The role of leptin in endothelial dysfunction and cardiovascular disease

Manuel Cruz González García
“.......but that's another story and shall be told another time”
Michael Ende, 1979

Dedicated to those who keep searching.
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>vi</td>
</tr>
<tr>
<td>List of papers</td>
<td>xi</td>
</tr>
<tr>
<td>Sammanfattning på svenska</td>
<td>xii</td>
</tr>
<tr>
<td>Resumen en español</td>
<td>xiv</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Why this thesis?</td>
<td>1</td>
</tr>
<tr>
<td>What to expect—and not to expect—from this thesis?</td>
<td>2</td>
</tr>
<tr>
<td>Obesity and CVD</td>
<td>3</td>
</tr>
<tr>
<td>The global epidemic of obesity</td>
<td>3</td>
</tr>
<tr>
<td>Obesity as an independent risk factor for CVD</td>
<td>3</td>
</tr>
<tr>
<td>Regional body fat distribution</td>
<td>4</td>
</tr>
<tr>
<td>Is BMI a reliable measure of obesity?</td>
<td>4</td>
</tr>
<tr>
<td>Does all accumulated fat have the same deleterious effect on health?</td>
<td>5</td>
</tr>
<tr>
<td>How does visceral fat promote heart disease?</td>
<td>6</td>
</tr>
<tr>
<td>The role of adipokines</td>
<td>7</td>
</tr>
<tr>
<td>Cardiometabolic effects of visceral adiposity</td>
<td>7</td>
</tr>
<tr>
<td>Visceral obesity, dyslipidemia, and insulin resistance</td>
<td>7</td>
</tr>
<tr>
<td>Visceral obesity and inflammation</td>
<td>8</td>
</tr>
<tr>
<td>Visceral obesity and hypertension</td>
<td>8</td>
</tr>
<tr>
<td>Endothelial function</td>
<td>9</td>
</tr>
<tr>
<td>Endothelium regulates vasomotor tone and vascular homeostasis</td>
<td>9</td>
</tr>
<tr>
<td>NO and other vasoactive factors</td>
<td>10</td>
</tr>
<tr>
<td>Endothelial-dependent and -independent vasodilatation</td>
<td>12</td>
</tr>
<tr>
<td>Antithrombotic and fibrinolytic function</td>
<td>12</td>
</tr>
<tr>
<td>Endothelial dysfunction (ED)</td>
<td>12</td>
</tr>
<tr>
<td>Mechanisms of endothelial dysfunction</td>
<td>13</td>
</tr>
<tr>
<td>Obesity and endothelial dysfunction</td>
<td>14</td>
</tr>
<tr>
<td>Effects of obesity on the microvascular system</td>
<td>14</td>
</tr>
<tr>
<td>Effects of obesity on the macrovascular system</td>
<td>16</td>
</tr>
<tr>
<td>Perivascular Adipose Tissue</td>
<td>16</td>
</tr>
<tr>
<td>Assessment of endothelial function</td>
<td>17</td>
</tr>
<tr>
<td>Conduit vs. resistance vessels</td>
<td>18</td>
</tr>
<tr>
<td>Techniques for assessing endothelial function</td>
<td>19</td>
</tr>
<tr>
<td>Invasive techniques</td>
<td>20</td>
</tr>
<tr>
<td>Non-invasive techniques</td>
<td>21</td>
</tr>
<tr>
<td>Fibrinolytic function</td>
<td>22</td>
</tr>
</tbody>
</table>
Leptin

Historical view
Leptin, the prototypical adipokine
Leptin signaling
Leptin receptors in vascular tissue
Concepts of leptin resistance and selective leptin resistance
Leptin resistance in the vascular tissue

Leptin and cardiovascular disease

Epidemiological evidence
Mechanisms of leptin that may promote CVD
Hyperleptinemia activates the SNS and/or ET-1
Leptin and inflammation
Leptin and oxidative stress
Hyperleptinemia may increase vascular tone and impair vasodilatation
Leptin and hypertension
Leptin and endothelial dysfunction/atherosclerosis
Leptin and type II diabetes
Crosstalk between leptin and insulin
Leptin and fibrinolysis

Aims

Methods

Design of this thesis
Ethical considerations
Subjects and methods
Study 1 (DISARM): Paper I
Study 3 (PIVUS): Paper II
Study 4 (Scottish study) and studies 5 and 6 (LIVFARM studies): Paper III
Anthropometry
Blood pressure
Metabolic measurements
Vascular and endothelial function
Strain-gauge forearm plethysmography
FBF determination
Brachial artery ultrasound
Radial artery applanation tonometry
Biochemical analysis
Statistical methods
Study 1
Study 2
Study 3
Study 4
Studies 5 and 6

Results and discussion
Abstract

Objective: Obesity has become the leading cause of mortality worldwide; however, the fundamental pathophysiology underlying this association remains unclear. The discovery of adipokines, i.e., cytokines produced by adipose cells (adipocytes), revealed that adipose tissue is a highly endocrine organ, thus opening new lines of investigation. The prototypical adipokine leptin increases in obesity, and leptin receptors are found in vascular cells. However, results are contradictory regarding the role of leptin in vascular and endothelial functions. Leptin has been shown to elicit vasodilation, but has also been linked with atherosclerotic and thrombotic disease. The main aim of the present thesis was to study the association of circulating levels of leptin with markers of endothelial function, and to analyze the effects of leptin infusion in vivo on vasomotor function and endogenous fibrinolysis.

Material: Four associative studies and two interventional studies were conducted. The former included DISARM (studies 1 and 2), the PIVUS study (study 3), and the Scottish post-infarction study (study 4). The DISARM studies and study 4, respectively, recruited 20 men and 83 men and women with stable ischemic heart disease. Study 3 included a random sample of 1016 subjects (54% women, 70 years old) living in the community of Uppsala, Sweden. For the interventional studies (studies 5 and 6), 10 healthy men were recruited for each study.

Methods: In all studies, endothelial function was estimated based on forearm blood flow (FBF) as measured by strain-gauge venous occlusion plethysmography, at rest or during infusion of vasodilators. In study 3, additional measurement techniques were used, such as brachial ultrasound flow-mediated dilation (FMD) and the aortic augmentation index (AoAIX) by tonometry in the radial artery. Fibrinolytic status was estimated based on basal and stimulated levels of tissue plasminogen activator antigen (t-PA), and by assessment of the endothelial release of t-PA (net t-PA release). Plasma leptin levels were measured by radioimmunoassay. In the associative studies, endothelial function and fibrinolytic status were related to circulating plasma leptin levels. In the experimental studies, exogenous leptin was administered in the brachial artery and endothelial function was assessed by strain-gauge plethysmography.

Results: In elderly men and women, leptin was independently associated with decreased endothelial-dependent and -independent vasodilatation, reflecting disturbed endothelial function in resistance vessels. This association was attenuated after adjustment for BMI, and when analyzed
among subjects with high plasma leptin levels. FMD (a measure of endothelial function in conduit vessels) was not associated with leptin. Exogenous leptin infusion did not alter vasomotor tone, but the endothelium-dependent and -independent vasodilatation was impaired during concomitant infusion of leptin and vasodilators. Infused leptin in the forearm did not affect blood pressure or pulse rate. Chronic hyperleptinemia, but not acutely induced hyperleptinemia, was associated with release of endothelial tissue plasminogen activator (net t-PA).

Conclusions: In humans, leptin was associated with impaired vasodilatation. However, this relationship was blunted after adjustment for BMI, suggesting that leptin could be the mediator between obesity and impaired vascular function. Furthermore, the observed lack of association in hyperleptinemic subjects may reflect a state of leptin resistance. The experimental result showing attenuated vascular reactivity following leptin infusion is in accordance with the results of the associative studies. The augmented net t-PA release in patients with chronic hyperleptinemia may indicate a state of “vascular activation,” which was not observed in healthy endothelium during a short period of leptin infusion.

This thesis addresses several controversial issues regarding the action of leptin on vascular tissue in humans. The final results indicate that the in vivo action of leptin on vascularity is complex and mediated by several mechanisms. Our findings suggest that leptin is an important mediator between obesity and endothelial dysfunction, and should stimulate further investigation of this matter.

Keywords: Obesity, cardiovascular disease, leptin, endothelial dysfunction, vasodilatation, strain-gauge plethysmography, forearm blood flow, net t-PA release, intraarterial.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ACEi</td>
<td>Angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AIX</td>
<td>Augmentation index</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>Ang I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AoAIX</td>
<td>Aortic augmentation index</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood–brain barrier</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>CAMs</td>
<td>Cellular adhesion molecules</td>
</tr>
<tr>
<td>CBF</td>
<td>Coronary blood flow</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computer tomography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DISARM</td>
<td>Diesel Inhalation Study in foreARMs</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>ED</td>
<td>Endothelial dysfunction</td>
</tr>
<tr>
<td>EDCF</td>
<td>Endothelium-derived contracting factor</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDV</td>
<td>Endothelial-dependent vasodilatation</td>
</tr>
<tr>
<td>EIDV</td>
<td>Endothelial-independent vasodilatation</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FBF</td>
<td>Forearm blood flow</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated dilation</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IMT</td>
<td>Intima media thickness</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JAK-2</td>
<td>Janus kinase-2</td>
</tr>
<tr>
<td>LIVFARM</td>
<td>Leptin Infusion for Vascular and Fibrinolytic function in foreARMS</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-nitro arginine methyl ester</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>L-N-monomethyl arginine</td>
</tr>
<tr>
<td>LR</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin-4 receptor</td>
</tr>
<tr>
<td>MetS</td>
<td>Metabolic syndrome</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>O2-</td>
<td>Superoxide anion radical</td>
</tr>
<tr>
<td>OGIS</td>
<td>Oral glucose insulin sensitivity test</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>ONOO-</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator-1</td>
</tr>
<tr>
<td>pAIx</td>
<td>Peripheral augmentation index</td>
</tr>
<tr>
<td>PAT</td>
<td>Peripheral applanation tonometry</td>
</tr>
<tr>
<td>PGI2</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
</tbody>
</table>
PIVUS  Prospective Investigation of the Vasculature in Uppsala Seniors

PP  Pulse pressure

PTP1B  Protein-tyrosine phosphatase 1B

PWA  Pulse wave analysis

PWV  Pulse wave velocity

RAS  Renin-angiotensin system

RI  Reflective index

ROS  Reactive oxygen species

SBP  Systolic blood pressure

SMC  Smooth muscle cell

SNP  Sodium nitroprusside

SNS  Sympathetic nervous system

SOCS3  Suppressor of cytokine signaling-3

STAT3  Signal transducer and activator of transcription 3

TNF-α  Tumor necrosis factor-alpha

t-PA  Tissue plasminogen activator

t-PA ag  Tissue plasminogen activator antigen

t-PA act  Tissue plasminogen activator activity

u-PA  Urokinase-type plasminogen activator

VCAM  Vascular cell adhesion molecule

VSMC  Vascular smooth muscle cell

WC  Waist circumference

WOSCOPS  West of Scotland Coronary Prevention Study
List of papers

This thesis is based on the following papers, which are referred to in the text using the Roman numerals I, II, and III.


Published papers and figures have been reprinted with the permission of the publishers.
Sammanfattning på svenska

**Bakgrund:** Förekomsten av fetma har ökat kraftigt med oförutsägbara hälsokonsekvenser för befolkningen. Fetma är kopplad till utveckling av hjärt- och kärlsjukdomar, men bakomliggande mekanismer är inte helt klarlagda. Fettväven är ett endokrint organ, som producerar hormoner, adipokiner, vilka har betydelse för bland annat insulinresistens och kärl (endotel) funktion. Ett av dessa är leptin, som upptäcktes av Friedman 1994, och som är inblandat i många olika metabola processer. I början associerades leptin främst med aptitkontroll och värme-produktion (termogenes) men har under senare år knutits till många olika organ och fysiologiska funktioner, bland annat till det vaskulära systemet. Cirkulerande leptinnivåer är generellt högre hos överviktiga individer, och det finns tecken på att leptin kan spela en viktig roll för kopplingen mellan övervikt och hjärt-kärlsjukdom. Många studier, men inte alla, har visat ett samband mellan höga leptinnivåer och insjuknande i hjärtkärlsjukdom. Emellertid finns det motsägande resultat avseende leptinets effekter på endotelfunktionen. En närmare analys av detta kan vara av stor betydelse för att bättre förstå sambandet mellan fetma och kranskärlssjukdom. Målet med denna avhandling var dels att analysera sambandet mellan leptin och olika mått på endotelfunktion (vasomotorakitivitet och fibrinolys (blodets levringssjukdom)), dels att studera effekten av intravaskulär tillförsel av leptin.

**Metoder och resultat:** fyra sambands- och två experimentella studier genomfördes. I alla studier värderades endotelfunktionen i resistenskärlen med så kallad underarmspletysmografi. Med denna teknik mättes underarmens blodflöde (FBF) i vila och under infusion av olika kärlvidgande substanser. Fibrinolysmarkörerna t-PA och PAI-1 samt t-PA frisättning efter farmakologisk stimulering ("net t-PA release") uppmättes. Studie 1 (DISARM) presenteras i delarbete I. Detta är en metodstudie där underarmspletysmografi beskrivs. I studierna 2 (DISARM-metab) och 4 (Skotska studien) analyserades sambandet mellan leptin och endotelfunktion hos hjärt-sjuka individer, 20 män respektive 83 män och kvinnor. Leptin var associerat med nedsatt kärlvidgning i båda studier, dock noterades ett mer utsatt negativt samband med både endotelberoende och -oberoende kärlvidgning i studie 4. Leptinnivåerna var också associerade med basal (t-PA och PAI-1) och stimulerad (net t-PA release) fibrinolys. Studie 4 presenteras tillsammans med de experimentella studierna 5 och 6 i delarbete III. I studie 3 (PIVUS) analyserades endotelfunktionen med tre olika metoder hos cirka ett tusen 70 åriga män och kvinnor i Uppsala. Metoderna var underarmspletysmografi, flödesmediert vasodilatation mätt med
ultraljud (FMD) samt analys av pulsvågskurvan i handledsartären med beräkning av s.k. ”augmentation index”. Resultaten presenteras i delarbete II. I korthet, höga leptin nivåer är associerade med nedsatt endotelberoende och -beroende kärlvidgning undersökt med pletysmografi och detta samband var oberoende av andra traditionella riskfaktorer för hjärtkärlsjukdom. Sambanden kvarstod ej efter justering för fetma, vilket kan tolkas som att leptin är ett viktigt mellanled mellan fetma och nedsatt kärlfunktion. Inget samband sågs mellan leptin och FMD. I de experimentella studierna LIVFARM 1 and 2 infunderades leptin intraarteriellt under 18 minuter respektive under mer än en timme hos friska män. I LIVFARM 2 tillfördes också vasoreaktiva substanser. Blodflödet bestämdes med pletysmografi, och basal samt stimulerad fibrinolys studerades. Intraarteriell leptintillförsel påverkade inte basalt blodflöde i någon av studierna. Infusion med vasoreaktiva substanser gav mindre endotelberoende och -beroende kärlvidgning vid samtidig infusion av leptin jämfört med samtidig infusion med koksalt. Leptin påverkade inte stimulerad (net t-PA release) fibrinolys.

**Diskussion och slutsatser:** Denna avhandling visar att leptin är associerat med nedsatt endotelberoende och -beroende kärlvidningsförmåga hos hjärtrussia individer under akut leptin tillförsel och hos hjärtsjuka med kronisk hyperleptinemi. Dessa samband sågs ej hos personer med hälftma vilket kan stödja konceptet leptinresistens. Leptin var associerat med pletysmografidata (FBF), men inte med ultraljudsdata (FMD). Detta kan bero på att leptin påverkar resistenskärl men inte konduktanskärl, eller att ultralju overstekniken inte lämpar sig väl för studier av äldre individer med ökad kärlstyrk. Stimulerad fibrinolys (net t-PA release) var associerad till leptininivåer hos hjärtsjuka personer med kronisk hyperleptinemi, men inte hos hjärtfriska personer efter kort leptin infusion. Detta kan tala för endotelaktivering i den första gruppen men inte i den andra.

Dessa resultat ökar kunskapen om leptinets effekt på endotelfunktionen och kommer förhoppningsvis att stimulera till fortsatta studier och, i förlängningen, till utveckling av farmakologiska metoder för modulering av leptinets eventuella negativa effekter. Ytterligare studier krävs för att verifiera resultaten från denna avhandling, helst inom olika viktkategori och under olika tidsintervall.
**Resumen en español**

**Introducción:** La epidemia de obesidad que afecta a la población mundial puede tener imprevisibles consecuencias para el nivel de salud global a corto y medio plazo. La obesidad y más en concreto, la obesidad central o visceral, ha sido asociada, entre otras complicaciones, con un incremento de la incidencia de enfermedad cardiovascular, aunque los mecanismos subyacentes aún no han sido bien establecidos. No obstante, el tejido adiposo se ha revelado en los últimos años como un órgano endocrino de gran actividad, al producir y secretar diferentes péptidos que ejercen su acción en tejidos distantes (efecto endocrino). Así, el adipocito (célula adiposa) es capaz de secretar proteínas, denominadas adipocinas que ejercen múltiples acciones en distintos órganos y de las cuales todavía queda mucho por conocer. La adipocina más conocida es la leptina. Los niveles circulantes de leptina están aumentados proporcionalmente al aumento del índice de masa corporal. Los efectos de la leptina son múltiples y desde su descubrimiento por Friedman en 1994 no han cesado de aparecer nuevos hallazgos, fundamentalmente asociados a su papel como regulador del metabolismo, pero también actuando en multitud de órganos y tejidos. Los efectos de la leptina sobre el tejido vascular y en particular sobre la función endotelial han sido objeto de intensa investigación. Muchos de los resultados obtenidos han sido difíciles de interpretar y de alguna forma contradictorios. Así, mientras ciertos ensayos in vitro han atribuido a la leptina efectos vasodilatadores, otros estudios han demostrado que la leptina está asociada a procesos inflamatorios, de activación del sistema nervioso central o de incrementos en los niveles de endotelina-1, los cuales conllevan un estímulo vasoconstrictor muy potente. En general, la mayoría de los estudios a nivel poblacional han demostrado que los niveles altos de leptina en sangre se asocian a enfermedad cardiovascular, infarto de miocardio o accidente cerebrovascular. En resumen, la leptina se ha perfilado como un posible factor clave en la asociación entre obesidad y enfermedad cardiovascular, aunque los efectos finales de la leptina sobre la función endotelial permanecen en gran medida desconocidos.

El objetivo general de esta tesis fue en primer lugar la de investigar la posible asociación de niveles de leptina plasmática con variables de función endotelial, entendida ésta como grado de vasodilatación y estudio de marcadores de fibrinólisis. Del mismo modo, analizar los efectos de la infusión directa de leptina sobre un vaso arterial en individuos jóvenes y sanos.
Material y métodos: Se realizaron seis estudios, cuatro de ellos asociantivos y dos experimentales. El primer estudio, denominado DISARM, conforma el primer artículo de ésta tesis. En este trabajo, se sentaron las bases metodológicas que se seguirán en el resto de estudios, en particular la técnica de pletismografía braquial. Esta técnica permite analizar la función endotelial mediante el análisis de la capacidad dilatadora de los vasos a través de la infusión intra-arterial de distintas sustancias vasodilatadoras, y el análisis de marcadores de fibrinólisis en las pruebas sanguíneas que se recogen varias veces durante el estudio. Para la realización del segundo estudio—DISARM-metab, a veinte pacientes con enfermedad cardiovascular tratada y estable, todos varones y participantes en el citado DISARM, se les cuantificaron niveles de leptina plasmática. Seguidamente, dichos niveles fueron correlacionados con las variables de función endotelial obtenidas mediante el estudio pletismográfico (grado de vasodilatación y fibrinolisis). El siguiente estudio correlativo de esta tesis, denominado Scottish o estudio número 4, fue realizado por nuestros colegas escoceses de la Universidad de Edimburgo, siguiendo éste un diseño similar al DISARM-metab, si bien en su estudio, el número de pacientes seleccionados, de ambos sexos, fue de 83. El siguiente estudio denominado PIVUS, o estudio número 3, conforma el artículo número II de esta tesis. En este estudio, se estimó la función endotelial mediante el empleo de distintas técnicas a más de mil individuos, que fueron seleccionados a través de registros poblacionales en la región de Uppsala. Este estudio fue realizado por el grupo de investigación de Uppsala, liderada por el Dr Lars Lind. En nuestro grupo analizamos los niveles de leptina plasmática en todos los participantes y lo asociamos con ciertas variables de función endotelial. De entre todas las variables recogidas en Uppsala, nosotros utilizamos los resultados de tres técnicas; la técnica de pletismografía brachial para medir el flujo sanguíneo brachial (FBF), el estudio de vasodilatación brachial mediado por flujo, valorado por técnica de ultrasonidos (FMD) y el análisis de la onda de pulso radial por tonometría arterial, mediante la variable denominada índice de aumento (AIx). En nuestros dos estudios experimentales, LIVFARM-1 y LIVFARM-2, las variables de la función endotelial fueron medidas en 17 jóvenes varones y sanos a través de la técnica pletismográfica, inyectándose durante la misma dosis crecientes de leptina humana recombinante a través de la arteria brachial. Estos dos estudios experimentales, junto con el estudio escocés antes citado, conformaron el artículo número III de esta tesis. En el primer estudio, o LIVFARM-1, la leptina fue inyectada durante 18 minutos, en tres intervalos de 6 minutos, con distintas (crecientes) concentraciones de leptina en cada intervalo. En el segundo, LIVFARM-2, una infusión de leptina o placebo (suero salino) fue inyectada de forma permanente y en solitario durante 60 minutos y transcurrido ese tiempo se dispuso la co-infusión de 4 sustancias vasodilatadoras, distribuidas aleatoriamente. Hay que reseñar
que cada individuo recibió al final del estudio ambas infusiones leptin y placebo, en días diferentes y con un orden de asignación aleatoriamente determinado. Los marcadores de fibrinolisis t-PA y PAI-1 fueron recogidos en plasma durante los dos estudios a intervalos pre-determinados.

**Resultados y discusión:** En todos los estudios asociativos pudo constatarse, en mayor o menor grado, que los niveles de leptina se correlacionaron con un descenso de la capacidad vasodilatadora. Además, esta asociación fue independiente de otros posibles factores de riesgo cardiovascular, incluidos en el denominado “Framingham score”. Del mismo modo, este hallazgo ocurrió tanto durante la administración de vasodilatadores que actúan a través del endotelio, p.ej la acetilcolina, la bradiquinina, o la sustancia P, como con aquellos que realizan su función de una forma independiente al endotelio, como fueron el nitroprusiato sódico o el verapamilo. Esto podría indicar que la acción inhibitoria de la leptina sobre la vasodilatación podría no depender exclusivamente del endotelio, y que sería a través de mecanismos más generales, como por ejemplo una estimulación de la respuesta inflamatoria vascular o una activación, bien local o bien sistémica, del sistema nervioso simpático autónomo los que producirían este efecto inhibidor. Del estudio 3, es interesante resaltar que, cuando la muestra se dividió en dos grupos, uno por encima y el otro por debajo de la mediana de leptina plasmática, la correlación negativa entre leptina y vasodilatación no pudo constatarse en aquellos individuos con mayores niveles de leptina circulante. Esto podría corroborar el concepto de resistencia selectiva a la leptina en el tejido vascular. Según esta teoría, apuntada en publicaciones previas, podría producirse un mecanismo de resistencia a la acción de la leptina sobre los vasos de aquellos individuos que presentaran una hiperleptinemia de forma crónica. Este mecanismo sería similar al que ocurre p.ej con los individuos obesos con niveles altos de insulina circulante y que manifiestan una resistencia a la acción de la insulina, siendo ésta una de las características del denominado síndrome metabólico. De éste estudio 3, también es interesante reseñar que con los datos de función endotelial valorada con la técnica de ultrasonidos (FMD), no pudo demostrarse ninguna asociación con alteraciones de la vasodilatación, al contrario que lo ocurrido con FBF o AIx. Es difícil saber si esto es debido a un déficit de ésta técnica para detectar cambios sutiles en la vasodilatación asociados a la leptina o si verdaderamente la leptina afecta fundamentalmente al FBF y no al FMD. Esto último implicaría que la acción de la leptina se manifestaría sobre todo sobre el tipo específico de vasos sanguíneos que refleja el FBF, es decir las arterias de resistencia, y no la de las arterias conductoras, que son de mayor calibre y diferente estructura, y cuya motricidad viene a ser reflejada fundamentalmente con la técnica de ultrasonidos (FMD).
En relación a los resultados de los estudios experimentales cabe destacar lo siguiente; en el primer estudio (LIVFARM-1), el tono basal del vaso (FBF basal) no fue alterado después de 18 minutos de infusión de leptina, lo que contradice estudios previos in vitro que reflejaban una acción vasodilatadora de la leptina. Los estudios in vitro e in vivo no son directamente comparables, ya que en el segundo caso intervienen muchos más mecanismos que pueden alterar la vasomotricidad y que no están presentes en un tubo de ensayo de laboratorio. En el segundo, y más complejo estudio (LIVFARM-2), el tono basal se mantuvo neutral después de 60 minutos de infusión pero, al igual que lo reflejado en los estudios asociativos, el grado de vasodilatación fue significativamente menor cuando los cuatro vasodilatadores (dependientes e independientes del endotelio) se infundieron junto a leptina, comparado a cuando éstos se administraron junto al placebo. En cuanto a la fibrinólisis, la infusión de leptina en sujetos sanos no alteró los marcadores de activación de fibrinólisis intravascular (en este estudio calculado por el índice “net t-PA release”), esto es liberación neta de t-PA en el endotelio. Sin embargo, en el estudio escocés (estudio 4), éstos niveles si que estaban aumentados en los pacientes con los niveles más altos de leptina circulante, lo que podrá indicar un estado de activación endotelial secundaria a la hiperleptinemia crónica.

Con esta tesis hemos aportado un mayor conocimiento sobre el papel de la leptina en la función endotelial y su implicación en el desarrollo de enfermedad cardiovascular asociada a la obesidad. Es posible especular sobre el desarrollo de medicamentos que pudieran influir (inhibir) sobre la acción vasopresora de la hyperleptinemia y que pudieran así ejercer un mecanismo beneficioso en la prevención de aterosclerosis. Sin embargo, se requiere más investigación al respecto antes de que tal posibilidad se haga realidad. En particular, sería interesante la realización de futuros estudios experimentales en humanos con distintas poblaciones (obesos, diabéticos, etc) y durante mayor tiempo de infusión, horas o incluso días o semanas.
Introduction

Obesity is an independent risk factor for atherosclerotic cardiovascular disease (CVD) [1], which is the leading cause of death and disability worldwide [2]. Adipose tissue is a complex, essential, and highly active metabolic and endocrine organ [3], and the underlying mechanisms by which excess adiposity causes vascular dysfunction are not well understood. Adipokines—the cytokines secreted by adipose tissue—have direct and indirect actions on vascular tissue, which may help explain this association. Leptin, traditionally considered the prototypical adipokine [4], is a pleiotropic hormone produced by adipocytes, which increases in parallel with the amount of fat mass tissue. Leptin is involved in regulating body weight, metabolism, and energy homeostasis [5]. Several epidemiological studies have linked hyperleptinemia with increased risk for CVD, independently of traditional risk factors [6, 7], but this evidence is controversial. Leptin has been associated with atherosclerotic and thrombotic disease [8] and leptin-deficient mice develop less atherosclerosis [9]; however, other studies in mice have suggested that leptin may have a protective effect against atherosclerosis [10], and, in women, low plasma leptin levels predict cardiovascular mortality [11]. Leptin receptors (LRs) are present in endothelial cells [12, 13], but the net effect of leptin on endothelial function remains unclear. Some in vitro studies have demonstrated that high leptin concentrations elicit direct vasodilation through distinct mechanisms [14], whereas other studies find that leptin is associated with vascular inflammation and oxidative stress [15, 16], which may lead to endothelial dysfunction.

Why this thesis?

Most of the evidence linking leptin with endothelial function has been obtained from in vitro and animal model studies. From the few in vivo human studies, we know that leptin affects vascular reactivity in humans [17], but not in all groups—for example, not in healthy adolescents [18]—suggesting that the net effect of leptin on cardiovascular pathophysiology in humans is complex and not completely understood.

The main objective of this thesis was to study the potential role of leptin in human endothelial function. We believe that this knowledge will improve our comprehension of obesity-associated heart disease—which, in turn, might lead to further development of new cardiovascular or antidiabetic drugs.
What to expect—and not to expect—from this thesis?

We aim to contribute to a better understanding of the association between leptin and development of the ED. Another indirect purpose of this thesis is to summarize actual knowledge regarding the mechanisms linking obesity and CVD, highlighting the role of adipokines, with special focus on leptin. Additionally, we have taken the opportunity to describe some of the most commonly used techniques for measurement of endothelial function.

The present studies were not designed to determine causality or to investigate the underlying mechanisms of action behind associations. However, diverse possible mechanisms will be discussed, especially in relation to our experimental study.
Obesity and CVD

The global epidemic of obesity

Through the adaptive process of evolution, the metabolism of *Homo sapiens* has developed the capacity to accumulate energy stores during periods of abundant resources. However, over the last few decades, especially in westernized societies, many people have a chronic positive energy balance. This is due to increased consumption of high-energy-density food products together with increasingly sedentary lifestyles involving substantially decreased energy expenditure. These factors, along with individual genetic predisposition, have contributed to development and further exacerbation of the obesity epidemic [19].

Obesity is increasingly a major health problem worldwide [2]. It is estimated that approximately 1.0 billion adults are overweight (body mass index (BMI) of 25–29.9 kg/m²) and 475 million are obese (BMI ≥ 30 kg/m²), representing about 23% and 10%, respectively, of the adult population worldwide [20]. Within the 27 member states of the European Union, approximately 60% of adults and over 20% of school-age children are overweight or obese [21]. In Sweden, the prevalence of obesity among adults has doubled during the last two decades, and is now approximately 10–15% in both men and women, while approximately 35% of women and 50% of men are overweight, according to estimates based repeated random samples of the population [22]. Two recent studies suggest that the growth of the obesity epidemic may be slowing down in northern Sweden; the Northern Sweden Monitoring of Trends and Determinants in CVD (MONICA) study found that BMI did not increase between 2004 and 2009 [23], and the Västerbotten Intervention Programme (VIP) reported a slower increase in obesity prevalence among middle-aged men and women [24].

Obesity as an independent risk factor for CVD

Obesity has been associated with several comorbidities or disorders, including CVD, type 2 diabetes mellitus (DM) [25], dyslipidemia, hypertension, stroke, sleep apnea, osteoarthritis, gall bladder disease, and certain types of cancer (e.g., breast and colon cancer) [26]. Large epidemiological studies have demonstrated that obesity is an independent risk factor for CVD. The Framingham Heart study has followed 5209 men and women aged 30 to 62 years for over four decades, and since its very first analyses, obesity has been found to be a significant independent predictor of CVD, congestive heart failure, and stroke after adjustment for risk factors [1].
With data from the Framingham study, Wilson et al. [27] showed that CVD risk (including angina, myocardial infarction, coronary heart disease (CHD), or stroke) was higher among overweight men (RR, 1.24; 95% CI, 1.07–1.44), obese men (RR, 1.38; 95% CI, 1.12–1.69), and obese women (RR, 1.38; 95% CI, 1.14–1.68), but not in overweight women, with overweight defined as BMI ≥ 25 but < 30, and obesity as BMI ≥ 30. These associations remained significant after adjustment for age, smoking, high blood pressure, high cholesterol, and diabetes. Overall, obesity appears to be a significant predictor of CVD, especially in younger subjects (<50 years old), with the greatest risk in the heaviest weight class. Similar results have been obtained in other large studies, such as the Nurses' Health Study [28], the Buffalo Health Study [29], and the Cancer Prevention study II [30].

**Regional body fat distribution**

The exploding obesity epidemic has become a key issue in CVD risk assessment and management. However, obesity is not included in most cardiovascular risk calculators. Instead, obesity is considered to be accounted for by other factors, such as triglyceride level. Such substitutions are made due to the existing difficulties and unanswered questions relating to defining obesity.

**Is BMI a reliable measure of obesity?**

BMI provides a simple and convenient measurement of obesity and therefore it has been used in most of the large epidemiological studies linking obesity with CVD. However, BMI also has several important limitations and can lead to the misclassification of certain individuals, such as those with increased muscle mass or elderly subjects. It has been proposed that waist circumference (WC) in combination with BMI may be a better indicator of health risk than BMI alone. WC is considered to also be a measure of abdominal fat (central obesity), and is particularly useful for classifying individuals with a BMI of 25–34 [31]. Several studies have further demonstrated the importance of considering WC in relation to hip circumference, as WC is strongly associated with CVD mortality only after adjustment for hip circumference and vice versa [32]. A recent metaanalysis shows that the inclusion of both waist and hip circumference may improve risk prediction models for cardiovascular disease and other outcomes [33].

Some studies have described a clustering of cardiovascular risk factors associated with obesity, referred to as metabolic syndrome (MetS). Patients with MetS classically display a constellation of symptoms, including hypertension, insulin resistance, truncal obesity, and dyslipidemia [34].
Information from the American Heart association (AHA) and updated guidelines from the National Cholesterol Education Program (NCEP ATP III) state that patients are considered to suffer from MetS if they exhibit elevated waist circumference (102 and 88 cm in Caucasian men and women, respectively, and 90 and 80 cm in Asian men and women, respectively), elevated triglycerides ($\geq 1.7$ mmol/L), decreased high density lipoprotein (HDL) cholesterol ($<1.03$ mmol/L for men, $<1.29$ mmol/L for women), elevated blood pressure ($>130/85$ mmHg) or use of medication for hypertension, and elevated fasting glucose ($\geq 5.6$ mmol/L) or use of medication for hyperglycemia [35, 36]. It is estimated that more than 30% of the adult population within the United States exhibits characteristics of this pre-diabetic, metabolic disorder [37]. This suggests that a significant proportion of the population is at an increased risk of CVD before exhibiting clinical signs (i.e., morbid obesity or type II DM).

**Does all accumulated fat have the same deleterious effect on health?**

Obesity has traditionally been defined as an excess of body fat to an extent that may adversely affect health. However, it is now known that obese individuals differ not only according to the degree of excess stored fat, but also according to the regional distribution of fat within the body. For example, abdominal visceral adipose tissue confers a greater risk of cardiovascular complications compared to subcutaneous adipose tissue [25]. In the mid-forties, the French physician Jean Vague hypothesized that regional body fat distribution could determine metabolic abnormalities, such as diabetes mellitus (DM) or cardiovascular disorders. This hypothesis was initially based on his observations of different fat distribution in men and women (described as android and gynoid type, respectively). However, it would take another 40 years before scientists used modern investigation methods to formally demonstrate important metabolic differences between obese individuals, showing the association of visceral fat depot with metabolic abnormalities promoting CVD development [38]. In 2001, Brochu et al. examined a population of obese subjects ($\text{BMI} \geq 30$ kg/m$^2$) and demonstrated significantly more visceral fat in insulin-resistant individuals than in insulin-sensitive subjects with normal glucose tolerance [39]. Thus, obesity defined solely based on BMI is unable to discriminate those individuals at high risk of developing cardiovascular disorders from the patients at low or moderate risk [40]. On the other hand, some non-obese overweight patients have a higher risk of developing CVD, but it cannot be detected based on BMI alone.
How does visceral fat promote heart disease?

Visceral obesity is defined as fat accumulation around the viscera and inside the intraabdominal solid organs. Visceral fat is composed of several types of adipose tissue, including mesenteric, epididymal, white adipose tissue, and perirenal fat. The condition of having excess visceral fat is known as visceral or central obesity, and is associated with a body type in which the abdomen protrudes excessively (“apple shaped”). This differs from an excess of subcutaneous and intramuscular fat, in which case the fat is mainly deposited on the hips and buttocks (“pear shaped”). Growing evidence indicates that visceral fat is not merely a marker of metabolic dysfunction but also a potential cause, and visceral obesity has been proposed as possible link between inflammation, hypertension, and CVD [41]. The pathophysiologic mechanisms underlying this link remain mostly unclear, although the association may be explained by many of the consequences of increased visceral fat, most of which are present in the definition of the MetS (Fig. 1).

Fig. 1. Visceral obesity may lead to development of atherosclerosis and hypertension, and is a major risk factor for CVD and type-2 DM. Figure adapted from Despres JP. Circulation. 2012 [42]. HDL: high-density lipoprotein; LDL: low-density lipoprotein; SNS: sympathetic nervous system; RAS: renin-angiotensin system.
The role of adipokines

In the late 1980’s and early 1990’s, it was discovered that adipocytes from white adipose tissue secrete two inflammation-associated signaling proteins: the complement-related factor adipsin [43] and the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) [44]. In 1994, Friedman et al. discovered leptin [45], which has a pivotal function in energy balance and metabolism. The discoveries of these three factors led to the current view of white adipose tissue as an active endocrine, paracrine, and autocrine organ [3], which functions in the uptake, storage, and synthesis of lipids, as well as secretes several hormones and a diverse range of other protein factors. The collective term “adipokines” is used for protein signals secreted from (and synthesized by) adipocytes, excluding signals released only by other cell types in adipose tissue, such as macrophages [46]. Adipokines have been proposed to be the molecular link between obesity and CVD [47]. However, our scientific and clinical understanding of vascular obesity-associated diseases is restricted by the versatile activity of adipokines, the multifaceted nature of ED, and the suspicion that adipokines may not contribute equally throughout the entire pathogenesis of vascular disease [48]. To date, more than 50 adipokines are known, and their number continues to rise [46].

Cardiometabolic effects of visceral adiposity

Visceral obesity, dyslipidemia, and insulin resistance

Hepatic lipoprotein metabolism may be impaired, in part, by the release of proinflammatory cytokines—like tumor necrosis factor-alpha (TNF-α) or interleukin-6 (IL-6)—by large intra-abdominal adipocytes and resident macrophages, combined with changes in the secretion of specific adipokines, particularly decreases in adiponectin [25].

Recent studies indicate that the renin-angiotensin system (RAS) is also important in the development of insulin resistance. Increased RAS activity has been demonstrated in obesity, both systemically and within adipose tissue, and this may relate directly to the adipose tissue mass. In obesity, the RAS seems to have a detrimental effect on insulin-induced glucose uptake. Chronic angiotensin II (Ang II) administration in rats causes insulin resistance in muscle and adipose tissue [49], whereas blocking the RAS improves insulin sensitivity in muscle of diabetic mice [50]. Accordingly, several previous clinical trials suggested that angiotensin receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEi) might decrease the risk for new-onset DM [51]. However, in a randomized study, the ACEi ramipril failed to protect against the development of diabetes [52].
Visceral obesity and inflammation

Intra-abdominal fat deposits may contribute to a pro-inflammatory state that is linked to clinical events. Several studies have documented significant associations between the amount of visceral adipose tissue and the circulating levels of IL-6, TNF-α [53], and cellular adhesion molecules (CAMs) [54]. In the liver, IL-6 promotes the secretion of acute-phase proteins, such as C-reactive protein (CRP) [55]. CRP is increased in subjects with visceral obesity and CRP levels are associated with coronary events, but the cause–effect relationship has not yet been elucidated. It remains unclear whether CRP is a risk marker or a risk factor of the athero-inflammatory process [56], although it is believed that it may help identify individuals at higher risk [57]. Additionally, adipose-derived secreted factors (adipokines) have been either directly or indirectly associated with inflammation [58].

Visceral obesity and hypertension

It is estimated that between 65% and 78% of hypertension cases can be attributed to obesity [59]. Furthermore, RAS activation has been shown to contribute to obesity-associated hypertension [60]. In addition to the liver, adipose tissue serves as an extra source of angiotensinogen [61], which is converted to angiotensin I (Ang I) by renin produced in the kidneys. Through the action of ACE, Ang I is then transformed to Ang II, a powerful vasoconstrictor factor. It has also been reported that Ang II-induced hypertension is associated with ED [62], and that reduction in body weight leads to reduced RAS activity in plasma and adipose tissue, following a decrease in blood pressure [63]. Ang II also contributes to the formation of large dysfunctional adipocytes that produce increased amounts of leptin and non-esterified free fatty acids (FFA), as well as reduced quantities of adiponectin. These findings suggest a vicious circle between the RAS and the dysfunctional adipose tissue that may be involved in obesity-associated hypertension [64]. Adipokines, such as leptin, may initiate and maintain hypertension through direct or indirect mechanisms (Fig. 2). The role of adipokines in ED will be further reviewed.
Endothelial function

Endothelium regulates vasomotor tone and vascular homeostasis

The endothelium is a single-cell lining that covers the internal surface of blood vessels, separating the circulating blood from the tissues, as well as covers cardiac valves and other body cavities. Its main function is to “sense” changes in hemodynamic signals and to respond by releasing different vasoactive substances [65]. Endothelial cells can release a variety of substances that help to maintain homeostasis, including the vasodilators nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF), and bradykinin (BK), as well as the vasoconstrictors Ang II, endothelin-1 (ET-1), thromboxane A2, and oxidant radicals [65]. The carefully regulated release of endothelium-derived relaxing and contracting factors modulates the vasodilation and vasoconstriction of vascular smooth muscle cells, thus maintaining vascular homeostasis and blood flow regulation.
NO and other vasoactive factors

NO, the key endothelium-derived relaxing factor, plays a pivotal role in maintaining vascular tone and reactivity and is the main determinant of basal vascular smooth muscle tone [66]. NO is synthesized from L-arginine by nitric oxide synthase (NOS). It is a volatile gas present in practically all tissues. Its low-molecular weight together with its lipophilic properties allows NO to easily diffuse across cell membranes, such as the endothelium intima, reaching the smooth muscular tissue of the arterial wall. Here it stimulates the soluble guanyl cyclase and increases intracellular cyclic guanosine monophosphate (cGMP), which in turn regulates the cytosolic calcium (Ca²⁺), causing smooth muscle fiber relaxation and therefore vasodilation [67] (Fig. 3).

There are three types of NOS isoenzymes: NOS I and NOS III are each constitutively produced at a low level (from neurological tissue and endothelial cells, respectively) and NOS II (iNOS) is inducibly expressed in macrophages and endothelial cells. The constitutive NOS isoenzymes respond to increases in intracellular Ca²⁺ and produce NO for short periods of time, being induced for example by vasodilators like acetylcholine (Ach) or BK. In contrast, iNOS is expressed due to the effects of pro-inflammatory cytokines, like TNF-α or IL-1, and can release several times more NO than the constitutive NOS isoenzymes [68]. iNOS has been also implicated in development of muscle insulin resistance in diet-induced obesity [69]. However, the most important stimulation for NO release comes from shear stress [70] caused by increased blood velocity. Shear stress leads to persistent NO production, which maintains constant vasodilatation proportional to the amount of NO released by the endothelium [71]. Via NOS stimulation, substances such as Ach, BK, or substance P, induce NO-release and consequently cause endothelium-dependent vasodilatation.

NO is also considered to be an antiatherogenic molecule [72]. Thus, beyond its vasodilator effect, NO also hinders coagulation by increasing blood flow, reduces vascular permeability, inhibits platelet adhesion and aggregation, inhibits leukocyte migration to the subendothelial space and adhesion, inhibits smooth muscle cell migration and proliferation, reduces the expression of adhesion molecules, reduces tissue oxidation and inhibits oxidation of low-density lipoproteins, inhibits activation of thrombogenic factors, and inhibits pro-atherogenic and pro-inflammatory cytokines [73].
Fig. 3. The L-arginine–NO system is an important endogenous vasodilator system. Endothelial NO synthase produces NO from the amino acid L-arginine. Endothelial release of NO is stimulated either by increased blood flow or by endogenous/exogenous agonists, activating guanylate cyclase in vascular smooth muscle cells and leading to vasodilation. Picture taken from Landmesser U and Drexler H. Curr Opin Cardiol. 2007.

The endothelium produces two other major vasodilating factors: prostacyclin (PGI2) and EDHF. Whereas NO is the principal endothelium-derived vasodilator in large conduit arteries, EDHF is more important in smaller resistance vessels [75]. The mechanisms of EDHF are heterogeneous, varying according to the type of vascular beds or animal, and are generally not well understood [76]. Calcium-activated potassium channels in the endothelial cell seem to be implicated in EDHF hyperpolarization [77]. In many experimental studies, EDHF-mediated relaxation is preserved or even upregulated when NO production is impaired or when NO becomes deficient. Thus, it has been proposed that EDHF may act as a back-up vasodilatory mechanism when NO becomes inefficient [75].

The endothelium also produces factors that induce vasoconstriction, including ET-1 and endothelium-derived contracting factor (EDCF). The latter is able to activate thromboxane receptors in smooth muscle cell. There is evidence that ET-1 and EDCF are upregulated in many diseases, such as DM, hypertension, and coronary artery disease [73].
**Endothelial-dependent and -independent vasodilatation**

Endothelium-dependent vasodilatation (EDV) is broadly defined as vasodilatation mediated by endothelium, either via NO or by other vascular relaxing factors (EDHF or prostacyclins). EDV may be induced by mechanical “shear stress” or by physiological or pharmacological agents, such as Ach, BK, or substance P. Endothelial-dependent vasodilatation is the most widely used clinical end-point for assessing endothelial function.

On the other hand, non-endothelial or endothelial-independent vasodilatation (EIDV) occurs when nitrates act as NO donors and directly release cGMP in the smooth muscle cells, causing vasodilatation that does not depend on the endothelial response. Calcium antagonists also interact with voltage-operated calcium channels, thereby inhibiting smooth muscle contractility and promoting EIDV. The term EIDV is often used in direct comparison with EDV.

**Antithrombotic and fibrinolytic function**

A healthy endothelium also functions in “thromboresistance,” and NO is a potent inhibitor of platelet adhesion and aggregation [78]. Additionally, endothelial mechanisms mediate the intravascular breakdown of fibrin (endogenous fibrinolysis), a critical component of maintaining vascular homeostasis. To carry out such functions, vascular endothelial cells synthesize and release various factors, such as the plasminogen activator, which converts plasminogen to plasmin, an enzyme that degrades fibrin (and fibrinogen). Tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) generate plasmin locally and, consequently, fibrinolysis is limited to the immediate environment [79]. Plasminogen activator inhibitor-1 (PAI-1)—a glycoprotein member of the superfamily of serine protease inhibitors (serpins)—is secreted by different cell types: principally by vascular endothelial cells but also by adipose tissue [80]. As the main inhibitor of t-PA and uPA, PAI-1 impairs fibrinolysis.

**Endothelial dysfunction (ED)**

ED can be defined as the disruption of the ability of the endothelial cell to maintain the delicate balance between the vasoactive substances necessary to maintain homeostasis. This imbalance predisposes the vasculature to vasoconstriction, platelet activation, mitogenesis, pro-oxidation, thrombosis, impaired coagulation, or vascular inflammation [81]. Alteration of endothelial function precedes the development of morphological
atherosclerotic changes, and can also contribute to lesion development and later clinical complications [82].

**Mechanisms of endothelial dysfunction**

Endothelial nitric oxide synthase (eNOS) normally helps to maintain the quiescent state of the endothelium but, in certain circumstances, it can switch to generate reactive oxygen species (ROS) instead of NO. This phenomenon is referred to as “eNOS uncoupling”. It leads to overproduction of ROS, particularly superoxide anion radical (O2-), which then binds NO to form peroxynitrite (ONOO-), a powerful oxidant agent that easily diffuses through cells and can alter protein function and damage a wide array of cellular molecules, including deoxyribonucleic acid (DNA) [83]. Endothelial ROS signaling may also be initiated by exposure to inflammatory cytokines and growth factors. Furthermore, the interaction between leukocytes and endothelium is important in atherosclerosis development. Overall, ED is related to an inflammatory process in the vessel, characterized by increased adherence of leukocytes (monocytes and T-lymphocytes) and lipids, as well as the apparition of “fatty streaks”, which is considered the first step in atherosclerotic plaque formation [84]. Endothelial NOS uncoupling may be considered part of “endothelial activation” and occurs in the context of diseases, such as atherosclerosis or diabetes. It is also observed in other situations, including hypoxia, passive smoking, and chronic exposure to air pollutants [75].

Other proposed mechanisms of ED are related to reduced eNOS expression, deficiency in L-arginine or its transport, excess endogenous eNOS inhibitor (ADMA), or deficiency in eNOS cofactor (tetrahydrobiopterin). Some of these mechanisms have been observed in patients and animal models with DM, hyperlipidemia, hypertension, aging, heart failure, or pulmonary hypertension [85]. Additionally, the endothelial progenitor cells (EPCs) produced by bone marrow have been the focus of endothelial research in recent years. Bone-marrow-derived endothelial stem cells and EPCs contribute to vascular injury repair in response to mechanical and chemical injuries. A relative EPC deficiency or diminished recruitment of EPCs to the site of tissue repair (as in type II diabetes) is also a condition associated with ED [86].

In summary, ED can be considered an integrative marker of the net effects of arterial wall damage from traditional and emerging risk factors, and of its intrinsic capacity for repair. The study of the ED is important, not only in relation to atherosclerosis initiation and progression, but also in relation to the transition from stable to unstable disease states [81].
Obesity and endothelial dysfunction

Obesity is associated with ED, and is a precursor of atherosclerosis and CVD [82], but the mechanisms underlying these associations remain relatively poorly understood. It remains unknown to what extent and through which mechanisms obesity promotes ED. This is a complex matter, partly because common obesity-related disorders (e.g., insulin resistance, dyslipidemia, and/or hypertension) are also associated with ED, and partly because of the etiology of ED is multi-factorial and depends on the affected segment(s) of the vascular tree. As discussed earlier, pathologies may vary between occurrences in small arterioles (located deep within the myocardium) and in the much larger conduit arteries. Therefore, it is critical to investigate the effects of obesity at the level of both the micro- and microvasculature to fully understand the mechanisms underlying obesity-induced coronary disease.

Under normal physiological conditions, myocardial oxygen consumption closely matches the oxygen delivered by the coronary blood flow. The myocardium has a very limited anaerobic capacity and is highly dependent on a continuous supply of oxygen from the coronary circulation to meet metabolic demands; thus, the microvascular coronary circulation plays an essential role in maintaining optimal cardiac function [87]. If the requirements for oxygen supply are not sufficiently met, the resulting underperfusion (ischemia) diminishes cardiac function within seconds [88].

The major coronary arteries serve as conduits for arterial blood flow between the ascending aorta and the smaller resistance arteries deep within the myocardium. In contrast to the coronary microvasculature, the larger epicardial arteries of the heart contribute very little to blood flow regulation [89]. In large coronary arteries, atherosclerosis is the primary obesity-associated pathology [90]. Large atherosclerotic lesions typically occur in the larger coronary arteries as opposed to in distal branches and microvessels [91].

Effects of obesity on the microvascular system

Impaired microvascular function has been demonstrated in the setting of obesity [92]. Obesity may affect microvascular circulation through both structural changes (decreased capillary density, also called rarefaction) [93] and functional changes, such as blunted vasodilation in response to classic endothelium-dependent vasodilators in skin and resistance vessels [92].

In particular, the coronary microvascular system is responsible for maintaining the myocardial oxygen supply. Obesity is characterized by
microvascular alterations, and the onset and severity of microvascular dysfunction appears to be associated with a progressive increase of abdominal fat [92]. Furthermore, patients with MetS have exhibited diminished coronary flow reserve (the difference between maximal and baseline coronary blood flow) [94]. This suggests that the ability to match coronary blood flow to myocardial oxygen demand is diminished with obesity, and that obese patients may have a limited capacity to vasodilate and provide sufficient oxygen during situations of increased myocardial metabolic demand. It is thought that this contributes to the increased CVD-related morbidity and mortality that is commonly observed in obese patients [95]. Additionally, Kiviniemi et al. demonstrated that waist-to-hip ratio is a negative independent predictor of coronary blood flow reserve in young healthy men [96]. The observed reduction in flow reserve could be related to impaired vasodilator capacity, enhanced vasoconstrictor responsiveness, and/or structural remodeling of the coronary microvasculature [97, 98]. Hence, ED and intensified vasoconstriction appear to be hallmarks of coronary circulation in cases of obesity.

Alterations in endothelial-mediated control of the coronary blood flow may occur due to activation of vasoactive neural–hormonal pathways [95]. Furthermore, it has been demonstrated that dysfunctional blood flow regulation in coronary arteries precedes obesity-associated metabolic alterations (changes in blood pressure, glucose tolerance, and insulin resistance or lipid metabolism) [99] or chronic inflammation [96]. This suggests that microvascular dysfunction is a primary component of disease progression. However, the mechanisms underlying obesity-associated microvascular dysfunction are complex and poorly understood. It has been proposed that altered eNOS production secondary to blunted phosphatidylinositol 3-kinase (PI3K) may increase oxidative stress in endothelial and vascular smooth muscle cells (VSMC) [100], and that upregulated ET-1 production or downregulated eNOS production may impair the balance between endothelial-derived vasodilator and vasoconstrictor substances.

Of the possible mechanisms, two are suggested to be primarily involved in the development of obesity-associated microvascular dysfunction [101]. The first relates to a state of obesity-related insulin resistance in which intracellular signaling disturbances may affect the normal balance between insulin-mediated vasodilator (via eNOS activity) and vasoconstrictor (via ET-1) effects. These insulin-signaling abnormalities would predominantly affect PI3K pathways and, correspondingly, eNOS activity, NO production, and vasodilatation. In this situation, vasoreactivity would be shifted from vasodilation toward vasoconstriction. One study demonstrated such a
change among obese hypertensive individuals, who showed insulin-induced vasoconstriction and increased ET-1-dependent vasoconstrictor tone, as well as decreased NO-dependent vasodilator tone [102].

The second mechanism is related to the concept of visceral adipose tissue as a highly endocrine organ, and particularly to the role of adipokines. Obesity has been associated with augmented production of ET-1 [103], angiotensinogen, leptin, resistin, and several inflammatory cytokines (e.g., TNF-α and IL-6) [104, 105], as well as with reduction of adiponectin [106]. Different studies have found associations between lipid and TNF-α infusion and decreased endothelium-dependent vasodilatation [107, 108]. Furthermore, studies performed in vitro and in animal models (dogs) have shown that leptin and resistin are associated with impaired vasomotor responses in both conduit and resistance coronary arteries, indicating direct effects on NO production, although vasomotor tone and systemic hemodynamics were unaffected [13, 109]. On the other hand, adiponectin is considered an anti-inflammatory and “protective” cytokine, with lower levels associated with obesity or increased fat mass [110].

**Effects of obesity on the macrovascular system**

Vascular atherosclerosis (i.e., coronary artery disease) may be considered a macrovascular disease [90]. As discussed above, endothelial injury is the initiating event in the development of atherosclerosis [111], and chronic ED precipitates the cascade of cellular events resulting in arterial plaque formation [82]. In the context of obesity, a vicious cycle of inflammation, lipid deposition, and smooth muscle proliferation results in early "fatty streaks" and atherosclerotic lesions that can further progress into complicated stenotic plaques with necrotic cores (vulnerable plaques) that are susceptible to rupture [112]. In the setting of high shear stress and turbulent blood flow (i.e., large coronary arteries), vessels are particularly vulnerable to injury if the vascular endothelium is impaired or dysfunctional [91]. Furthermore, the onset of obesity or MetS (i.e., inflammation, hyperlipidemia, hyperglycemia, and hypertension) compromises the natural endothelial response to blood flow, potentially initiating atherosclerosis development, particularly in large coronary arteries [113].

**Perivascular Adipose Tissue**

Perivascular adipose tissue—the local visceral adipose tissue surrounding a vessel—may also contribute to unfavorable cardiometabolic complications [114, 115] and may be involved in atherosclerosis development [116]. In vitro and ex vivo studies have shown that perivascular adipose tissue induces
artery wall inflammation by secretion of pro-inflammatory proteins. Furthermore, coronary atherosclerosis is typically found in segments of coronary arteries that are surrounded by perivascular adipose tissue, where pro-inflammatory cytokines and adipokines may be expressed and secreted at higher levels. These observations suggest that atherogenesis in the vascular wall is stimulated from “outside to inside”. In this process, it has been proposed that the coronary vasa vasorum might help to traffic harmful adipokines between perivascular adipose tissue and the vascular lumen [48].

Little is currently known about the physiological and pathophysiological functions of perivascular adipose tissue, although some studies have shown that perivascular adiposity may affect eNOS phosphorylation, subsequently impairing NO-mediated vasodilatation [117]. Adipokines might also directly inhibit vasodilatory pathways distal in the arteriole, thereby reducing blood flow in the nutritive capillary. Hence, adipokines released from fat depots would have local rather than a systemic vasoregulator effect—a mechanism termed “vasocrine” signaling [118]. However, no investigation has yet elucidated any direct causal link between perivascular adipose tissue and coronary artery disease.

**Assessment of endothelial function**

Monitoring disturbed vasomotion (vasoreactivity) associated with endothelial activation in response to stimuli (either by drugs or shear stress) has become the standard method of studying endothelial function.

An ideal test for assessment of endothelial function would be safe, noninvasive, reproducible, cheap, and standardized between laboratories [119]. Unfortunately, there is no single method fulfilling all of these requirements. Assessment of ED by infusing coronary circulation with different agonists is a direct measurement technique, but its high cost and invasive nature limits its use in patients with overt CVD, in healthy individuals, and for repeated testing during follow-up. Therefore, several different methods have been developed, which may individually characterize the multiple facets of the endothelial biology. Characteristics of each test are summarized in Table 1.
Table 1. Methods for Clinical Assessment of Endothelial Function (adapted from Deanfield et al. 2007 [81]).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Non-Invasive</th>
<th>Repeatable</th>
<th>Reflects biology</th>
<th>Predicted Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac catheterization (Change in CBF)</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Strain-gauge venous plethysmography (Change in FBF)</td>
<td>−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ultrasound FMD (Change in brachial artery diameter)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tonometry-PWA (Change in AIX)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>PWA + pharmacological testing (Change in RI)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Repeatable = the ability to obtain consistent results when measuring the same event with the same measuring instrument; Reflects biology = the results reflect the dynamic biology of the endothelium through the natural history of atherosclerotic disease; Predicted outcome = the results define subclinical disease processes and provide prognostic information for risk stratifications in the later clinical phase; − = insufficient evidence in the literature; + = supportive evidence in the literature; CBF = coronary blood flow; FBF = forearm blood flow; FMD = flow-mediated vasodilation; PWA = pulse wave analysis; AIX = augmentation index; RI = reflective index.

**Conduit vs. resistance vessels**

At this point, it is necessary to expatiate on the definitions of two types of vessels. The conduit vessels—i.e., the aorta and its main branches—are elastic arteries that offer little resistance to blood flow. There is little pressure drop throughout this system, and the vessels are able to expand when blood is intermittently ejected into them from the heart, and to constrict again as blood flows out of them into the smaller vessels. The combination of a distensible large vessel and a downstream resistance (the arterioles) transforms an intermittent cardiac ejection into a continuous capillary flow [120]. Thus, since coronary arteries are considered conduit arteries, all techniques measuring ED in peripheral conduit arteries have
been accepted as surrogate markers of coronary ED. There are two main methods for assessing endothelial function in conduit arteries: ultrasound measure of flow-mediated dilation (FMD) [121] and peripheral applanation tonometry (PAT) in the radial artery [122]. Several studies have shown a close relationship between endothelial function in the coronary arteries and the peripheral circulation (often measured in the brachial artery) [123], even in patients with normal coronary arteries [124].

Small arteries and arterioles are called resistance vessels, and are responsible for determining blood pressure. Arterioles have a diameter of between 20 and 30 µm, and are the vessels at the end of the arterial tree. In relation to their diameter, they have very thick walls, which are mainly comprised of smooth muscle. The degree of contraction of this muscle regulates the vessel diameter and, consequently, the amount of blood flowing through it. Arterioles are normally partially contracted and are responsible for the largest pressure drop in the circulation. The degree of contraction in these vessels can also be modified or regulated by external factors, such as chemical products (metabolites), stimuli from the sympathetic nervous system (SNS), and various hormones that reach the muscle fibers in the walls of the arterioles and cause relaxation or contraction. This local vasoreactivity can enable an organism to control local blood flow to match the tissue energy requirement and to regulate arterial blood pressure. Most studies have measured vasomotor function in small resistance vessels through estimation of the FBF, namely by venous pressure plethysmography [125].

Despite the differences between these types of vessels, there is a fairly good correlation between endothelial function in the subcutaneous small arteries and in the brachial artery [126].

**Techniques for assessing endothelial function**

No single technique of testing endothelial function is representative of all vascular beds, and each method presents both advantages and limitations (summarized in Table 1). For simplicity, we have divided these techniques into two categories: invasive and non-invasive. Additionally, the analysis of the fibrinolytic function represents another way to study endothelial function.
Invasive techniques

Intracoronary infusion of vasoactive agents

In 1980, Furchgott and Zawadzki performed their classical experiment which demonstrated that a direct Ach infusion in coronary arteries exerted NO-related vasodilatation in vessels with intact endothelium [127]. However, Ach also caused paradoxical vasoconstriction and decreased coronary blood flow in subjects with disrupted, activated, or dysfunctional endothelium, resulting from a direct muscarinic smooth muscle vasoconstrictor effect [71]. Since then, intracoronary infusion of vasoactive agents combined with quantitative angiography has been considered the method of choice for direct quantification of endothelial function in the coronary arteries [128]. This technique is invasive but simple. Coronary blood flow velocity is recorded in the proximal segment of a coronary artery using a Judkins catheter, usually in the left anterior descending (LAD) coronary artery. This is followed by co-infusion of saline with adenosine, Ach, or any other vasoactive substance (e.g., BK, serotonin, or substance P). Finally, endothelial function is evaluated using dose–response curves as a measure of coronary blood flow.

Although simple and effective, the high expense and the invasive nature of coronary assessment limit its use for patients with overt CVD, in healthy individuals, and for repeated testing during follow-up studies. For this reason, other “indirect” ways to assess ED have been developed.

Intrabrachial infusion of vasoactive agents

The technique of intracoronary infusions of vasoactive agents can also be applied in the brachial artery, which is more accessible than the coronary vessels and presents fewer potential complications [129]. Endothelial function in the human coronary has been shown to be closely related to peripheral circulation [123]. Venous occlusion plethysmography is a classical method, first described over a century ago by Hewlett & van Zwaluwenburg [130]. This technique was originally developed as a non-invasive method to assess limb blood flow through the analysis of venous outflow curves, which reflect volume changes in response to temporary occlusion of venous blood flow [131]. Initially, these curves were evaluated to document venous outflow obstruction in patients with deep venous thrombosis [132]. Since then, venous occlusion plethysmography has been extensively used to study human vascular physiology in vivo, especially after the introduction of computerization and the advent of mercury-in-silastic strain-gauges. This method of assessment is most powerful when combined with intra-arterial
administration of receptor agonists or antagonists, usually into the forearm vascular bed, permitting a detailed study of the vascular physiology [133]. This technique may now be considered as a “gold standard” in the assessment of vascular function in health and disease, enabling assessment of the effects of new vasoactive drugs and hormones in humans in vivo [134]. Advantages of this approach include that vessels are studied in their physiological environment under the influence of neuronal, circulating, and local mediators [125], and that drugs can be administered at subsystemic doses to minimize disturbances to systemic physiology [133].

This technique measures changes in forearm resistance vessel tone, and provides the opportunity to evaluate endothelial pathophysiology in the preclinical stage of disease. Intrabrachial infusion of vasoactive agents mainly evaluates EDV in forearm resistance arteries. A reduced EDV has been found in patients with CHD, hypertension, hypercholesterolemia, and DM [135-138], and has also been observed to predict future cardiovascular events [139]. Further details regarding the procedure of the plethysmography technique will be reviewed in the “Materials and methods” section.

**Non-invasive techniques**

**Ultrasound**

Another method of EDV assessment is the ultrasound-based method in the brachial artery during hyperemia [121, 140]. This technique is based on the principle that increased blood flow in peripheral arteries during hyperemia leads to increased shear stress stimuli, increased NO production, and vasodilation. The vasodilatory response of the brachial artery to increased shear stress is called FMD, and reflects the NO production ability of vascular endothelium [141]. Moreover, the comparison of FMD produced after administration of nitrates may help to elucidate the changes in smooth muscle function or arterial compliance that may affect the observed changes in FMD [140]. FMD is attenuated in patients with CHD and in those with major risk factors [83, 123], and can predict future cardiovascular events [142]. Recent studies show an association between decreased FMD and the coronary risk as expressed by the Framingham score index [143, 144]. This technique mainly evaluates EDV in a conduit artery, and has the advantages of being non-invasive, and safer and faster than invasive methods, while its results are closely correlated with endothelial function in the coronary arteries. However, it also seems to be highly operator dependent, requires excellent patient cooperation, and has relatively poor resolution relative to arterial size [129]. The procedure requires a quiet room set at a constant
temperature, 12 hours of fasting, and no previous vasoactive medication or smoking. Thereafter, in a comfortable arm position, the brachial artery is imaged above the antecubital fossa in the longitudinal plane, using a transducer (frequency, 7–12 MHz) attached to a high-quality ultrasound system.

**Tonometry**

Applanation tonometry is a non-invasive method for obtaining the arterial pressure waveform of an artery. A small pencil-probe-like tonometer is positioned over the maximal arterial pulsation to minimally flatten (applanate) the arterial wall against the underlying bone. The tip of the tonometer is directly impacted by intra-arterial pressure, allowing accurate recording of the pressure waveform.

In general, pulse wave analysis (PWA) provides a measure of aortic stiffening or arteriosclerosis, which is primarily considered as a degenerative process secondary to age-related changes in the vessel. The features of the pulse wave curve may vary depending on physiological conditions, such as heart rate, height, age [145], and BMI or body fat [146]. Furthermore, some pathological conditions may increase arterial stiffness, such as chronic renal insufficiency [147], hypertension, and DM [148]. PWA can be used to estimate two important parameters: the augmentation index (AIx) and the pulse wave velocity (PWV). Both are independent predictors of adverse cardiovascular events, including mortality [149].

**Fibrinolytic function**

Consistent findings between the peripheral (forearm) [150] and coronary circulations [151] support the notion that the forearm model is a reasonable surrogate of endogenous fibrinolysis in the coronary vessels. The fibrinolytic system protects from intravascular thrombus formation, and the endothelium also plays a vital role in controlling coagulation and fibrinolysis. Injury or inflammation may cause the endothelium to become pro-coagulant through downregulation of its anticoagulant functions and reduced fibrinolytic activity that can enhance atherogenesis. Prolonged presence of residual thrombus over a disrupted plaque provokes smooth muscle cell (SMC) migration and new connective tissue production, leading to plaque expansion [152].

Endothelial t-PA—a 68-kDa serine protease generated principally in the endothelium—is the main fibrinolysis activator in the intravascular compartment. t-PA catalyzes the conversion from plasminogen to plasmin,
which is involved in the dissolution and proteolytic degradation of fibrin to soluble fibrin degradation products. The basal concentration of t-PA antigen (t-PA ag) has been associated with CHD risk, but fibrinolysis onset is mainly determined by the rapidity and magnitude of t-PA release, which is used as a measure of endothelial function [153]. t-PA may be released to the circulation through a constitutive and a regulated pathway. In the former, t-PA is continuously synthesized and secreted from the endothelium, whereas the latter pathway involves t-PA release from small dense vesicles, allowing a rapid and substantially local increase of t-PA concentration in response to various stimuli. The t-PA concentration in human plasma is 3–10 ng/mL, with a half-life of 3 to 5 min.

The proportion of active t-PA varies with the presence of its main inhibitor, plasminogen activator inhibitor type 1 (PAI-1). PAI-1 is a glycoprotein belonging to the serpins, which is produced in multiple cell types, including endothelial cells, SMC, platelets, macrophages, hepatocytes, and adipocytes. Plasma t-PA circulates in a stable 1:1 complex with PAI-1 (the t-PA/PAI-1 complex), as well as in an active uncomplexed form. The sum of the complexed and uncomplexed t-PA is denoted t-PA ag; hence, increased t-PA ag partly reflects the concomitant increase of plasma PAI-1 concentration. Most t-PA circulates as part of the t-PA/PAI-1 complex [154], which restricts fibrinolysis by limiting the activation of plasminogen to plasmin [155]. About 20% of steady-state plasma t-PA circulates in its active form (t-PA act) [156]. Reduced t-PA act, expressed in IU per 100 mL of forearm tissue per minute, is associated with cardiovascular risk [157].

In summary, high concentrations of t-PA/PAI-1 complex, t-PA ag, and PAI-1 antigen reflect deteriorated fibrinolytic activity, whereas high t-PA act indicates high fibrinolytic activity. However, the acute endothelial release of t-PA in vivo is more relevant than the basal t-PA concentration. Systemic inflammation [158], oxidative stress [159], or SNS activation [160] may alter plasma levels of released t-PA. It is difficult to measure basal t-PA concentration in systemic plasma, partly due to the rapid clearance in the liver. One possible way to systemically measure acute release of t-PA is by using intravenous infusion of BK, substance P, methacholine, or desmopressin. BK releases t-PA without affecting release of PAI-1 and without involving NOS. Thus, assessment of dynamic t-PA release after BK infusion provides a measure of endothelial function that is distinct from assessment of NO-mediated responses. It is important to remember that ACE may influence BK-induced t-PA release. In fact, ACE inhibition might enhance endothelial t-PA release through augmentation of endogenous BK or by increasing BK receptor expression [161]. Therefore, ACE-blocker/ARB
treatment must be stopped for at least one week before assessment of net t-PA release.

Local availability of active t-PA depends on the extent of local t-PA release rather than on the amount of t-PA or PAI-1 entering the tissue in arterial blood [162]. Thus, direct assessment of the local capacity for acute t-PA release within individual vascular beds provides a better representation of defense against arterial thrombus. This method also avoids potential systemic confounders related to differences in t-PA and PAI-1 clearance rates or eventual SNS activation. Another benefit of measuring t-PA release locally in the forearm is the relatively low rate of clearance, which occurs mainly in the liver.

**Leptin**

**Historical view**

In 1950, a study described an extremely obese mouse presenting hyperphagia, infertility, and metabolic disturbances, including early onset of DM and insulin resistance. This condition was considered an autosomal recessive syndrome, with spontaneous genetic mutation in chromosome 6. This gene was defined as *ob*, and was expressed exclusively in adipocytes. The obese mice carrying the homozygous mutation in the *ob* gene were called *ob/ob* mice. In 1994, Friedman et al. identified and cloned the protein encoded by the *ob* gene [45], located on chromosome 7 in humans, and the gene product of this gene was called leptin, from the Greek word "leptos", which means thin. *Ob/ob* mice do not produce functional leptin, and thus exhibit increased appetite and reduced energy expenditure (decreased mobility and thermogenesis), resulting in a chronic positive energy balance. Administration of exogenous leptin normalizes body weight and all metabolic and endocrine abnormalities in these animals [163].

The leptin receptor (LR; also known as the *ob* receptor) is the product of the *db* gene. The *db/db* mouse presents a deletion in the long isoform of the LR (LRb), resulting in leptin resistance [164]; this mouse is used as a model of obesity, diabetes, and dyslipidemia. The LR was found in 1995 and is considered a member of the cytokine receptor family [165]. LR expression patterns vary in different tissues, with greater expression in the hypothalamus, where the LR is responsible for generating intracellular signals in response to leptin, and thereby regulating appetite and body weight [163].
**Leptin, the prototypical adipokine**

Leptin is a 167-amino acid peptide with a molecular weight of 16 kDa, formed by a four-helix bundle motif similar to that of a cytokine (resembling the structure of IL-6). It is produced primarily in adipose tissue, and is therefore included in the adipokine family, together with adiponectin, resistin, tumor necrosis factor-alpha, and others. However, leptin is also expressed in other tissues, including the placenta, ovaries, mammary epithelium, bone marrow, and lymphoid tissues. After its discovery in 1994, leptin was initially considered a satiety factor, and it was demonstrated that circulating leptin communicates the state of body energy repletion to the CNS in order to suppress food intake and permit energy expenditure [166]. However, later studies have shown that, in addition to its central actions in the hypothalamus, leptin may affect multiple target organs. Leptin shows immune properties [167]; acts on reproduction, with fetal and placental mechanisms [168]; is postulated to regulate bone mass and cartilage [169]; and affects vascular function, with demonstrated effects on atherosclerosis, angiogenesis, and oxidative stress [170].

Leptin concentration reflects the amount of body fat; however, adipocytes exhibit no significant leptin storage. Basal secretion of leptin occurs at a stable rate. Pulsatile levels follow a circadian rhythm in both obese and lean individuals, with the highest levels between midnight and early morning and lowest levels in the early afternoon to mid-afternoon [4]. In human serum, leptin circulates as free leptin in a monomeric form, or partially bound to proteins. The fraction of free/total leptin is not constant, and its balance is mediated by the metabolic state. In lean subjects, most leptin (approximately 45%) circulates in the bound form, restricting the availability of leptin to exert its effects on food intake [171], whereas in obese subjects, the majority of leptin circulates as free leptin (only 20% bounded). A small proportion of leptin is also tissue-bound, helping to maintain stable plasma concentrations [172]. The half-lives of free and protein-bound leptin are similar to those of other peptide hormones, approximately 3-4 minutes and 71 minutes, respectively [173].

Independently of BMI, non-menopausal women present higher plasma leptin levels than men, although these levels decline after menopause. These sexual differences may be partly due to differences in sex hormones (especially estrogens) [174], the amount of fat mass and body fat distribution [175, 176], and/or the rates of clearance in the kidney (clearance rates are lower in lean and obese women compared to obese men) [177]. However, it has not been clearly demonstrated that the clearance rate differs between
Lean and obese individuals [178]. Leptin is degraded within the kidneys and, consequently, higher levels are seen in individuals with renal failure.

Beyond obesity, other factors have been associated with increased leptin levels, such as estrogens, glucocorticoids, inflammation, infections, certain cytokines and growth hormones (GH), or certain toxins (e.g., toxin-A from *Clostridium difficile*). Leptin levels may also fluctuate with acute changes in caloric intake. Chronic starvation, acute fasting, androgens, catecholamines, or chronic exposure to IL-1, IL-6, and TNF-α have been documented to decrease leptin levels.

Leptin has been used in various research studies, and as treatment in humans in rare cases of genetically caused leptin deficiency with severe and early-onset obesity. The pharmacokinetic properties of leptin have been studied, with assays indicating a time to maximal concentration and half-life of approximately three hours [179].

**Leptin signaling**

The LR, produced by the *db* gene, is a leptin-binding transmembrane protein. It is mainly located throughout the CNS, but also detected in a wide variety of target organs and cells, including the kidneys, pancreas, bowels, vascular endothelium and vascular smooth muscle [180], gonads, prostate, adipose tissue, choroid plexus, liver, lung, skeletal muscle, bone marrow, spleen, and hematopoietic cells (particularly macrophages) [172, 181, 182]. The biologically active soluble form of LR determines the rate of circulating free leptin (free leptin index), which in turn is regulated by factors such as gender, adiposity, sex hormones, or exogenous leptin administration [183].

The *db* gene encodes at least six different LR isoforms: the “long” form (LRb, also called OB-Rb), the four “short” forms (LRa, -c, -d, and -f) and the soluble form (LRe) [184]. All receptor isoforms share the same extracellular hormone-binding domain, but differ in the structure and signaling potential of their intracellular domains. The short isoforms LRa, LRe, LRd, and LRf trigger signaling mechanisms, such as mitogen-activated protein kinases (PI3K/protein kinase B (also known as Akt), or adenosine monophosphate (AMP)-activated protein kinase (AMPK). LRa and LRe seem to play important roles in transporting leptin across the blood–brain barrier (BBB) [185].

The long isoform LRb contains the biggest intracellular fragment and is primarily responsible for leptin signaling [186]. After ligand binding, LRb recruits the cytosolic Janus tyrosine kinase 2 (JAK2), which is a signal
transducer and activator of transcription 3 (STAT-3) protein. Phosphorylated (activated) STAT-3 translocates to the nucleus and regulates gene expression [187]. Through hypothalamic expression, LRb and the downstream JAK2-STAT-3 pathway are involved in regulation of energy homeostasis and anorectic effects [188]. However, the role of LRb in regulating extrahypothalamic signal transduction remains mostly unclear.

The soluble isoform LRe lacks both intracellular and transmembrane domains. It circulates in the plasma, and is the primary isoform that binds leptin in circulation, thus regulating leptin clearance and half-life [188]. Several studies have indicated that the LRe also increases insulin sensitivity by acting on the Insulin receptor substrate (IRS)-PI3K pathway. This interaction could partly explain the observed inverse relationship between the soluble receptor and insulin resistance [189].

**Leptin receptors in vascular tissue**

Immunocytochemical studies of vascular tissue first revealed that leptin and two LR isoforms, long (125 kDa) and short (100 kDa), were present in endothelial cells and smooth muscle of umbilical veins and arteries [12]. In 2005, Knudson et al. further showed that the long isoform LRb was expressed in human coronary artery endothelial cells [13]. The activation of LRs in the endothelium may start a cascade of several signal transduction pathways. Of particular interest, the resulting activation of eNOS (through mechanisms involving AMPK and protein kinase B/Akt) further activates eNOS, increasing its activity and favoring NO production.

**Concepts of leptin resistance and selective leptin resistance**

Most obese individuals (except those with congenital leptin deficiency) present higher leptin levels than lean individuals, and are resistant or tolerant to the effects of leptin [190]. Elevated leptin levels in obesity are typically insufficient to prevent dysregulation of energy balance [191]. Except in rare cases of monogenic genetic syndromes, leptin resistance appears to usually be multifactorial [192]. LRa and LRc-mediated transport may be impaired in cases of obesity, partly due to saturation of the transporter across the BBB [193]. Additional factors, such as hypertriglyceridemia, may also impair BBB transport [194]. Obesity-associated hyperleptinemia has a negative feedback effect on leptin signaling, which is mediated by leptin signaling inhibition via the long isoform LRb. This process may involve different pathways, e.g. through upregulation of the suppressor of cytokine signaling 3 (SOCS3), or by dephosphorylating the Janus kinase and Signal
transducer and activator of transcription 3 (JAK2/STAT3) through Protein-tyrosine phosphatase 1B (PTP1B) [195].

Although most cases of human obesity are associated with hypothalamic leptin resistance, little is known about the peripheral effects of leptin in obese individuals. Another hypothesis, which may be relevant to both central and peripheral leptin resistance, involves leptin interaction with circulating factors in the blood, such as CRP, which is elevated in obesity states. CRP may inhibit the binding of leptin to LRs, blocking its ability to signal in cultured cells. Furthermore, CRP has been correlated with increased adiposity and plasma leptin levels, suggesting a systemic self-induced negative feedback that may lead to leptin resistance in the obese state [196].

**Leptin resistance in the vascular tissue**

According to the classic concept of “selective” leptin resistance [197], the appetite-suppression effect of leptin is attenuated in obese subjects, whereas other activities, such as the sympathetic activity or cardiac remodeling, remain intact [198].

It remains unknown whether peripheral leptin resistance occur not only in CNS but also in vascular tissues. Obesity-induced leptin resistance has been demonstrated in the vascular wall, but not in the myocardium. High leptin concentrations significantly attenuated Ach-mediated vasodilation in coronary rings in control dogs. However, these effects were not observed when the same leptin concentrations were administered to dogs fed a high-fat diet, suggesting that mechanisms of leptin resistance may also occur in vascular tissue [199]. Leptin resistance in peripheral tissues is not as well studied as that in neuronal circuits involving the inhibition of food intake, but several mechanisms have been proposed. It has been demonstrated that hyperleptinemia downregulates LR expression [200, 201], although this finding is not universally observed in hyperleptinemic states [202]. As mentioned above, SOCS3 may play an important role in leptin resistance. SOCS3 synthesis is stimulated by STAT-3 in response to prolonged LR stimulation, but it is unclear whether SOCS-3 mediates vascular leptin resistance *in vivo* [203]. Finally, leptin-mediated NO formation in rats is diminished in a setting of obesity, independently of insulin levels, insulin sensitivity, plasma glucose, lipid profile, or blood pressure—indicating that this is a primary effect and not secondary to obesity-associated metabolic and/or hemodynamic abnormalities [204]. These findings suggest that chronic hyperleptinemia (as in obesity states) may evoke resistance to the acute NO-mimetic effect of leptin, thus inducing leptin resistance in the vascular tissue.
Leptin and cardiovascular disease

Epidemiological evidence

Leptin has been proposed as a molecular link between visceral obesity and CVD, although contradictory results may be found in literature. For example, one study in mice suggested that low leptin may be protective against atherosclerosis [10], while low plasma leptin levels predicted cardiovascular mortality in women during a 7-year follow-up period [11]. The overall evidence indicates that both elevated circulating leptin levels and genetically-mediated lack of leptin are associated with unfavorable risk factors or cardiovascular outcomes, such as coronary ED and atherosclerotic CHD [13, 205], hypertension [206], myocardial infarction [207], restenosis after coronary angioplasty [208], and heart failure in the absence of established CHD [209]. Hyperleptinemia has been linked with increased number of cardiovascular events in several large epidemiological studies [210-212], but not all [213]. The largest of these studies was the WOSCOPS (West of Scotland Coronary Prevention Study), a nested case-control study with 377 male cases and 783 controls, which reported an approximately 20% increase of CHD per leptin level increase of one standard deviation, after adjustment for several established cardiovascular risk factors [7]. A recent metaanalysis study linked leptin to CVD, finding moderate associations that were largely dependent on BMI [205].

Mechanisms of leptin that may promote CVD

Leptin has been determined to be involved in various pathophysiological mechanisms for CVD development. Hyperleptinemia has been linked with the development of hypertension and ED/atherosclerosis [170, 214], two main pathophysiological conditions associated with CVD development. However, the net influence of leptin on CVD is complex and several other factors must be considered, including age, degree of hyperleptinemia, exposure time, and the coexistence of other deleterious factors. For example, leptin has the potential to be beneficial or detrimental, depending on the context. It also remains unclear whether the various actions of leptin on the heart are enhanced or suppressed in hyperleptinemic individuals. Furthermore, leptin may affect vascular function differently within a setting of obesity or morbid obesity, indicating two different pathophysiological entities rather than a simple augmentation of body weight [215]. Fig. 4 shows several potential actions of leptin related to the development of CVD, which will be further discussed in detail.
Hyperleptinemia activates the SNS and/or ET-1

A very recent study demonstrated that experimental hyperleptinemia acutely increases vasoconstrictory sympathetic nerve activity in healthy humans [216]. Leptin binds to its receptors in various regions of the CNS—including the hypothalamus—activating neural pathways, decreasing appetite, and increasing SNS activity and energy expenditure [217]. SNS activation by leptin may occur through both local peripheral actions and centrally mediated effects on the hypothalamus—although intact LRs and CNS signaling are still required for this response [218]. Leptin-mediated increases in SNS activity may be among the principal mechanisms evoking obesity-related hypertension. Individuals with the rare genetically mediated leptin deficiency (homozygous children and adults) present not only morbid obesity, but also decreased SNS activity and postural hypotension [219]. In 1997, Dunbar et al. showed that both intravenous infusion and intracerebroventricular administration of leptin in rats increased the mean arterial pressure (MAP) and heart rate, and that blockade of the adrenergic system inhibited this pressor response to leptin [220]. In this regard, the
melanocortin system of the CNS has been implicated in blood pressure regulation by leptin [221]. Greenfield et al. demonstrated a lower prevalence of hypertension in obese subjects with a loss-of-function mutation in the melanocortin 4 receptor (MC4R) gene compared to in obese controls with the intact gene, again implicating melanocortinergic signaling in the control of systemic hemodynamics [222].

ET-1 is a 21-amino acid peptide that is mainly produced by the vascular endothelium through the actions of an endothelin-converting enzyme on the endothelial cell membrane. ET-1 causes prolonged smooth muscle-mediated vasoconstriction and hypertension, affects cardiac stimulation in a manner that can indirectly modify cardiovascular function, stimulates aldosterone secretion, decreases renal blood flow and glomerular filtration rate, and releases atrial natriuretic peptide (ANP). Because of its powerful vasoconstrictor properties and its effects on intracellular calcium, ET-1 has been implicated in the pathogenesis of hypertension, coronary vasospasm, and heart failure. Leptin has been shown to induce ET-1 in endothelial cells in vitro [223], and stimulates ET-1 activity in lean subjects in vivo [224].

**Leptin and inflammation**

Several reports have also demonstrated that leptin possesses pro-inflammatory cytokine-like properties [15]. LRIs have been observed in human T-lymphocytes, suggesting a role of leptin in the modulation of lymphocyte stimulation and pro-inflammatory response [225]. Leptin may regulate pro-inflammatory responses by activating immune cells, such as monocytes and macrophages. It has been demonstrated that high plasma leptin levels up-regulate the expression of monocyte cytokines, such as TNF-α and IL-6 [226, 227]. Leptin levels are also correlated with CRP in both lean and obese subjects [228, 229]. Furthermore, CRP can impact atherosclerosis development by hampering endothelial NO production—either directly, by decreasing endothelial NO expression [65], or indirectly, by increasing ROS production and thus inactivating NO [230]. CRP may also have effects relating to VSMC activation or the stimulation of monocyte adhesion to the endothelial surface [231]. In patients with CVD, CRP levels determine systemic NO bioavailability [230]. Leptin may regulate CRP production in the liver both through its effects on IL-6 and by direct regulation [198, 232].

In summary, pro-inflammatory mechanisms associated with hyperleptinemia may lead to impaired systemic bioavailability of NO and reduced vasodilatation [158], suggesting a causal association with adverse cardiovascular outcomes.
**Leptin and oxidative stress**

ROS production is enhanced in obesity. Leptin induces oxidative stress in human endothelial cells [16] and mitochondrial superoxide anion production in aortic endothelial cells via augmented fatty acid oxidation [233]. Excess superoxide production may quench NO, thus favoring the formation of ONOO-, which reduces local NO bioavailability [234]. This may result in the attenuation of dilator responses to NO-mediated agonists, such as Ach.

**Hyperleptinemia may increase vascular tone and impair vasodilation**

As discussed above, leptin-mediated SNS activation, vascular inflammation, and/or oxidative stress may lead to increased vascular tone and impaired vasodilation. Impaired availability of endothelium-derived NO to exert vasodilation is a hallmark of ED, which contributes to the pathogenesis of arterial hypertension and atherosclerosis. Paradoxically, although obesity is associated with impaired vasodilatation and ED [235, 236], isolated *in vitro* samples have shown that leptin has a stimulatory effect on NO production [237] and that NO-dependent vasodilatation is promoted via eNOS [238]. In aortic artery rings of rats, the vasodilatory effect of leptin was abolished by endothelial denudation and by the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), suggesting that leptin may induce vasodilatation by endothelium-dependent mechanisms. These processes have been shown to involve distinct NO-mediated endothelial mechanisms and EDHF [14].

These paradoxical results led to the development of the “counterbalance theory” [75]. According to this theory, under normal physiological conditions *in vivo*, the simultaneous vasodilatory and vasopressor effects of leptin counteract each other, making leptin’s net hemodynamic effect neutral [239]. However, in obese hyperleptinemic individuals or subjects with previous endothelial damage, this balance could be disrupted. In such cases, hyperleptinemia may alter vasodilatation capacity and raise blood pressure through SNS activation [216] and pro-inflammatory effects [15], along with co-existing impairment of NO synthesis or NO activity [204]. Supporting this proposal, in obese rats, it has been demonstrated that acute leptin infusion exerted hypertension, which was at least partly related to impaired vascular NO and EDHF production characteristic of obesity [240]. Accordingly, previous studies have also shown that the hypertensive effects of leptin are greatly exacerbated by reduced NO synthesis [241], as is often observed in obese subjects with ED. This theory may be applied in a context of chronic hyperleptinemia.
It is likely that, with only short exposures, the leptin-mediated increase in SNS activity may not be sufficient to cause marked peripheral vasoconstriction and to acutely raise blood pressure. Additionally, impaired leptin-induced NO production might be compensated by EDHF. However, chronic increases in plasma leptin, comparable to those found in severe obesity, can raise blood pressure. This effect is partly because the contribution of EDHF to the hemodynamic effect of leptin becomes inefficient, as well as partly due to the activation of leptin-induced renal mechanisms.

**Leptin and hypertension**

The cause–effect relationship between leptin and high blood pressure in humans has not yet been clearly demonstrated; however, chronic hyperleptinemia in humans is associated with hypertension in both men and women [242, 243], and in normotensive and hypertensive individuals [214]. Shankar et al. showed a positive association between hyperleptinemia and hypertension after adjusting for multiple covariates, including age, sex, race/ethnicity, education, smoking, BMI, DM, and serum cholesterol [244]. Furthermore, a recent study showed that leptin predicted new-onset hypertension in 620 women and 300 normotensive individuals over a 10-year time period in the Copenhagen City Heart study [245]. However, results regarding the overall effect of leptin on blood pressure have been controversial. For example, some studies have shown that chronic leptin infusion exerts an increase in blood pressure in rats [246], whereas other results have shown that acute injection of leptin in rats had little effect on BP despite SNS activation [247].

Leptin-mediated SNS activation, through either hypothalamic or extra-hypothalamic mechanisms [218], may increase vascular tone, playing an important role in hypertension development. However, it has been noticed that hyperleptinemia-mediated BP rises tend to occur slowly. In principle, this observation may rule out a setting of generalized peripheral vasoconstriction, and instead point to slow-acting renal mechanisms. In fact, some studies have demonstrated that long-term leptin administration increases both renal sympathetic nerve activity and blood pressure, promoting sodium retention and decreasing natriuresis [248]. However, in lean rats, leptin-mediated increase of renal sodium excretion was not followed by blood pressure changes [249]. It has been proposed that the lack of effect after acute leptin administration in vivo is related to the concept of selective leptin resistance, as well as to the previously mentioned “counterbalance theory”. Following this reasoning, the acute effects of leptin would include both vasodilator effects together with SNS stimulation,
resulting in an overall neutral effect. However, in chronic hyperleptinemic conditions (such as in obesity), resistance to the NO-mimetic vasodilatory effect of leptin but not to the SNS stimulation could alter this in vivo effect of leptin on blood pressure such that it no longer neutral [75].

Leptin has also been associated with hypertension via other mechanisms; for example, leptin increases ROS generation in endothelial cells, which decreases the amount of bioactive NO [250]. As mentioned earlier, leptin stimulates the secretion of proinflammatory cytokines such as TNF-α and IL-6 [251], and increases the release of ET-1 [223], which may promote hypertension Interestingly, one study demonstrated a reduction in serum leptin levels with the use of ACEi and ARBs, suggesting a potential interaction between leptin and the RAS for hemodynamic regulation in obesity [252].

**Leptin and endothelial dysfunction/atherosclerosis**

Leptin may alter vascular tone and vasoreactivity. These changes together with altered fibrinolysis are considered main features of ED. Various mechanisms have been proposed to underlie this association, including SNC activation [217], altered vascular tone and vasopressor effects [18], enhancement of platelet aggregation and arterial thrombosis [253], impairment of fibrinolysis [254, 255], pro-angiogenic actions and systemic inflammation [228], or promoting oxidative stress [16].

**Leptin and type II diabetes**

The relationship between increasing obesity rates and prevalence of overt type II DM has not yet been clarified. For example, data from the Northern Sweden MONICA Survey indicates that the prevalence of general and abdominal obesity increased from 1986 to 1999, but the prevalence of DM remained stable [256], and a similar pattern has been reported from another part of Sweden [257]. However, an association has recently been described between leptin and increased fasting and post-load glucose levels [256], and it has been shown that leptin predicts future development of DM in men [258, 259].

**Crosstalk between leptin and insulin**

Plasma leptin levels positively correlate with fasting plasma insulin [260]. Additionally, evidence suggests an interrelationship between adipose tissue and the pancreatic β-cell, which has led to the concept of the adipoinsular axis, a dual hormonal feedback loop involving leptin and insulin [261].
According to this framework, under some conditions, leptin negatively regulates insulin signaling [262] and hyperleptinemia is associated with lower insulin sensitivity, independent of changes in body weight [263].

Studies have investigated a possible crosstalk between insulin and leptin in the modulation of vascular tone and NO production [264]. Interactions between leptin and insulin could have important implications regarding the vascular disorders associated with metabolic syndrome. It is possible that disruption of either insulin or leptin signaling, or both, may lead to ED and ultimately to hypertension and atherosclerosis development. However, further studies are needed to fully elucidate the complex mechanistic interplay between leptin and insulin as related to ED.

**Leptin and fibrinolysis**

Impaired fibrinolysis is a hallmark of ED and plays a role in the development of atherothrombotic disease [265]. It increases the risk of myocardial infarction [266] as well as the possibility of reinfarction and death after myocardial infarction [267]. Additionally, fibrinolytic factors are elevated in obese subjects [268, 269]. Little is known about the role of leptin in fibrinolytic function, although investigations have demonstrated associations between the two [254]. For example, leptin levels (adjusted for age, BMI, and insulin) correlate positively with serum fibrinogen and PAI-1 activity, and negatively with t-PA act in both men and postmenopausal women, but not in premenopausal women [255]. Leptin also correlates with PAI-1 in patients with CVD, and PAI-1 expression in human coronary artery endothelial cells is upregulated in vitro by leptin [270]. The association of leptin with PAI-1 is not entirely independent of confounders [271]. However, increased PAI-1 and t-PA ag (complex t-PA/PAI-1) may eventually lead to a pro-thrombotic state due to an overall reduction in “free” t-PA act because of the concomitant rise of PAI-1 [272].
Aims

The overall aim of this thesis was as follows:

• To investigate the role of leptin on endothelial function.

Specific aims were as follows:

• To study the associations between plasma leptin levels and endothelial function (vasodilatation and fibrinolysis) in humans.

• To study the characteristics of these associations in different populations.

• To study the role of leptin on endothelial function in chronic vs. acute hyperleptinemia.

• To study the association of leptin with different variables of vascular function (measured using different assessment techniques).

• To study the effects of direct leptin infusion on basal vascular tone and endothelial function in vivo in healthy male volunteers.

• To interrelate results from both associative and experimental studies.
Methods

Design of this thesis

This thesis comprises six different studies. The main results of these studies were previously presented in the three papers included in this thesis (Fig. 5).

The first study, corresponding to paper I, was called DISARM, which is an acronym for Diesel Inhalation Study in foreARMs. This study aimed to analyze the effect of diesel exhaust on endothelial function, and is included in this thesis due to its methodology. Specifically, DISARM used forearm venous plethysmography to investigate endothelial function, which was the principal technique of vascular assessment applied in further studies in this thesis.

Study 2, called DISARM-metab, analyzed the association of plasma leptin levels with the results of endothelial function from DISARM.

DISARM and DISARM-metab were further complemented by two additional associative studies. The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study analyzed associations between leptin and endothelial function in a community-based population, while the Scottish study explored these associations in patients with known CVD. The results of the PIVUS and Scottish studies are presented in papers II and III, respectively.

Finally, the experimental Leptin Infusion for Vascular and Fibrinolytic function in foreARMs (LIVFARM) studies subjected healthy men to
intraarterial infusion of human recombinant leptin, corresponding to studies 5 and 6. The LIVFARM results are presented in paper III.

**Ethical considerations**

The DISARM study included exposure to a potentially hazardous agent and required careful ethical consideration. Our group has used diesel-exhaust exposure in earlier studies in patients with asthma and chronic obstructive pulmonary disease. In these studies, some participants reported irritation in eyes and throat. Beyond this, no serious complications were reported. The concentrations used were similar to those measured along an ordinary Swedish highway. In respect to the LIVFARM study, the literature did not report any serious effects of systemically administered leptin over longer periods [273]. In both studies, all participants got extensive information and written consent was obtained. Ethical approval was obtained from the respective ethics committees of Umeå, Upsala, and Edinburgh Universities.

**Subjects and methods**

This section presents the design of each study, including a description of the participants and the methods. Table 2 provides an overall representation of the subjects and methods from each study.

Table 2. Overview of the studies in this thesis.

<table>
<thead>
<tr>
<th>STUDY</th>
<th>SUBJECTS</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>Patients with known CVD n = 20 and n = 85 in papers I and III, respectively</td>
<td>FBF</td>
</tr>
<tr>
<td>Paper I</td>
<td></td>
<td>Fibriolytic function</td>
</tr>
<tr>
<td>Paper II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>General population n = 1016</td>
<td>FBF, FMD, Alx</td>
</tr>
<tr>
<td>Paper II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5, 6</td>
<td>Healthy men n = 17 10 volunteers in each study, 3 of them participated in both studies</td>
<td>FBF</td>
</tr>
<tr>
<td>Paper III</td>
<td></td>
<td>Fibriolytic function</td>
</tr>
</tbody>
</table>

**Study 1 (DISARM): Paper I**

Study 1 was a randomized, double-blind, cross-over study assessing the effect of dilute diesel exhaust inhalation on myocardial, vascular, and fibrinolytic function in a population of patients with stable CHD. After a systematic medical history review, 20 male volunteers were recruited—each with a verified ischemic CVD event (unstable angina pectoris event or myocardial infarction older than 6 months), with successful coronary stenting, and stable (symptom free) at inclusion time. These subjects were exposed to filtered dilute diesel exhaust or filtered air for one hour in an exposure chamber. All subjects were fitted with 12-lead Holter electrocardiographic monitors, and during each exposure they performed two 15-minute periods of exercise on a bicycle ergometer, separated by two 15-minute periods of rest. The electrocardiographic recordings were analyzed by calculating the ST-segment deviation, comparing the ST segment during each 15-minute exercise test with the average ST segment from the 15-minute period immediately before the start of the exposure. The ischemic burden during each exercise test was calculated as the product of the change in ST-segment amplitude and the duration of exercise. Six to eight hours after exposure, vascular function was measured by strain-gauge venous pressure plethysmography. Heart rate and blood pressure were measured with a semiautomated, noninvasive oscillometric sphygmomanometer.

**Study 2 (DISARM-metab)** was basically a substudy of study 1. Data obtained under diesel exposure were excluded, and the results from the vascular study under air exposure were examined for association with the plasma circulating levels of leptin. Data from the vascular study were also examined for associations with anthropometric measures, estimated values of insulin sensitivity, and measures of percentage body fat and fat distribution.

**Study 3 (PIVUS): Paper II**

The PIVUS study was initiated by Professor Lars Lind in 2001 at the University of Uppsala, Sweden, to investigate the predictive power of different measurements of endothelial function and arterial compliance. The study recruited a random sample of 1016 subjects (70 years old, 54% women) living in the community of Uppsala. The study also included other measurements of cardiac function and atherosclerosis, such as carotid and heart ultrasound or magnetic resonance imaging (MRI). Data were analyzed for associations between circulating plasma leptin levels and several representative parameters of vasomotor activity—particularly, between
leptin and measures of vasodilatation, such as FBF, FMD, and arterial stiffness (AoAIX). Total and partial analyses of the correlations were adjusted by gender, anthropometric measures, traditional CVD risk factors, and insulin resistance. Finally, all observed associations were tested after stratification under and above the median value for leptin levels and central obesity (WC), and analyses were performed restricted in those participants with neither CVD history nor medication.

**Study 4 (Scottish study) and studies 5 and 6 (LIVFARM studies): Paper III**

The Scottish study was conducted by Dr. Simon Robinson, from the group of Professor David Newby of Edimburgh (Scotland). This study was a substudy of a previously reported cohort of patients [274]. Eighty-three patients with angiographically proven coronary artery disease, who were stable at the time of inclusion, were investigated using forearm plethysmography. Data were analyzed for associations of FBF and fibrinolytic function with circulating leptin levels.

The LIVFARM studies (studies 5 and 6) were conducted in Umeå (Sweden). These experimental studies were designed to investigate the effects of leptin on endothelial function in healthy males. Endothelial function was assessed by forearm plethysmography, before and during hyperleptinemia exogenously induced by intraarterial infusion of human recombinant leptin. Ten healthy volunteers participated in each study, with three of them participating in both studies (n = 17). The LIVFARM studies were executed in two steps. In study 5 (LIVFARM-1), the volunteers received escalating doses of intra-arterial recombinant human leptin, and each dose was infused for six minutes, and FBF was assessed at each step. Study 6 (LIVFARM-2) was designed as a double-blind randomized crossover study in which volunteers received intra-arterial infusions of either leptin or saline on two separate occasions, with at least two weeks between visits.

**Anthropometry**

In each of our studies, the height and the circumference of the waist (at the umbilicus level) and hip (maximum over the buttocks) were recorded for all of the participants. BMI was calculated as weight/height (kg/m²). BMI and WC were used as measures of obesity and central obesity, respectively.

In study II, total fat mass and percentage body fat were measured by dual-energy x-ray absorptiometry (DXA), a non-invasive method that uses x-ray attenuation to assess bone mineral density, fat mass, and bone-free lean mass. These tissues yield different rates of attenuation, which are used to
calculate the percentage of each tissue component [275]. Compared to multi-slice computer tomography (CT), DXA provides accurate and reliable estimations of total percent body fat ($r = 0.85$), fat mass ($r = 0.94$) [276], and abdominal fat mass ($r = 0.86$) [277].

**Blood pressure**

In all studies, blood pressure was measured with the subject in the supine position, using a mercury sphygmomanometer (Omron Corporation, Tokyo, Japan) on the contralateral arm with respect to the arm used for vascular function assessment.

**Metabolic measurements**

Participants in studies 2, 4, and 6 underwent the oral glucose tolerance test (OGTT), which measured fasting glucose and insulin levels at 30, 60, 90, and 120 minutes after glucose load. Furthermore, the oral glucose insulin sensitivity (OGIS) test was used to assess insulin sensitivity based on the OGTT results. These results provide an index that is in good agreement with the index of insulin sensitivity obtained from the glucose clamp [278]. In study 3, insulin resistance was estimated using homeostasis model assessment (HOMA), which calculated insulin resistance (IR) as follows: $\text{IR-HOMA} = \frac{\text{fasting insulin (pmol/L)}}{22.5 * e^{-\ln \text{fasting glucose (mmol/L)}}}$ [279]. Since, mathematically, $e^{-\ln X} = 1/X$ [280], this formula can be simplified as $\text{IR-HOMA} = \frac{\text{fasting insulin} \times \text{fasting glucose}}{22.5}$. This index is appropriate for use in large epidemiological studies where only fasting insulin and glucose values are available [281].

**Vascular and endothelial function**

The method of choice for assessing endothelial function was through the assessment of FBF by strain-gauge forearm venous pressure plethysmography. In study 3, vascular/endothelial function was also measured by ultrasound FMD of the brachial artery and by radial artery applanation tonometry.

**Strain-gauge forearm plethysmography**

Venous pressure plethysmography is a highly reproducible technique, for which previous studies have shown a variation of 5–8% in both short-term evaluation (two-hour intervals) and in the longer term (three-week intervals) [282]. Walker et al. assessed the intra-subject variability and determined the coefficient of variation (CV) for FBF across resting conditions and with a
range of agonists to be between 24–27% [283]. Some potential side effects have been reported, such as median nerve injuries and brachial artery bleeding [284].

Strain-gauge plethysmography measures the FBF in a limb (generally the forearm), using a strain-gauge, which is a stretchable tube containing a liquid metal, such as mercury. In both experimental and contralateral forearms, the strain-gauge is simultaneously placed around the widest part of the forearm, in two lines separated by approximately 1 cm [285]. Since forearm length between the occlusion and collecting cuff is constant, volumetric changes are directly proportional to circumferential changes measured by the strain-gauge [125]. The strain-gauge measures forearm circumference changes in this segment per time, which reflect eventual changes in volume, giving a measure of artery flow in this segment \( dV/dt = \text{flow} \). The electric resistance in the strain-gauge increases along with the circumference of the forearm, because the tube becomes thinner and longer. If the gauge length is made equal to the resting circumference of the forearm, then changes in resistance are directly proportional to the changes in forearm volume:

\[
\Delta V_c = \Delta R_{sg} \times V_c / R_{sg}
\]

In this equation, \( V_c \) = volume in the segment under the strain-gauge loop; \( R_{sg} \) = resistance in the strain-gauge; \( \Delta V_c \) means change/increase in volume in the segment under the strain-gauge loop; \( \Delta R_{sg} \) means resistance change/increase in the strain-gauge; and \( \Delta V_c \) is transformed in artery flow \( (dV/dt = \text{flow}) \) per 100 g tissue. Hence, FBF is expressed in mL/minute * 100 g tissue.

To assess vasodilatory capacity, the brachial artery is infused with various vasodilator substances. The neurotransmitter Ach acts on muscarinic receptors, and is the most commonly used agonist for evoking NO. To evaluate NO availability, Ach infusion may be repeated under simultaneous constant intra-arterial infusion of the NOS inhibitor L-N-monomethyl arginine (L-NMMA). BK is a vasodilator peptide stimulating synthesis and release of NO, prostacyclin and EDHF. BK is one of the most potent stimuli for endothelial release of tPA \textit{in vivo} [286]. Substance P is neuropeptide found in the neural tissue of the central, peripheral, and enteric nervous systems [287]. Substance P is a potent vasodilator when given intra-arterially [288] and acts through an endothelium-dependent mechanism [289]. This mechanism is partly mediated by the L-arginine/NO-release pathway [290] and contributes to the induction of t-PA release \textit{in vivo} in humans [291]. Thus, both BK and substance P infusion enables assessment
of endogenous fibrinolysis in vivo. SNP is a non-endothelium-derived vasodilator that acts as an exogenous source of NO, and is used to examine the relaxing ability of vascular smooth muscle cell. In general, infusion of Ach, BK, or substance P provides indexes of EDV, whereas nitroprusside or verapamil infusions give a measure of EIDV.

**FBF determination**

At base level and during the infusion of vasodilators, FBF may be referred to in terms of absolute volume increase/time, expressed as artery flow per 100 grams of tissue (mL/minute * 100 g tissue). Alternatively, FBF can be calculated as a percent change in volume/flow with respect to the baseline FBF value at a given concentration, using the following formula:

\[
\% \text{ Change in vasodilatation} = 100 \times \frac{\text{FBF (for a given substance at a given concentration)}}{\text{basal FBF (predose)}} - \text{basal FBF}
\]

In general, our studies performed forearm venous occlusion plethysmography according to standardized procedures [125, 134] (Fig. 6).

FBF was recorded in the dominant and non-dominant forearms using mercury-in-silastic strain-gauges (EC-4 strain-gauge Plethysmograph, D. E. Hokanson). The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper’s Needle Works Ltd, Birmingham, UK) under local anesthesia with 1% lignocaine (Xylocaine;
Astra Pharmaceuticals Ltd, Kings Langley, UK). The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK), and patency was maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). After cannulation, the patient was kept at constant room temperature (22–24°C), and normal saline was infused at a constant intra-arterial rate of 2 mL/min.

To obtain plethysmographic recordings, upper arm cuffs were placed above the elbow and were intermittently inflated to 40–50 mmHg pressure (sufficient to shut the veins but not the arteries) for 10 s of every 15 s. This was sufficient to halt blood flow through venous occlusion, while not changing the rate of arterial inflow. As blood flows into the limb, it causes the limb to swell, and the rate of swelling reflects the increase of volume, providing a measure of the arterial flow rate at that instant (Fig. 7).

The rate of swelling reflects the volume increase, giving a measure of arterial flow rate.

The flow of the hands is quite variable; therefore, during measurement periods, the hands were excluded from the circulation by rapid inflation of wrist cuffs to a pressure of 200 mmHg using E20 Rapid Cuff Inflators (D. E. Hokanson Inc., Washington, USA).

Analogue voltage output from an EC-4 strain-gauge Plethysmograph (D. E. Hokanson) was applied to the widest part of the forearm. Data were processed by a MacLab® analogue-to-digital converter and Chart TM® software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a MacIntosh computer (Apple Computers Inc., Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph. Throughout the plethysmography study, blood pressure in the dominant arm was
monitored at intervals using a semiautomated noninvasive oscillometric sphygmomanometer (Omron Corporation, Tokyo, Japan).

After a 30-minute period of constant saline infusion, 8–10 subsequent FBF measurements were performed and the mean baseline FBF was estimated. Then, different vasodilator substances were infused in the brachial artery at a constant infusion rate. For each vasodilator, three increasing doses were infused. For each single dose, a dose–response curve was registered (Fig. 8).

Following infusion with each vasodilator drug, saline was infused for 30 minutes at a constant rate. This “wash-out” period allowed the vascular endothelium to return to base levels before testing the next drug. Dose–response curves were registered in both the infused arm and the contralateral non-infused arm, using the latter as a control. This design allowed us to rule out the possibility of any systemic effect of the infused drugs.

Non-infused arm

Infused arm

Fig. 8. Dose-response curves, showing measures of FBF under vasodilator infusion. Measurements were taken simultaneously in infused and non-infused arms. The gradient of the curve in the infused arm reflects the increasing resistance in the strain-gauge, which corresponds to an increase in volume that, in turn, calculated per units of time, reflects an increase in blood flow.

Forearm plethysmography in studies 1 and 2

Following the standard procedure, dose–response curves were registered during the period of cuff inflation. From these curves, absolute FBF values were extracted from the program Chart TM and calculated using a template spreadsheet (Excel 2002; Microsoft, USA). The last five flow recordings in each 3-minute measurement period were calculated and averaged for each arm. A single operator analyzed all data collected during the forearm
plethysmography study. FBF responses were reported as absolute blood flow responses (mL/100 mL tissue/min) in the infused and the non-infused arm.

The vasodilators used for this study were BK (Clinalfa AG, Switzerland) at 100, 300, and 1000 pmol/min, and Ach (Clinalfa AG) at 5, 10, and 20 µg/min for the assessment of EDV. SNP (David Bull Laboratories, UK) at 2, 4, and 8 µg/min, and verapamil (Abbott UK Ltd.) at 10, 30, and 100 µg/min were used for assessing EIDV. In these studies, the grade of vasodilatation was defined as the % of change in FBF, as previously defined.

**Forearm plethysmography in study 3**

FBF was measured by venous occlusion plethysmography (Elektromedicin, Kullavik). The pharmacological tools included ACH at 25 and 50 µg/min (to assess EDV) and SNP at 5 and 10 µg/min (to assess EIDV). Only the values corresponding to the maximal concentrations (50 µg/min and 10 µg/min, respectively) were used. As in study 1, vasodilatation was defined as % change in FBF. EDV was equal to FBF during infusion of Ach at 50 µg/min, minus resting FBF, divided by resting FBF. EIDV was equal to FBF during infusion of 10 µg/min of SNP, minus resting FBF, divided by resting FBF.

**Forearm plethysmography in study 4**

The plethysmography procedure used in this study differed from others in that substance P, instead of BK, was used to assess EDV. Substance P (Clinalfa AG, Läufelfingen, Switzerland) was infused in three increasing doses: 2, 4, and 8 pmol/min.

**Forearm plethysmography in studies 5 and 6**

In study 5, after positioning of the cuffs and strain-gauge and cannula insertion in the brachial artery, base (resting) FBF was measured over 30 minutes. Next, recombinant human leptin (Sigma-Aldrich, Saint-Louis, Missouri, USA) was infused at 0.1, 1, 10, and 100 ng/kg/min, based on a previous study [292]. For the sake of simplicity, we assumed an average participant weight of 80 kg, and thus chose infusion doses of 80, 800, and 8000 ng/min for a six-minute infusion/dose. The lowest dose (8 ng/min) was eventually excluded from further analysis because it resulted in an imperceptible increase in plasma leptin levels.

In study 6, all volunteers were exposed in different days—at least 2 weeks between visits—to either a continuous leptin infusion or saline in a randomized order. Over 60 minutes, recombinant human leptin (or saline)
was infused at a constant dose of 800 ng/min, which was the dose from study 5 that exerted physiological plasma leptin levels like those found in obesity. Within the first 15 minutes of continuous infusion—considered as stabilizing phase after arterial cannulation—no FBF recordings were made. After this period, five basal FBF measuring were recorded with 7-8 minutes intervals. Thereafter, four vasodilators (Ach, SNP, BK, and verapamil, each at three increasing concentrations as in study 1) were infused along with the intra-arterial leptin or saline infusion. Each drug infusion was performed for 6 minutes/dose, with FBF recordings within the last 3 minutes, with a 15-minute saline wash-out period between each agent. The different drugs were administrated in randomized order, except that verapamil was always administered at the end because of its long-lasting vasomotor effects (Fig. 9).

Protocol of LIVARM 2

For these studies, absolute FBF instead of % FBF change was measured and analyzed. By this, we expected to increase our ability to detect possible changes in resting FBF during the first hour of leptin infusion alone. Leptin infusion was well tolerated in both studies, with no adverse events detected.

**Brachial artery ultrasound**

In study 3, EDV was also measured by FMD. For this purpose, the brachial artery was assessed at 2–3 cm above the elbow using external B-mode ultrasound imaging (Acuson, 7.0 MHz linear transducer, Acuson Mountain...
View). The cuff was inflated for 5 min to a pressure of at least 50 mmHg above SBP. FMD was defined as the maximal brachial artery diameter recorded at 30–90 s following cuff release, minus the diameter at rest, divided by the diameter at rest. The coefficient of variation for repeated measurements of brachial artery diameter is about 3–4% in short-term (two-hour interval) and in longer term (three-week interval) repeated measurements [144].

The diameter of the brachial artery was initially determined at rest, as the average diameter from all measured baseline images. The base blood flow was estimated by time-averaging the pulsed Doppler velocity signal obtained from a mid-artery sample volume, according to the following formula:

\[ Q = \frac{V \pi D^2}{4}, \text{ which can be simplified as } Q = V \times A \]

\[ Q = \text{blood flow}, \ V = \text{Doppler-velocity}, \ D = \text{diameter of the brachial artery at measured point}, \ A = \text{area of the brachial artery at measured point}, \ \pi = \text{the number pi, which is approximately equal to 3.1416}. \]

Next, the arterial flow was interrupted by a cuff that was placed on the proximal forearm and inflated to either 200 mmHg or 50 mmHg greater than the systolic blood pressure (SBP). The release of ischemia by cuff deflation after five minutes led to increased blood flow, resulting in a vasodilatory effect on the brachial artery, which is also called “reactive hyperemia.” Simultaneous electrocardiographic triggering was used to measure the artery diameter at the same period of the cardiac cycle. End-diastolic images were digitally captured at baseline and for two minutes after cuff deflation, although the brachial artery was continuously monitored from 30 seconds before to 120 seconds after ischemia cuff release. FMD was defined as the percent change of the brachial artery diameter from rest to 60 seconds after ischemia cuff release. FMD by reactive hyperemia was calculated by subtracting the baseline brachial artery diameter from the brachial artery diameter at 60 seconds, which was obtained by averaging all of the measurements from images obtained between 55 and 65 seconds after cuff deflation [143].

\[ \text{FMDmm} = \text{FMD at 60 seconds} - \text{FMD baseline diameter} \]

FMD is often presented as the relative (or percent) change from baseline:

\[ \text{FMD\%} = \frac{\text{FMD mm}}{\text{baseline diameter brachial artery}} \times 100. \]
Finally, study 3 included calculation of AoAlx, the surrogate measure of vascular stiffness. In this study, PWV was not estimated, but it will be briefly discussed.

The pulse waveform was calculated as the sum of forward and reflected waves. There are two types of pulse waves: central and peripheral (Fig. 10A and 10B). In central arteries (aortic and those close to the heart, such as subclavia or carotid), the shape of the aortic pulse wave is a result of the ventricular ejection and the physical properties of the arterial system. Several parameters can be extracted from the central aortic waveform (Fig. 10A), including the first systolic inflection (first “shoulder” or anacrotic notch; P1), the systolic peak (P2), and the relative difference between these two (P2 − P1) in terms of augmentation pressure (AG). The amount of augmentation increases as the arteries stiffen. The pressure decreases immediately after the peak, but the ejection duration can be calculated based on the dicrotic notch, a typical incision caused by short regurgitation at the end of systole that represents the closure of the aortic valve. The central arterial pressure waveform is usually derived from the recorded (peripheral) radial pulse waveform that is computer-synthesized by sphygmocardiograph (SpygmoCor).

The pulse pressure (PP)—i.e., the pressure difference between the systolic and diastolic pressures (SBP − DBP)—is by definition the pressure change to create the pulse. PP is directly proportional to stroke volume and inversely proportional to the compliance of the aorta. In elderly subjects, increased arterial stiffness leads to increased wave reflection, which increases central PP. Thus, PP increases as the aorta becomes more rigid.

The pressure changes in peripheral arteries are different from those in the aorta; the curve is more rounded, with no incision in the increasing part due to the attenuation of higher frequencies (Fig. 10B). The peripheral pulse wave has two parts: primary and dicrotic. Peripheral arterial waveforms have higher systolic pressure, lower diastolic pressure, and wider PP when compared with central aortic pressure. The difference in PP is dependent on age, with a greater difference seen in younger subjects compared to in the elderly [293]. The variation coefficient of this technique is approximately 5% [294]. Fig. 10 shows representative central and peripheral pulse waves.
Fig. 10. (A) Computer synthesized central pulse wave. (B) Peripheral (radial) pulse waves. P1 = first systolic inflection, P2 = systolic peak, PP = pulse pressure (SBP–DBP).

From the analysis of pressure wave forms, two important parameters can be estimated: the augmentation index and the pulse wave velocity. Both are independent predictors of adverse cardiovascular events, including mortality [149].
**Augmentation index (AIX)**

The AIX is an expression of arterial pressure increase caused by the reflected wave or, in other words, the amount of augmentation pressure quantified in terms of relative change over the whole pulse. AIX is defined as the difference between the first systolic (P1) and second pressure peak (P2), expressed as a percentage of the pulse pressure (PP). Increased aortic pressure will thus provide an estimate of the stiffness of the arterial system in its complexity, and can be expressed numerically. AIX can be evaluated either as the central/aortic augmentation index (AoIx) or in radial/peripheral arteries (pAIX) [146]. These measures are expressed in different forms but both convey similar information.

**AoIx**

An increase of the aortic pressure provides an estimation of the stiffness of the arterial system. Central augmented pressure is calculated as the difference between the early and late systolic peaks (ΔP = P2 − P1) of the estimated central pressure waveform. AoIx is calculated as the augmented pressure expressed as a percentage of the PP (Fig. 10A).

\[
AoIx = \frac{P2}{P1} - \frac{SBP - DBP}{100}.
\]

**pAIX**

pAIX is calculated as the ratio of the amplitude of the late systolic peak (P2) to the amplitude of the early systolic peak (P1) (Fig. 10B).

\[
pAIX = \frac{(P1 - P2)}{P1} * 100%.
\]

**Pulse wave velocity (PWV)**

The rate at which aortic pressure waves travel is partly determined by vascular stiffness. As large arteries stiffen with age or disease processes, both PWV and the amplitude of the reflected wave increase. PWV is a parameter used to measure pulse wave spreading in the artery system, and is calculated by the following formula:

\[
PWV = \frac{L}{PTT}
\]

L = length of the distance between the two spots between which the velocity is measured, PTT = pulse transit time, referenced to the R wave of the electrocardiogram.
Carotid-femoral PWV has been proposed as the gold standard for arterial stiffness measurement, because it is a more direct measure of stiffness, has good reproducibility, and requires little technical expertise; there is also evidence from a number of large prospective independent studies supporting its use as an independent predictor of vascular outcomes [295].

Biochemical analysis

In studies 1 and 2, serum CRP was measured using an immunonephelometric assay, and cholesterol levels, triglycerides, hematocrit, white cell count (WCC), and platelets were evaluated according to the standard laboratory techniques of the hospital. In study 4, CRP concentrations were measured with an immunonephelometric assay, and the hematocrit level was determined by capillary tube centrifugation (at baseline and during infusion of BK at 1000 pmol/min). Samples were collected, immediately stored on ice, and centrifuged at 2,000 g for 30 min. Platelet-free plasma and serum were stored at −80°C until assayed.

Analysis of plasma leptin

In all studies, leptin levels in plasma were analyzed using a double-antibody radioimmunoassay (RIA) method with rabbit antihuman leptin antibodies [296]. The leptin detection level in this assay was 0.5 ng/mL. Total CV for leptin was 4.7% at both low (2–4 ng/mL) and high (10–15 ng/mL) levels.

Fibrinolytic factors

Blood samples (10 mL) were collected into acidified buffered citrate (Stabilyte tubes, Biopool International) for t-PA assays, and into citrate (BD Vacutainer) for PAI-1 assays. Plasma t-PA ag and PAI-1 antigen concentrations were determined using enzyme-linked immunosorbent assays (ELISA; Coaliza®, Chromogenix), while fibrinolytic t-PA act was assayed using a photometric method (Coatest t-PA, Chromogenix AB) [297, 298]. The detection range for t-PA ag was 1.5–30 ng/mL and the intra- and inter-assay CVs were 8% and 10%, respectively. According to the manufacturer, the within- and between-assay CVs for t-PA act were 7.0% and 5.3 %, respectively, at low levels (0.43–0.48 IU/mL), and 3.9% and 5.2% at mid-range levels (1.25–1.32 IU/mL) [225]. Concentrations of PAI-1 and PAI-1 ag were determined using enzyme-linked immunosorbent assays [299]. The CV was 9% according to the manufacturer.

Except for in study 3, plasma t-PA (activity and antigen) and PAI-1 antigen concentrations were measured during forearm plethysmography studies. In
studies 1 and 6, t-PAac, t-PA ag, and PAI-1 were measured at baseline; following BK infusion at 100, 300, and 1000 µg/min; and at the end of the study. In study 4, these measurements were made after infusion of substance P at 2, 4, and 8 pmol/min. In study 6, t-PA and PAI-1 were also measured at 60 minutes after infusion with leptin/saline.

In studies 1, 4, and 6, the estimated net release of t-PA ag was calculated by the venous–venous approach. Net t-PA release was defined as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused FBF) and the concentration difference between the infused (t-PA inf) and non-infused arms (t-PA non-inf) [298]. This value is expressed in ng/100 mL of forearm tissue/minute.

Net t-PA release = FBF \( (101 - \text{hematocrit}/100) \times (t\text{-PA inf} - t\text{-PA non-inf}) \)

**Statistical methods**

Table 3 summarizes the statistical methods used in this thesis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Statistical method</th>
<th>Program</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ANOVA</td>
<td>GraphPad Prism 5</td>
<td>when appropriate</td>
</tr>
<tr>
<td>2</td>
<td>Bivariate and partial correlation</td>
<td>SPSS 15.0</td>
<td>Adjustments for BMI, waist circumference, % fat mass (DEXA), and grade of insulin sensitivity (OGIS)</td>
</tr>
<tr>
<td>3</td>
<td>Bivariate and partial correlation</td>
<td>SPSS 20</td>
<td>Adjustments for sex, BMI, Framingham score, and HOMA-IR</td>
</tr>
<tr>
<td></td>
<td>Univariate and multivariable linear regression</td>
<td>SPSS 20</td>
<td>Analysis after stratification for waist circumference and leptin levels</td>
</tr>
<tr>
<td></td>
<td>Stepwise method</td>
<td>SPSS 20</td>
<td>Analysis after stratification for waist circumference and leptin levels</td>
</tr>
<tr>
<td>4</td>
<td>ANOVA</td>
<td>GraphPad Prism 5</td>
<td>Statistical analysis conducted by our colleagues in Edimburgh, Scotland</td>
</tr>
<tr>
<td></td>
<td>Non-parametric chi-square when appropriate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spearman correlations</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Statistical significance was indicated by two-sided $P < 0.05$ in all studies.

**Study 1**

Continuous variables are reported as mean ± SEM. GraphPad Prism (GraphPad Software, California, USA) was used for statistical analyses, including analysis of variance (ANOVA) with repeated measures and two-tailed Student’s $t$-test, where appropriate. Statistical significance was indicated by $P < 0.05$.

**Study 2**

Normality distributions were checked and data were ln-transformed when necessary. Correlation analyses (Pearson and partial correlation) and univariate and multivariable linear regression analysis were performed where appropriate. Statistical analyses were performed using SPSS 15.0 (IBM Corp, Somers, NY, US) and significance was assigned at the 5% level.

**Study 3**

Baseline characteristics were expressed as geometric means with 95% CI. Leptin, vasodilatation (EDV, EIDV, and FMD), and arterial stiffness variables (AIx) were ln-transformed to achieve normality. Correlation analyses (Pearson and partial correlation) and univariate and multivariable linear regression analysis were used to test for associations of leptin with measures of endothelial function, arterial stiffness, and blood pressure. These analyses were adjusted by sex, Framingham score (when appropriate), and HOMA index, and were repeated after stratifications for waist circumference and leptin levels. These associations were further explored with a step-wise method, including BMI, sex, SBP, LDL and HDL cholesterol, triglycerides, HOMA-IR, and CRP as explanatory variables. Finally, a restricted analysis was performed in only subjects without medication or known CVD.
**Study 4**

Basic characteristics were presented as means and standard errors of the mean (SEM) if normality was present. If not, data were ln-transformed and presented as geometric means with 95% confidence intervals. In the associative study, patients were divided into tertiles based on leptin levels, and characteristics were compared between tertiles using parametric (ANOVA) and non-parametric (chi-square) tests as appropriate. The associations between circulating leptin levels and baseline variables were assessed with Spearman’s correlation analysis.

**Studies 5 and 6**

Data relating to FBF, fibrinolytic markers, leptin, and blood pressure were analyzed using ANOVA with repeated measures, or with non-parametric tests (Friedman or Wilcoxon) when appropriate. Changes in FBF were further analyzed with a linear mixed model (LMM) analysis. The model included a random intercept to correct for the dependency of repeated measurements within subject, and for the fixed factors—including time (increasing concentrations of the vasoactive substance), infusion (leptin or saline), and the time-by-infusion interaction. The model was also adjusted for order (leptin followed by saline, or saline followed by leptin) and baseline values. The statistical software programs SPSS v.20 and GraphPad Prism were used, and significance was assigned at the 5% level.
Results and discussion

For the purpose of simplicity, this section is structured according to the three papers of this thesis. Study 2, which has not been published in any paper, is discussed as additional findings from the patient cohort of study 1, paper I.

Study 1 (Paper I)

Results

The main finding in this study was the detection of worsened myocardial ischemia—as reflected by a greater maximum ST-segment depression (in lead II) and total ischemic burden—in all subjects during exercise under exposure to diesel exhaust compared to in subjects during exercise under exposure to filtered air ($P = 0.003$ and $P < 0.001$, respectively).

Furthermore, neither endothelium-dependent nor endothelium-independent vasodilatation was affected by inhalation of diesel exhaust or filtered air. Basal plasma concentrations of t-PA ag, t-PA act, and PAI-1 remained unaffected during exposure to diesel exhaust. Finally, BK caused dose-dependent increases in plasma t-PA concentrations and net t-PA release, and these increases were suppressed 6 to 8 hours after exposure to diesel exhaust ($P = 0.009$) compared to after air exposure.

Discussion

In this study, we demonstrated that transient exposure to dilute diesel exhaust (at concentrations that may occur in any urban milieu) exacerbated exercise-induced myocardial ischemia, and impaired endogenous fibrinolytic capacity in men with stable and asymptomatic CVD. This finding supports epidemiological observations of increased rates of myocardial infarction in individuals with high exposure to traffic [300]. No effect on vasomotor function was demonstrated after 5–6 hours of diesel exposure, in contrast to our previously report of impaired vasomotor function in healthy volunteers after 2 hours of exposure [301]. In light of these contradictory results, a detrimental vasomotor effect at an earlier point in time cannot be excluded. Additionally, the patients with CVD may have had preexisting vasomotor or endothelial dysfunction and/or been taking medications that could influence vasomotor function, thus hindering the ability of our method to demonstrate further impairments of the vascular function after diesel exhaust exposure.
The association between circulating levels of leptin and agonist-mediated vasodilatation in this cohort was further investigated (study 2, not published).

**Study 2**

**Results**

In general, the participants were middle-aged males without DM and with predominantly single-vessel coronary artery disease. They were of normal weight or moderately overweight. There were no differences in resting heart rate, blood pressure, or FBF in the non-infused arm (data not shown). A dose-dependent increase in blood flow was observed with each vasodilator ($P < 0.001$ for all comparisons).

Bivariate correlations showed that high leptin levels were associated with decreased vasodilatation after infusion of SNP ($s = -0.57, P = 0.017; s = -0.60, P = 0.01$; and $s = -0.66, P = 0.004$, respectively, for the increasing SNP doses; Table 4 and Fig. 11). This association did not remain after adjustment for BMI, % fat mass, or OGIS. This pattern was not observed after infusion of Ach, BK, or Verapamil (Table 4).

Table 4. Bivariate (Spearman) and partial correlation between leptin and vasodilatation.

<table>
<thead>
<tr>
<th>Type of VD</th>
<th>Vasodilator</th>
<th>Dose</th>
<th>Adjusted for</th>
<th>$s$</th>
<th>$P$</th>
<th>$r$</th>
<th>$P$</th>
<th>$s$</th>
<th>$P$</th>
<th>$r$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDV BK (%ChBK)</td>
<td>100</td>
<td>0.31</td>
<td>0.40</td>
<td>0.60</td>
<td>$0.006^{**}$</td>
<td>-0.06</td>
<td>0.80</td>
<td>0.30</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>0.36</td>
<td>0.20</td>
<td>0.60</td>
<td>$0.006^{**}$</td>
<td>-0.2</td>
<td>0.30</td>
<td>0.30</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.28</td>
<td>0.30</td>
<td>0.50</td>
<td>0.05</td>
<td>-0.2</td>
<td>0.30</td>
<td>0.20</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ach (%ChAch)</td>
<td>5</td>
<td>-0.18</td>
<td>0.50</td>
<td>-0.3</td>
<td>0.27</td>
<td>-0.2</td>
<td>0.45</td>
<td>-0.2</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-0.25</td>
<td>0.30</td>
<td>-0.2</td>
<td>0.40</td>
<td>-0.1</td>
<td>0.70</td>
<td>-0.13</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-0.19</td>
<td>0.50</td>
<td>-0.5</td>
<td>$0.04^{**}$</td>
<td>-0.3</td>
<td>0.20</td>
<td>-0.07</td>
<td>0.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDV SNP (%ChSNP)</td>
<td>2</td>
<td>-0.57</td>
<td>0.017$^{**}$</td>
<td>-0.04</td>
<td>0.80</td>
<td>-0.07</td>
<td>0.80</td>
<td>-0.11</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-0.6</td>
<td>0.01$^{**}$</td>
<td>-0.14</td>
<td>0.60</td>
<td>-0.13</td>
<td>0.60</td>
<td>-0.27</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-0.66</td>
<td>0.004$^{**}$</td>
<td>-0.39</td>
<td>0.13</td>
<td>-0.34</td>
<td>0.18</td>
<td>-0.48</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil (%ChVp)</td>
<td>10</td>
<td>0.04</td>
<td>0.80</td>
<td>0.60</td>
<td>0.01$^{*}$</td>
<td>-0.4</td>
<td>0.07</td>
<td>0.20</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>-0.13</td>
<td>0.60</td>
<td>0.60</td>
<td>0.01$^{*}$</td>
<td>-0.3</td>
<td>0.18</td>
<td>0.02</td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>-0.19</td>
<td>0.40</td>
<td>0.60</td>
<td>0.01$^{*}$</td>
<td>-0.2</td>
<td>0.40</td>
<td>0.08</td>
<td>0.70</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

VO = vasodilator; EDV = endothelial dependent vasodilatation; EDIV = endothelial independent vasodilatation

All variables were log transformed before analysis. = Spearman’s correlation; = Pearson’s correlation

%ChBK = percent change in flow-mediated vasodilatation during bradykinin infusion; %ChAch = percent change during acetylcholine infusion

%ChSNP = percent change during sodium nitroprusside infusion; %ChVp = percent change during verapamil infusion

% fat tissue measured by DXA; IS = insulin sensitivity, measured by OGIS
We also found a positive association between leptin and basal circulating levels of PAI-1 and t-PA ag ($P < 0.001$ for both), which remained statistically significant after adjustment for BMI but not after adjustment for fat mass. Leptin was not associated with net t-PA release (Table 5). Inflammation markers, such as white cell count or serum CRP, were not associated with leptin level (data not shown).

Table 5. Bivariate (Spearman) and partial correlation between leptin and fibrinolysis

<table>
<thead>
<tr>
<th>Variables of fibrinolysis</th>
<th>Adjusted for</th>
<th>s</th>
<th>P</th>
<th>r</th>
<th>P</th>
<th>r</th>
<th>P</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>BMI</td>
<td>%fat tissue</td>
<td>IS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1 ag</td>
<td></td>
<td>0.71</td>
<td>&lt;0.001***</td>
<td>0.44</td>
<td>0.05</td>
<td>0.08</td>
<td>0.73</td>
<td>0.54</td>
<td>0.01*</td>
</tr>
<tr>
<td>t-PA ag</td>
<td></td>
<td>0.52</td>
<td>0.03*</td>
<td>0.70</td>
<td>0.003**</td>
<td>0.21</td>
<td>0.44</td>
<td>0.66</td>
<td>0.005**</td>
</tr>
<tr>
<td>Net t-PA release</td>
<td>BK 100</td>
<td>-0.11</td>
<td>0.70</td>
<td>-0.21</td>
<td>0.50</td>
<td>0.37</td>
<td>0.23</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BK 300</td>
<td>-0.29</td>
<td>0.30</td>
<td>-0.08</td>
<td>0.70</td>
<td>-0.08</td>
<td>0.70</td>
<td>-0.21</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>BK 1000</td>
<td>0.08</td>
<td>0.80</td>
<td>-0.09</td>
<td>0.70</td>
<td>-0.09</td>
<td>0.70</td>
<td>-0.21</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001

BK = bradykinin; Net t-PA release = endogenous t-PA released after BK infusion (ng/100mL tissue/min); PAI-1 ag = plasminogen activator inhibitor-1 antigen (ng/mL); t-PA ag = tissue plasminogen activator antigen (ng/mL); BMI = body mass index; %fat tissue measured by Dual-energy X-ray absorptiometry (DXA); IS = insulin sensitivity measured by Oral Glucose Insulin Sensitivity (OGIS)


**Discussion**

This associative study provided the first indications that leptin might be associated with blunted vasodilatation capacity, at least in relation to EIDV. This correlation did not remain significant after adjustment for measures of obesity. In this study, plasma leptin was associated with circulating levels of t-PA ag and PAI-1, but t-PA act was not measured. In summary, we found no evidence suggesting any association of leptin with endogenous fibrinolysis.

This study had several important limitations. The design was deficient, the study included a relatively small number of participants, and the results were somewhat contradictory, making it difficult to draw clear conclusions. However, these results led us to further explore the association between leptin and endothelial function.

**Study 3 (Paper II)**

**Gender-stratified analysis**

Compared to women, the men were more centrally obese, and had higher diastolic blood pressure (DBP) and blood glucose. In contrast, women had higher SBP, total LDL and HDL cholesterol, and circulating leptin levels than men.

**Leptin and vasodilatation**

Leptin was inversely associated with EDV and EIDV, even after adjustment for sex ($r = -0.18$, $P < 0.001$ and $r = -0.14$, $P < 0.001$, respectively). Leptin was not associated with EDV, as measured by ultrasound brachial technique (FMD). Leptin was associated with EDV only in subjects with low waist circumference (without abdominal obesity) or in those presenting low leptin levels. On the other hand, leptin was associated with EIDV independently of abdominal measurement and at both low and high leptin levels. However, these associations did not remain significant after BMI adjustment.

Linear regression analysis showed that leptin was strongly associated with EDV and EIDV but not with FMD. These correlations remained significant after adjustment for sex, traditional CV risk factors represented in the Framingham score, and HOMA index; however, no associations remained significant in the fully adjusted model that included BMI.
Leptin and arterial stiffness

Leptin was associated with AoAIX (r = 0.14, P < 0.001), although this association did not remain significant after adjustments for sex, Framingham score, and HOMA index. Linear regression analysis showed this association in the univariate analysis, but not in the adjusted multivariable analysis.

Leptin and blood pressure

Leptin was associated with SBP and DBP (r = 0.18, P < 0.001 and r = 0.20, P < 0.001, respectively) irrespective of sex and insulin resistance. These associations were attenuated after adjustment for BMI.

All of the above-mentioned associations remained unaltered after restricting the analysis to only subjects with no reported CVD or medication.

Discussion

The main finding of this study was the indication that hyperleptinemia was associated with impaired vasodilatation. This association was found in both men and women, and remained after adjustment for traditional CVD risk factors represented by the Framingham risk score (including age, LDL and HDL cholesterol levels, blood pressure, presence of DM, and smoking [302]). This suggests that the action of leptin on vasomotor reactivity may be independent from other potential obesity-associated risk factors. Nevertheless, all of the detected associations were strongly attenuated after adjustment for BMI, indicating that leptin could be the mediator between obesity and impaired vascular function.

Although it is not possible to make causal inferences from this associative study, several interesting observations may be discussed. For example, in the stratified analysis (above and below the median value of BMI or leptin level), leptin was not associated with decreased EDV among subjects with high BMI or high plasma leptin. This suggests that, in the context of high BMI and hyperleptinemia, the contribution of leptin to the normal regulation of endothelial function may be blunted. This result supports the concept of selective leptin resistance in the vascular wall, and is in accordance with previous experimental findings. In particular, Knudson et al. previously demonstrated that acutely raised hyperleptinemia attenuated Ach-mediated coronary dilation, both in anesthetized dogs and in vitro in isolated canine coronary rings [13]. However, in hyperleptinemic dogs that were fed a high-
fat diet, exogenous leptin did not significantly alter vascular responses to ACh in vivo or in vitro [199].

We demonstrated the association of leptin with impaired vasodilatation in resistance vessels (plethysmography-assessed FBF), but not in conduit arteries (EDV measured by FMD). In this regard, it is plausible to hypothesize that leptin may differentially affect various types of arteries, particularly considering that the signaling pathways may differ between conduit and resistance arteries. There are two possible explanations for this finding. The first possibility takes into account the deterioration of arterial compliance in elderly subjects, which could mitigate the ability of the ultrasound technique (FMD) to detect subtle leptin-related changes in vasodilatation. The second explanation is that leptin truly has differential effects on different types of arteries. In this regard, it is important to highlight the existence of an EDHF (non-NO/non-PGI2) of yet unknown origin that would be essential for the physiological control of resistance artery tone [75].

The association of leptin with EIDV is also noteworthy. There is evidence suggesting that leptin’s vascular actions may not be endothelium dependent [303]. Our finding supports the hypothesis that leptin acts on the smooth muscle cell function of the vascular wall, either directly on the VSMC or indirectly, for example by activating the SNS. In this regard, LRs have been detected in VSMC [304], and leptin has been shown to promote vascular remodeling by inducing smooth muscle cell proliferation, migration, hypertrophy, and calcification—although the direct effects of leptin on VSMC have not yet been systematically studied [180].

Measurements of arterial stiffness are very important, particularly considering that arterial stiffness may serve not only as a means of assessing cardiovascular risk [305] but also as an indicator of treatment benefit [306]. In our study, leptin was positively associated with arterial stiffness, although this association did not remain after adjustment for traditional risk factors (Framingham score) and insulin resistance. This finding is partially in line with the results of a recent study that showed a positive independent association between leptin and carotid–femoral PWV (another measure of arterial stiffness), as well as a negative association of PWV with both resistin and adiponectin [307]. However, in contrast to our findings, the previous report showed that leptin’s association with arterial stiffness remained significant after adjustment for each other and for conventional risk factors, such as hypertension. Furthermore, the association between adiposity and PWV disappeared completely after adjusting for leptin, indicating that leptin could be the mediator between obesity and vascular stiffness [307]. One
possible explanation for the different results after adjustment is that, although AIx and PWV are both measures of arterial stiffness and correlate positively with each other [308], they differ in some aspects. For example, AIx is more influenced by heart rate and blood pressure than PWV, and vasoactive drugs may affect aortic AIx independently from aortic PWV [309].

Leptin was associated with high SBP and DBP, irrespective of sex and insulin. This result is in accordance with emerging evidence suggesting a direct relationship between chronic hyperleptinemia and hypertension in both men and women, independent of BMI and grade of insulin resistance [242, 243]. Shankar and Xiao recently studied 5,599 Americans, and found that hyperleptinemia was positively associated with hypertension after adjusting for multiple covariates, including age, sex, race/ethnicity, education, smoking, BMI, DM, and serum cholesterol [244].

Finally, when our study of association was restricted to only healthy elderly individuals (i.e., participants without known CVD and without cardiovascular medication), the main results were basically unaltered. This may suggest that leptin-related impaired vasodilatation occurs independently of the presence of previous ED, which is presumed in patients with known CVD.

Studies 4, 5, and 6 (Paper III)

Results

Study 4 showed that plasma leptin concentrations were correlated with BMI ($r = 0.73$, $P < 0.001$). BMI and sex differed between tertiles (both $P < 0.001$), with more obese subjects and females in the highest tertile. Plasma leptin concentrations were correlated with C-reactive protein ($r = 0.26$, $P = 0.02$). Basal FBF did not differ between leptin tertiles. All three vasodilators caused dose-dependent increases in infused FBF ($P < 0.001$), but the patients with the highest leptin concentrations had lower responses to Ach ($P = 0.004$), substance P ($P < 0.001$), and SNP ($P = 0.02$) compared to the patients belonging to the lower tertiles.

Resting arterial pressure did not differ between leptin tertiles. Plasma leptin concentrations correlated with basal concentrations of t-PA ($r = 0.41$, $P < 0.001$) and PAI-1 antigen ($r = 0.42$, $P < 0.001$). Moreover, substance P caused a dose-dependent increase in the plasma concentrations of t-PA ag and act ($P < 0.001$), and the estimated net release of both t-PA ag ($P < 0.001$) and activity ($P = 0.03$) was greatest in the highest plasma leptin tertile.
In studies 5 and 6, a total of 17 male volunteers were recruited, with a mean age of 26.4 ± 1.4. Their mean BMI and waist circumference was within the upper limit of normality: 24.9 ± 0.6 and 84.9 ± 2.2 (mean ± SEM), respectively. Leptin level was 4.7 ± 0.9. SBP and DBP were 140 ± 3.7 and 69 ± 2.8, respectively.

Intra-arterial leptin infusion achieved hyperleptinemic plasma levels in the infused arm ($P < 0.001$), as well as in the non-infused arm to a lesser extent ($P = 0.004$). Neither 18 minutes (in study 5) nor 60 minutes (in the study 6) of continuous leptin infusion altered the base FBF levels in both arms (Fig. 12).

![Study 5](image1.png)  ![Study 6](image2.png)

Fig. 12. Plasma leptin concentration increased during intraarterial leptin infusion, but base FBF was not affected.

The response to vasodilators was reduced during co-infusion of leptin compared to during co-infusion with saline, although this response was not significant with all vasodilators: BK, $P = 0.07$; SNP, $P = 0.009$; Ach, $P = 0.64$; and verapamil, $P = 0.03$ (Fig 13). Taking together the responses to the four vasodilators and adjusting for differences at baseline showed that the decreased response during leptin co-infusion was highly significant ($P = 0.001$).
Overall, FBF was reduced following concomitant infusion with leptin (●) vs. saline (○). Continuous lines represent FBF in the infused arm, whereas dashed-lines show FBF in the non-infused arm. From left to right: First peak = FBF during BK-infusion, Second peak = FBF during AcH-infusion, Third peak = FBF during SNP-infusion, Forth peak = FBF during verapamil infusion. Within the experiment, the vasodilators were infused in random order—except for verapamil, which was always infused last.

Heart rate, SBP, and DBP did not significantly change in study 5 during the infusion of leptin ($P = 0.95$, $0.86$, and $0.14$), or in study 6 during leptin infusion ($P = 0.35$ to $0.97$) or the infusion of arterial vasodilators ($P = 0.74$ to $0.99$).

In study 5, a short leptin infusion of 15–20 minutes did not affect circulating levels of t-PA ag, t-PA act, or PAI-1 ag. In study 6, circulating levels of t-PA ag and t-PA act were not significantly altered during 60 minutes of leptin infusion ($P = 0.72$ and $P = 0.25$, respectively). They were also not affected by the type of co-infusion (leptin vs. saline): $P = 0.43$ for t-PA ag and $P = 0.10$ for t-PA act. As expected, t-PA act increased during BK infusions in both infused and non-infused arms ($P < 0.0001$ and $P = 0.009$, respectively). Interestingly, this activity was significantly increased during the co-infusion of leptin compared to with saline ($P = 0.002$). Neither net t-PAag nor net t-PA act release differed between leptin or saline co-infusions ($P = 0.40$ and $P = 0.23$, respectively).

---

Fig. 13. Overall, FBF was reduced following concomitant infusion with leptin (●) vs. saline (○). Continuous lines represent FBF in the infused arm, whereas dashed-lines show FBF in the non-infused arm. From left to right: First peak = FBF during BK-infusion, Second peak = FBF during AcH-infusion, Third peak = FBF during SNP-infusion, Forth peak = FBF during verapamil infusion. Within the experiment, the vasodilators were infused in random order—except for verapamil, which was always infused last.
Discussion

Paper III demonstrated that intraarterial leptin infusion achieved hyperleptinemic plasma levels. Furthermore, the results showed that hyperleptinemia did not alter basal vascular tone in vivo, but was associated with reduced endothelium-dependent and -independent vasodilatation in both healthy volunteers after short leptin overload, as well as in hyperleptinemic patients with known CVD. Significant augmentation of the intravascular release of t-PA (net t-PA release) was only observed in patients with known CVD and chronic hyperleptinemia, not in healthy males during exogenous leptin infusion.

One of the main results of this paper was that exogenous-induced hyperleptinemia did not evoke any change in basal blood flow. Previous studies—most executed either experimentally in vitro or in animal models—have indicated that leptin may induce vasodilatation through NO, EDHF release, and/or activation of endothelial NO synthase (eNOS) [75]. Other studies have postulated that the physiological “vasodilatory” effect of leptin demonstrated in vitro, may become ineffective or be annulated in vivo models. For example, a direct intracoronary infusion of leptin at physiologic and supra-physiologic concentration ranges had no effect on coronary flow in anesthetized dogs [13]. More recently, Wolsk et al. demonstrated in a group of young, healthy, lean males that infusions with recombinant human leptin did not affect abdominal adipose tissue flow (measured using the 133-Xenon wash-out method) or leg blood flow in the femoral artery (measured using the Doppler ultrasound method) [310].

However, not all in vivo studies have followed this pattern. Nakagawa et al. and Matsuda et al. each showed in peripheral vessels and in coronary arteries, respectively, that a short leptin infusion elicited vasodilatation in humans in vivo [292, 311]. Using a methodology similar to ours but with a 10-fold lower infused leptin concentration that only exerted a subtle augmentation in plasma leptin levels, Nakagawa’s study observed significant increases in FBF, reflecting significant vasodilatation from the very beginning of the experiment. In contrast, in study 6, we found no demonstrable effect on basal FBF after 60 minutes of leptin infusion, despite the achievement of a >20-fold increase in plasma leptin concentrations. We firmly believe that our result is more in accordance with previous observations, and that direct local infusion of leptin at physiologic and supra-physiologic concentration ranges does not significantly affect the basal vascular tone. Our findings may be in line with the theory of a “counteracting effect of leptin,” in which the vasodilatory effects of leptin (mediated by NO
and EDHF) [14] are counterbalanced \textit{in vivo} by simultaneous leptin-mediated vasoconstriction [75, 170].

Another main finding of paper III was that, in both studies 4 and 6, EDV and EIDV were negatively associated with chronic and acutely exogenously induced hyperleptinemia, respectively. As previously mentioned, we are aware that the design of these studies does not allow us to infer causality. However, we will present some mechanistic reflections based on the present results.

Previous studies have demonstrated that vascular inflammation can have the effect of blunting vasodilatation. For example, inflammation induced by \textit{Escherichia coli} endotoxin or \textit{Salmonella typhi} vaccination reportedly decrease EDV (without affecting AIX) [312]. Furthermore, other findings have shown that intravascular administration of TNF-\textit{a} exerted similar effects in healthy individuals [313] as in patients with CVD [108], supporting the view that different inflammatory stimuli may induce harmful effects on the vasculature. Leptin promotes inflammatory vessel changes by modulating the production of TNF-\textit{a} and IL-6 [226]. Study 4 showed that leptin was also associated with levels of the common inflammation marker CRP, as has been previously reported [228]. CRP is known to decrease the expression of endothelial NO synthase [314] and to increase ROS production [230], thus interfering with NO production and bioavailability. CRP has also been associated with decreased EDV and EIDV in resistance vessels, but not in conduit arteries [315]. Unfortunately, no inflammatory markers were analyzed in our infusion studies.

Another possible mechanism by which leptin may affect vasodilatation is through SNS activation. It has been demonstrated that leptin mediates SNS activity [218], and early SNS activation following leptin infusion has been described [316], which may lead to reduced vascular reactivity, increased vasoconstrictor tone, and decreased NO availability [317]. Unfortunately, in designing our experimental studies, we did not consider the possibility of measuring SNS activity. For this reason, in our analysis we examined possible variations in heart rate and blood pressure, in order to detect possible effects of SNS activation. In study 6, blood pressure and pulse remained unaltered during around 3 hours of leptin infusion, and in the associative study 4, we detected no differences in blood pressure or pulse among the different leptin tertiles. However, the absence of heart rate and blood pressure variations during leptin infusion and in patients with higher circulating leptin levels does not entirely rule out the possibility of an underlying SNS stimulation. In fact, a recent study demonstrated that acute experimental hyperleptinemia in healthy men elicited significant central
nervous excitatory effects on vasoconstrictory sympathetic outflow (measured by muscle sympathetic nerve activity), with no significant changes in blood pressure and heart rate [216]. This is in line with several previous studies showing increased overall sympathetic outflow without any systemic changes in blood pressure and pulse [247].

An alternative mechanism explaining the impaired vasodilatation associated with leptin could be through the stimulation of ET-1, a potent vasoconstrictor released by the endothelium. Leptin has been shown to induce endothelial cell release of ET-1 in vitro [223], as well as in lean subjects, but not in patients with obesity-related metabolic syndrome [224].

In study 6, leptin was found to be associated with impaired vasodilatation in response to both endothelial and NO-mediated mechanisms (Ach and BK infusion), as well as in relation to exogenous (SNP infusion as an external NO donator) and endothelium-independent stimuli (SNP and Vp-infusion). Theoretically, these results could be explained by any of the above-mentioned mechanisms, either alone or in some combination.

The results exposed in paper III suggest that altered vasoreactivity associated with leptin can be demonstrated in both cases of acute leptin exposure and in the context of chronic hyperleptinemia. These findings show that leptin could be a mediator of altered vasodilatation, contributing to atherosclerotic development [212, 318], and favoring the development of hypertension [207] and CVD [319].

The role of leptin in fibrinolytic function remains unclear. The results of studies 4 and 6 may suggest that the effect of leptin on fibrinolytic function may differ depending on the underlying status of the endothelium (patients with CVD vs. healthy individuals) and/or the time of exposure to leptin (chronic hyperleptinemia vs. acute leptin infusion).

Basal levels of t-PA ag and t-PA act were significantly higher in hyperleptinemic patients in study 4, but remained unaltered during leptin infusion in study 6. This may suggest a state of permanent endothelial activation in the former situation. In any case, it must be noted that neither basal plasma t-PA nor PAI-1 concentrations control the local vascular fibrinolytic capacity, which is instead determined by the acute release of t-PA from the endothelium [162]. In study 6, BK-stimulated release of t-PA act was significantly augmented during the concomitant leptin infusion, which would be in line with the results of study 4. This finding could be explained by several different mechanisms, such as vascular inflammation [158] or SNS activation [160], which may theoretically be associated with the
presence of hyperleptinemia. However, the increased BK-stimulated t-PA act under leptin infusion does not necessarily imply an augmented fibrinolytic capacity. The estimation of endothelial net t-PA release is the pivotal measurement for assessing the intrinsic fibrinolytic capacity of the vascular wall [153]. Net t-PA release was augmented in the highest leptin tertile (possibly underlying a state of endothelial activation), but remained unaltered following leptin infusion. Thus, we did not find evidence suggesting that leptin per sé played any direct role in the endogenous activation of the fibrinolytic system in the vascular wall.

In summary, the results of studies 4 and 6 revealed common tendencies. The impaired EDV and EIDV found in the associative study were confirmed under leptin infusion. These findings were in line with other previous results obtained in animal models. The effects on fibrinolysis were less clear. In this regard, it is necessary to highlight the inherent difficulties of analyzing t-PA and PAI-1 in cross-over studies. The variation in t-PA and PAI-1 levels within a day and between different days, principally due to circadian and hemodynamic factors, adds some uncertainty in the interpretation of these factors in this kind of study.
General discussion

In this section, we discuss the research process, the link between objectives and conclusions, the strengths and limitations of this thesis, and the paths for future research with some possibilities for therapeutic approaches.

Research process

Here we tell the short story of this thesis, with the intent of describing its evolution from the beginning, particularly the reason that it was started and how the methodology and conforming studies were chosen. During this process, some new questions and hypotheses arose, which opened the door to new lines of investigation (marked in the text with the symbol **). Some of these will be briefly discussed under the paragraph “Further studies”.

This thesis was basically started with the general aim of shedding light onto the complex and intriguingly relationship between visceral obesity and CVD. From the beginning, we realized that a project with such a broad goal would demand a narrowing of focus on a more specific question. In this regard, we concentrated our interest towards the study of endothelial function, which is universally recognized as an early pathophysiological state in the development of vascular atherosclerosis and CVD. Following previous studies, our investigation of endothelial function was based on the analysis of vasomotor reactivity and circulating basal or stimulated markers of fibrinolysis.

The unavoidable first step of this study was to learn a technique for assessing endothelial function. Forearm strain-gauge plethysmography is a reliable and affordable method, and had been widely described in many other studies and even used within our group for research in Umeå. This is the principal reason for including paper I (corresponding to study 1) in this thesis. It was a laborious process to recruit volunteers (which is not always straightforward), to design the protocol, and to learn and implement the plethysmography technique, but these steps were necessary for the further execution of the experimental studies 5 and 6. The results of paper I were not directly related to the aims of this thesis, but they might open the door to new lines of investigation (**1).

Study 2 represented an additional analysis of the data obtained from study 1, becoming the first study in this thesis to address the association of leptin with endothelial function. The results of study 2 gave some first indications of an association between leptin and impaired vasodilation after
stimulation with pharmacologic agonist. Study 2 was also useful in the design of further studies. For example, we found no supplementary benefits in the use of DEXA measures (amount of total fat tissue) in addition to the use of waist circumference or BMI as markers of obesity. Thus, we decide that BMI and waist circumference would be the variables of choice to represent obesity in further studies (**2). Although investigating the effects of insulin/insulin resistance was mentioned in the initial planning of this thesis, study 2 highlighted the complexity of vascular disorders in the context of metabolic syndrome, leading us to drop this focus from further studies. At this point, we decided to focus our efforts on the effects of leptin, deciding that the important interplay between leptin and insulin was beyond the scope of this thesis (**3). After the first analysis of the results, it became clear that the characteristics of the studied population (men with known CVD) and the low number of participants would make it difficult to generalize our findings.

Study 3 provided the opportunity to analyze leptin associations in a larger community-based population of both men and women. We were also able to analyze not only FBF but also other techniques of vascular assessment. Each technique delivers different information regarding vascular function and may thereby reflect changes in different types of vessels (e.g., conduit vs. resistance vessels). We obtained different results depending on the technique used for vascular assessment. Further investigation is required to address the remaining question of whether this finding represented true differentiation in the action of leptin on different types of vessel or if it was a “technique-dependent” result (**4).

In study 4, conducted by our colleagues in Scotland, data regarding endothelial function in patients with known CVD were correlated to leptin. The goal was to perform a study similar to study 2, but in a bigger cohort of patients.

In studies 5 and 6, we made the jump from correlational to experimental research. Non-obese, young, healthy males were recruited for these studies. This selective sex inclusion (only men) was primarily based on inherited “tradition” from our Scottish research partners, from whom we learned the plethysmography technique. Their inclusion of only men was based on two facts. First, women have a menstrual cycle that makes it more difficult to control the study process. The participants often undergo plethysmography studies two times or more during different weeks, and EDV and fibrinolytic function may vary according to menstrual cycle phase [320, 321], which could potentially impact the final results. Second, women generally have a smaller brachial artery diameter than men, making it more difficult to
cannulate the artery within the allowed three attempts (a general rule in this kind of study). Although these inconveniences can be worked around, they would add difficulties to the performance of the study.

Because of the novelty of study 6, we did not include subjects who were overweight, or exhibited impaired glucose tolerance or DM. Thus, the results of this study in healthy volunteers can be used as a reference study for further studies in other groups. The intraarterial coinfusion of leptin and vasodilators in humans was a novel intervention in the spring of 2007 (the year in which this study was executed), although the vasomotor effects of leptin infusion in the peripheral brachial artery in healthy men had been previously tested. Nakagawa et al. in 2002 [292] and Matsuda et al. in 2003 [311] had also tested the effects of leptin on the brachial artery and the coronary arteries, respectively. From our point of view, the results of the former study were somewhat controversial. Nevertheless, Nakagawa's study tested the concentrations of leptin infusion, and their data were used to determine the concentrations used in our protocol in order to achieve plasma levels like those found in obesity and to evaluate the possible vasomotor or fibrinolytic changes.

To provide an associative context for our novel experimental study, we decided, in collaboration with the Scottish members of our research group, to merge studies 4, 5, and 6 into a single paper. This strategy was an excellent opportunity to compare the effects of chronic vs. acutely induced hyperleptinemia, and the effects on patients with CVD vs. healthy volunteers. The juxtaposition of the studies also provided a frame of reference to hypothesize on some of the possible mechanisms underlying the effects of leptin on endothelial function (**5).

**Aims and conclusions**

Table 6 shows how our initial objectives correspond with the final conclusions, and indicates the respective studies/papers from which each conclusion comes from.
Table 6. Aims and conclusions.

<table>
<thead>
<tr>
<th>AIM</th>
<th>CONCLUSION</th>
<th>STUDIES</th>
<th>PAPER</th>
</tr>
</thead>
<tbody>
<tr>
<td>To study the association between plasma leptin levels and endothelial function in humans.</td>
<td>Hyperleptinemia was independently associated with blunted vasodilation. This association was attenuated after adjustment for BMI. The association of leptin with impaired EDV was blunted in subjects with higher circulating plasma leptin levels. Leptin was associated with SBP and DBP. Hyperleptinemia was associated with increased t-PA ag, PAI-1, and net t-PA release in patients with CVD.</td>
<td>2, 3, 4</td>
<td>II, III</td>
</tr>
<tr>
<td>To study different characteristics of this association: a. In different populations.</td>
<td>a. In an elderly community-based population and in patients with known CVD, leptin was associated with decreased EDV and EIDV, independently of sex.</td>
<td>3, 4</td>
<td>II, III</td>
</tr>
<tr>
<td>b. Chronic vs. acute exogenous hyperleptinemia.</td>
<td>b. Both chronic and acute exogenous hyperleptinemia were independently associated with EDV and EIDV.</td>
<td>2, 3, 4</td>
<td>II, III</td>
</tr>
<tr>
<td>To study this association with different techniques of vascular assessment.</td>
<td>Leptin was independently associated with decreased FBF, as a measure of EDV and EIDV in resistance vessels. Leptin was not associated with FMD, which represents a measure of EDV in a conduit vessel. Leptin was associated with increased AIx, but this association did not remain after adjustments for traditional risk</td>
<td>3</td>
<td>II</td>
</tr>
</tbody>
</table>
factors of CVD and/or measures of insulin resistance.

| To study the net effects of direct infusion of leptin in vivo in healthy volunteers. | Short intra-arterial infusion of leptin in healthy males induced obesity-related and pharmacological plasma leptin levels. | 5, 6 | III |
| In healthy males, acute induced hyperleptinemia did not alter basal vasomotor tone. | 5, 6 | III |
| Acute exogenous hyperleptinemia was independently associated with EDV and EIDV. | 6 | III |
| Acute exogenous hyperleptinemia did not affect either basal levels of t-PA or endothelial net t-PA release. | 6 | III |

| To develop a hypothesis regarding a possible mechanistic mechanism for this association. | Further mechanistic studies are warranted, but vascular inflammation and/or SNS activation may underlie the effects of leptin on the endothelial function. | 3, 4, 6 | II, III |

**Strengths and limitations**

Generally speaking, cross-sectional studies may be considered as hypothesis generators, which are unable to determine cause–effect associations. In our eyes, the inclusion of experimental studies in this thesis is an important strength, especially considering their novelty. The experimental studies provided the opportunity to “test” some of the hypotheses raised from the associative studies, particularly the effect of leptin on vasodilatation. Another strength of this thesis lies in the huge amount of unresolved questions that were generated during the process and execution of these studies, which may be interpreted in terms of “possibilities,” new challenges, or new directions for future research.

Most of the limitations of this thesis are related to the study design or were generated due to methodological reasons. For example, the gender perspective was not conveniently addressed in most of our studies. In our first two associative studies (studies 1 and 2) and in the experimental ones
(studies 4 and 6), only men were subjected to endothelial assessment by forearm plethysmography. This is, of course, a major limitation that hinders further generalization to the general population.

Another gender-derived limitation occurs in study 4, in which leptin tertiles were generated without any stratification by sex. As women have higher leptin concentrations than men [255, 322], there was an increased frequency of females in the higher plasma leptin tertiles. In this regard, females are known to exhibit greater stimulated endothelial release of t-PA, a finding which may explain their lower incidence of thrombotic events [323]. Although our study design is insufficient to explore this interaction in detail, it is theoretically possible that gender differences in acute fibrinolytic capacity may be related to the higher leptin concentrations in females. However, it should be noted that when restricting our analysis to males, we still observed a greater t-PA release with higher leptin concentration, suggesting this is not solely a gender effect. The relationships between leptin and the fibrinolytic system demand further research (**6).

Another limitation comes from the possibility that our results may strictly be applicable to normal or overweight individuals, but not in a setting of morbid obesity. In this regard, a recent study demonstrated that the effects of leptin on endothelial function may differ depending on the grade of obesity [215] (**7). The analysis of our results is limited by the relative lack of measurements in our studies. It is clear that other interesting questions remain, relating to the concept of leptin resistance in vascular tissue. For example, it is unclear how long it would take leptin infusion to exert a possible state of leptin resistance (we only examined 18 minutes in study 5, and 1–3 hours of leptin infusion in study 6) (**8).

There also exists another group of limitations that are much more difficult to solve, namely, the difficulties that arise when trying to reflect a physiological phenomenon in a laboratory. For example, the aim of study 6 was to obtain knowledge regarding the physiologic role of leptin in human metabolism. However, acute (and also chronic) supplemental studies may not reflect normal physiology, because endogenous secretion of leptin is pulsatile and may be affected by different factors, including food intake and fasting [324], exercise [325], disease exacerbation [326], or the menstrual cycle [327].

**Further studies**

Further research in this field could include investigations of additional associations between leptin and different markers of endothelial function or arterial distensibility, such as circulating markers of endothelial function or
measures of intima media thickness (IMT) of the carotid artery. The gender factor is also an important issue to investigate, considering that different rates of free circulating leptin are present in men and women.

(**1) Effects of diesel exhaust on endothelial function

This is a very important matter of study and has become a major public health issue in recent years. In fact, our research group in Umeå has conducted and is still carrying out several studies in this field. There are many remaining questions that warrant further studies, for example, regarding whether diesel exhaust affects endothelial function differently in diabetics or selected overweight hyperleptinemic individuals. In this regard it could be worth noting the observation that, during diesel exposure, the significant negative association between leptin and SNP did not remain.

(**2) Ideal measurement of obesity

Which measure of obesity is most adequate in vascular or endothelial studies? Do we need to overcome the issue of the strong interrelationship between leptin and measures of obesity, especially BMI? Is it necessary to make any adjustment in this kind of study? In all of our studies, the association of leptin with vasodilatation was blunted after the adjustment for BMI. One possible explanation is that leptin underlies the causal pathway between BMI and vasoreactivity. Thus, leptin could be considered as “the messenger” between fat tissue and the vessel. However, statistically speaking, it seems difficult to adjust two variables for each other when they are so closely related ($r = 0.73$, $P < 0.001$ for the association of leptin with BMI in studies 3 and 4).

(**3) Interplay between insulin and leptin in the modulation of vascular tone

Both insulin and leptin have shown vasodilator effects through enhancement of endothelial NO release. Theoretically, they could interact with each other or potentiate each other’s effects. In such a case, the concept of insulin resistance would be in some way related to the concept of leptin resistance. We believe this to be an important potential area of further research. Further analysis in special populations, such as in glucose intolerant or diabetic individuals, would be of great interest.

(**4) Suitability of various measurement techniques
Are all measures of endothelial function reliable for the study of the vascular effects of visceral fat? Do the results depend on the type of vessel (conduit vs. resistance) and/or on the underlying state of the vessel (young vs. elderly individuals)? In light of our controversial results regarding FMD and Aix, we believe that this is also an interesting area for further study.

(**5) Mechanistic studies

This is an almost virgin field, waiting to be explored by intrepid and fearless researchers. In our studies, causality was not examined. Such an approach would have required a different design with some complementary assessments. For example, important information could have been obtained from our studies if they had included an extended analysis of inflammatory markers (e.g., CRP), any measures of SNS activation (e.g., by neurographic methods or spillover noradrenalin measurements) [328], analysis of circulating markers of endothelial activation (e.g., soluble vascular cell or intracellular adhesion molecules) [329], or measures of circulating levels of vasoconstrictors (e.g., ET-1 or Ang II).

(**6) Relationships between leptin and fibrinolytic system

This is generally a poorly understood issue. An increasing number of studies suggest an association of hyperleptinemia with dysfibrinolysis. However, the strength of possible gender effects or possible bias in this association remains unclear.

(**7) Details of BMI-related variation in leptin action

Further studies are needed to investigate the possible differences in the action of leptin on the endothelial function among overweight, obese, and super-obese (BMI > 45) individuals.

(**8) The concept of leptin resistance in vascular tissue

How long does it take for infused leptin to create a state of leptin-resistance in the vascular tissue? Seconds? Hours? Weeks? It would be very interesting to perform experiments in humans with a longer period of leptin infusion (days or weeks). Would this show an unopposed cumulative vasodilator effect of leptin? Or would the induction of leptin resistance occur with further impairment in vasodilatation?
**Therapeutic implications**

Our aim in the present thesis was to explore the physiological actions of leptin, not the potential pharmacological effects. However, from our studies, we did not find any indication of beneficial effects of high circulating levels of leptin. On the contrary, our findings promote speculation about the possibility that hyperleptinemic obese individuals may benefit from further development of leptin antagonist peptides that block leptin activity, receptor antagonists, or substances that increase leptin clearance—especially in cases showing ED or early signs of atherosclerosis.

In this regard, some intriguing questions arise. Can we prevent atherosclerosis development by blocking leptin’s vascular effects? Does the demonstrated leptin-associated impairment of vasodilatation play any role in a clinical setting? For example, do obese (hyperleptinemic) individuals manifest lower clinical responses to nitrates following an angina attack? And what are the antihypertensive effects of calcium-antagonist drugs in overweight/obese individuals? Is there any role of leptin-antagonist substances in the treatment of ischemic heart disease and/or hypertension?

These questions are very difficult to address, and go far beyond the aim of this thesis...

...but that is another story and shall be told another time [330].

77
Conclusions

1. In an elderly community-based population and in patients with known CVD, leptin was associated with decreased FBF (both EDV and EIDV), independently of sex. This suggests that the action of leptin on vasomotor function is not exclusively endothelium/NO-dependent.

2. In an elderly community-based population, leptin was not associated with FMD (reflecting EDV in a conduit vessel), indicating that leptin may exert its effect on specific vessel segments (resistance vessels). An alternative possibility is that FMD assessment is not a sensitive enough method to measure changes in an elderly population with stiff vessels and diminished vascular compliance.

3. Leptin was associated with increased AIx, but this association did not remain significant after adjustments for traditional risk factors of CVD and/or insulin resistance.

4. Leptin was independently associated with higher SBP and DBP.

5. The association of leptin with blunted vasodilatation was attenuated when adjusted for obesity (BMI), indicating that leptin could be the mediator between obesity and impaired vascular function.

6. The association of leptin with impaired EDV, but not with EIDV, was blunted in subjects with higher circulating plasma leptin levels. This may reflect a state of “selective” leptin resistance.

7. In healthy males, a short intra-arterial infusion of leptin increased the plasma leptin level, resulting in impaired EDV and EIDV, but no effect on basal vasomotor tone.

8. In patients with CVD, hyperleptinemia was associated with elevated fibrinolysis markers, particularly with stimulated endogenous t-PA release (net t-PA release). This may reflect an underlying state of fibrinolysis activation. In contrast, acute induced hyperleptinemia in healthy men did not affect endothelial net t-PA release.
Final comments

Obesity-associated cardiovascular disorders: the never-ending story?

In the fabulous book, “The Never Ending Story” written by Michael Ende and first published in 1979 [330], the main protagonist of the story, Bastian Balthasar Bax, finds throughout his tale, that it is impossible to describe all of the different characters and situations in one single book.

In many ways, something similar occurs with the protagonist of our story, leptin. The complexity and wide reach of this cytokine's action can sometimes make it difficult to follow “the red thread” of the story. During the course of preparing this thesis, many questions arose, some of which were not yet answered in the scientific literature. Hopefully, this thesis can be seen as inspiration for further possible studies.

This thesis ends here, but the story hopefully doesn’t.
Acknowledgements

Many people have assisted with the realization of this project. In this short section, I would like to name some of the persons and institutions that have directly or indirectly helped me throughout this journey.

First of all, I want to mention my friend and main tutor, Assoc. Professor Stefan Söderberg. Thanks for initiating and guiding my research, and for your never-ending enthusiasm and patience. Integrity, exactitude, and carefulness in the research process are ensured when Dr. Söderberg is in the boat. I will miss our discussions and hard statistical work at your kitchen table.

Professor Anders Blomberg, co-supervisor, for your contagious energy, kindness, and capacity to always make the “right choice.” Special thanks for your support in the communication with our Scottish partners.

Professor Tommy Olsson, co-supervisor, for allowing me to participate in the seminars of your research group, for wise comments and advice, and for your support “behind the curtains.”

Professor Thomas Sandström, for allowing me to participate in the studies of your group, and for giving me the opportunity to initialize the learning of the plethysmography technique. By extension, acknowledgments to the Respiratory Medicine and Allergy Department (NUS) for economic support when needed.

Dr. Stefan Barath for Graph Pads guiding and supplying me with several pictures taken from our plethysmography studies.

Dr. Nicholas Mills and Professor David Newby, co-authors and colleagues from the University of Edinbigh, for fruitful collaboration and valuable suggestions in the design of our common studies. Thanks Nick for the professional and friendly plethysmography instruction, and thanks both for your incredible hospitality during our stay in Edinburgh.

Dr. Simon Robinson for your generosity in sharing the results of the Scottish study and supplying me with additional data when needed.

Dr. Håkan Törnqvist, co-author and first plethysmography study partner. Maybe you have now realized that Real Madrid is, after all, the best team in the world.
Maria Eriksson, statistician, for advice and statistical support. A light in dark times.

Associate Professor Lars Lind, co-author, for your incredibly exhaustive and well-done work in the Uppsala project (PIVUS), and for your generosity in sharing the data with us, and for always quickly answering my e-mails.

Margot Johansson, Frida Holmström, Annika Johansson, and Veronika Sjögren, for your invaluable practical work with volunteers and assistance during long plethysmography journeys...sometimes until late afternoon/evening...

All volunteers who participated in this thesis, especially to those who participated in the studies conducted in Umeå.

Ulf Näslund, Krister Lindmark, as clinical chiefs, and all of my colleagues at the Department of Cardiology, NUS for your comprehension and support in allowing me to be absent from my clinical duties when research time was required.

Kerstin Rosenqvist, for your help and support with many practical things, especially during the last period of administrative requirements and formalities.

Miguel San Sebastian and Anna-Karin Hurtig for your active listening followed by friendly feedback, in a genuine coaching style. True friends are hard to find, difficult to leave, and impossible to forget.

Andreu Jacob, for the innovative creation of Léptima, the Sound Track of this thesis. It is an honor to me that this prestigious Spanish artist, composer & musician has been interested in my work, describing the magnitude of the obesity epidemic and the effects of leptin on the endothelial function...with music!!

The Västerbotten Council and the University of Umeå for financial support through “ALF-medel”.

The Swedish Heart and Lung Foundation and the local Heart and Lung Association in Sollefteå and Örnsköldsvik for financial support.

Last but foremost, to my family. My wife Virginia, partner in life and best friend, for her love and unconditional support. I do not regret any minute of the adventure that we started together years ago. Thanks to my
children just for being around: the “Gonzalez-Zazo brothers” Alvaro, David & Miguel, who are my principal reason for living. Thanks to my parents, Manuel and Felisa for unconditionally staying by my side and for teaching me that worthy aims in life usually demand effort and determination; to my brothers Javier, Rafael, and my sister Elena and their respective families and to my brother Miguel, always present in our minds and living in our hearts.

........but that's another story and shall be told another time.
References


hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density lipoprotein receptor. The Journal of biological chemistry 2001;276:37402-8.


Payne GA, Contribution of perivascular adipose tissue to coronary vascular dysfunction, In, Faculty of Indiana University, Indiana University, 2010.


[149] Nichols WW. Clinical measurement of arterial stiffness obtained from noninvasive pressure waveforms. American journal of hypertension 2005;18:3S-10S.


[151] Newby DE, McLeod AL, Uren NG, Flint L, Ludlam CA, Webb DJ, Fox KA and Boon NA. Impaired coronary tissue plasminogen activator


[163] Lilja M, Trends in obesity and type 2 diabetes; ethnic aspects and links to adipokines, In, Department of Public Health and Clinical Medicine, Umeå, Umeå University, 2011.


[225] Eriksson M, Adipocyte-derived hormones and cardiovascular disease, In, Department of Public Health and Clinical Medicine, Medicine, Umeå, University of Umeå, 2010.


[229] van Dielen FM, van’t Veer C, Schols AM, Soeters PB, Buurman WA and Greve JW. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese


[232] Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J and Feve B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. European cytokine network 2006;17:4-12.


106


[267] Jansson JH, Olofsson BO and Nilsson TK. Predictive value of tissue plasminogen activator mass concentration on long-term mortality in


