Sex differences in atherosclerosis and exercise effects

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“It would be so nice if something made sense for a change”

Alice’s Adventures in Wonderland
by Lewis Carroll

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ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death globally, with atherosclerosis being the main cause of cardiovascular diseases. Atherosclerosis is an inflammatory disease of the blood vessel wall, which over time will cause thickening and hardening of the vessel wall. Atherosclerosis can result in catastrophic vascular events, such as myocardial infarction and stroke. There are distinct sex differences in CVD mortality at different ages, before menopause women have a lower mortality of CVD in comparison to men, which equalises after menopause. In addition to sex differences in the incidence of CVD, there are also distinct sex differences in the phenotype of atherosclerotic plaques, with men generally developing more severe and vulnerable plaques that are at risk of rupture.

This thesis aimed to investigate the sex differences in atherosclerosis, in particular how the proteome and pathophysiology differs. In addition, we sought to investigate the potential benefit of an exercise programme, in reducing CVD risks, using a randomised controlled trial including postmenopausal women.

Sex differences in atherosclerosis were first investigated via proteomic analysis of human carotid endarterectomy samples. Initially, five intraplaque biopsies were taken from distinct atheroma regions, including; internal control, fatty streak, plaque shoulder, plaque centre, and fibrous cap. Protein extracts from these biopsies were subjected to analysis by mass spectrometry. The novel sampling method was successful in reducing the effect of plaque heterogeneity, a limitation in previous proteomic studies of atherosclerosis, and a number of previously unreported proteins were identified in human carotid atheroma. In addition to this, with the inclusion of multivariate statistical modelling, it was found that 43 proteins significantly discriminated the carotid atheroma between men and women. These proteins were grouped by function, and it was found that atheroma from men was associated with the increased abundance of inflammatory response proteins, including phospholipase-A2 membrane associated and lysozyme C, and atheroma from women was associated with increased abundance of blood coagulation, complement activation, and transport proteins, notably including; antithrombin-III, coagulation factor XII, and afamin. In addition, differences were also

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observed in the abundance of iron metabolism related proteins. These sex differences were further expanded upon from a pathophysiological perspective. Immunohistochemistry stainings of ferritin and transferrin receptor 1 were found significantly increased in the atheroma from men. Moreover, the levels of plasma haemoglobin were also significantly increased in men and were associated with the development of more vulnerable and severe plaque types. The more vulnerable and severe plaque types were also associated with significantly greater macrophage infiltration. In summary, these results are indicative of men developing atheroma with greater inflammation that are more vulnerable, due to increased iron and inflammatory proteins and macrophage infiltration, whereas atheroma from women develop with less inflammation and a more stable phenotype.

The randomised controlled clinical trial aimed at investigating the effects of resistance training (RT), over a 15-week period, in postmenopausal women. Plasma samples were obtained at week-0 and week-15 of the study period, and analyses were performed primarily using a series of immunoassays. Results showed that women participating in RT, with good compliance, were associated with significant decreases in plasma levels of ferritin, lipids, and inflammatory adipokines. These results suggest that the use of regular RT may be a beneficial intervention in reducing the levels of body iron, lipids, and inflammation, all of which are risk factors for the development of CVD. However, validation studies are required in a larger cohort of postmenopausal women, in addition to the inclusion or complementary studies in middle-aged men.

In summary, the works included in this thesis further expand on the current knowledge of sex differences in atherosclerosis, and also provides information on the potential of an exercise intervention to beneficially reduces the effects of known risk factors of CVD.

POPULAR SCIENTIFIC SUMMARY

Cardiovascular diseases (CVD), such as heart attacks and strokes, are the biggest causes of death globally. The main cause of CVD is called atherosclerosis, which is a process where the blood vessels become narrower and stiffer due to stored fats and dying cells, and inflammation in the blood vessel wall. Atherosclerosis can affect major arteries including the coronary and carotid arteries, which provide oxygenated blood to the heart and brain, respectively. The amount of CVD-related deaths is disproportional between the sexes at different ages; before menopause, fewer women die of CVD than men, which then equalises after menopause. In addition, atherosclerosis in men seems to develop with a greater risk profile, being more vulnerable to rupture and blocking the artery, than atherosclerosis in women. The reasoning behind these differences are largely unclear and more research is needed to understand why such differences occur.

This thesis aimed to investigate the sex differences in atherosclerosis further from two main perspectives, including; proteomics and pathophysiology. Proteomics is the study of all proteins within a specified cell, tissue, or organism, and pathophysiology is the study of physiological processes associated with a specified disease or injury. In addition, works in this thesis also evaluated the potential of an exercise programme aimed to reduce the CVD risk profile in postmenopausal women, a population group that is at risk of developing CVD.

Proteomic analyses were performed on atherosclerotic carotid arteries obtained from both men and women, from these, five biopsies were taken from distinct regions of each artery. Proteins were extracted from these biopsies and broken down into smaller components called peptides. These are analysed using mass spectrometry, a method that measures the peptide masses, creating a mass pattern. Since every protein has a specific mass pattern, a database search is made to match the mass patterns to find out which proteins are in the sample. Taking samples from multiple regions in the atherosclerotic artery allowed for more proteins to be identified in the samples, in comparison to previous studies using similar methods. When analysing the data from proteomic analyses to identify sex differences, it was found that proteins within 16

groups discriminated the protein profile between men and women. Atherosclerotic arteries from men had a higher abundance of proteins involved in inflammation, which is a key promoter of atherosclerosis development. Atherosclerotic arteries from women had a higher abundance of proteins involved in blood coagulation and complement activation, both systems play roles in blood clot formation, and transport proteins. In addition, differences in proteins related to iron metabolism in the body were found, and expanded experiments were performed to study these further. Immunohistochemistry, a method which stains sections of tissues for specific proteins that can then be viewed under the microscope, was used to stain for the expression of iron-related proteins, ferritin and transferrin receptor-1, and macrophages, a cell type associated with inflammation and vulnerability in atherosclerosis. The results of these experiments found that levels of iron-related proteins were higher in atherosclerotic arteries from men, compared to women, and that these high levels were associated with higher levels of macrophages and greater severity of atherosclerotic artery. In summary, a number of proteins were identified that discriminate the atherosclerosis protein profile between men and women, which in addition to immunohistochemistry differences can contribute to the understanding of atherosclerosis development and vulnerability.

An exercise programme was used to evaluate its effect on reducing potential CVD risks in a group of postmenopausal women. The exercise programme was based on 15 weeks of resistance training and women included were randomised into two groups to either receive the exercise training or be a control group and not alter their physical activity levels. Blood samples were taken at two time points in the trial, weeks 0 and 15, and analysed for a wide variety of markers, including fats, iron-metabolism markers, and inflammation markers. To summarise, the trial found that women who participated in the exercise, with good compliance, were associated with significant reductions in the blood levels of fats, ferritin (an iron transport protein), and inflammatory markers derived from body fat – termed adipokines.

In summary, the works included in this thesis further expand on the current knowledge of sex differences in atherosclerosis, and also provides information on the potential of an exercise programme that could beneficially reduce risk of CVD.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt- och kärlsjukdomar (CVD), såsom hjärtinfarkt och stroke, är den ledande dödsorsaken över hela världen. Huvudorsaken till CVD är ateroskleros (även kallat åderförfattning/åderförkalkning), vilken är en process där blodkärlen blir trängre och stelare på grund av inlagrat fett, döende celler och inflammation i blodkärlsväggarna. Ateroskleros kan påverka stora artärer, inklusive krans- och karotidartärer, vilka förser syrerikt blod till hjärtat respektive hjärnan. Antalet dödsfall till följd av CVD är oproportionellt mellan män och kvinnor i olika åldersgrupper. Före klimakteriet dör färre kvinnor än män av CVD, vilket jämnas ut efter klimakteriet. Hos män utvecklas dessutom ateroskleros med större riskprofil, som är känsligare för bristningar och blockerande av blodflöde än ateroskleros hos kvinnor. Orsakerna till skillnaderna är oklara och mer forskning behövs inom området.

Denna avhandling avser att undersöka könsskillnader i ateroskleros utifrån två perspektiv, proteomik och patofysiologi. Proteomik är läran om alla proteiner inom en specificerad cell, vävnad, eller organism, och patofysiologi är läran om fysiologiska processer associerade med specificerad sjukdom eller skada. Avhandlingen utvärderar dessutom potentialen av ett träningsprogram ämnat att reducera CVD-riskprofilen hos kvinnor efter klimakteriet, vilka är inom riskgruppen att utveckla CVD.

Proteomikanalys utfördes på aterosklerotiska karotidartärer från män och kvinnor, där fem biopsier togs från distinkta regioner i varje karotidartärsprov. Proteiner extraherades från biopsierna och klyvdes ner till mindre beståndsdelar som kallas peptider. Dessa analyserades sedan med masspektrometri vilket är en metod som initialt bryter ner proteinerna i mindre fragment och mäter dess massa som summeras i ett massmönster. Då varje protein ger ett specifikt massmönster, görs en efterföljande databassökning som matchar mätresultatets massmönster för att få fram vilka proteiner som finns i provet. Prover från flera regioner i den aterosklerotiska artären tillät identifikation av fler proteiner än vad som gjorts i tidigare studier med liknande metoder. Proteomikanalyserna visade på 16 proteingrupper som urskilde proteinprofilen mellan män och kvinnor. Aterosklerotiska artärer från män hade en större majoritet av proteiner involverade i inflammation, vilka har en nyckelroll i

utvecklingen av ateroskleros. Aterosklerotiska artärer från kvinnor hade en större andel proteiner involverade i blodkoagulering och komplementaktivering, två mekanismer delaktiga i bildning av blodproppar, men även proteiner involverade i transport av olika ämnen i kroppen. Vidare upptäcktes skillnader i proteiner kopplade till ämnesomsättning av järn i kroppen, där utökade experiment genomfördes för att vidare studera skillnaderna. Immunohistokemi är en metod som färgar delar av vävnader för specifika proteiner och som sedan kan studeras i mikroskop. Denna metod användes för att färga in de järnrelaterade proteinerna, ferritin och transferrinreceptor-1, samt makrofager som är en celltyp kopplad till inflammation och sårbarhet i aterosklerotiska artärer. Resultaten av dessa experiment visade att nivåer av järn-relaterade proteiner var högre i aterosklerotiska artärer hos män, jämfört med kvinnor, och att dessa höga nivåer kunde relateras till fler makrofager och stelare aterosklerotiska artärer. Sammanfattningsvis identifierades proteinprofiler där ett flertal proteiner skilde mellan män och kvinnor, vilket tillsammans med patofysiologiska skillnader skulle kunna bidra till vidare förståelse för bildandet av ateroskleros och dess sårbarhet.

Ett träningsprogram tillämpades för att utvärdera dess effekt på att minska risken för CVD i en grupp kvinnor efter klimakteriet. Träningsprogrammet innefattade 15 veckors resistensträning där de inkluderade kvinnorna slumpades in i två grupper. Ena gruppen tilldelades träningsprogrammet i fråga medan den andra blev kontrollgrupp och därmed inte skulle förändra sin fysiska aktivitetsnivå. Blodprover togs vid två tillfällen under försöket, vecka 0 och vecka 15, och analyserades för ett brett urval av markörer, inklusive fetter, järnomsättnings- samt inflammationsmarkörer. Försöket visade att kvinnor som deltog i träningen med god följsamhet, hade kopplade minskade nivåer av fetter, ferritin (ett järntransportprotein) och adipokiner (inflammationsmarkörer från kroppsfett) i blodet

Sammanfattningsvis så bidrar arbetet i denna avhandling till kunskaperna om könsskillnader i ateroskleros och ger även information om potentialen för ett träningsprogram som skulle kunna reducera riskerna för CVD.

LIST OF PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

Paper I

Wenzhao Liang*, **Liam J. Ward***, Helen Karlsson, Stefan A. Ljunggren, Wei Li, Mats Lindahl, & Xi-Ming Yuan.

Distinctive proteomic profiles among different regions of human carotid plaques in men and women. *Scientific Reports*. 2016; 6:26231.

**Authors contributed equally*

Paper II

Liam J. Ward, Patrik Olausson, Wei Li, & Xi-Ming Yuan.

Proteomics and multivariate modelling reveal sex-specific alterations in distinct regions of human carotid atheroma. *Biology of Sex Differences*. 2018; 9:54.

Paper III

Xi-Ming Yuan*, **Liam J. Ward***, Claes Forssell, Nabeel Siraj, & Wei Li.

Carotid atheroma from men has significantly higher levels of inflammation and iron metabolism enabled by macrophages. *Stroke*. 2018; 49:419-425.

**Authors contributed equally*

Paper IV

Liam J. Ward, Mats Hammar, Lotta Lindh-Åstrand, Emilia Berin, Hanna Lindblom, Marie Rubér, Anna-Clara Spetz Holm, & Wei Li.

Resistance training is associated with significantly reduced plasma levels of ferritin and atherogenic lipids, but not oxidative stress markers, in postmenopausal women: a randomised controlled trial. *Manuscript*.

Paper V

Liam J. Ward, Sigrid Nilsson, Mats Hammar, Lotta Lindh-Åstrand, Emilia Berin, Hanna Lindblom, Anna-Clara Spetz Holm, Wei Li, & Marie Rubér

Resistance training is associated with significantly reduced plasma adipokine levels in postmenopausal women: a randomised controlled trial. *Manuscript*.

PAPERS OUTSIDE OF THIS THESIS

Fredrik Iredahl, Veeranjanyulu Satta, **Liam J. Ward**, Johannes Hackethal, Simon Farnebo, Erik Tesselaar, & Folke Sjöberg.

Modeling perfusion dynamics in the skin during iontophoresis of vasoactive drugs using single-pulse and multiple-pulse protocols. *Microcirculation*. 2015; 22:446-453.

Xi-Ming Yuan, Nargis Sultana, **Liam J. Ward**, Bijar Ghafouri, & Wei Li.

Autophagy induction protects against 7-oxysterol induced cell death via lysosomal pathway and oxidative stress. *Journal of Cell Death*. 2016; 9:1-7.

Wei Li, Nargis Sultana, **Liam J. Ward**, Monika Pawlik, Efrat Levy, Stefan Jovinge, Eva Bengtsson, & Xi-Ming Yuan.

Autophagy dysfunction and regulatory cystatin C in macrophage death of atherosclerosis. *Journal of Cellular and Molecular Medicine*. 2016; 20(9):1664-1672.

Liam J. Ward, Stefan A. Ljunggren, Helen Karlsson, Wei Li, & Xi-Ming Yuan.

Exposure to atheroma-relevant 7-oxysterols causes proteomic alterations in cell death, cellular longevity, and lipid metabolism in THP-1 macrophage. *PLoS One*. 2017; 12(3): e0174475.

Sara Oppi, Stefanie Nusser-Stein, Przemyslaw Blyszczuk, Xu Wang, Simone Marzolla, Elena Osto, **Liam J. Ward**, Xi-Ming Yuan, Alaa Othman, Thorsten Hornemann, Zoran Rancic, Stefanie D. Krämer, Dongryeol Ryu, Maaïke H. Oosterveer, Thomas F. Lüscher, & Sokrates Stein.

Macrophage NCOR1 protects from atherosclerosis by blocking the proatherogenic oxLDL-PPAR γ -CD36 pathway. *Manuscript in submission*.

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ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
AT-III	Antithrombin-III
BMI	Body mass index
CCD	Charge-coupled device
CRP	C-reactive protein
CVD	Cardiovascular disease
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FXII	Coagulation factor XII
Hb	Haemoglobin
HDL	High-density lipoprotein
HRP	Horseradish peroxidase
IFN- γ	Interferon- γ
IHC	Immunohistochemistry
IL	Interleukin
IPH	Intraplaque haemorrhage
LC	Liquid chromatography
LDL	Low-density lipoprotein
m/z	Mass-to-charge ratio
MALDI-TOF	Matrix-assisted laser desorption/ionisation time-of-flight
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
MS	Mass spectrometry
nLC-MS/MS	Nano-liquid chromatography tandem-mass spectrometry
NO	Nitric oxide
oxLDL	Oxidised low-density lipoprotein
ROS	Reactive oxygen species
RT	Resistance training
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Serpin	Serine protease inhibitor
SHBG	Sex hormone binding globulin
SMC	Smooth muscle cell
sPLA ₂ -IIA	Phospholipase A ₂ , membrane associated
TfR1	Transferrin receptor 1
TNF- α	Tumour necrosis factor- α

INTRODUCTION

Cardiovascular disease

Cardiovascular disease (CVD) relates to all diseases of the heart and blood vessels, with the etymology deriving from the Greek word “*kardia*” and Latin word “*vascularis*”. CVD encompasses a variety of diseases originating from the heart, including; heart failure, valvular heart disease, cardiac dysrhythmias, etc., and the blood vessels, including; coronary/peripheral artery disease, cerebrovascular disease, aortic aneurysm, etc.

In 2016, it was estimated that 17.8 million people died from CVD, 31% of all deaths, making it the greatest cause of death globally.¹ The two most common causes of death, and in turn common CVDs, are ischaemic heart disease (colloquially: “*heart attack*”) and stroke accounting for 16% and 10% of deaths, respectively.¹ CVD disproportionately affects low- and middle-income countries, accounting for more than 75% of CVD deaths. In high-income countries, such as those in Western Europe and North America, CVD mortality has declined, however, it still remains the most common cause of death.^{1,2}

This thesis, and the accompanying works, were produced in Sweden. Thus, specific figures relating to CVD mortality in Sweden are as follows: In 2016, it was estimated that 31.6 thousand people died from CVD, 34% of all deaths. As with the global trend, CVD is the greatest cause of death in Sweden.¹

Risk factors

There are several risk factors associated with the development of CVD that have been studied extensively in epidemiology. The Framingham Heart Study, commissioned in 1948, was one of the largest cardiovascular epidemiology studies and has contributed to some of the most significant insights to CVD risk factors. This study first identified major CVD risk factors such as high blood cholesterol, high blood pressure, smoking, obesity, physical inactivity, and diabetes.³ Recently, the

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INTERHEART case-control study, which included over 29,000 participants from 52 countries across all inhabited continents, investigated the relationship between myocardial infarction and several risk factors.⁴ The INTERHEART results provided evidence that smoking, abnormal lipids, hypertension, diabetes, abdominal obesity and psychosocial factors were positively associated with the risk of myocardial infarction, where fruit/vegetable consumption and physical activity were negatively associated with risk of myocardial infarction. These associations were found in both men and women, irrespective of age, across all regions of the world.⁴

Sex differences

In social terms, CVD has always been thought to be the disease of men. Though factually incorrect, this misconception is rooted in the incidence of CVD across the population at different ages. In general, CVD presents itself in men at a younger age than women. The global CVD mortality for persons aged 30-59 years shows a distinct bias with an approximate 65:35 ratio between men and women.¹ However, when reassessing the CVD mortality in the older population, 60+ years, this bias somewhat balances out at an approximate 50:50 ratio between men and women (Figure 1).¹

Figure 1 | Cardiovascular disease (CVD) mortality ratios by age and sex – global 2016

Global 2016 mortality rates for CVD (all), and the two most common CVDs; ischaemic heart disease and stroke. Ratios divided by sex, and by the two age groups with the greatest CVD mortality. Data for these figures were obtained from the World Health Organisation.¹

Atherosclerosis

Atherosclerosis is the leading cause of CVD, including coronary artery disease, cerebrovascular disease, and peripheral vascular disease. The term atherosclerosis comes from the Greek words “*athere*” and “*sclerosis*” meaning gruel and hard, respectively, and was originally coined by German pathologist Félix Marchand in 1904.⁵ Atherosclerosis describes the thickening and hardening of arteries, which over time can lead to reduced and/or restricted blood flow. In simple terms: arterial thickening occurs from the build-up of a plaque in the intima of the arterial wall, which then gradually expands narrowing the arterial lumen. In advanced cases, plaques can rupture into the lumen causing thrombosis, blood clot formation, occluded the artery and restricting blood flow (Figure 2).

Figure 2 | The atherosclerotic plaque.

Illustration depicting the progression of plaque formation in the artery. (Top) Normal artery, with normal blood flow; (Middle) Atherosclerotic artery with plaque build-up, causing a reduced blood flow; (Bottom) Atherosclerotic artery with a ruptured plaque, causing thrombosis and blocking blood flow.

Atherogenesis

The process in which an atherosclerotic plaque forms within a blood vessel is called atherogenesis. Atherosclerosis is multifaceted, with many factors contributing to the initiation and progression of atherogenesis; however, one key component is common throughout the process: inflammation. Atherosclerosis is a progressive inflammatory disease of the vessel wall.⁶ Inherently, inflammation involves both branches of the immune system, innate and adaptive immunity, and both are involved, in some capacity, in atherogenesis.⁷

One of the most well-studied theories in atherogenesis is the ‘response-to-injury’ hypothesis, initially developed by Russell Ross, proposing that atherogenesis starts from a point of activation and injury/dysfunction of the endothelial cell layer on the inner side on the blood vessel.^{6, 8} The injury to endothelial cells can derive from either mechanical, physiological, or immunological causes, such as laminal sheer stress, exposure to CVD risk factors (e.g. free radicals), or infection, respectively. These disturbances can activate the endothelial cells inducing a phenotype promoting the expression of proinflammatory cytokines, adhesion molecules, and chemoattractants.⁹ Activation implies resolution, which is possible, however, in the event of uncontrolled or prolonged endothelial cell activation this can lead to cell apoptosis (irreversible damage) or dysfunction (increased permeability).¹⁰ In both instances, increases in vascular cell adhesion molecule-1 (VCAM-1) promotes the recruitment of circulating immune cells to the endothelium.¹⁰ Recruited immune cells, monocytes and T-cells, migrate from the endothelium to the intima under the stimulation of chemoattractants, such as monocyte chemoattractant protein-1 (MCP-1) and interferon-inducible T-cell chemoattractant (ITAC).^{11, 12}

Monocytes and differentiated macrophages, are innate immune cells that play pivotal roles in atherogenesis (Figure 3). Monocytes that migrate into the intima of the vessel wall differentiate into macrophages, which have been seen to accumulate in human atherosclerotic lesions.¹³⁻¹⁵ Herein, macrophages express scavenger receptors, such as macrophage scavenger receptor types I and II (MSR1), scavenger receptor class B member 1 (SRB1), and cluster of differentiation 36 (CD36), amongst others.¹⁶ Scavenger receptors recognise various forms of lipoproteins that are subsequently engulfed by macrophages. MSR1 is highly expressed in tissue macrophages and has a high affinity for modified forms of low-density lipoprotein (LDL), such as oxidised-LDL (oxLDL) or acetylated-LDL.¹⁷ SRB1 and CD36 also have the capacity to bind oxLDL, as well as other lipoprotein fractions such as high-density lipoprotein (HDL) and very-low-density lipoprotein (VLDL).¹⁶ Accumulation of oxLDL then occurs inside the macrophages as the proinflammatory microenvironment of the atherosclerotic lesion inhibits lipid efflux.¹⁸ This accumulation results in a

Figure 3 | The pivotal role of macrophages in atherogenesis

Schematic overview of atherogenesis within the intima layer of arterial walls. Initiating from monocyte recruitment, infiltration, and maturation to macrophages with the uptake of modified/oxidised-LDL (mod-LDL) to form foam cells. Foam cell accumulation and apoptosis forms the necrotic core, with a fibrous cap made from collagen fibres and migrating vascular smooth muscle cells (VSMC). MCP-1: monocyte chemoattractant protein-1, VCAM-1: vascular cell adhesion molecule-1, VEGF: vascular endothelial growth factor, M-CSF: macrophage colony-stimulating factor, MMP: matrix metalloproteinases

morphological change of macrophages to foam cells, which are unable to leave the intima and further contribute to inflammation in the atherosclerotic plaque. The build-up of these lipid-laden foam cells in the intima produces the first visible sign of atherosclerosis – a fatty streak, a white/yellow discolouration of the vessel wall. Though macrophages are the most studied population of cells known to develop into foam cells, it must also be noted that foam cells from non-myeloid origins, such as endothelial and smooth muscle cells (SMC), have also been found in atherosclerotic lesions.¹⁹ In normal conditions, foam cells would undergo cell death, and resulting debris would be cleared from the environment via efferocytosis. In advanced atherosclerotic lesions, this efferocytosis is impaired, due to the increasingly inflammatory microenvironment, resulting in apoptotic foam cells undergoing secondary necrosis.²⁰ Macrophages, SMC, and other cells types have been demonstrated to undergo apoptosis in atherosclerotic lesions.^{14, 15, 21, 22} Apoptotic or post-apoptotic necrotic foam cells release a number of inflammatory cytokines and growth factors, creating a feedback loop perpetuating inflammation and foam cell formation, in addition to the release of matrix metalloproteinases (MMP) and lysosomal cathepsins that degrade the extracellular matrix.^{15, 22-25} These processes ultimately lead to the formation of a necrotic core in an atherosclerotic lesion.

The necrotic core constitutes the major component of advanced atherosclerotic plaques, which grows over time, thickening the vessel wall and restricting the blood flow. Necrotic core formation represents irreversible changes to the normal structure of the intima with the degradation of extracellular matrix (ECM) and deposition of lipids and cell debris, termed “gruel”.²⁶ As the necrotic core expands and the intima thickens, SMC migrate from the media layer of the vessel to the top of the necrotic core and, in combination with a collagenous matrix, forms a fibrous cap over the necrotic core. This fibrous cap hardens and helps to stabilise the atherosclerotic plaque from rupture into the lumen of the vessel.

In vulnerable atherosclerotic plaques, this fibrous cap is thinned by proteolytic enzymes released by foam cells, such as cathepsins and MMPs. Additionally, in atherosclerotic lesions, there are pronounced areas of hypoxia, specifically around the

necrotic core. These areas of hypoxia can stimulate the release of hypoxia-induced factor 1 α (HIF1 α).²⁷ HIF1 α can stimulate angiogenesis of the *vaso vasorum*, small vessels that supply blood to the artery wall towards the hypoxic areas of the atherosclerotic plaque.²⁸ As described, the atherosclerotic microenvironment is highly inflammatory and has a high level of proteolytic activity; such new vascularisation is prone to rupture causing intraplaque haemorrhage (IPH) – a risk factor for plaque destabilisation and rupture.^{27, 28}

Plaque vulnerability

Atherosclerosis is a chronic progressive disease that can originate from adolescence at a subclinical level through to mid-life and beyond where it may present clinically via CVD. Lesions are nominally classified in terms of lipid accumulation in the early stages (types I-III) and then by plaque vulnerability in the latter stages (types IV-VI). Type I lesions, which can occur from adolescence, comprise of isolated macrophage foam cells within the vessel wall. Type II lesions are characterised by the formation of fatty streaks. Type III lesions are characterised by the presence of fatty streaks with small extracellular lipid pools. Type IV lesions are the first type with a pronounced extracellular lipid/necrotic core. Type V lesions develop a fibrous cap above the lipid/necrotic core. Type VI lesions are complicated lesions that may contain IPH and/or have ruptured.²⁹

As implied with the classification, the higher the classification the greater vulnerability the atherosclerotic plaque has to rupturing, potentially causing a catastrophic event such as myocardial infarction or stroke. There are a number of determinants that affect the vulnerability of the atherosclerotic plaque, with major ones including: the level of inflammation, the thickness of the fibrous cap, and the level of proteolytic enzymes.³⁰ These determinants are very much intertwined when describing plaque vulnerability. Inflammation is the driving force of atherogenesis, aptly described above, and as such, a greater inflammatory profile propagates the recruitment of immune cells to the atherosclerotic microenvironment. Activated immune cells, such as neutrophils and macrophage foam cells, as well as SMC release proteolytic enzymes into the microenvironment. The increasing proteolytic activity in the plaque serves to

degrade the ECM and thin the fibrous cap, leading to plaque vulnerability. Several proteolytic enzymes are found present at increased levels in atherosclerotic plaques, of which MMPs are some of the most studied. In healthy arteries, there is little or no MMP activity, whereas in arteries with atherosclerosis MMP activity is present. In vulnerable plaques, the activity of MMP-8 and MMP-9 are significantly greater than in stable plaques.^{31, 32} MMP-8 and -9, which degrade gelatin and collagen fibres, have been shown to have predictive power for cardiovascular events in population-based studies.³³⁻³⁶

In addition, the role of iron and associated oxidative stress has been implicated in atherosclerotic plaques.³⁷⁻⁴¹ Free iron, and other transition metals, can catalyse the formation free radicals, including reactive oxygen species (ROS) production.⁴² Free radicals have one or more unpaired electrons and have an increased reactivity, which is a risk factor for atherosclerosis.⁴³ Iron is found in numerous molecules biologically, notably the haem-proteins – haemoglobin, hemopexin, haptoglobin – and iron transport proteins – ferritin and transferrin.⁴⁴ Within the proteolytic environment of the atherosclerotic plaque these proteins can be broken down, releasing free iron with the potential to increase oxidative stress. Increased ROS levels stimulate a number of mechanisms in atherogenesis, including; endothelial dysfunction, oxidation of LDL, immune cell migration and differentiation, and cathepsins and MMP release.^{15, 22, 24, 45}

Plaque rupture and thrombus formation

Key determinants of plaque rupture include a thin-fibrous cap, a large necrotic core, IPH, inflammatory cell infiltration, and low SMC density in the fibrous cap.^{46, 47} Plaque rupture exposes the underlying vascular and necrotic core elements to the blood stream, which stimulate the recruitment of platelets and also activates a protein called tissue factor.^{48, 49} Platelets adhere to molecules exposed by rupture, such as collagen, and become activated. Activated platelets in turn release molecules to facilitate platelet aggregation and activation of circulating coagulation factors. Activated coagulation factors perpetuate the coagulation cascade, of which tissue factor is the first step of the extrinsic pathway (Figure 4).⁴⁹ The coagulation cascade ends with thrombin catalysing the conversion of fibrinogen to fibrin, which constitutes the matrix for the formation of

a thrombus.⁴⁹ Moreover, thrombin creates a feedback loop with a number of coagulation factors in the intrinsic pathway (Figure 4). The formation of a thrombus at the site of plaque rupture poses a severe risk for blocking blood flow or breaking away and blocking blood flow downstream in a smaller vessel, potentially causing an ischaemic event like myocardial infarction or stroke.

Figure 4 | The coagulation cascade

The extrinsic and intrinsic pathways of the coagulation cascade. The coagulation cascade is primarily made up from a number of coagulation factors, denoted by their respective roman numeral and upon factor activation is suffixed with an 'a'.

Sex differences

As mentioned previously, there are distinct sex differences in the mortality rates of atherosclerosis-related CVD in the population. In 1981, Jerome L. Sullivan presented the “iron hypothesis” that hypothesised that men and postmenopausal women were at greater risk of CVD due to higher levels of stored iron, compared to premenopausal women.⁵⁰ Moreover, distinct sex differences have also been observed in the development and pathophysiology of atherosclerotic plaques. The PROSPECT study,

evaluating plaque morphology in coronary artery disease, observed that plaques from women had less plaque rupture with smaller necrotic cores than men.⁵¹ Additionally, analysis of carotid IPH showed an age-specific sex difference in that men are more likely to have IPH at younger ages than women, albeit with increasing age post-menopause the likelihood of IPH equalises between the sexes.⁵² Moreover, IPH is a risk factor for the destabilisation of atherosclerotic plaque providing an increase in the availability of iron.

Proteomics in atherosclerosis research

Proteomics is the study of the entire complement of proteins expressed within an organism, tissue, or cell – both qualitatively and quantitatively.⁵³ Proteomics is to transcriptomics and genomics, as proteins are to RNA and DNA. The proteomics field has rapidly grown since the mid-1990s and is now a staple field of research used in molecular biology.

“Proteins are central to our understanding of cellular function and disease processes, and without a concerted effort in proteomics, the fruits of genomics will go unrealized”

– Ian Humphrey-Smith, co-founder of HUPO.⁵⁴

Proteomics has been shown to be a powerful tool in the analysis of proteins within atherosclerotic lesions. A variety of proteomics techniques have been used, from conventional gel-based studies, including two-dimensional electrophoresis (2-DE) and western blot,⁵⁵⁻⁵⁷ to more complex mass spectrometry (MS) techniques, including tandem-mass spectrometry.⁵⁸ Exploiting a combination of these techniques has also been used to gain a comprehensive overview.^{59, 60} Recently, with the development of proximity extension assay proteomic chips, based on antigen-antibody reactions, have also been implemented for analysis in a large cohort to facilitate high-throughput.⁶¹ Detailed information on the type of proteomic methodologies used in this thesis are included in the Comment of Materials and Methods section.

Different approaches have been taken for the proteomic analysis of atherosclerosis in humans. A number of studies have focussed on the tissue protein profiling of atherosclerosis, increasing the number of known proteins in plaques.^{58-60, 62} Other studies have investigated the differences between plaque types, from early/advanced plaques and non-complicated/complicated plaques.^{57, 63-65} Additionally, studies have analysed the plaque secretome, by culturing plaques in media, to investigate the potential of identifying circulating biomarkers that are sourced from plaques.⁶⁶⁻⁶⁸

The use of proteomics in CVD and atherosclerosis research has been increasing over the past decade. A number of studies have used proteomics to investigate sex differences and overall CVD risk, mostly from biological fluids, such as plasma/serum and urine.⁶⁹ In parallel, advancements in the proteomic profiling of atherosclerotic tissue have also occurred.⁶² However, as aptly reviewed, studies have yet to focus on identifying sex differences in the atherosclerotic plaque tissue proteome, until “now” (**Paper I**).⁶⁹

Exercise effects on atherosclerosis

Lack of physical activity, and the adoption of a sedentary lifestyle, is a major modifiable risk factor for the development of CVD.^{3, 4} Physical inactivity leads to an increase in visceral fat accumulation and activation of inflammatory and oxidative stress pathways, all of which are risk factors for CVD, atherosclerosis and metabolic disorders.^{70, 71} Globally, according to the world health organisation (WHO), it is estimated globally that approximately 23% and 32% of adult (18+) men and women are insufficiently active, respectively.⁷² In general, physical activity has long been accepted as beneficial to maintaining good health and has shown to be a useful tool in both primary and secondary interventions of CVD.⁷³ Moreover, physical activity is one of the most cost-effective strategies of intervention.⁷⁴

In Sweden, it is estimated that approximately 23% and 27% of adult men and women are insufficiently active, respectively.⁷²

Exercise intervention studies rely heavily on the analytical measurements of circulating markers in the blood and the measurement of physical body parameters. Various circulating markers are implicated in disease mechanisms and physical body parameters, such as blood pressure, weight, body mass index (BMI) etc., can be used as a measure of overall health. Exercise-induced changes in circulating marker and/or physical body parameters can be indicative of potential changes in disease processes in the body.

Exercise has been shown to exert beneficial effects on molecular and cellular processes that are central in atherogenesis and plaque vulnerability, including inflammation, endothelial cell dysfunction, and macrophage foam cell formation. Exercise-induced regulation of inflammatory cytokines is a well-studied area. As comprehensively reviewed, exercise induces reductions in circulating inflammatory cytokines and chemokines, such as tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin-6 (IL-6), MCP-1, as well as c-reactive protein (CRP), in patients with atherosclerosis-related CVD.⁷⁵ Exercise is a promising therapy for endothelial cell dysfunction by promoting nitric oxide (NO) bioavailability. NO bioavailability is linked with injury to the endothelial cell layer in blood vessels at the early stage of atherogenesis and NO bioavailability is impaired with increasing levels of ROS and oxLDL.⁷⁶ Macrophage foam cell formation can be indirectly reduced by the effects of exercise, which can promote the clearance of cholesterol, including LDL, to the liver.⁷⁷

The type of exercise used as an intervention can also exhibit different outcomes on patient health in a trial. In general, both moderate aerobic and strength/resistance training (RT) are seen to be beneficial in reducing the circulating inflammatory profile and have been advised to alleviate CVD risk factors.^{78, 79} However, intensive prolonged aerobic exercise can be detrimental in increasing the circulating inflammatory profile.⁸⁰ The type and duration of exercise is therefore an important consideration in exercise intervention.

AIMS

Papers I & II

To investigate the proteomes from distinct regions of human carotid atherosclerotic plaques and identify protein alterations in relation to sex and plaque region.

Paper III

To investigate inflammation and iron metabolism in human atherosclerotic plaques and how these differ between sexes.

Papers IV & V

To investigate if supervised resistance training amongst postmenopausal women can alter cardiovascular disease risk markers, including; body iron status, oxidative stress, lipid profiles, and inflammation, adipokine and immune markers.

COMMENTS ON MATERIALS AND METHODS

Patient cohorts

Linköping Carotid Study (Papers I-III)

The Linköping Carotid Study is a prospective clinical pathology study in which carotid atherosclerotic plaques are collected from patients undergoing carotid endarterectomy at Linköping University Hospital. The study was approved by the Regional Ethical Review Board, Linköping, Sweden (2003-449) and all procedures were carried out in accordance with accepted guidelines. Written informed consent was obtained from all patients included in this study.

Proteomic analyses carried out in **Papers I and II** used carotid endarterectomy samples from 26 patients, 13 men and 13 women. In **Paper I**, samples from 3 men and 3 women were used for two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis. In **Papers I-III**, samples from 10 men and 10 women were used for nano-liquid chromatography tandem MS (nLC-MS/MS; hereafter referred to as ‘tandem-MS’). In **Paper III** carotid endarterectomy samples from 62 consecutive patients, 39 men and 23 women, were used for immunohistochemistry (IHC) analyses.

In proteomic analyses for **Papers I and II** carotid endarterectomy samples were further processed to excise predefined regions of interest, including: internal control, fatty streak, plaque shoulder, plaque centre and fibrous cap (Figure 5). These intra-lesion biopsies were taken to reduce the effect of heterogeneity in atherosclerotic tissues. Additionally, we hypothesised that using such intra-lesion biopsies would provide insights into protein localisation, abundances, and in turn could potentially shed light on functional implications in atherogenesis and plaque vulnerability. From each patient, all four intra-lesion regions and internal control were taken from the same carotid artery. These biopsies were used for 2-DE and MALDI-TOF MS analysis, and three intra-lesion regions were used to tandem-MS analysis (internal control, fatty streak and plaque centre).

Figure 5 | Carotid endarterectomy sampling

Whole carotid endarterectomy samples (above) were processed and intra-lesion biopsies were taken from 5 predefined regions of interest (below): (1) internal control, (2) fatty streak, (3) plaque shoulder, (4) plaque centre, and (5) fibrous cap.

Strength “Styrka” Study (Papers IV-V)

The Strength (Swedish: “Styrka”) Study is a registered clinical trial (clinicaltrials.gov identifier: NCT01987778) conducted at Linköping University Hospital, Sweden. The study was approved by the Regional Ethical Review Board, Linköping, Sweden (2013/285-31 and 2013/338-32) and all procedures were carried out in accordance with accepted guidelines. Written informed consent was obtained from all participants prior to entry to the trial.

The Strength Study is an open, parallel, randomised controlled intervention trial where healthy postmenopausal women ($n = 65$) were recruited to take part in a 15-weeks exercise programme. Women were randomised to join a control group with unchanged physical activity, or a resistance training (RT) intervention group. The exercise programme was based on supervised RT targeting all major muscle groups, utilising either seated resistance machines or body-weight exercises. A full description of the trial design, inclusion- and exclusion-criteria and resistance training programme can be found in the paper by Berin, et al.⁸¹ Venous blood samples were taken at week-0 and week-15 of the trial period. Plasma was isolated from samples and stored at -70°C for investigations.

Proteomics

Protein extraction (Paper I-III)

The first step in the proteomic workflow is to obtain a protein extract that is suitable for experimentation. Protein extraction methods can be used on a wide variety of samples, including; plasma, cells, and tissues. The method of protein extraction used in **Papers I-III** was a liquid-phase separation using the acid guanidinium thiocyanate-phenol-chloroform extraction; commonly known by the brand names TriZol (Invitrogen, UK) or TRI reagent (Sigma-Aldrich, MA, USA). This method was first developed for the extraction of RNA but is also suitable for the isolation of DNA and proteins in the same procedure.⁸²

Two-dimensional gel electrophoresis (Paper I)

Two-dimensional gel electrophoresis (2-DE) is a method of protein separation, used prior protein identification techniques, that is widely used for proteome analyses.⁸³ Indicative of its name, 2-DE combines separation in two dimensions; separating by isoelectric point, the pH where a molecule carries a net electrical charge of zero, and by molecular weight (Figure 6). The first dimension, called isoelectric focussing (IEF), uses an immobilised pH gradient (IPG) gel strip that is rehydrated with solution containing denatured proteins. Once electricity is applied to the strip, the proteins migrate towards their respective isoelectric points.⁸⁴ The second dimension, separation by molecular weight, uses sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The IPG strips from the first dimension are transferred onto a polyacrylamide gel and subjected to an electrical current. This allows for the separation of proteins according to size, as lower molecular weight proteins will migrate through the polyacrylamide gel faster than higher molecular weight proteins.

Figure 6 | Two-dimensional gel electrophoresis.

Representation of the principles of 2-DE. Proteins are first separated by isoelectric focussing (IEF) on immobilised pH gradient (IPG) strips. Proteins are then transferred to polyacrylamide gels for a second separation according to molecular weight (SDS-PAGE). The resulting gels can be stained for proteins, and relative amounts of proteins can be determined.

SDS-PAGE gels can then be stained to visualise the protein separation. Several staining procedures are available that vary with sensitivity, silver staining is relatively sensitive,⁸⁵ whereas fluorescent stains have a broader sensitivity.⁸⁶ 2-DE experiments in **Paper I** used the SYPRO Ruby fluorescent staining procedure.⁸⁷ The fluorescent intensities of the protein staining are proportional to the relative protein abundance.

MALDI-TOF mass spectrometry (Paper I)

Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (MS) is a method used for protein identification and is commonly used downstream of 2-DE. The development of this technique led to the Noble Prize in Chemistry 2002 being awarded, in part, to engineer Koichi Tanaka.⁸⁸

Figure 7 | MALDI-TOF mass spectrometry

Schematic representation of a MALDI-TOF mass spectrometer. Peptide-matrix samples are spotted onto the target plate. Laser pulses evaporate and ionise into the electrostatic field accelerating them through the flight tube to the detector. The travel-time from laser pulse to detector corresponded to peptide size and is used to calculate the mass-to-charge ratio (m/z).

Separated protein spots are excised from 2-DE gels, de-stained, and exposed to an endopeptidase (e.g. trypsin) to digest proteins in preparation for MS analysis. The resulting peptides are combined with an acidic matrix solution. Common matrix solutions include α -cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid, the latter matrix performs better for low-abundant proteins from 2-DE.⁸⁹ The peptide-matrix mixture is then spotted onto a target plate where the mixture dries forming crystalline structures. In the mass spectrometer, these crystals are hit by a laser and absorb energy, this subsequently leads to the evaporation and ionisation of singly-charged peptides in the matrix. An electrostatic field accelerates these charged peptides through the flight tube, in a vacuum, to a detector (Figure 7). Herein, only the peptide mass can affect the travel-time, which is used to calculate the mass-to-charge ratio (m/z) that corresponds to the peptide size.⁹⁰ The MALDI-TOF MS spectrum can be used to generate a peptide mass list that is subjected to database searching, called peptide-mass fingerprinting, which results in protein identification.

Tandem-mass spectrometry (Papers I-III)

The use of nano-liquid chromatography tandem-MS (nLC-MS/MS, herein referred to as tandem-MS), was used in **Papers I-III**. We have specifically used a label-free workflow that is widely used and recommended for potential biomarker discovery in proteomic studies.^{91, 92} The method consists of two major systems; a liquid chromatography (LC) system, and a hybrid-MS system containing both a linear ion-trap and an Orbitrap (orbital ion trap).

In preparation for tandem-MS, protein samples are reduced, alkylated, and digested with the use of dithiothreitol, iodoacetamide, and an endopeptidase (e.g. trypsin) respectively. The resulting peptide mixtures are lyophilised and reconstituted in an acidic solution, in this case 0.1% formic acid, that aides in ionisation. These samples are then loaded into the LC system in a reverse-phase LC column, which separates the peptide samples by size and hydrophobicity, via an increasing organic solvent concentration gradient. The peptides are then introduced to the hybrid-MS system via electrospray ionisation, displacing ionised peptides from a liquid to a gaseous state. The hybrid-MS system comprises of a linear-ion trap and an Orbitrap. The initial scan of peptides occurs in the Orbitrap, with high resolution, of which the most intense ions will be selected for fragmentation. These precursor ions are fragmented in the linear ion trap via collision-induced dissociation, which collides ions with an inert gas whereby the kinetic energy is converted to internal energy that fragment the amino acid backbone.⁹³ The fragments are then analysed according to their respective m/z , which can then be compared to the theoretical MS data.

Multivariate statistical modelling (Paper II)

Proteomics, like the other -omics methodologies, can produce large datasets from a low number of subjects. It is becoming more apparent that complex conditions and diseases do not occur from alterations in a single biomarker, but more likely the result of alterations in multiple biomarkers. Traditional univariate statistics compares changes between individual variables, assuming independence from one another, and disregarding any potential interrelationships.⁹⁴ Conversely, multivariate statistical

modelling, based on component analyses, is capable of handling interrelated variables more akin to biological systems.⁹⁵ Moreover, multivariate statistical modelling is more appropriate to handle such datasets comprised of low subject-to-variable ratios, reducing the number of false positives without the loss of statistical power.⁹⁵

An example of a multivariate model is orthogonal partial least squares-discriminant analysis (OPLS-DA) (**Paper II**), which can be used to predict which variables are responsible for discrimination between two predefined groups. OPLS-DA indicates what variables drive the differences between the groups, with each variable being assigned a ‘variable influence on projection’ (VIP) value. The greater the VIP value, the greater the influence on the group discrimination.^{95, 96} These types of multivariate models are useful in reducing such large datasets to so-called biomarker “fingerprints” that can be further explored in more detail.⁹⁶

Immunohistochemistry (Papers I & III)

Immunohistochemistry (IHC) is a method to visualise the distribution of specified proteins within a tissue. IHC is based on an antigen-antibody reaction that can produce a visible staining on a tissue section (Figure 8). In principle, a primary antibody for a specific antigen of interest is used, then a secondary antibody conjugated to an enzyme for chromogen conversion. A commonly used enzyme is horse-radish peroxidase (HRP) that catalyses the oxidation of 3',3'-diaminobenzidine (DAB), a common chromogen, to produce a brown pigmentation.⁹⁷ The resulting pigmentation can be examined via light microscopy and images digitised. For quantification of IHC, a percentage of total pixel area can be calculated by recording both positive- and negative immunostained pixels for each image. All histological samples should be assessed by at least two observers, who are blinded to the clinical characteristics and classifications of the samples.

Figure 8 | Antigen-antibody reaction

Sample antigens are immobilised to a solid support, or present on a tissue sections, and are exposed to a specific primary antibody. A complementary horseradish peroxidase (HRP)-conjugated secondary antibody is then incubated to bind. The HRP enzyme is used to catalyse a substrate or chromogen to produce a light or staining that can be measured.

Western blot (Paper II)

Western blot is comparable to the second-dimension separation in the 2-DE methodology with the inclusion of an antigen-antibody reaction (Figure 8).⁹⁸ Protein samples are denatured, and negatively charged, via boiling in an SDS-containing running buffer. The samples are then separated by molecular weight by SDS-PAGE and transferred to a membrane for antibody probing. Membranes are blocked, with buffers containing non-fat milk or bovine serum albumin, to prevent non-specific binding prior incubation with the primary antibody. As with the previously described IHC methodology, the principle of the antigen-antibody reaction is the same; incubation with the primary antibody, and then incubation with a secondary antibody conjugated to HRP (Figure 8). In Western blot, HRP catalyses the oxidation of luminol in enhanced chemiluminescence solution, the result of which emits light that can be imaged using a charged coupled device (CCD) camera.⁹⁹ Western blot, much like 2-DE, is merely a semi-quantitative method in that relative values can be deduced from measures of optical density.⁹⁸ Herein, however, this method was used only as a qualitative measure of protein expression.

Enzyme-linked immunosorbent assay (Papers IV & V)

As with the prior methods, IHC and Western blot, enzyme-linked immunosorbent assay (ELISA) runs on the premise of an antigen-antibody reaction. ELISA can be run on protein extracts and/or biological fluids, run within a microtiter plate, and is based on colourimetry. Multiple types of ELISA methods exist, including: direct, sandwich, reverse, indirect, and competitive, the latter two types were used in **Papers IV and V**.

Figure 9 | Enzyme-linked immunosorbent assay (ELISA)

Representation of the two ELISA methods used in **Papers IV and V**. Indirect ELISA is a dual antibody method, an antigen-specific primary antibody and a complementary enzyme-conjugated secondary antibody. Competitive ELISA utilises two antigens, the native sample antigen and a known amount of enzyme conjugated-recombinant antigen, to compete to bind the primary antibody. Enzyme substrate is then added to produce a blue precipitate and stop solution to turn yellow, which can be measured spectrophotometrically.

The indirect ELISA method is based on the more conventional antibody-antigen reaction, as previously described in the Western blot method. Antigens, from standards and samples, are coated onto the microtiter wells. A specified primary antibody is added, followed by the addition of an enzyme-conjugated secondary antibody (Figure 9). Competitive ELISA, as the name suggests, is based on a competitive antigen-antibody reaction. Initially, the microtiter plate is coated with the specified primary antibody. Then the sample, with the native antigen of interest, and enzyme-conjugated

recombinant antigen, the competing antigen, are added to the microtiter wells. As a known amount of recombinant antigen is added to each microtiter well, the amount of native antigen in the sample will determine the binding ratio between native and recombinant antigen. After incubation, all unbound antigens are washed away. To measure the level of antigen-antibody complexes in microtiter wells an enzyme substrate is added, 3,3',5,5'-tetramethylbenzidine (TMB) is used for HRP enzyme-conjugates, the results of which produces a blue coloured precipitate. The enzyme-substrate reaction is stopped with the addition of an acidic stop solution that turns the blue precipitate yellow, which can be measured spectrophotometrically at 450 nm (Figure 9).¹⁰⁰ The light absorbance, measured in optical density values, is equivalent to the concentration of the molecule of interest and can be calculated from a known standard curve.

Multiplex bead array (Paper V)

Multiplex bead array, commercially and commonly referred to as Luminex, is an immunoassay method with similar reaction principles to ELISA methodology. The major difference is that Luminex is a bead-based immunoassay, that can simultaneously measure multiple analytes in a single sample. The principle is that capture antibodies are coated to either polystyrene or magnetic beads that are dyed to distinct spectral regions. As each set of beads is uniquely dyed, different beads can be coated with different antibodies and mixed together to add to each microtiter well, thus allowing for multiple analytes to be measured simultaneously. To analyse the antibody-conjugated beads that have bound antigens, a biotin-conjugated secondary antibody is added. Streptavidin-phycoerythrin is used to detect biotinylated antibody complexes, which when run through a dual-laser detection system is excited and induces a light emission. The concentration of analytes can be calculated using a standard curve of known concentrations.

Statistical analyses (Papers I-V)

The Shapiro-Wilk test (**Papers I-V**) is a test of normality to analyse distribution amongst continuous variables. Shapiro-Wilk test was used as a prerequisite to other statistical tests used for continuous variables. The null hypothesis for the Shapiro-Wilk test is that data are normally distributed. Should the p-value be less than the chosen alpha level, e.g. 0.05, then the null hypothesis is rejected, and the data are non-normally distributed. Non-parametric tests, that do not assume normal distribution, are therefore preferred for further analyses.

Mann-Whitney *U* test (**Papers I-V**), Wilcoxon signed rank test (**Papers IV-V**), and Spearman's rank correlation (**Papers III-IV**) are all examples of non-parametric statistical analyses used within this thesis. Mann-Whitney *U* and Wilcoxon signed rank tests both compare the differences in distribution between two samples, evaluating differences between independent or paired samples, respectively. Spearman's rank correlation is used to assess the relationship between the rankings of two variables within a sample set.

Chi-squared test (**Papers I and III**) is a statistical analysis applied to categorical/nominal variables. Chi-squared is used to evaluate if differences between variables occur by chance or not.

RESULTS AND DISCUSSION

This section will present results and discuss them from **Papers I-V** that build the foundation of this thesis. Herein, only selected results are highlighted, complete results and discussions can be found in the original papers.

“Deconstructing” the atheroma proteome (Paper I)

The impact of plaque heterogeneity on the atheroma proteome has yet to be fully understood. Thus, in **Paper I**, one aim was to increase the amount of information obtained from proteomic analysis by attempting to reduce the effect of plaque heterogeneity. Deconstructing the carotid atheroma, by taking intraplaque biopsies, and analysing them separately allowed for more information to be obtained from proteomic analyses – as established in the 2-DE protein mapping presented in **Paper I**. The sampling method allowed for the mapping of 15 proteins (Table 1 and Figure 10) that had not previously been mapped before, by 2-DE and MS, in comparison to previous protein maps of carotid atherosclerotic plaques.^{55, 59, 60, 62, 101, 102}

Table 1 | Newly mapped proteins in relation to carotid atherosclerotic plaque.
Following a literature review of previous studies utilising 2-DE/MS on carotid atherosclerotic lesions, it was found that 15 protein identities had not previously been reported.

Spot No.	Protein name	UniProt Accession No.
21	Serpin B12	Q96P63
23	EH domain-containing protein 4	Q9H223
24	Procollagen C-endopeptidase enhancer 1	Q15113
33-36	Fibrinogen beta chain fragment	D3DP13
38-42	Biglycan	P21810
49-51, 61	Rab-39	Q14964
56	ADP-ribosylation factor-like protein 15	Q9NXU5
60	IGK@ protein	Q6PJF2
63, 84	Rab-35	Q15286
64	Synaptophysin-like protein 2	Q5VXT5
65-66	Hepatoma-derived growth factor	P51858
85	Calmodulin	P62158
88	SH3 domain-binding glutamic acid-rich-like protein	O75368
90	Synoviolin	E9PN88
91	Protein S100-A11	P31949

Figure 10 | Newly mapped proteins in a 2-DE protein map of carotid atherosclerosis.
2-DE protein map of carotid atherosclerotic plaque. All marked spots were identified by MALDI-TOF MS (**Paper I**), and blue spots represent newly mapped proteins in comparison to previous 2-DE protein maps of carotid atherosclerotic plaque. Mw – molecular weight; pI – isoelectric point.

This method of sampling, and 2-DE/MS analysis, allowed us to better characterise the atheroma proteome. The ability to localise proteins, and abundances, to specific areas in the atherosclerotic lesions can shed light on functional implications in atherogenesis and plaque vulnerability. From the 2-DE/MS, a total of 18 proteins were found with significantly differed abundances when comparing the internal control region to each of the four plaque regions (fatty streak, plaque shoulder, plaque centre, fibrous cap). As we had compiled this data from both men and women, we proceeded to breakdown this data further to identify if these differed protein abundances were generalised across sexes, or if any showed sex specificity (to be discussed in the next section). From a generalised viewpoint, nine proteins displayed differed abundances

consistently in specific atheroma regions, irrespective of sex, and were termed ‘site-specific’. Site-specific alterations were found predominantly in the plaque shoulder and plaque centre regions, with nine proteins showing a significant reduction in abundance when comparing the internal control to the plaque shoulder/centre regions (Figure 11).

Many of these proteins have functions involved with ECM remodelling and SMC function. The reduced abundance of mimecan and increased abundance of

Figure 11 | Site-specific protein alterations in carotid atheroma.
Heat map depicting the differential protein abundance of nine proteins in distinct regions of atheroma from six carotid endarterectomy samples (equal sex ratio). The colour scale represents the relative abundance per protein, with red indicating high abundance through to blue indicating low abundance.

fibrinogen β fragment is indicative of ECM remodelling in the plaque shoulder and plaque centre regions. Mimecan functions to regulate collagen fibril growth and organisation, thus dysregulated mimecan can lead to a lack of adequate ECM formation.¹⁰³ Fibrinogen has a major role in blood coagulation, once cleaved to fibrin by thrombin (Figure 4), however, it can also bind a number of growth factors, e.g. fibroblast growth factor and vascular endothelial growth factor, making it an important factor in vascular ECM physiology.¹⁰⁴ Proteomic analysis has previously identified fibrinogen fragments in atheroma,⁵⁷ which can be an indicator of fibrinolysis in advanced lesions. Reduced abundances of actin, transgelin, and heat shock protein 27 (hsp- β 1) were present in the atheroma regions and all have described functions in vascular SMC function. Actin cytoplasmic 1 (beta-actin), the specific actin type identified in this study, is required for actin filament formation in the cytoskeleton and cell motility in vascular SMC.¹⁰⁵ LDL present in the intima during atherosclerosis

induces the dephosphorylation of heat shock protein 27 from actin, inhibiting actin polymerisation.¹⁰⁶ Additionally, actin-binding protein transgelin is known to be down-regulated in atherosclerosis,¹⁰⁷ and is an early marker for SMC differentiation and repressor of MMP-9.¹⁰⁸ Taken together these results may indicate that in advanced atheroma regions there is an increased level of ECM remodelling and degradation, reduced SMC migration, and SMC dysfunction – which can all contribute to plaque vulnerability.

Overall, the method to reduce the impact of tissue heterogeneity in atherosclerotic lesions, developed in **Paper I**, was successful. This result provided the basis to expand the study utilising a MS methodology with greater sensitivity: tandem-MS. Results of the expanded tandem-MS were, in part, used to validate the 2-DE/MS results in **Paper I**, however, the majority of this investigation results are presented in **Papers II and III** – from a sex difference perspective. To streamline the project and increase the number of samples that could be analysed, the tandem-MS investigation only used intraplaque biopsies from three regions; internal control, fatty streak and plaque core. These regions were selected because the internal control is needed to control for individual variation, the fatty streak is a ‘representation’ of early atherogenesis, and the plaque core had the majority of altered protein abundances – as seen in the results from **Paper I**. The fibrous cap was omitted because not all atherosclerotic plaques contain a large enough fibrous cap for analysis and the plaque shoulder was omitted as the patterns of altered protein abundances were similar to that of the plaque core.

Sex differences in the atherosclerosis

In **Paper I**, statistical comparisons were performed comparing each atheroma region to the internal control region to identify proteins with altered abundance. In this paper we first observed sex differences in proteins that were significantly altered in atheroma regions. Proteins observed to only show significant alterations in one sex were deemed ‘sex-specific’ (‘gender-specific’ in **Paper I**). In atheroma from men both cathepsin D and ferritin light chain showed sex-specific alterations, and in atheroma

from women apolipoprotein (apo) A-IV, transthyretin, ferritin light chain, and heat shock cognate 71kDa showed sex-specific alterations. Interestingly, both sexes showed sex-specific alterations in ferritin light chain, however, the abundances were opposing between sexes in all lesion regions, with an opposing significant increase and decrease in the fibrous cap of men and women, respectively.

These observations provided the motivation to investigate sexual dimorphism in the atheroma proteome further. **Paper II** was focussed specifically on sex differences in the atheroma proteome, and **Paper III** investigates pathophysiological differences, specifically inflammation and iron metabolism, in atherosclerosis.

Proteomic perspective (Paper II)

Paper II focussed solely on revealing the differences in the atheroma proteome between men and women, utilising tandem-MS and multivariate statistical modelling. Tandem-MS analyses of the distinct atheroma regions identified more than 1000 unique proteins per biopsy protein extract. Having analysed samples from 10 men and 10 women, with three region biopsies each, this resulted in a data matrix of over 60,000 data points. To appropriately analyse such a dataset, with a low subject-to-variable ratio, multivariate statistical

Figure 12 | Sex differences in the atheroma proteome by discriminating functional groups.
Multivariate statistical modelling, using OPLD-DA, revealed 43 proteins that discriminate the atheroma proteome between sex, grouped into 16 function groups according to reported ‘biological process’. (Human atheroma, n = 20, equal sex distribution)

Figure 13 | Proteins with significant altered abundances between sexes.

Conservative univariate statistical analysis revealed three proteins that show an overall significant altered expression between carotid atheroma from men (n = 10) and women (n = 10). Bonferroni adjusted p-values are used. LFQ – label-free quantification. (Data mean ± SD).

modelling, in the form of OPLS-DA, was implemented with the categorical variables defined as ‘men’ and ‘women’. Multivariate statistical modelling revealed 43 proteins that discriminated the atheroma proteome between men and women, these proteins were then grouped in 16 functional groups based on the reported ‘biological process’ from the UniProt consortium database (Figure 12).

To further filter the sex differences in the atheroma proteome, conventional univariate statistics was employed on the remaining 43 proteins. This was performed to see if the individual proteins significantly differed between sexes on their own, without taking co-variance into account. This additional filter resulted in 18 individual proteins showing significantly altered abundances between sexes, including proteins in the following functional groups: blood coagulation, cell cycle, complement activation, inflammatory response, iron homeostasis, peptidase activity (negative), response to ROS, transcription, transport, and unknown. Furthermore, upon p-value correction for multiple comparisons, using Bonferroni adjustment, three proteins retained significance with a greater abundance present in the atheroma from women; blood coagulation proteins coagulation factor XII (FXII) and antithrombin-III (AT-III), and transport protein afamin (Figure 13).

As with **Paper I**, the data obtained was from distinct atheroma regions, and with that, we wanted to see the sex-discriminating proteins had any atheroma region

differences. Thus, univariate statistics was used to compare the matched atheroma regions between men and women. The results of this analysis showed that 10 proteins, from five functional groups, also displayed significant differences in abundance between specific atheroma regions (Figure 14). The majority of atheroma regional differences between sexes were found in the plaque centre region, eight proteins, and lesser so in the internal control, four proteins, and fatty streak, only afamin. FXII, AT-III and afamin showed significant differences in more than one region site, with all showing an increase in abundance in atheroma regions from women (Figure 14).

Figure 14 | Sex-discriminant proteins with significantly altered abundances between matched atheroma regions.

Heat map depicting the five functional groups with proteins that show significantly altered abundances in one or more regions from carotid atheroma (n = 20, equal sex). Colour scale represents relative abundance per protein, with blue representing low abundance through to red representing high abundance. Bars represent a significant difference, $p < 0.05$, between matched atheroma regions.

To summarise, and select some of the key results, when looking at sex differences in carotid atheroma: the increases of FXII, AT-III and afamin are the most prominent differences in the atheroma from women, alongside the increases in inflammatory response proteins, phospholipase A2 membrane associated (sPLA₂-IIA) and lysozyme C, in the atheroma from men.

FXII and AT-III, a serine protease and serine protease inhibitor (serpin), respectively, both function in the blood coagulation system and are found at increased abundances in atheroma from women. Interestingly, these two proteins have somewhat opposing functions, with FXII being associated with plaques with greater inflammatory cytokine release and susceptibility to plaque instability,¹⁰⁹ and AT-III inhibiting thrombin and aiding plaque stability.¹¹⁰ Previously, in a large cohort study, AT-III has been observed at increased levels in women.¹¹¹ The answers as to whether the increased abundance in women is beneficial in aiding plaque stability, or not, may lie in the disruption of the clotting cascade at varying levels. FXII conversion is at the beginning of the intrinsic pathway of the coagulation cascade, whereas thrombin lies at the end of the coagulation cascade and converts fibrinogen to fibrin to create the thrombus (Figure 4). Inhibition of thrombin by AT-III, and also heparin cofactor 2, can inhibit thrombus formation.¹¹⁰ This ‘stop’ at the end of the coagulation cascade may inadvertently cause the increased abundance of FXII at the beginning of the cascade, however, more in-depth studies are required to investigate this and the effects on other factors in the coagulation cascade in relation to thrombosis and atherosclerosis. It must be noted that a number of serpins have been identified in this study with overall increased abundances in atheroma from women, including; alpha-1-antichymotrypsin (serpin A3), AT-III (serpin C1), plasma protease C1 inhibitor (serpin G1), heparin cofactor 2 (serpin D1), and corticosteroid-binding globulin (serpin A6).

Afamin is the fourth member of the albumin gene family and is a vitamin-E transport protein.¹¹² The majority of vitamin E transport takes place in the lipoprotein system, wherein a proportion of afamin is associated to HDL.¹¹³ HDL is commonly referred to as the ‘good’ cholesterol and increased levels of afamin may be an indication of higher levels of HDL in the atheroma from women.

Although no conservative significant increases were found in the atheroma from men, there were a number of proteins with more liberal significance. Amongst these, two inflammatory response proteins showed significant increases between sexes, overall and with regional differences in the plaque centre region; sPLA₂-IIA and lysozyme C. It should come to no surprise that proteins related to inflammation would become a focus in a study related to atherosclerosis and indeed, inflammatory response proteins were the most discriminant functional group for the atheroma from men (Figure 12). These have both been attributed to atherosclerotic disease. sPLA₂-IIA functions to hydrolyse LDL in the vascular wall promoting foam cell formation and inflammation.¹¹⁴ A similar protein, lipoprotein-phospholipase A2, also display sexual dimorphism with increased abundance, also stimulating inflammation, in atheroma from men.¹¹⁵ Lysozyme C has been proposed as a possible plasma biomarker for atherosclerotic disease, as the plasma levels are correlated with disease severity.¹¹⁶ Conversely to afamin, lysozyme C has been found associated with LDL, the ‘bad’ cholesterol, at increased levels in patients with metabolic syndrome.¹¹⁷ Thus, increased lysozyme C could be an indication of higher levels of LDL in the atheroma from men.

Pathophysiology perspective (Paper III)

Paper III investigated the sex differences in carotid atheroma in relation to inflammation and iron metabolism, complimented with relevant results from the multivariate proteomic analyses in **Paper II**. The primary results from this study were based on serum analyses and IHC analyses of sections of human carotid atheroma from men and women having undergone carotid endarterectomy. Carotid atheromas from 62 patients were divided into two groups by sex, men (n = 39) and women (n = 23). To complement these findings results from two discriminating protein groups, iron homeostasis and haemoglobin/haptoglobin, were also presented.

Haemoglobin (Hb), the iron-containing oxygen transport protein in red blood cells, at high blood concentrations has been identified as a risk factor for major atherosclerotic events.¹¹⁸ Herein, serum analysis revealed that men had significantly higher levels of Hb than women (Figure 15A). Hb levels were also significantly associated with men, independent of potential confounders, such as age, hypertension,

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statin therapy, diabetes, and smoking. Increased abundances of Hb, subunits α and β , were also observed in the atheroma from men on proteomic analysis (Figure 15B). This association of Hb and male sex led us to categorise the patients by serum Hb levels, using the mean serum Hb level as a cut-off, into the high (Hb > 140 g/L) or low (Hb $\leq$ 140 g/L) groups. After categorisation, it was found that the majority of men had high levels of Hb (Figure 15C), that high Hb levels were indicative of high-risk plaques with rupture (Type 3; Figure 15E), and that these high-risk plaques were more commonly found in men (Figure 15D). Extracellular and/or serum-free Hb can contribute to atherosclerosis, being a source of labile iron upon its breakdown in the vessel wall.

Figure 15 | Men have significantly greater amounts of haemoglobin, which is associated with more severe plaques.
(A) Men have significantly higher levels of haemoglobin (Hb) in the serum than women (n = 39/23 men/women) (Data mean $\pm$ SD). (B) Protein abundance of Hb, subunits α and β combined, were non-significantly increased in atheroma from men. (n = 20, equal sex distribution) (Data mean $\pm$ SD).; (C) Significantly more men presented with high levels of serum Hb (> 140 g/L) than women (high/low Hb: n = 25/12 men, n = 5/16 women); (D) Significantly more men presented with more advanced plaque (type 2 and 3) than women (type 1/2/3: n = 5/9/16 men, n = 5/1/5 women); (E) Type 3 plaques are associated with high serum Hb (> 140 g/L) (type 1/2/3: n = 4/4/16 with high Hb, n = 5/6/4 with low Hb).

Hb, as well as the Hb-scavenger proteins haptoglobin, have also been shown to associate with HDL in patients with coronary heart disease but not in healthy individuals.¹¹⁹ These associations with HDL were observed to impair normal HDL function and promote a more proinflammatory nature.¹¹⁹ Another haem-scavenger protein hemopexin, is thought to be antiatherogenic due to its observed capacity to prevent Hb-induced oxidation of LDL.¹²⁰ Interestingly, from the proteomic analyses we found that Hb subunit- α and Hb subunit- β were increased in abundance in the atheroma from men (Figure 16). Moreover, hemopexin was found at significantly increased abundance in the atheroma from women, alongside iron homeostasis group member – serotransferrin (Figure 16).

In addition, we also investigated the expression of ferritin and transferrin receptor 1 (TfR1) by IHC in sections of carotid atheroma from both men and women. Results from both of these stainings revealed that atheroma from men had significantly greater expression of both ferritin and TfR1, compared to women. Previously, our group had demonstrated a correlation with ferritin and TfR1 expression with the macrophage infiltration in carotid atheroma,¹²¹ hence herein, we further investigate the differences in macrophage

Figure 16 | Iron-related proteins with differential abundance between sexes.

Proteomic analyses of carotid atheroma (n = 20, equal sex distribution) revealed sex differences in the protein abundances of haemoglobin (Hb), subunits - α and - β , hemopexin and serotransferrin. LFQ – label-free quantification. (Data mean $\pm$ SD)

Figure 17 | Atheroma from men had significantly higher expression of both M1 and M2 macrophages.

Immunohistochemistry (IHC) analysis was performed in sections of carotid atheroma from both men ($n = 19$) and women ($n = 40$). IHC staining for CD86, a proinflammatory M1 macrophage marker, and CD163, an anti-inflammatory M2 macrophage marker. (Data mean $\pm$ SD).

infiltration and subtype between sexes. Macrophage markers were used for the IHC analyses, including pan-macrophage marker – CD68; proinflammatory macrophage M1 subtype marker – CD86; and an anti-inflammatory macrophage M2 subtype marker – CD163. Results from macrophage IHC showed that atheroma from men possessed higher levels of overall macrophage infiltration, as well as both M1 macrophages and M2 macrophages (Figure 17). These results were consistent with previous findings that women have less macrophage infiltration, and potentially lower inflammatory reaction in atherosclerosis.¹²² Correlation analyses showed that macrophage expression colocalised with the ferritin and TfR1 expression in atheroma from both men and women. Additionally, correlations were found between CD68 and activated macrophage subtype markers CD86 and CD163. The correlations may suggest that ferritin and TfR1, and by association labile iron, may be potential markers for macrophage activation in atherosclerosis.

In summary, this study showed that carotid atheroma from men, compared to women, had more advanced plaques, which were associated

with significantly higher levels of macrophage infiltration and high iron stores in the circulation and plaques. These findings help contribute to the understanding of sex differences in atherosclerosis, specifically in plaque vulnerability.

Resistance training in postmenopausal women

Physical inactivity is a major risk factor for the development of CVD and regular physical activity is a concept well-known to boost overall health in the population. As we had identified a number of potential markers related to atherosclerotic plaque development and plaque vulnerability, we aimed to investigate if physical activity could alter selected parameters that could be of potential benefit for reducing the development of CVD. For **Papers IV-V**, we had access to a physical exercise clinical trial called the “Strength study”, based at the Division of Children’s and Women’s Health at Linköping University, which was centred around the effects of resistance training (RT) amongst postmenopausal women experiencing vasomotor symptoms. Obviously, with access to only postmenopausal women, this present a major caveat in our overall aim of this thesis to study sex differences in atherosclerosis and CVD. However, epidemiology has shown that after menopause there is a remarkable increase in stroke risk in women, which approximately doubles during the 10 years after menopause.^{1, 123, 124} Thus, these results may provide valuable insights into the effectiveness of RT as a potential intervention for CVD.

The clinical trial was a randomised controlled intervention trial, over a period of 15 weeks, and included 65 sedentary postmenopausal women who were randomly assigned into either a control group or RT intervention group. Clinical characteristics were recorded at baseline, and body anthropometrics and blood samples were obtained at two time-points in the trial at week-0 and week-15. At the conclusion of the study period, samples from 55 women were available for analysis – with seven women voluntarily removing themselves from the trial, and samples from three women were missing one or more timepoints. A flow chart detailing the clinical trial is presented in Figure 18.

As with all intervention trials, compliance must be taken into consideration. In the present study, the level of compliance to the RT-programme was set at an average of at least two RT sessions per week, excluded time off due to illness. At the completion of the intervention period, participants with good compliance were 15 out of 26 women (58%). To account for compliance, the RT group was analysed in two forms; the first

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including all RT women (all-RT), and secondly just including those women with good compliance (compliant-RT).

A series of assays were performed on the isolated plasma samples obtained from week-0 and week-15, including measurements of cytokines, chemokines, adipokines, myokines, iron status markers, lipids, hormones, and oxidative stress markers. A total of 51 analytes were investigated in plasma samples, however, not all analytes were detectable or presented within detectable ranges in enough participants for statistical consideration. A list of analytes that were measured within detectable ranges and were subjected to further statistical analysis are listed in Table 2.

Figure 18 | Participant inclusion, allocation, and division of sample analyses. Flow chart of the clinical trial set-up. A total of seven participants voluntary withdrew during the study period, as well as a further three participants being excluded from analyses due to missing plasma samples. Flow chart also includes the division of samples taken forward for analyses in **Paper IV** and **V**. Reduced sample numbers for lipid and iron analysis was due to the requirement of an extension of ethical permits.

Table 2 | Detectable analytes measured in plasma from participants in the Strength Study.

Plasma analytes that were measured within detectable ranges of assays used, and that were carried forward for statistical analyses.

Group	Detected analytes
Adipokines	Adiponectin, adipisin, lipocalin-2, plasminogen activator inhibitor-1 (PAI-1), resistin.
Hormones	Leptin, sex hormone binding-globulin (SHBG), testosterone.
Iron status	Ferritin, iron, transferrin, transferrin saturation.
Lipids	Total cholesterol, LDL, HDL, triglycerides, non-HDL, apolipoprotein-A1, apolipoprotein-B.
Myokines	Brain-derived neurotrophic factor (BDNF), oncostatin-M, osteonectin.
Oxidative stress	Protein carbonyl, 4-hydroxynonenal, total antioxidant capacity.
Cytokines	MCP-1, tumour necrosis factor (TNF).
<i>Other</i>	CRP, MMP-2, MMP-9

Once all measures were obtained, a series of statistical comparisons were performed, primarily comparing the two time-points in each control and RT groups, and between the study groups using a normalised value representing the change-over-time, calculated by divided the week-15 value by the week-0 value to obtain a percentage. RT exercise was associated with a number of significant alterations in plasma analytes, including those to be discussed herein; lipids and iron status (**Paper IV**), and adipokines and hormones (**Paper V**).

RT associated effects on iron status and lipids (Paper IV)

As introduced, lipid levels and iron status pose risks for the development on progression of CVD. Increased serum ferritin levels have been associated with the risk of CVD.¹²⁵⁻¹²⁸ Recent studies have reported associations between increased ferritin levels and increases in lipids, including total cholesterol, triglycerides, and LDL, in patients with CVD.^{129, 130} Thus, in the Strength study, we aimed to analyse if RT could alter the plasma lipid profile and iron status in postmenopausal women.

Figure 19 | Compliant-RT associated reductions in lipids and ferritin.

Women performing RT with good compliance (n=15) had associated significant reductions in plasma levels of total cholesterol, LDL, non-HDL, and ferritin when comparing week-15 and week-0 values. (Data is median $\pm$ IQR).

By comparing values from week-0 and week-15 in the control, all-RT and compliant-RT groups, we found that participants performing RT with good compliance had significant reductions in plasma levels of total cholesterol, LDL, non-HDL, and ferritin (Figure 19). In contrast, no significant reductions were observed for participants in the control group, and in the all-RT group only a significant reduction in ferritin was retained ($p = 0.01$). When analysing the change-over-time values comparing the study groups, ferritin levels were reduced significantly more in both all-RT ($p = 0.03$) and compliant-RT ($p = 0.01$) groups when compared to the control group. Overall, these results showed an RT-associated reduction in selected plasma lipids and ferritin levels.

Exercise regimes, in postmenopausal women, have previously demonstrated associations with alterations in iron status and lipid profile. Aerobic exercise has produced decreases in body iron burden, including ferritin, iron, and transferrin saturation,^{131, 132} and has shown to have beneficial outcomes in altering the lipid profile in postmenopausal women.^{132, 133} RT trials have been utilised to a lesser extent overall, and specifically in postmenopausal women the majority of these trials have investigated the effect of RT in women who were overweight or obese. Results from these RT trials are somewhat contradictory in regard to the effect on lipid profile. A similar RT intervention study, over 12 weeks, in obese postmenopausal women also showed beneficial reductions in total cholesterol, LDL, and non-HDL.¹³⁴ However, another RT intervention

trial in obese/overweight postmenopausal women showed no alterations in lipid profile,¹³⁵ and using a combined aerobic-RT regime showed no additional benefit of RT in altering the lipid profile.¹³⁶

Correlation analyses were also performed based on the results of reduced ferritin and altered lipid profile. Correlations were performed for the data at week-0, week-15, and also a change-over-time value (week-15 minus week-0) for control and RT groups. The majority of correlations were observed when analysing the values representing change-over-time. A negative correlation between ferritin and HDL was found in all participants groups (Table 3). Additionally, positive correlations between ferritin and triglycerides, non-HDL, and apolipoprotein-B were found in the all-RT group when correlating the change-over-time (Table 3). In the context of the raw plasma measurements, this meant that in the all-RT group there were correlated reductions in ferritin, triglycerides, non-HDL, and apolipoprotein-B, and a contrasting increase in HDL levels. Overall, these changes in lipids can suggest an association of RT counteracting the atherogenic lipid profile with complementary reductions in ferritin. A large observational study investigating the age and sex relationships between serum ferritin and lipids levels, included a subgroup of 3539 women (mean age 39 years).¹³⁰ Whilst this study covered a wide range of ages, 20-70 years, similar correlations were observed, including positive correlations between ferritin and total cholesterol and

Table 3 | Correlation analyses of the change-over-time of plasma ferritin and lipid levels.

Correlation analyses (Spearman) of the change-over-time values, week-15 minus week-0, for all participant groups in the Strength study; control, all-RT, and compliant-RT.

Parameters	Control (n = 21)		All-RT (n = 21)		Compliant-RT (n = 15)	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Ferritin + ↓						
TC	-0.04	0.88	0.11	0.65	-0.15	0.60
LDL	0.08	0.72	0.36	0.13	0.17	0.55
HDL	-0.63	< 0.01	-0.49	0.03	-0.56	0.04
Triglycerides	-0.13	0.59	0.52	0.02	0.48	0.08
Non-HDL	0.07	0.77	0.048	0.04	0.32	0.26
Apo-A1	-0.31	0.17	-0.15	0.54	-0.38	0.19
Apo-B	-0.21	0.38	0.52	0.02	0.27	0.35

TC – total cholesterol; LDL – low-density lipoprotein; HDL – high-density lipoprotein; Apo – apolipoprotein

triglycerides, as well as LDL, and a negative correlation between ferritin and HDL.¹³⁰ This study also shows a clear increase in the serum ferritin levels in women with advancing age, albeit still significantly lower than men in all age groups.¹³⁰ The molecular mechanisms linking increased iron burden and CVD, or metabolic syndrome, is unclear. However, one of the most probable is the relationship between iron and oxidative stress, with iron being a catalyst for ROS production in atherosclerosis.^{41, 43} This relates back to the original ‘iron hypothesis’, by Sullivan, suggesting the sex differences in CVD are related to the iron load in the body.⁵⁰ In the Strength study, we also analysed the plasma levels of oxidative stress markers, including protein carbonyl, lipid peroxidation, as well as total antioxidant capacity. However, no RT-associated effects were observed in plasma oxidative stress markers.

RT associated effects on adipokines and hormones (Paper V)

After menopause, a number of physiological alterations occur that can affect CVD risk, including but not limited to: reduced energy expenditure, redistribution of fat, increase in systemic inflammation, and a shift in sex hormone production.¹³⁷⁻¹⁴⁰ Thus, in the Strength study, we also wanted to analyse plasma levels of selected inflammation markers, hormones, adipokines and myokines. Whilst the majority of inflammation-related cytokines were undetectable in the assays used, we were able to measure a number of adipokines and hormones that displayed significant differences over the course of the trial. Results of plasma analyses of adipokines revealed significant differences in three adipokines, including adiponectin, lipocalin-2 and resistin. When comparing the levels of adipokines from the two-points, week-0 and week-15, adiponectin levels were significantly altered in all three subgroups, control, all-RT and compliant-RT, lipocalin-2 was significantly reduced in both RT groups, and resistin was significantly reduced in the compliant-RT group (Figure 20).

Adipokines are cytokines secreted from adipose tissue and can be measured systemically and locally, by blood sampling or adipose tissue culturing, respectively.^{141, 142} Adipokines can be proinflammatory or anti-inflammatory, with adiponectin being anti-inflammatory, and lipocalin-2 and resistin being proinflammatory.

Adiponectin is produced by both white and brown adipose tissue and circulates in a variety of isoforms, which can exert differing biological effects.^{143, 144} In the Strength study, women participating in RT had associated decreases in plasma adiponectin levels. This observation seems to run contradictory to providing a potential benefit, as an increase in anti-inflammatory factors would be perceived as beneficial in the context of inflammation-driven diseases. Akin to the Strength study, another exercise intervention study, in obese postmenopausal women, observed significant decreases in adiponectin levels whilst still retaining an overall benefit.¹⁴⁵ Currently, there is debate whether high levels of adiponectin are physiologically beneficial, as high levels of adiponectin in high-risk CVD populations have been associated with CVD mortality.^{146, 147} Here, it must be reiterated that postmenopausal women are at an increased risk of CVD incidence,¹²³ therefore high adiponectin levels may not be beneficial. Expanded analyses into the effect of exercise on particular adiponectin isoforms, such as high-molecular-weight adiponectin and adiponectin receptors, as these may discern the mechanisms responsible for altered adiponectin levels.

Figure 20 | RT was associated with significant reductions in plasma adipokine levels.

Postmenopausal women taking part in a 15 weeks RT programme had associated reductions in the plasma levels of three adipokines, both adiponectin and resistin levels were significantly reduced in the all-RT group ($n = 26$) and compliant-RT ($n = 15$) groups; levels of resistin were significantly reduced in the compliant-RT group. Additionally, a significant increase in adiponectin levels was observed in the control group ($n = 29$). (Data is median $\pm$ IQR).

Lipocalin-2 and resistin are both proinflammatory adipokines, and in the Strength study, significant decreases in plasma levels were observed in women who participated in RT (Figure 20). Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is mainly produced by adipocytes in white adipose tissue.¹⁴⁸ Lipocalin-2 has been associated with inflammatory and metabolic disorders, such as obesity, metabolic syndrome, and CVD.¹⁴⁹⁻¹⁵² Resistin is mainly produced in peripheral blood macrophages, with a lesser proportion being secreted by adipocytes in white adipose tissue.¹⁵³ Resistin has been found to directly interact with mechanisms related to atherosclerosis, via the promotion of lipid accumulation in macrophages,¹⁵⁴ stimulating foam cells formation, and also by endothelial cells activation.^{155, 156} Lipocalin-2 and resistin have both been identified as independent predictors of cardiovascular events, which in addition to CRP level could provide better predictions for CVD.^{151, 157} To our knowledge, the Strength study is the first to show reduced plasma levels of lipocalin-2 in association with RT in postmenopausal women. Whereas, similar results for decreases in plasma resistin levels have been seen in elderly obese postmenopausal women participating in a periodical RT regime.^{158, 159}

In addition to the described RT-associated alteration in adipokines, significant changes in testosterone and sex hormone-binding globulin (SHBG) were observed in the Strength study. Comparing the plasma levels at week-0 and week-15, a small significant increase in testosterone levels were observed in women in both the all-RT and compliant-RT groups. Moreover, when analysing the change-over-time values, both testosterone and SHBG levels were seen to increase significantly more in the all-RT and compliant-RT groups, respectively (Figure 21). The observed increases in plasma testosterone levels run contradictory to most other exercise trials in postmenopausal women, which show trends to physical activity reducing testosterone levels. The differences may be the result of the type of exercise employed. It must be noted, that the absolute change in testosterone levels was small when comparing week-0 and week-15, thus this small increase may lack clinical significance.

Figure 21 | RT over the course of 15-weeks was associated with significantly greater increases in plasma levels of testosterone and sex hormone binding globulin (SHBG)

Postmenopausal women randomised in control (n = 29) or RT groups, presented as both all-RT (n = 26) and compliant-RT (n = 15). Plasma levels were measured at week-0 and week-15. Statistical analysis was performed using 'change-over-time' values, week-15 normalised to baseline (% of corresponding week-0 value). (Data is median $\pm$ IQR).

As previously mentioned, the Strength study only studied postmenopausal women, a limitation of the overall aim of this thesis. However, specific limitations must also be noted, the foremost being that the primary outcome of the Strength study was to investigate if RT could alleviate vasomotor symptoms, hot flushes, in postmenopausal women, and as such power calculations were based on this primary outcome variable. The aforementioned investigations relating to iron status, lipid profile, and inflammation are essentially secondary outcomes to this clinical trial, and as such the participant number that is relatively low for conclusive analysis of these analytes. Thus, additional studies with a larger participant group are required to confirm the results presented. In future studies, diet should also be accounted for, especially in the context of iron status and oxidative stress.

To summarise, the Strength study as a whole showed RT-associated alterations in a number of plasma analytes, including; ferritin, atherogenic lipids, and adipokines. The results taken together suggest that RT may be a beneficial intervention to potentially alleviate inflammation and atherogenesis in postmenopausal women, however, a larger study is needed with more women and the inclusion of diet must be taken into consideration.

Highlights (Papers I-V)

As is evident, the works included in this thesis fall into two projects, with **Papers I-III** related to the proteomics and pathology of atherosclerosis, and **Papers IV-V** related to a clinical trial in postmenopausal women. Whilst from the onset these two projects seem independent, and arguably are, there are coherent concepts throughout.

To briefly highlight the major results from **Papers I-V**; we have found the following:

- I. Proteomic analysis of distinct atheroma regions reduces the effect of plaque heterogeneity and identifies differences between sexes, such as apolipoprotein A-IV, cathepsin D, ferritin light chain and transthyretin.
- II. Sexual dimorphism is present in the atheroma proteome, with particular focus on proteins related to blood coagulation and inflammatory response. Men having greater levels of lysozyme C and sPLA₂-IIA, and women greater levels of serpins and afamin.
- III. Atheroma iron stores and inflammatory macrophage infiltration differ between sexes and relate to plaque severity and vulnerability. Men developing more advanced and ruptured lesions associated with higher levels of inflammatory macrophages, and higher iron stores in the blood and plaques.
- IV. RT intervention is associated with reduced ferritin and lipids, including total cholesterol, LDL and non-HDL, in postmenopausal women.
- V. RT intervention is associated with reduced inflammatory adipokines, lipocalin-2 and resistin, in postmenopausal women.

Looking at these results, three concepts are highlighted – sex, iron, and inflammation. These are the major concepts that sew the illusive “*red thread*” throughout the projects. These concepts are all heavily involved with a hypothesis that aims to explain, in part, the development of CVD and atherosclerosis – the “iron hypothesis”. Originally introduced in 1981, this hypothesis set out to explain sex differences observed in the heart disease risk and how levels of stored iron in the body may be associated with the distinct increase in body iron seen women after

menopause.⁵⁰ Over the next decades, this hypothesis has been further explored resulting in the better understanding of the role of iron in atherosclerosis, and the related oxidative stress and subsequent inflammation mechanisms.^{38-41, 160, 161} Whilst, I do not believe the “iron hypothesis” can explain atherosclerosis in its entirety; the results from **Papers I-V** adds to the knowledge of sex differences, how iron and inflammation mediators may be involved, and how they can be potentially be modulated, in atherosclerosis.

CONCLUDING REMARKS

Atherosclerosis-related CVD are the largest killers in the world, with distinctive differences in the incidence, mortality, and pathology between men and women. Exploring these sex differences is of key relevance in understanding the mechanisms driving atherosclerosis, and to help develop therapies for prevention and intervention. This thesis was aimed at investigating sex differences in atherosclerosis, with reference to the atheroma proteome and pathophysiology, and also the effects of exercise in postmenopausal women, a group whom are at high risk of CVD development.

Proteomic analyses of atheroma revealed distinctive differences between the atheroma from men and women, as well as distinctive differences between different regions of atherosclerotic lesions. Protein groups found to discriminate the atheroma proteome between men and women included proteins related to iron-metabolism, inflammatory response, blood coagulation, complement activation, and transport. Iron metabolism and inflammation were further explored from a pathophysiological perspective, where it was found that atheroma from men had higher levels of iron metabolism and inflammatory macrophages, which were associated with more advanced and ruptured plaque, when compared to atheroma from women. These sex differences in proteome and pathophysiology indicate that atheroma from men develop with a greater level of inflammation and more vulnerable phenotype, than atheroma from women that develop with less inflammation and more stable phenotype. These studies provide new insights into the atheroma proteome and vulnerability, which with further investigations could provide new candidate biomarkers for either, or both, men and women for therapeutic targeting.

The clinical trial aimed at investigating the effects of RT in postmenopausal women, was successful in identifying RT-associated decreases in ferritin, lipids, and inflammatory adipokines. These results suggest that the use of regular RT may be a beneficial intervention in reducing the levels of ferritin, lipids, and inflammation, all of which are risk factors for the development of CVD. However, validation studies are

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required in a larger cohort of postmenopausal women, in addition to the inclusion or complementary studies in middle-aged and older men.

In summary, the works including in this thesis further expand on the current knowledge of sex differences in atherosclerosis, with novel insights in the atheroma proteome, and also provides information on the potential of an exercise intervention in beneficially reducing the effect of known risk factors of CVD.

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WE WILL MISS YOU LIKE HELL, AND
WE'RE GONNA KILL YOU IF YOU
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- Cynthia Veenstra & Jelena Gacic,
Linköping 2010

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*“Would you tell me, please, which way
I ought to go from here?”*

*“That depends a good deal on
where you want to get to.”*

“I don’t much care where –”

“Then it doesn’t matter which way you go”

Alice and The Cheshire Cat
Alice’s Adventures in Wonderland
by Lewis Carroll

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