped.</td>
</tr>
<tr>
<td>Blood Pressure Regulation: Lower Body Negative Pressure</td>
<td>You will lie on a bed that is attached to a Lower Body Negative Pressure tank. This tank is made-up of a metal chamber connected to a standard vacuum cleaner. The tank is applied to your lower body (legs) and sealed around your waist. We will use the vacuum cleaner to create a graded gentle negative pressure in the tank that will draw blood into your legs. Three different grades of negative pressure will be applied, for a minimum of ten minutes each, with a five minute rest in between each grade. The blood returns to the body very quickly on turning the tank off. If you are uncomfortable the test will be stopped.</td>
</tr>
<tr>
<td>Blood Pressure Responses: 24 hour recording</td>
<td>We will give you a 24-hour blood pressure recording device that is about the size of a small portable radio. You will wear it on a belt around your waist. A blood pressure cuff, attached to the monitor by a thin tube, is worn under your clothes, on your upper arm, without anyone seeing it. You will be asked to not exercise during the recording period. Measurements of blood pressure and heart rate will be made at 15 minute intervals</td>
</tr>
</tbody>
</table>
during daytime hours and at 60 minute intervals during night-time hours. You will be given instructions on how to use this device properly at the time.

*Dietary Log*

In addition to the tests described above, you will be required to complete one 3-day dietary log. If you are menstruating regularly, you will complete the three-day dietary during the follicular phase (days 1-14) of your ‘study’ menstrual cycle (i.e., when we are running our tests). If you are not menstruating at all, you will complete the three-day dietary log during days 1-14 of the 30-day monitoring period that you are not wearing an estrogen patch. You will be given verbal and written instructions on how to measure food intake as accurately as possible.

*Risks and /or side effects*

The possible risks and discomforts of the procedures are as follows:

Some of the questions we will ask you relate to your eating habits and dietary preferences. We understand that these questions are of a sensitive nature and it is important for you to know that answering them is optional should you feel uncomfortable sharing such information.

Drawing blood by means of a catheter could cause some bruising or clotting at the insertion site, but these are neither common nor serious occurrences. Every precaution possible (i.e., use of a trained nurse, sterile equipment) will be taken to minimize this risk.

The risk of heart attack or other cardiac events occurring as a direct result of a maximal exercise test is considered low (less than 1 in 10,000), particularly in young and healthy individuals. A physician will be available should the need for medical attention arise.

Inflation of an arm blood pressure cuff for a time of 4.5 minutes is not linked with any risks, but may be uncomfortable, causing tingling in the area of the inflated blood pressure cuff. These sensations will go away as soon as the blood pressure cuff is released.

The nitroglycerin may cause a headache which will go away within a few minutes. Sometimes the nitroglycerin can cause blood pressure to fall and make you lightheaded. This improves within a few minutes. We will measure your blood pressure continuously during this procedure.

Micronuerography has been used in well over 1,500 experiments worldwide with only three known complications, the worst of which was a slight weakness of the muscle of the leg lasting 2 to 6 months. You may notice a mild ache in the muscles of the left leg for a day to two afterwards.
During the first Lower Body Negative Pressure measure (-10 mmHg) you should feel nothing. During the second (-20 mmHg) and third measures (-40 mmHg), your blood pressure may fall to a small amount and you may feel light headed or dizzy. These sensations will go away as soon as the Lower Body Negative Pressure is stopped. We will measure your blood pressure continuously during this procedure.

Having a DEXA scan will expose you to a small amount of radiation. This level of exposure is about 1/6th of a regular chest x-ray, and is not considered to be dangerous or harmful. The DEXA technician will ask you to remove any body piercings before the scan. The technician will talk to you about any unremovable piercings or any internal metals or plastics, such as joint replacements, metal plates, pins or screws at the time. If you have recently undergone barium tests/exams (within 2 weeks), or who have had a nuclear medicine scan or injection with an x-ray dye (within 1 week), you cannot have a DEXA scan. If this applies to you, please advise the study investigator, Dr Paula Harvey or the study co-ordinator, Miss. Emma O'Donnell, immediately.

Estrogen patches are used regularly in standard clinical practice and as with all drugs, side effects may occur. For the estrogen skin patch as used in this study, these include minor skin irritation at the site of the patch, breast soreness, headache, a bloated feeling in the abdomen, weight gain, irritability and nausea. These effects may occur in about 5-10% of women. There are some pre-existing conditions which may be made worse by estrogen therapy which include gallstones, endometriosis, and some cancers including breast cancer and cancer of the uterus (i.e., womb). For this reason non-menstruating participants with a history of any of these conditions will not be able to be included in this study. The progestogen, which will be given at the end of the course of estrogen, may cause a "withdrawal bleed" resembling a light menstrual period when the tablets are stopped. The progestogen may also cause similar temporary side effects to the estrogen including fluid retention, a bloated feeling in your abdomen, headache and nausea.

**Pregnancy**

Estradiol, the type of estrogen contained in the skin patches will used in this study, has been shown to have toxic (poisonous) effects on developing embryos (i.e., on a developing baby up until 8 weeks of pregnancy). Abnormal urogenital tract development (i.e., feminization of male fetuses) has been reported when estrogen is taken in high doses. While the type and dose of estrogen in the skin patch is low, if you are planning on becoming pregnant, or become pregnant, during the study period, please advise the investigators immediately.

Measurement of body composition using dual energy x-ray absorptiometry (DEXA) methods exposes you to a very low dose of radiation (2-4 millirem; a millirem is a unit of radiation).
The National Council on Radiation Protection and Measurements and the American College of Doctors and Gynecologists both agree that the potential health risks to a fetus (i.e., a developing baby) are not increased from most standard x-ray and medical tests with a radiation dose below 5,000 millirem. However, we will do a pregnancy test immediately before your DEXA scan to reduce the possibility of exposing a developing fetus to any unnecessary radiation.

**Benefits**
You will not receive any medical benefit from your participation in this study. Information learned from this study may benefit future studies looking at the effects of estrogen on heart and blood vessel health, particularly in physically active premenopausal women that have stopped menstruating.

**Confidentiality**
All information obtained during the study will be held in strict confidence. You will be identified with a study number only. No names or identifying information will be used in any publication or presentations. No information identifying you will be transferred outside the investigators in this study or this hospital. The University Health Network Research Ethics Board (a group that oversees the ethical conduct of research studies) may look at the study information for auditing purposes.

**Participation**
Your participation in this study is voluntary. You can choose not to participate or you may withdraw at any time without consequence.

**Compensation**
If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your health insurance for any injury or illness that is directly a result of participation in this study. In no way does signing this consent form waive your legal rights nor does it relieve the investigators or involved institutions from their legal and professional responsibilities.

**Compensation for time**
You will be compensated for your time during Study Visits 1, 2, 3, and 4 at the rate of $25 per hour. Compensation does not include travel time to and from the laboratory for these study visits. If you withdraw or are withdrawn from the study before its completion, you will receive a pro-rated payment.

**Questions**
If you suffer any side effects or other injuries during the study, or if you have any general questions about the study, please call the doctor in charge of this study, Dr Paula Harvey at 416-340-4755. You may also call the study co-ordinator, Miss. Emma O’Donnell, at the same number or at 416-978-0762.

If you have any questions about your rights as a research participant, please call Dr. R. Heslegrave, Chair of the University Health Network Research Ethics Board at (416) 340-4557. This person is not involved with the research project in any way and calling him will not affect your participation in the study.

Consent
I have had the opportunity to discuss this study and my questions have been answered to my satisfaction. I consent to take part in the study with the understanding I may withdraw at any time without affecting my medical care. I have received a signed copy of this consent form. I voluntarily consent to participate in this study.

__________________________  ___________________________  ________________________
Participant’s Name          Participant’s Signature  Date
(Please Print)

I confirm that I have explained the nature and purpose of the study to the participant named above. I have answered all questions.

__________________________  ___________________________  ________________________
Name of Person              Signature                   Professional
obtaining consent           relationship to patient
IF THIS CONSENT HAS BEEN VERBALLY TRANSLATED:

I confirm that I have verbally translated this consent form for the study participant noted above, and in my opinion the study participant has understood what I have explained to them.

<table>
<thead>
<tr>
<th>Name of Translator</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Language of Translation</th>
<th>Relationship to Participant (if applicable)</th>
</tr>
</thead>
</table>
Prior to commencing the study, same day test-retest measures of Doppler ultrasound methods of brachial artery diameter measurement (n=7) and calf blood flow (n=8) were conducted to establish the intra-reliability of these methods. Measures were collected by one assessor (EOD) at ≥ 2 hours apart. Measures of reliability included: paired students t-test, Pearson correlation coefficient, intraclass correlation coefficient (ICC), and coefficient of variance (CV). The subjects were assumed to be a random sample from a larger population. Paired students t-tests provides the limits of agreement for paired sample means, and provides confidence intervals for the difference between a pair of means (1). The Pearson correlation measures the correlation or strength of linear dependence between two measures, thereby providing an estimate of the degree of association, or agreement, between the measures (1). ICC estimates the average correlation between all possible pairs within the subject taken by the same observer. The ICC is based on mean squares obtained by employing a model of analysis of variance (ANOVA) (2). Finally, the coefficient of variance, a normalized measure of dispersion, was calculated for each individual subject for the 2 repeat trials (3). An average CV for each subject and each outcome variable was then calculated. All calculations were determined using SPSS (SPSS version 20; SPSS Inc., Chicago, IL). Table 1 presents the means ± SEM for each trial, the Pearson correlation, the ICC, the paired students t-test, and the CV for each primary outcome variable measured in the studies. All reported values are consistent with that previously reported in the literature (3-6).
Table 1. Same day test-retest reliability for brachial artery and calf blood flow measures.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Trial 1*</th>
<th>Trial 2*</th>
<th>Mean*</th>
<th>r</th>
<th>P</th>
<th>CV (%)</th>
<th>ICC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial Diameter Rest (mm)</td>
<td>7</td>
<td>0.315 ± 0.04</td>
<td>0.319 ± 0.04</td>
<td>0.317 ± 0.02</td>
<td>0.97</td>
<td>&lt;0.001</td>
<td>1.53</td>
<td>0.95</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.835, 0.994)</td>
</tr>
<tr>
<td>FMD diameter (mm)</td>
<td>7</td>
<td>0.328 ± 0.02</td>
<td>0.333 ± 0.01</td>
<td>0.331 ± 0.02</td>
<td>0.96</td>
<td>0.001</td>
<td>2.60</td>
<td>0.92</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.684, 0.987)</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>7</td>
<td>8.2 ± 0.7</td>
<td>8.6 ± 0.7</td>
<td>8.4 ± 0.7</td>
<td>0.73</td>
<td>0.041</td>
<td>10.58</td>
<td>0.71</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.147, 0.932)</td>
</tr>
<tr>
<td>Calf BF Rest (ml/min/100ml)</td>
<td>8</td>
<td>4.3 ± 0.7</td>
<td>4.1 ± 0.6</td>
<td>4.2 ± 0.6</td>
<td>0.91</td>
<td>0.002</td>
<td>11.31</td>
<td>0.93</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.663, 0.987)</td>
</tr>
<tr>
<td>Calf BF Pk (ml/min/100ml)</td>
<td>8</td>
<td>55.3 ± 3.0</td>
<td>58.3 ± 3.6</td>
<td>56.8 ± 3.2</td>
<td>0.82</td>
<td>0.012</td>
<td>4.48</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.476, 0.976)</td>
</tr>
</tbody>
</table>

*Values are mean±SEM
17 β-estradiol, Progesterone and Prolactin

Blood samples were collected into vacutainers containing no additive. Serum was analyzed using an automated one-step chemiluminescent immunoassay (ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). Analytical sensitivity of the assays are reported as: 17 β-estradiol, ≤25 pg/ml; progesterone, ≤0.1 ng/ml; and prolactin, ≤0.6 ng/ml.

Free T3

Blood samples were collected into vacutainers containing heparin. Serum was analyzed using an automated two-step immunoassay based on paramagnetic microparticle chemiluminescent techniques (ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). Analytical sensitivity of the assay is reported as ≤ 0.1 pg/ml.

Free Testosterone

Blood samples were collected into vacutainers containing no additive. Serum was analyzed with a competitive $^{125}$I-radioimmunoassay using the Coat-A-Count system (Siemens, Los Angeles, CA, USA). Analytical sensitivity of the assay is reported as ≤0.15 pg/ml.
Sex Hormone Binding Globulin

Blood samples were collected into vacutainers containing no additive. Serum was analyzed using a solid phase sandwich immunoradiometric assay (Cisbio Bioassays, Codolet, France). SHBG is sandwiched between a monoclonal antibody labelled with iodine$^{125}$ and a monoclonal antibody coated onto the inner walls of the assay tubes. Analytical sensitivity of the assay is reported as <0.5 nmol/L.

Glucose

Blood samples were collected into vacutainers containing sodium fluoride additive. Serum was analyzed using an automated quantitative enzymatic assay based on Hexokinase/G-6-PDH methodology (ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). Analytical sensitivity of the assay is reported as ≤ 5mg/dL (0.28 mmol/L).

Insulin

Blood samples were collected into vacutainers containing ethylenediaminetetraacetate (EDTA). Serum was analyzed using an automated one-step immunoassay based on paramagnetic microparticle chemiluminescent techniques (ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). Analytical sensitivity of the assay is reported as ≤1.0 µU/ml.
High density lipoprotein cholesterol (HDLc) and Total Cholesterol (TC)

Blood samples were collected into vacutainers containing heparin. For HDL, serum was analyzed using an automated quantitative and colorimetric assay (ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). For TC, serum was analyzed using an automated enzymatic assay ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). Limit of detection for both assays is reported as $\leq 5 \text{ mg/dL}$.

Triglyceride

Blood samples were collected into vacutainers containing heparin. Serum was analyzed by direct colorimetric procedures using a glycerol phosphate oxidase assay (ARCHITECT®, Abbot Laboratories, Abbot Park, IL, USA). Limit of detection is reported as $\leq 5 \text{ mg/dL}$.

Low density lipoprotein cholesterol (LDLc)

LDLc was determined by the method of Friedewald (7). In brief, LDLc was calculated as total cholesterol minus HDLc minus very-low-density lipoprotein (VLDL) cholesterol (estimated as triglyceride divided by 5).

Renin, Aldosterone and Angiotensin II

Blood for the measurement of the RAAS components was collected into pre-chilled tubes containing ethylenediaminetetraacetate (EDTA). An angiotensinase inhibitor (0.1ml Bestatin Solution; Buhlmann Laboratories AG, Switzerland) was added to the angiotensin II samples.
After centrifugation, plasma samples were stored at -70\(^\circ\) C until analysis. Active plasma renin was measured by a two-site immunoradiometric assay (Cisbio Bioassays, Codolet, France). Assay sensitivity is reported as \(\leq 1\) pg/ml. Angiotensin II was measured by a competitive \(^{125}\)I radioimmunoassay (Bulmann Laboratories AG, Switzerland) after plasma samples were extracted on phenylsilica columns. Assay sensitivity is reported as 0.68 pg/ml. Aldosterone was measured by a competitive \(^{125}\)I radioimmunoassay using the Coat-A-Count system (Siemens, Los Angeles, CA, USA). Assay sensitivity is reported as \(\leq 11\) pg/ml.
Since the primary question involves the effects of estrogen deficiency on mechanisms of cardiovascular regulation, sample size calculations were determined for each study.

### Brachial Artery FMD%

\[ N \text{ (sample size)} = \left[ \frac{(Z_a + Z_b) \times SD}{\text{diff}} \right]^2 \]

- \( Z_a = 1.96 \) (Z alpha is the alpha error set at 0.05 with a critical value of 1.96 SE)
- \( Z_b = 0.84 \) (Z beta is the beta error set at 0.2 with a critical value of 0.84 SE)
- SD is 4
- Diff (difference) is approximately 3
- N=14

Expected responses between ExFHA and ExOv women were derived from Zeni and colleagues (8) and Rickenlund and colleagues (9) who reported approximately 3-5% lower FMD% in ExFHA women versus ExOv women. Standard deviation was reported to be between 3-4%. From these values, it was determined that 14 subjects in each group would provide 80% power for detecting a difference of 4% in the FMD% response between ExFHA and ExOv women.

### Peak Calf Blood Flow

\[ N \text{ (sample size)} = \left[ \frac{(Z_a + Z_b) \times SD}{\text{diff}} \right]^2 \]

- \( Z_a = 1.96 \) (Z alpha is the alpha error set at 0.05 with a critical value of 1.96 SE)
- \( Z_b = 0.84 \) (Z beta is the beta error set at 0.2 with a critical value of 0.84 SE)
- SD is 10
- Diff (difference) is approximately 8
- N=12
Expected responses between ExFHA and ExOv women were derived from O'Donnell et al., (10) who reported approximately 8 ml/min/100ml lower peak calf blood flow responses in ExFHA versus ExOv women. Standard deviation was reported to be between 10 ml/min/100ml. From these values, it was determined that 12 subjects in each group would provide 80% power for detecting a difference of 8 ml/min/100ml in peak calf blood flow responses between ExFHA and ExOv women.

**Neurohumoral and Renin-Angiotensin System Mechanisms of Blood Pressure Regulation**

\[
N = \frac{Z_a + Z_b \times SD}{\text{diff}}^2
\]

Za is 1.96 (Z alpha is the alpha error set at 0.05 with a critical value of 1.96 SE)
Zb is 0.84 (Z beta is the beta error set at 0.2 with a critical value of 0.84 SE)
SD is 11
Diff (difference) is approximately 8
N=15

Based on studies examining the effects of estrogen status on hemodynamic responses to lower-body negative pressure (LBNP) in PMW (11) it was determined that in order to provide 80% power for detecting a mean change in mean arterial pressure (MAP) of approximately 8 mmHg with a standard deviation of 11 mmHg during -40 mmHg LBNP that a sample size of 15 subjects in each group would be required.

**Autonomic Control of Heart Rate**

\[
N = \frac{Z_a + Z_b \times SD}{\text{diff}}^2
\]

Za is 1.96 (Z alpha is the alpha error set at 0.05 with a critical value of 1.96 SE)
Zb is 0.84 (Z beta is the beta error set at 0.2 with a critical value of 0.84 SE)
SD is 200
Diff (difference) is approximately 150
N=14
Based on studies examining the effects of estrogen status on parasympathetic modulation of HR (HF, ms\(^2\)) in PMW (12) it was determined that in order to provide 80% power for detecting a mean change in baseline HF of approximately 150 (ms\(^2\)) with a standard deviation of 200 (ms\(^2\)) that a sample size of 14 subjects in each group would be required.

**Total Sample Size**

Volunteers were recruited to complete three studies on separate study days. To allow for a potential dropout rate of 20% based on previous studies of this population due to a combination of subclinical hormonal abnormalities, such as anovulation (detected on determination of menstrual status via a urinary ovulation hormone kit, Clearblue Easy), and unforeseen circumstances (10, 13), a total of 24 volunteers will be required to be recruited for each study group.
References for Appendices


