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EFFECTS OF WEIGHT LOSS, PHYSICAL TRAINING AND ANTI-INFLAMMATORY THERAPY ON ENDOTHELIAL FUNCTION IN VIVO

Robert Bergholm

ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.


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ABBREVIATIONS

ACE  angiotensin converting enzyme
ACH  acetylcholine
Ang II  angiotensin II
ANOVA  analysis of variance
Apo  apolipoprotein
BH4  tetrahydrobiopterin
BMI  body mass index
BW  body weight
CAD  coronary artery disease
CRP  C-reactive protein
CVD  cardiovascular disease
DMARD  disease modifying antirheumatic drug
DBP  diastolic blood pressure
EDHF  endothelium-derived hyperpolarizing factor
eNOS  endothelial nitric oxide synthase
ESR  erythrocyte sedimentation rate
ET-1  endothelin-1
FFA  free fatty acids
FBF  forearm blood flow
FMD  flow-mediated dilatation
GDM  gestational diabetes mellitus
GTN  glyceryl trinitrate
Hb  hemoglobin
HbA1c  glycosylated hemoglobin A1c
HDL  high-density lipoprotein
IDL  intermediate-density lipoprotein
ICAM-1  intercellular adhesion molecule-1
IL-6  interleukin 6
iNOS  inducible nitric oxide synthase
LDL  low-density lipoprotein
L-NMMA  NG-monomethyl-L-arginine
MRI  magnetic resonance imaging
NO  nitric oxide
NOS  nitric oxide synthase
OGTT  oral glucose tolerance test
ox-LDL  oxidized low-density lipoprotein
PAI-1  plasminogen activator inhibitor-1
PET  positron emission tomography
PGI2  prostacyclin
PGH2  prostaglandin H2
PPAR  peroxisome proliferator-activated receptor
RF  rheumatoid factor
SBP  systolic blood pressure
SEM  standard error of mean
SNP  sodium nitroprusside
TF  tissue factor
TFPI  tissue factor pathway inhibitor
TG  triglyceride
TNFα  tumor necrosis factor α
t-PA  tissue plasminogen activator
TRAP  total peroxyl radical trapping potential
TXA2  thromboxane A2
VCAM-1  vascular cell adhesion molecule-1
VLDL  very low-density lipoprotein
VO2max  maximal oxygen uptake
vWF  von Willebrand factor
WHR  waist to hip ratio
ABSTRACT

Introduction: Cardiovascular disease is the leading cause of death in Western countries. Endothelial dysfunction is considered an early step in the development of atherosclerosis and an independent predictor of cardiovascular morbidity and mortality. Epidemiological studies have revealed several important environmental and genetic risk factors associated with atherosclerosis. Obesity, physical inactivity and a number of inflammatory rheumatic diseases including rheumatoid arthritis have all been associated with an increased risk of cardiovascular disease. The present studies were undertaken to investigate effects of 1) moderate weight loss achieved by a hypocaloric diet combined with orlistat or placebo on endothelial function in vivo, and 2) physical training on endothelial function in vivo, and to assess whether 3) patients with rheumatoid arthritis (RA) have impaired endothelial function compared to normal subjects, and whether 4) vascular function in this group of patients can be improved with anti-inflammatory therapy.

Subjects and methods: In study I, in vivo endothelial vascular function was measured in 47 obese women. The women were randomised into two groups using either orlistat or placebo. Both groups were designed to lose 8% of body weight during a similar time period. In study II, endothelial function and antioxidant status were measured before and after 3 months of training in 9 healthy men. In study III, forearm vascular function was compared between 20 patients with RA and 33 normal subjects. In study IV, the effect of 6 months of anti-inflammatory therapy on endothelial function was assessed in 10 patients with newly-diagnosed RA. Vascular function was determined from forearm blood flow responses to intra-arterial infusions of endothelium-dependent vasodilator acetylcholine and –independent vasodilator sodium nitroprusside in all studies.

Results: Moderate weight loss improved endothelium-dependent vasodilatation and decreased LDL cholesterol significantly in the orlistat but not in the placebo group. Intense physical training decreased circulating antioxidants, except ascorbic acid, and impaired endothelium-dependent vasodilatation. Patients with RA had blunted responsiveness to nitric oxide. Basal blood flow was increased in proportion to inflammatory activity. Six months of anti-inflammatory therapy decreased both clinical and laboratory markers of inflammation and improved vascular function in newly-diagnosed patients with RA.

Conclusions: Moderate weight loss does not improve endothelial function unless a simultaneous and significant reduction in LDL cholesterol is achieved. Intense physical training may impair vascular function by increasing oxidative stress. Patients with RA have blunted responses to endothelium-dependent and –independent vasodilators compared to normal subjects. This vascular dysfunction is reversible with anti-inflammatory therapy.
INTRODUCTION

Cardiovascular disease is the leading cause of death in Western countries (1). Epidemiological studies have revealed several important environmental and genetic risk factors associated with atherosclerosis. Our understanding of the pathogenesis of atherosclerosis has improved greatly during the recent years and now there is increasing evidence that both inflammatory components as well as endothelial dysfunction are important factors in this process (2). Endothelial dysfunction has recently been recognised as an early marker of atherosclerosis and a predictor of cardiovascular morbidity and mortality (2,3).

Obesity has been shown to increase the risk of cardiovascular disease and there are data suggesting that obesity is associated with mild chronic inflammation as judged from increased levels of circulating C-reactive protein (CRP) and other circulating markers of inflammation (4). No studies have examined effects of weight loss on endothelial function.

Physical activity is associated with a low risk of cardiovascular disease (CVD) (5). In conditions where endothelial dysfunction is present, physical training seems to improve vascular function (6,7,8,9). Strenuous aerobic physical training increases oxidative stress, which could have harmful effects on vascular function. Effects of high-intensity physical training on endothelial function in healthy subjects are unknown.

CVD is the major cause of excessive mortality in patients with RA (10,11,12,13). Chronic inflammation in RA has been proposed to be one of reasons causing premature atherosclerosis (14). Inflammation has also been linked to endothelial dysfunction but whether patients with RA have endothelial dysfunction and whether anti-inflammatory therapy improves vascular function has so far not been studied.

In present studies we determined whether identical amounts of weight loss with or without inhibition of fat absorption with orlistat improves endothelial function in premenopausal women with a history of gestational diabetes. The effect of intense physical training on endothelial function, antioxidants and circulating lipids was studied. In patients with RA, endothelial function was compared to normal subjects. Effects of 6 months anti-inflammatory therapy on endothelial function in patients with newly-diagnosed RA was also studied.
2. REVIEW OF THE LITERATURE

2.1 Endothelial function in vivo

2.1.1 Normal function of the endothelium

The cardiovascular system is lined by a monolayer of elongated cells – the endothelium. This thin and permeable layer between the circulating blood and vessel wall was first described in 1660 by Malphigi, who was studying capillary circulation in the lung of a frog (15). Until the end of the 19th century, the endothelium was considered as an inert, inactive membrane, only separating blood from tissue. In 1891, Heidenhain suggested that the endothelium was an active secretory organ with selective permeability (15). It took, however, several decades before his theory was accepted and proved to be correct. In animal studies intravenous infusions of acetylcholine (ACh) caused vasodilatation but when strips of blood vessels were incubated with ACh in vitro, no relaxation was seen. This puzzled scientists until extensive work by Furchgott and colleagues lead to the discovery of the endothelium-dependent relaxation of blood vessels. They found that relaxation by ACh appeared only when the thin and easily damaged layer was intact and attached to the vessel wall. They showed that muscarinic receptors on endothelial cells, when stimulated with ACh, released an endothelium-derived relaxing factor (EDRF), which relaxed the underlying vascular smooth muscle in rabbit aortic rings (16). Some years later EDRF was identified as the free radical nitric oxide (NO) (17), which is synthesized by endothelial cells from L-arginine (18). This process could be blocked specifically with an arginine analogue, L-NMMA (19), which is still used to measure NO-dependent vasodilatation.

We know today that the endothelium plays a crucial and active role in many biological processes. In an adult man the vascular endothelium covers over 3000 m$^2$, weighs 1.5 kilograms, and is thus the biggest endocrine organ in the human body (20). The endothelium regulates vascular tone, platelet activation, monocyte adhesion, lipid transport, immune responses, vessel growth and remodelling (Table 1). The endothelial cells produce a wide range of substances such as NO, prostacyclin (PGI$_2$), endothelin (ET-1), vascular endothelial growth factor (VEGF), interleukins, tissue plasminogen activator (t-PA), tissue factor pathway inhibitor (TFPI), angiotensin converting enzyme (ACE) and von Willebrand factor (vWF) (20). Additionally, adhesion molecules like intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin are synthesized (20)(Table 1).
Table 1. Normal functions of the endothelium (modified from Abdu et al (21))

<table>
<thead>
<tr>
<th>Function</th>
<th>Mediator</th>
<th>Test of Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasoregulation</td>
<td>NO, EDHF, PGI₂, ET-1, Ang II, TXA₂</td>
<td><em>Endothelium-dependent vasodilatation:</em> Invasive tests using ACh, bradykinin, substance P, serotonin; Non-invasive test using shear-stress (FMD); Measurement of the AgI after salbutamol</td>
</tr>
<tr>
<td>Coagulation</td>
<td>PGI₂, TXA₂, vWF, TF fibrinogen, thrombomodulin</td>
<td>Measurement of plasma fibrinogen, vWF, thrombomodulin levels</td>
</tr>
<tr>
<td>Fibrinolysis</td>
<td>t-PA, PAI-1</td>
<td>Measurement of circulating t-PA and PAI-1 antigens and activities</td>
</tr>
<tr>
<td>Inflammation</td>
<td>P- and E-selectin, VCAM, ICAM, TNFα, IL-6</td>
<td>Measurement of plasma or serum levels of CRP, E-selectin, fibrinogen, ICAM-1, IL-6, TNFα, VCAM</td>
</tr>
</tbody>
</table>

Agl, augmentation index; Ang II, angiotensin II; CRP, C-reactive protein; EDHF, endothelium-derived hyperpolarizing factor; ET-1, endothelin-1; FMD, flow-mediated dilatation; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin 6; L-NMMA, N-nitro-L-arginine; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PGI₂, prostacyclin; TNFα, tumor necrosis factor α; t-PA, tissue plasminogen activator; TF, tissue factor; TXA₂, thromboxane A₂; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor.

Vascular tone is regulated by a complex interplay involving nerves, shear stress, circulating factors and the endothelium. In the resistance arteries the endothelium releases several substances, which regulate vascular smooth muscle function. Relaxation is induced by NO, endothelium-derived hyperpolarizing factor (EDHF), and PGI₂ (17,22,23) (Fig. 1). Contracting factors include ET-1, angiotensin II (Ang II), thromboxane A₂ (TXA₂), and prostaglandin H₂ (20,24,25). PGI₂, TXA₂, and prostaglandin H₂ (PGH₂) are formed from arachidonic acid in the endothelium (26) (Fig. 1). Extensive work is done in laboratories worldwide to solve the mystery of EDHF. There are preliminary indications that EDHF could be a natriuretic peptide, C-natriuretic peptide is for the moment the strongest candidate (27).
Figure 1. This figure shows endothelium-dependent relaxing and contracting systems and some factors influencing endothelial function. Acetylcholine (ACh) binds to muscarinic receptor (M) and activates nitric oxide (NO) synthesis via the L-arginine pathway. NO causes relaxation of the smooth muscle cells by increasing the synthesis of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). Sodium nitroprusside (SNP) is a exogenous NO-donor. Insulin, bradykinin, and methacholine are examples of substances increasing the release of vasodilators from the endothelium while cytokines and hypoxia increase the release of vasoconstrictors. Relaxation is induced by NO, endothelium-derived hyperpolarizing factor (EDHF), prostacyclin (PGI₂). Contracting factors are endothelin-1 (ET-1), thromboxane A₂ (TXA₂), prostaglandin (PGH₂), angiotensin II (Ang II). N⁰-monomethyl-L-arginine (L-NMMA) and asymmetric dimethylarginine (ADMA) competitively block NO-synthesis. O₂⁻= superoxide; Arg=L-arginine, Cit=L-citrulline, eNOS=endothelial nitric oxide synthase, Ca²⁺= ionised calcium, AT1= type 1 angiotensin II receptor, ACE=angiotensin converting enzyme.
Of all the molecules synthesized by the endothelium, NO is the most widely studied. NO is a free radical released from the endothelium when L-arginine is deiminated to L-citrulline by the L-arginine system (Fig. 1). The reaction is catalysed by nitric oxide synthase (NOS) and can be specifically and competitively blocked by L-NMMA (19). Several isoforms of NOS have been identified, but only two have been found in the endothelium; inducible NOS (iNOS) and endothelial NOS (eNOS) (28). eNOS contributes to NO production at rest and when shear stress stimulates the vessel wall (29). The increase in eNOS activity evoked by shear stress contributes to the phenomenon of flow-mediated vasodilatation, an important autoregulatory mechanism by which blood flow increases in response to exercise (30). eNOS is stimulated by several receptor-dependent agonists like bradykinin, ACh, methacholine, carbachol, substance P, thrombin, adenosine 5’-diphosphate, and muscarinic agonists (31). The activation of eNOS is Ca\(^{2+}\) dependent. Tetrahydrobiopterin (BH4) is an essential cofactor for the synthesis of NO. Reduced availability of BH4 seems to be involved in the development of endothelial dysfunction in atherosclerosis (32). The iNOS enzyme is expressed in endothelial cells, macrophages and smooth muscle cells during inflammation and stimulation by cytokines (33). Activation of iNOS is Ca\(^{2+}\) independent. Smooth muscle cells are relaxed when NO binds to guanylate cyclase rising the intracellular cyclic guanosine monophosphate (34) (Fig. 1).

2.1.2 Definition of endothelial dysfunction

Endothelial dysfunction can be defined as loss of one or more of the normal functions of the endothelium (Table 1) (20,35). Clinically, endothelial cell dysfunction can be manifested as generalized or local vasosspasm, thrombosis, atherosclerosis, and restenosis (Fig. 2). Impaired vasodilatory responses to endothelial-dependent vasoactive agents are used as markers of a dysfunction in endothelial vasoregulation. Diminished responses to intra-arterial infusions of endothelium-dependent vasodilators, such as ACh can theoretically be due to diminished synthesis of NO, impaired sensitivity of smooth muscle cells to endogenously formed NO, and increased destruction of NO. Comparison of responses to endothelium-dependent vasodilators and endothelium-independent vasodilators (SNP or GTN) forms the basis of an endothelial function test. Endothelial dysfunction has been found in a multiple of diseases and conditions (Table 2).
Figure 2. In normal, healthy endothelium there is balance between the release of vasodilatory and vasoconstrictive factors as well as pro- and anti-inflammatory, -thrombotic, -atherogenic, and -coagulant factors. Increasing risk factors shift the balance towards vasoconstrictive, thrombotic, and atherogenic status. NO=nitric oxide, PGJ$_2$=prostacyclin, EDHF=endothelium-derived hyperpolarizing factor, ET-1=endothelin-1, TXA$_2$=thromboxane A$_2$, PGH$_2$=prostaglandin H$_2$, O$_2^-$ = superoxide. Modified from Verma et al. (35).
### Table 2. Conditions associated with impaired endothelial function

<table>
<thead>
<tr>
<th>Endocrine diseases</th>
<th>Lipid abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acromegaly</td>
<td>Hypercholesterolemia</td>
</tr>
<tr>
<td>Hypopituitarism</td>
<td>High LDL cholesterol</td>
</tr>
<tr>
<td>Polycystic ovary syndrome</td>
<td>Low HDL cholesterol</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>Type 1 and 2 diabetes</td>
<td>Hypertriglyceridemia</td>
</tr>
<tr>
<td>The insulin resistance syndrome</td>
<td>High Lp(a)</td>
</tr>
<tr>
<td></td>
<td>Small dense LDL particles</td>
</tr>
</tbody>
</table>

**Cardiovascular diseases**

<table>
<thead>
<tr>
<th>Atherosclerosis</th>
<th>Vasculitides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>Kawasaki disease</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>Chagas’ disease</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>Behcet’s disease</td>
</tr>
<tr>
<td>Syndrome X and variant angina</td>
<td>Wegener’s granulomatosis</td>
</tr>
<tr>
<td>Transplantation atherosclerosis</td>
<td>Primary Raynaud’s phenomenon</td>
</tr>
<tr>
<td>Ischemia/reperfusion</td>
<td>Polyarteritis nodosa</td>
</tr>
<tr>
<td>Cardiopulmonary bypass</td>
<td></td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td></td>
</tr>
<tr>
<td>Family history of CAD</td>
<td></td>
</tr>
</tbody>
</table>

**Other conditions**

| Obesity                                   |                                 |
| Pre-eclampsia                             |                                 |
| Postprandial dyslipemia                   |                                 |
| Active and passive smoking                |                                 |
| Hyperglycemia                             |                                 |
| Aging                                     |                                 |
| Male gender                               |                                 |
| Elevated homocysteine                     |                                 |
| Elevated ADMA                             |                                 |
| Renal insufficiency                       |                                 |
| Postmenopause                             |                                 |
| Depression                                |                                 |
| Inflammation                              |                                 |
2.1.3 Measurement of endothelial function

2.1.3.1 Invasive techniques

One way of assessing endothelium-dependent vasodilatation is to infuse endothelium-dependent pharmacological agents and measure the change in vessel diameter or blood flow. Several different endothelium-dependent agonists such as substance P, bradykinin, serotonin, methacholine, carbachol, thrombin and ACh have been used to stimulate the endothelium to produce relaxing factors (36,37). Of these ACh is the most commonly used agent. ACh is an endogenous neurotransmitter present in cholinergic synapses and neuroeffector junctions in the central and peripheral nervous system, acting via nicotinic and cholinergic receptors. It is used by ophthalmologists to constrict pupils during surgery of the eye and in research involving the endothelium. When infused intra-arterially, ACh binds to the muscarinic receptors on the endothelium activating eNOS to catalyse NO production (Fig. 2). ACh dilates, however, not only via NO, but also by stimulating the release of EDHF and prostacyclin and by inhibiting the release of the vasoconstricting norepinephrine presynaptically. Although the vasodilatory effects predominate in most vascular beds, ACh exerts vasoconstrictive abilities by direct effects on smooth muscle cells by activating the cyclo-oxygenase system to produce the vasoconstrictors prostaglandin H₂ and TXA₂. If the endothelium is damaged or absent, ACh causes vasoconstriction by stimulating directly the smooth muscle cells (38). Additionally, ACh is rapidly hydrolyzed by acetylcholinesterase when administered in the circulation (39). This metabolic instability may explain why ACh responses may be altered by basal flow and forearm length (40). In normal subjects, intra-arterial infusion of 7.5-15 µg/min of ACh increases the forearm blood flow (FBF) to 3 to 6 times over the basal blood flow within a few minutes.

To measure the endothelium-independent vasodilatation, intra-arterial infusion of sodium SNP or oral GTN are mostly used. SNP is a NO-donor, thus directly activating guanylate cyclase in the smooth muscle cells (41). SNP dilates both arteries and veins. In normal subjects, intra-arterial infusion of 3-10 µg/min of SNP increases forearm blood flow 3-6 times over the basal blood flow. The blood flow response to ACh relative to SNP provides a measure of endothelial function. L-NMMA, an arginine analogue, which is a specific and competitive inhibitor of NO synthesis, is often used to measure the contribution of NO to endothelium-dependent vasodilatation. When infused alone, use of L-NMMA allows determination of the fraction of basal blood flow that is NO-dependent, which in normal subjects is ranging from 20 to 50% (42,43). When co-infusing L-NMMA with ACh, it is possible to measure the portion of ACh induced vasodilatation that is NO-dependent.
Forearm occlusion plethysmography is mostly used to measure the vasodilatory responses to intra-arterially infused substances (44). Blood flow is measured in both forearms simultaneously. The non-infused arm serves as a control arm for the experimental arm. The results can be expressed in absolute units of blood flow (ml/dl forearm·min) or as the per cent change in flow, or as a ratio of flow in the experimental and control arm. Although there is no consensus as to how the blood flow data should be expressed, the ratio is recommended as it minimizes the intersubject variability and variations in blood flow caused by factors and stimuli not related to the infusion of drugs (45,46,47). Blood flow responses to infusions of ACh (48) or other vasoactive substances (49,50,51) have also been measured in coronary arteries. Vasodilatation in those studies has been measured using coronary angiography, Doppler or PET (51,50,49).

Figure 3. Simultaneous measurement of forearm blood flow during infusion of 7.5 μg/min acetylcholine (ACh) in the experimental arm and saline in the control arm. Arterial inflow is determined by drawing a tangential line across the first few pulses following the occlusion of venous return.
Another approach to study endothelial function is to measure the vasodilatation in peripheral arteries such as brachial or superficial femoral arteries after a standardized hyperaemic flow stimulus using a high-resolution ultrasound device. After the release of an occlusion cuff, a sudden increase in flow increases endothelial cell shear stress which activates NO synthesis (52,53,54). This flow-mediated increase in arterial diameter is endothelium-dependent in dogs (52) and can be blunted by local L-NMMA infusion in humans (53). Sublingual GTN is usually used to measure the endothelium-independent vasodilatation with this technique. This method is highly operator-dependent (55,56). There is some correlation between forearm intra-arterial infusion plethysmography technique and flow-mediated dilatation (FMD) but only the former has been shown to predict cardiovascular events in patients with hypertension (57) and in patients with CAD (58). Impaired response of intracoronary infusion of ACh has been shown to be predictive for cardiovascular events in patients with CAD (3,59,60) and interestingly also in subjects with angiographically normal coronary arteries (59). Anderson et al. found a correlation between endothelial function measured with FMD and responses to intracoronary infusion of ACh (61). In a recent study by Monnink et al. (62) coronary diameter response to ACh, forearm blood flow response to ACh, and brachial artery flow-mediated dilative responses to postischemic hyperemia were compared. The effect of ACh on forearm resistance vessels was significantly related to the effect of ACh in coronary conduit vessels, whereas, FMD was neither related to forearm blood flow (FBF) nor to the coronary response (62).

Ischemia-induced vasodilatation in upper extremities is another approach to study vasodilatation. The forearm circulation is interrupted for 2-10 min with a proximal occlusion cuff. After the cuff release, reactive hyperemia is measured. Only 16% of the vasodilatation induced by 5-minutes of ischemia can be blocked by L-NMMA (63), indicating that mainly other factors than endothelial NO are responsible for the vasodilatation.

Radial artery pulse-wave analysis by applanation tonometry combined with administration of vasoactive agents is a new method to assess endothelial function (64,65). With this technique the augmentation index, which produce a measure of the tone or stiffness of arteries greater than those controlling peripheral vascular resistance, is measured before and after administration of endothelium-dependent and –independent agents. The method has good reproducibility and the response to β2-adrenergic-agonists (salbutamol) inhalation correlates with FBF response to ACh infusion (64,65). The decrease in the augmentation index induced by salbutamol but not GTN can
be partly blocked with L-NMMA (64,65). Impaired responses to β2-agonists have been found in patients with hypercholesterolemia (64), coronary artery disease (65) and type 2 diabetes (66).

2.1.3.3 Circulating markers of endothelial function
Many circulating molecules have been proposed as markers of endothelial damage or activation (67). These include soluble forms of E-selectin, P-selectin, vascular cell adhesion molecules (VCAMs), intercellular adhesion molecule-1 (ICAM-1) and von Willebrand factor. These markers are often elevated in patients who have or are at risk of CVD (68). A recent meta-analysis concluded, however, that the predictive value of measurement of these soluble cell adhesion molecules for future coronary events is poor (69). Other possible markers of endothelial function include ET-1, ACE-activity, TFPI, different NO metabolites and PGI₂ metabolites. Their role as markers of endothelial function is unclear. In patients with RA, elevated serum concentrations of ICAM-1, VCAM-1, and E-selectin have been found (70) but they seem to correlate with synovial inflammation (71). Measurement of serum CRP with highly sensitive assay is perhaps the marker that predicts CVD the best (72) but to what extent CRP reflects endothelial dysfunction is unclear.

2.1.4 Endothelial dysfunction as a predictor of cardiovascular disease
The current concept of the pathogenesis of atherosclerosis is that endothelial dysfunction represents a key early step in the development and progression of atherosclerosis (2). Several recent studies have shown that endothelial dysfunction, measured in the forearm (58,57) and coronary vascular bed (3,59,60), is an independent predictor of vascular events in patients with hypertension or CAD. Interestingly, impaired coronary endothelium-dependent vasodilatation has recently been shown to predict cardiovascular events even in patients without CAD or hypertension (3). Atherosclerosis develops both in the forearm and human coronary arteries, and the severity of these atherosclerotic lesions has been found to be significantly interrelated (73). Several studies have found a good correlation between endothelium-dependent vasodilatation in forearm and coronary arteries (74,75). This relationship is also supported by treatment studies. For example lipid-lowering drugs improve endothelium-dependent dilatation both in the forearm (76,77,78) and coronary (79,80) vessels. Flow-mediated dilatation of the brachial artery has also been shown to correlate with that in the coronary arteries (49,81). Taken together, these data show that endothelial dysfunction, measured either in the peripheral resistance vessels or in the coronary arteries is a predictor of cardiovascular disease and cardiovascular events.
2.2 Causes of variation in endothelial function in human

2.2.1 Gender and age

Women seem to develop atherosclerosis later than men. The mean onset of symptomatic CAD is 10 years later in women (82) than in men. Endothelial-dependent vasodilatation deteriorates with increasing age, while no major changes has been shown in the endothelium-independent vasodilatation (83,84). In men the impairment can be detected after the age of 40, whereas deterioration in endothelial function in women starts 10-15 years later (85,86). This difference has been attributed to estradiol (87). The menstrual cycle influences the endothelial function (88). Endothelial function is worse in post– than premenopausal women. Data regarding the effect of postmenopausal hormone replacement therapy on endothelial function has been contradictory. It seems that oral (89,90,91,92) but not transdermal (89,93) estradiol hormone replacement therapy in postmenopausal women improves endothelial function.

2.2.2 Body weight and composition

Several studies have suggested that obesity is associated with endothelial dysfunction (94,95,96,97,98,99). In these studies, both obesity (94) and associated metabolic abnormalities such as dyslipidemia (100), hypertension (97), insulin resistance (96) and accumulation of fat in intra-abdominal rather than subcutaneous depots (95,98,99) have correlated with altered vascular function. Interestingly, low birth weight is associated with reduced flow-mediated dilation but not with endothelium-independent dilation in young adults (101). The exact cause of endothelial dysfunction in human obesity is, however, unclear.

2.2.3 Physical activity

Based on observational follow-up studies there seem to be an inverse dose-response relation between increasing physical activity and CVD incidence and mortality (5). Multiple mechanisms exist to explain protective effects of physical activity on cardiovascular health, but the relation between the amount of physical activity and endothelial function in humans is poorly understood. In most (102,103,104,105) but not all (106,107,108) animal studies exercise training has improved responsiveness to ACh and other endothelium-dependent vasodilators. Data on effects of physical training on endothelial function in humans are sparse and controversial. In the study by Utriainen et al., physical fitness defined by maximal oxygen uptake did not correlate with endothelium-dependent vasodilation in the brachial arteries in normal subjects in a cross-sectional study (109). Green et al. found that 4 weeks of handgrip exercise training did not change responses to metacholine in the forearm resistance vessels i.e. at a location exposed to
exercise hyperemia (110). The same group also reported that tennis players have similar
vasodilatory responses to ACh in both forearms, despite enhanced ischemia-induced peak
vasodilatory capacity in the trained forearm (111). Interestingly, immobilisation of the forearm
for 6 weeks by casting after a bone fracture in forearm or hand had no effect on forearm
endothelium-dependent or -independent vasodilatory responses (112). On the other hand,
Kingwell et al. found 4 weeks of bicycle training to significantly enhance the vasoconstrictive
response to a moderate but not high dose of L-NMMA, in forearm resistance vessels of sedentary
males (113). In healthy subjects results are conflicting but in patient groups with diseases, which
are associated with endothelial dysfunction at baseline have shown the best improvement after an
exercise program. In diseases like CAD (7), peripheral arterial disease (114), and chronic heart
failure (115) physical training has improved endothelial function. Elderly athletes have been
shown to have better forearm endothelial function than age-matched sedentary controls (116).
The enhanced vasodilatation to ACh in athletes could partly be explained by their better lipid
profile (117).

2.2.4 Smoking
Cigarette smoking is a major risk factor for CVD (118,119) and for rheumatoid factor (RF) -
positive RA (120,121). Both active and passive smoking impairs endothelium-dependent
vasodilatation in a dose-dependent manner (122,123,124). Smoking one cigarette impairs
endothelial function for approximately 90 minutes (125). Cessation of chronic smoking restores
endothelial function (126), but it is unclear how long period of time is needed for the endothelium
to recover. Smoking is associated with an increased incidence of positive RF in subjects without
(127,128) and with RA (120), independent of the amount of smoking. The increased risk of
developing seropositive, but not seronegative RA requires a long duration of smoking and it
persists for years after cessation (121).

2.2.5 Lipid abnormalities
Hypercholesterolemia due to elevated serum LDL cholesterol has in several studies been shown
to be associated with endothelial dysfunction both in forearm (100,129,130) and coronary
(131,132) vessels independent of the presence of CAD. This impairment in endothelial function
appears long before structural vascular changes or symptoms occur (133).
Oxidized LDL (ox-LDL) impairs endothelial function even more than native LDL in animals (134). In humans the susceptibility of LDL to oxidation has been associated with impaired coronary reactivity (135,136).

Small LDL size has also been associated with endothelial function in healthy men (137) and in patients with type 2 diabetes (138). Insulin resistance and the accompanying hypertriglyceridemia are the main causes of small LDL size.

In humans HDL is an important anti-atherogenic lipoprotein, which is a carrier of cholesterol from the periphery to the liver. It has been suggested that there is a direct association between HDL and endothelial function (139), and, indeed Spieker et al. recently reported that 4 hours infusion intravenous reconstituted HDL normalized endothelium-dependent vasodilatation while response to SNP was unchanged in hypercholesterolemic men (140). Previously it has been shown that HDL counteracts the inhibitory effect of LDL on endothelium-dependent vasodilatation (141). It has been hypothesized that the antioxidant properties of HDL may be important in maintaining normal endothelial function and that low levels of HDL increases oxidation of LDL and impairs endothelial function (142).

Plasma free fatty acids (FFA) increase basal blood flow in the forearm and attenuate the response to intra-arterial infusion of ACh (143). Fatty acid composition of serum lipids may also be related to endothelium-dependent vasodilatation in healthy subjects (144).

2.2.6 Type 1 and 2 diabetes mellitus
Mortality from cardiovascular disease is 2-4 times higher in patients with type 2 diabetes as compared with general population (145). Diabetes is associated with several metabolic abnormalities and cardiovascular risk factors such as hyperglycemia, hypertension, and increased serum levels of FFA, ox-LDL, triglycerides and low levels of serum HDL cholesterol, and insulin resistance. All these abnormalities have been associated with impaired endothelial function (96,129,142,146,147,148,149). It is therefore not very surprising that diabetic subjects, especially those with type 2 diabetes have endothelial dysfunction (96,150,151,152). The data regarding type 1 diabetes are not consistent. There are studies showing either impaired (153,154,155) or normal (156,157) endothelium-dependent vasodilatation.
2.2.7 Hypertension
Hypertension has been associated with impaired endothelial function in most (43,147,158,159,160,161), but not all (162) studies. The mechanisms underlying endothelial dysfunction in hypertension are poorly understood.

2.2.8 Vasculitides
Vasculitides are a heterogeneous group of diseases characterized by inflammation of vessels of different diameter. Typical features include fibrinoid necrosis of the vessel wall and inflammatory infiltrates of leukocytes and vessel occlusion (163). Several studies have reported impaired endothelium-dependent vasodilatation in vasculitides, such as Kawasaki disease (164,165,166,167), primary Raynaud’s phenomenon (168), thromboangitis obliterans (169), Behcet’s disease (170), Wegener’s granulomatosis, and polyarteritis nodosa (171). Anti-inflammatory therapy has been shown to restore vascular endothelial function in patients with Wegener’s granulomatosis and polyarteritis nodosa (172). Circulating markers of inflammation and endothelial function have also been found to be increased in vasculitides. These include elevated levels of homocysteine, ET-1 (173), PAI-1 and VCAM-1 (174) in Behcet’s disease, affecting veins and arteries. Expression of ICAM-1, VCAM-1, E-selectin, and TNFα in the vessel wall have been increased in patients with thromboangitis obliterans, which is a periferal vasculitis causing occlusive thrombosis (175).

RA is associated with chronic inflammation and an increased risk for development and mortality of CVD (10,11,12,14,176). It is unknown, however, whether endothelial function is impaired in these patients. Elevated levels of circulating markers of endothelial activation or dysfunction such as, ICAM-1, VCAM-1, and E-selectin have been measured in patients with RA but they seem to originate from the inflamed joints and not from the arteries (70,71).

2.2.9 Other
Women with previous gestational diabetes appear insulin resistant and have an increased risk of developing type 2 diabetes (177). While some studies have reported endothelial dysfunction in these women (178,179,180), others have not (181). Polycystic ovary syndrome (PCO) has been associated with either impaired (182) or normal (183) endothelial function.

Homocysteine is toxic to the endothelium (184) and decreases the bioavailability of NO by inhibiting metabolism of ADMA (185). ADMA is an endogenous and competitive inhibitor of
eNOS, and serum levels of ADMA are correlated with the severity of endothelial function (186,187). Elevated plasma homocysteine concentration is associated with an increased risk of atherosclerosis of coronary, peripheral, and cerebral arteries (188). Treatment with folic acid decreases circulating homocysteine concentrations and improves endothelial function (189).

2.3 Treatment of endothelial dysfunction

2.3.1 Diet

Amount and composition of dietary fat
An ordinary mixed meal with 34% fat transiently impaires endothelium-dependent vasodilation in healthy subjects compared to a fat-free meal (190). In another study, 28 days of Mediterranean and low-fat diets improved endothelial function in hypercholesterolemic men compared to a saturated fat rich diet (191). Replacement of dietary saturated fatty acids by trans fatty acids lowers serum HDL and impairs endothelial function (192). The exact mechanism by which high-fat diets induce impairmment in vascular reactivity is not clear. Elevated triglycerides after a fatty meal has been suggested to impair flow-mediated dilatation in young healthy men (193), but opposite effects have also been reported (194).

There are data suggesting that nutrients, which have antioxidant and/or cell membrane stabilizing properties, can protect endothelial cells. Red wine, olive oil and fish are examples of such of nutrients. In a recent study, antioxidant polyphenols in olive oil and red wine inhibited endothelial activation in vitro (195). A high-fat diet-induced endothelial dysfunction has also been shown to be counteracted by red wine in human volunteers (196).

Supplementation of fish oil, rich of omega-3 fatty acids, for 3 weeks improved endothelium-dependent vasodilatation in coronary arteries in heart transplant recipients compared to heart transplant recipients who did not receive fish oil (197). Similarly 4 months treatment of hypercholesterolemic subjects with 4 g/day marine omega–3 fatty acids improved flow-mediated vasodilatation in the brachial artery (198).

Vitamins
The effect, of antioxidant supplementation on CVD is still a matter of debate. Recent data from HOPE and Heart Protection Study indicated that several years of vitamin C, vitamin E, and β-carotene supplementation in high risk subjects had no effect on cardiovascular events (199,200).
On the other hand, the ASAP study suggested that intake of vitamins E and C for 6-years slows down the progression of atherosclerotic vascular disease in hypercholesterolemic patients (201).

Data concerning antioxidant supplementation on endothelial function are also contradictory. 12 weeks of vitamin E, vitamin C and β-carotene supplementation compared to placebo did not affect brachial vascular reactivity in healthy subjects, even though susceptibility of LDL to oxidation in vitro decreased (202). In another study, acute infusion of high doses of vitamin C to hypercholesterolemic (203) and hypertensive (204) patients improved endothelial function. Smokers have benefitted acutely from oral vitamin C but a supplementation with 1 g/day for 8 weeks had no effect on FMD in the brachial artery (205). Vitamin C supplementation has also improved FMD in patients with CAD (206) and chronic heart failure (207). However, in patients with hypertension, acute or chronic vitamin C had no effect on brachial artery endothelium-dependent, FMD or on endothelium-independent, NTG-mediated dilatation (208). In healthy subjects, vitamin C has improved or had no effect on FMD (209).

Brachial artery FMD has improved by vitamin E supplementation in patients with coronary spastic angina, high remnant lipoprotein, smokers, and type 1 diabetes, but not in healthy subjects (209).

Folic acid
Folic acid, or folate, is a micronutrient found in many green leaf vegetables, such as spinach, and in some animal products, such as egg yolk. Adequate intake of folic acid plays a role in the prevention of CVD. Folate is a regulator of plasma concentrations of homocysteine, which seems to be an independent risk factor for CVD (210). Hyperhomocysteinemia causes endothelial dysfunction, which can be restored by acute and chronic treatment with folic acid in both hypercholesterolemic patients and in healthy subjects (189,209,211). Interestingly, in children with type 1 diabetes and early endothelial dysfunction, low folate levels have been associated with endothelial dysfunction (212).

Phytoestrogens
Phytoestrogens have been recently proposed to be anti-atherogenic substances, which improve vascular function. In a placebo-controlled study, a four-week therapy with genistein, which is an isoflavonoid found in soybeans, improved flow-mediated endothelium dependent vasodilation in healthy postmenopausal women (213).
L-arginine

L-arginine is the substrate for NOS in the production of NO. Oral L-arginine administration improves endothelial function in hypercholesterolemic patients and in patients with cardiovascular disease but not in diabetic or healthy subjects (209). There are no clinical trial data on effects of supplementary arginine on cardiovascular disease.

2.3.2 Weight loss

Although weight loss is the cornerstone of antidiabetic therapy, there are no data on effects of weight loss on in vivo endothelial function in humans. Ziccardi et al. reported recently that endothelial activation as judged from increased concentrations of ICAM-1, P-selectin, and VCAM-1 is associated with visceral adiposity (214). In this study a 10% weight loss caused significant improvement in vascular responses to i.v. arginine infusion and decreased circulating levels of both adhesion molecules and cytokines. The i.v. arginine infusion test does not, however, measure specifically endothelial function if no control substance is administered. Theoretically, weight loss might improve endothelial function since several studies have shown the beneficial effects of weight loss on various cardiovascular risk factors including cytokines (215,216,217), adhesion molecules, blood pressure (218,219,220,221), sympathetic nerve activity (222,223), cardiac parasympathetic activity (224), lipids (225,226,227,228,229), blood glucose (230,231), insulin sensitivity (215,232,233,234), coagulation factors (235) and incidence of diabetes (236,237). Weight loss with diet (238) with or without different weight reducing agents such as sibutramine (239,240) and orlistat (241,242,243,244,245,246,247,248,249) reduce also cardiovascular risk factors, but no data are available on endothelial function. Oxidized LDL, one of the most potent inhibitors of endothelium-dependent vasodilatation decreases during weight loss (250). So does also intra-abdominal fat, which has been associated with endothelial dysfunction (232,233). Weight loss increases circulating concentrations of adiponectin, which in mice is a regulator of endothelial function (251).

2.3.3 Physical activity

In patients with peripheral arterial disease 6 months of aerobic exercise was reported to significantly improve flow-mediated brachial vasodilatation (114). Four weeks of daily handgrip training has been shown to improve endothelial function in patients with chronic heart failure (6). In another group of patients with chronic heart failure 4 weeks of bicycle training improved endothelium-dependent vasodilatation in the forearm significantly (115). In hypercholesterolemic patients, 4 weeks of bicycle training decreased DBP and infusion of L-NMMA caused a greater
vasoconstriction, whereas forearm vasodilatory responses to SNP or ACh remained unchanged (252). Physical training, consisting of 3 weekly exercise bouts for 12 weeks improved FMD in patients with the metabolic syndrome (8). Coronary endothelial function was markedly improved in patients with CAD after 4 weeks of bicycle ergometer training (10 min 6 times a day) (7). In patients with CAD, 10 weeks of leg exercise 3 times a week improved FMD in the legs but not in the forearm (9). Six months of regular stationary cycling improved flow-mediated dilatation in heart transplanted recipients (253). The response to intra-brachial infusion of ACh and FMD improved after combined aerobic and resistance exercise training 3 times/week for 8 weeks in type 2 diabetic subjects (254). Twelve weeks of brisk walking 5 to 7 times/week improved forearm endothelium-dependent vascular relaxation in both normotensive and hypertensive subjects (255). Finally, it has been shown that physical activity prevents age-related impairment in NO availability (116) and endothelium-dependent dilatation (256) in elderly athletes.

In summary it can be said that most conditions where endothelial dysfunction is present physical activity seem to improve it. There is evidence that increasing physical activity lowers CVD incidence and mortality in a dose-dependent fashion (5,257), but the amount of physical activity that is optimal for the prevention CVD is, unknown (5).

Exercise increases oxygen consumption and it has been estimated that 2-5% of oxygen consumed by cells can by utilised via an alternative pathway in which highly reactive oxygen species are produced, such as hydroxyl and superoxide radicals (258,259). These radicals are known to cause direct tissue destruction and lipid peroxidation but also to decrease the bioavailability of NO. Skeletal muscle tissue is very well adapted to high metabolic stress. However, during extremely high intensity exercise or endurance exercise the formation of reactive oxygen species may exceed the capacity of the protecting scavenger systems (258,259). These data raise the possibility that too much physical training causes endothelial dysfunction by increasing oxidative stress. In our study the fast progression from little physical training to very intensive training may have caused more oxidative stress than a training program with slowly increasing intensity. On the other hand, regular training seems to reduce oxidant release and leads to an adaptation of antioxidative mechanisms, which may contribute to a limitation of exercise-induced oxidative stress (258).
2.3.4 Lipid-lowering therapy

Several studies have shown that cholesterol lowering in patients with hypercholesterolemia improves endothelial function in both coronary arteries (79,132,260,261,262) and forearm resistance vessels (76,77,78,263,264). Statins, which are HMG-CoA reductase inhibitors and inhibit cholesterol synthesis in the liver, have provided strongest evidence for beneficial effect of lipid lowering. Heart Protection Study showed convincingly that statin therapy (simvastatin 40 mg daily) reduces vascular events irrespective of initial cholesterol concentrations (265). There are some indications that statins possess endothelium-friendly properties beyond their lipid lowering effects. Ten days of 10 mg atorvastatin daily improved ACh-stimulated forearm vasodilatation in postmenopausal women with normal serum lipids, without changing lipid levels (263). In vitro experiments with statins show up-regulation of eNOS and increased NO release from endothelial cells by ACh (266,267). Statins also have anti-inflammatory properties, judged from their ability to decrease circulating CRP and cytokines (268,269).

Fibrates lower triglycerides and 14 days of fenofibrate in hypertriglyceridemic subjects has recently been shown to improve vascular smooth muscle function, improving both endothelium-dependent and –independent vasodilatation (270). In type 2 diabetic subjects the use of gemfibrozil for 3 months improved FMD and decreased triglycerides (271).

2.3.5 Antihyperglycemic therapy

Insulin therapy induces several changes that potentially could enhance endothelial function. Such changes include decreases in serum triglycerides (272,273), FFA (148,272,274) and glucose concentrations (275). A supra-physiological dose of insulin increases blood flow in a dose-dependent manner in healthy subjects (276). This insulin-induced vasodilatation is NO-dependent (277,278). Insulin also enhances ACh dependent vasodilatation in forearm resistance vessels (279).

One year of insulin therapy, irrespective of the oral agent used, improved glycemic control and decreased serum E-selectin concentrations (280). Insulin therapy for 3 months improved glycemic control (HbA1c from 10.3 to 8.2%) and forearm reactivity to hyperemia, and there was a significant negative correlation between change vascular reactivity and change in HbA1c (281). In the study by Vehkavaara et al. (282) 6 months of insulin and metformin combination therapy increased blood flow responses to both intra-arterial ACh and SNP, whereas these measures remained unchanged by 6 months of metformin therapy alone. In this study insulin therapy
decreased HbA\(_1c\) from 9.0 to 7.6%. Rask-Madsen et al. thereafter reported that 2 months of insulin therapy improved forearm insulin-stimulated endothelial function in patients with type 2 diabetes and ischemic heart disease and decreased HbA\(_1c\) from 10 to 7.5% (283).

Twelve weeks of metformin, 500 mg twice daily, improved both forearm endothelial function and insulin resistance in diet-treated type 2 diabetic subjects (284), while SNP responses were unchanged. On the contrary, a reduction of HbA\(_1c\) from 10.8 to 8.0% in 20 weeks by metformin or glipizide or a combination of both did not change FMD in patients with type 2 diabetes (285).

Thiazolidinediones are PPAR-\(\gamma\)–agonists and insulin sensitizers in liver and muscle. In obese insulin resistant subjects, 8 weeks of troglitazone did not affect vascular responses to ACh, SNP or L-NMMA, despite an improvement in insulin sensitivity (286). Troglitazone reduces LDL oxidation and lowers plasma E-selectin concentration in type 2 diabetic subjects (287). There are no published data regarding effects of thiazolidinediones on endothelial function. Gemfibrozil, a fibrate with some PPAR-\(\gamma\)-agonist activity has improved FMD and insulin sensitivity in a study where type 2 diabetic subjects were treated for 3 months (271).

2.3.6 Anti-hypertensive therapy

Anti-hypertensive therapy decreases the incidence of stroke, heart and renal failure and mortality (288). Effects of blood pressure lowering on endothelial function appear to depend on the drug used. Use of \(\beta\)–blockers (289) or diuretics (289) have not had beneficial effects on endothelial vasodilatation. In contrast, there is some evidence that angiotensin-1 (AT1) receptor antagonists reverse functional changes in resistance arteries (290) and improve endothelial function of epicardial coronary arteries in patients with essential hypertension (291). Forearm endothelial function has improved in two studies with AT1 receptor antagonists. In the first study candesartan was used for 6 weeks (292), in the second irbesartan for 3 months (293). Calcium antagonists have had variable effects on endothelial function (289,294,295,296,297,298). Most (289,293,297,299,300,301) but not all (302) studies have reported an improvement in endothelial function during treatment with ACE-inhibition.

2.3.7 Anti-inflammatory therapy

Results from recent studies suggest that anti-inflammatory therapy may improve endothelial function in conditions where inflammation is present. Active use of disease modifying antirheumatic drugs (DMARD), especially methotrexate, has been suggested to decrease
cardiovascular mortality (303). Selective COX-2 inhibitors have been found to improve endothelial function (304). Suppression of inflammation by cyclophosphamide and methyl prednisolone in primary systemic vasculitis restored vascular endothelial function (171). Statins have anti-inflammatory effects that seem to improve endothelial function even without lipid lowering (305).

2.4 Rheumatoid arthritis and risk of cardiovascular disease

In recent years, CAD has been recognized as the major cause of excess morbidity and mortality in patients with RA (10,13,176,306,307,308,309). This is true also for other inflammatory diseases with arthritis such as systemic lupus erythematosus and systemic vasculitis (310,311). The increased incidence of cardiovascular events in RA is not explained by traditional cardiovascular risk factors (12,14). Several studies have shown that patients with RA have increased atherosclerosis in carotic arteries when compared to control subjects (312,313). The symptoms, progression and prognosis of a RF-negative disease are often milder than a RF-positive disease (314,315,316). Interestingly, RF independent of arthritis is a risk factor for cardiovascular death and its role may be directly pathogenic (316,317). Because of parallels between inflammatory/autoimmune diseases and atherosclerosis, it has been suggested that various inflammatory mediators may contribute to vascular dysfunction in patients with RA (318). The similarities include increases in circulating concentrations of adhesion molecules, proinflammatory cytokines and acute phase proteins in both patients with RA as well as in subjects with cardiovascular risk factors or overt CVD (318,319,320). Seropositivity for rheumatoid factor seems to be associated with increased cardiovascular mortality in patients with RA (314,315) but also in patients with RF-positive inflammatory polyarthritis independent of the fulfillment of the diagnostic criteria for RA (321). A retrospective study of patients with RA who were followed since diagnosis, showed that high inflammatory activity predicted subsequent cardiovascular events (322). In humans, local administration of TNF-α and IL-1β increases basal NO-dependent venodilatation but impairs endothelium-dependent venodilatation induced by bradykinin (323). These experimental data suggest that inflammatory disorders could predispose to CVD via blunting of endothelium- and NO-dependent vasodilatation.

Indirect measurements of NO production in patients with RA have suggested that the production of endogenous NO is increased rather than diminished due to activation of the iNOS (324,325,326,327,328,329). The INOS has been found to be overactive in circulating monocytes (330,331) and in vitro cultures of inflammatory synovium and cartilage (332). Interestingly, an
increase in iNOS activity inhibits eNOS activity and responses to endothelium-dependent
vasodilators such as ACh in experimental models of sepsis (333,334) but whether this occurs in
RA (335,336) is unknown. The finding of increased basal flow and the fraction of basal flow
which is inhibited by L-NMMA in vivo is consistent with several reports of increased NO
production by various indirect measurements in vitro (275,332,330,337,338,339,340,341,
342,343). The serum concentration of TNFα has been shown to correlate with enhanced
mitochondrial radical production in patients with RA (344). Attenuation of the endothelial
vasodilatory response to ACh has been restored by specific inhibitors of iNOS such as L-NG-(1-
iminoethyl)-lysine (345).

Patients with RA have lower LDL-cholesterol than the normal subjects (346,347). Recently Hurt-
Camejo et al. reported that RA patients have higher levels of small dense LDL despite a lower
concentration of total LDL cholesterol (347). LDL particles from RA patients also had
significantly higher binding affinity to glycosaminoglycans, which suggests that LDL particles
are prone to become trapped in the vessel wall matrix and be prone to oxidation.

There are currently no studies examining endothelial function in patients with RA.
3. AIMS OF THE STUDY
The present studies were undertaken to examine in vivo endothelial function in humans, and to specifically answer the following questions:

1) How do similar amounts of weight loss induced by a hypocaloric diet with or without inhibition of fat absorption with orlistat influence endothelial function in obese women with previous gestational diabetes? (I)

2) Does 12 weeks of intense physical training influence forearm vasodilatory responses to endothelium-dependent and -independent vasodilators, circulating antioxidants and plasma lipids and lipoproteins in healthy subjects? (II)

3) Do patients with rheumatoid arthritis have endothelial dysfunction? (III)

4) Can endothelial dysfunction in patients with RA be ameliorated by anti-inflammatory therapy? (IV).
4. SUBJECTS AND STUDY DESIGN

Baseline characteristics of the subjects are shown in Table 3. Aims and designs of the studies are listed below. Written informed consent was obtained from all subjects. The Ethics Committee of the Department of Medicine in the Helsinki University Central Hospital approved all the studies.

Study I

Aim: To determine whether moderate weight loss (8%) achieved by hypocaloric diet combined with orlistat or placebo improves in vivo endothelial function in obese women with previous gestational diabetes.

Design: Obese premenopausal women (Table 3) with a history of gestational diabetes were randomised to receive orlistat (120 mg t.i.d.) or placebo with a hypocaloric diet, designed to induce 8% weight loss over 3-6 months. Blood flow responses to intrabrachial artery infusions of endothelium-dependent ACh and -independent SNP vasoactive agents, body composition and serum lipids were determined before and after weight loss.

Study II

Aims: To test the hypothesis that intensive physical training impairs endothelial function in vivo by exposing tissues to repeated bouts of oxidative stress. We examined the effect of a 12-week intense endurance-training period on vasodilatory responses to endothelium-dependent and -independent vasoactive drugs in forearm resistance vessels.

Design: Nine recreational males runners (Table 3) training for a marathon run volunteered for the study. In each subject, in vivo endothelial function, maximal oxygen uptake (VO2max) and body composition were measured before and after a 12-week training period. Blood samples for determination of circulating antioxidants, lipid and lipoprotein concentrations were taken before the endothelial function test and at 3 months. Before participating in the study, the subjects had exercised regularly once a week. The training program consisted of 4 one-hour running sessions per week. The intensity of training was adjusted to correspond to 70–80% of each subject’s VO2max. Training was not allowed for 36 h before the studies. All subjects were healthy as judged by history, physical examination, and standard laboratory tests, and did not use any drugs.

Study III

Aim: To examine whether endothelial dysfunction characterizes patients with RA.

Design: Twenty patients (Table 3) who fulfilled the 1987 ACR criteria for RA (348) and 33 normal subjects received intra-brachial artery infusions of endothelium-dependent ACh and -
independent SNP vasodilators to determine the integrity of arterial responsiveness to NO. Basal flow and its % decrease by L-NMMA, an inhibitor of both endothelium-independent nitric oxide synthase iNOS and endothelium-dependent NOS (eNOS) was used to determine the contribution of iNOS and eNOS-dependent NO to basal flow. Prior to the vascular function study, venous blood samples were taken for measurement of serum lipids, IL-6, TNFα and CRP concentrations and the erythrocyte sedimentation rate (ESR).

Study IV
Aims: To determine whether endothelial dysfunction characterizes patients with newly-diagnosed rheumatoid arthritis compared to normal subjects and whether it is reversible with 6 months of anti-inflammatory therapy.

Design: Ten patients (Table 3 and publication IV) who fulfilled the 1987 ACR criteria for RA (duration of symptoms ≤ 18 months) were studied before and 6 months after initiation of therapy. Vascular function test, serum lipids and lipoproteins and markers of inflammation were measured. Before the first vascular function study, no patient had received treatment with disease modifying antirheumatic drugs (DMARD) or oral prednisone. A total of 33 matched normal subjects were studied as a control group. Eight of the patients were RF-positive. None of the patients had detectable levels of anti-nucleolar or anti-centromere antibodies. One of the patients had rheumatoid vasculitis and rheumatoid nodules. No other patient had extra-articular symptoms or signs of secondary Sjögren’s syndrome. Two of the patients had erosions at the time of diagnosis. None of the patients or normal subjects had hypertension or a history of cardiovascular disease and none of the normal subjects used any drugs. After the basal study, the patients were started with DMARD if they had a clinically active disease. Non-steroidal anti-inflammatory drugs were prescribed as symptomatic therapy. Treatment of the RA patients consisted of methotrexate in 5 patients, and cyclophosphamide in 1 patient with rheumatic vasculitis. Four patients were on low dose (5-7.5 mg) prednisone and 7 used non-steroidal anti-inflammatory drugs. Prior to the endothelial function tests the patients were instructed not to take acetylsalicylic acid or other non-steroidal anti-inflammatory drugs for one week.
Table 3. Baseline characteristics of the study groups.

<table>
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<th>STUDY</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<td>BMI (kg/m²)</td>
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<td>32±1</td>
<td>26±1</td>
<td>27±1</td>
<td>26±1</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>15±2</td>
<td>36±1</td>
<td>32±1</td>
<td>32±2</td>
<td>34±1</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>114±3</td>
<td>125±2</td>
<td>137±5</td>
<td>133±7</td>
<td>135±4</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>73±2</td>
<td>82±1</td>
<td>76±2</td>
<td>76±3</td>
<td>81±1</td>
</tr>
<tr>
<td>S-Cholesterol (mmol/l)</td>
<td>4.03±0.10</td>
<td>5.26±0.11</td>
<td>5.18±0.24*</td>
<td>5.10±0.46</td>
<td>5.80±0.17</td>
</tr>
<tr>
<td>S-LDL cholesterol (mmol/l)</td>
<td>2.40±0.12</td>
<td>3.33±0.10</td>
<td>3.16±0.21*</td>
<td>3.24±0.34*</td>
<td>3.85±0.15</td>
</tr>
<tr>
<td>S-HDL cholesterol (mmol/l)</td>
<td>1.36±0.04</td>
<td>1.29±0.04</td>
<td>1.53±0.08</td>
<td>1.44±0.07</td>
<td>1.44±0.08</td>
</tr>
<tr>
<td>S-Triglycerides (mmol/l)</td>
<td>0.73±0.10</td>
<td>1.41±0.10</td>
<td>1.33±0.13</td>
<td>1.20±0.23</td>
<td>1.24±0.10</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>-</td>
<td>-</td>
<td>41±6**</td>
<td>40±7***</td>
<td>8±1</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>-</td>
<td>-</td>
<td>30±6**</td>
<td>29±10***</td>
<td>4±1</td>
</tr>
<tr>
<td>S-TNF-α (ng/l)</td>
<td>-</td>
<td>-</td>
<td>2.1±0.3***</td>
<td>3.1±0.5***</td>
<td>1.5±0.2</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 for patients with rheumatoid arthritis (RA) vs. normal subjects. BMI= body mass index; ESR= erythrocyte sedimentation rate; CRP= C-reactive protein; S-TNFα= serum tumor necrosis factor α.
5. METHODS

5.1 Endothelial function

Endothelial function was assessed in forearm resistance vessels by measuring FBF responses to intra-arterial infusions of endothelium-dependent ACh and -independent SNP vasodilators and L-NMMA, an arginine analogue, which blocks generation of NO by both iNOS and eNOS (19). Since ACh stimulates NO production via eNOS but not iNOS (334) the blood flow response to ACh was used as a measure of endothelial function. The % decrease in blood flow below basal by infusion of L-NMMA reflects the contribution of NO produced both via iNOS and eNOS to basal flow (333,349).

The study was begun after a 10-12 hour fast at 7.30 a.m. An indwelling cannula was inserted in an antecubital vein for blood sampling. A 27 G unmounted steel cannula (Coopers Needle Works, Birmingham, UK), connected to an epidural catheter (Portex, Hythe, Kent, UK), was inserted into the left brachial artery. All drugs were infused at a constant rate of 1 ml/min with infusion pumps (Braun AG, Mesungen, Germany). Subjects rested supine in a quiet environment for 30 min after needle placement before blood flow measurements were begun. Normal saline was first infused for 18 min (Fig. 4). Drugs were then infused in the following sequence: SNP (Nitropress, Abbott Labs, North Chigaco, IL) 3 (low dose) µg/min and 10 (high dose) µg/min, ACh (Miochol, OMJ Pharmaceuticals, San Germán, P.R.) 7.5 (low dose) and 15 (high dose) µg/min and L-NMMA (Clinalfa, Läufelfingen, Switzerland) 4 µmol/min. Each dose was infused for 6 min, and the infusion of each drug was separated by infusion of normal saline for 18 min, during which blood flow returned to basal values. Forearm blood flow was recorded for 10 s at 15 s intervals during the last 3 minutes of each drug and saline infusion period with mercury-in-rubber strain-gauge venous occlusion plethysmography (EC 4 Strain Gauge Plethysmograph, Hokanson, Bellevue, WA), which was connected to a rapid cuff inflator (E 20, Hokanson), an analog-to-digital converter (McLab/4e, AD Instruments Pty Ltd, Castle Hill, Australia) and a personal computer. Blood flow measurements were performed simultaneously in the infused (experimental) and control arm. Means of the final five measurements of each recording period were used for analysis. Blood pressure and heart rate were measured before and after the endothelial function test. Blood flow results during infusion of vasodilators SNP and ACh are in studies I, III, and IV reported as a ratio of blood flow in the experimental arm divided with the blood flow in the control arm to correct for any differences in basal flow. In study II, which was the first study conducted, blood flow results in experimental arm were not divided with the blood flow in the control arm.
Figure 4. Design of the endothelial function test. The infusion rate was 1 ml/min in all studies. The saline infusion is depicted with slashed-line bars and infusion of vasoactive agents with white bars. Two concentrations of sodium nitroprusside (SNP), 3 µg/ml and 10 µg/ml, and two concentrations of acetylcholine (ACh) 7.5 µg/ml and 15 µg/ml were infused. In studies II and IV, N-monomethyl-L-arginine (L-NMMA) 4µmol/ml was infused in the end of the endothelial function test.

5.2 Reactive hyperemia

Reactive hyperemic blood flow (used in study II) provides a measure of structural and functional properties of arterial and arteriolar vascular smooth muscle and of vasodilatory capacity (350). Prior to the endothelial function test, FBF was occluded by inflating a cuff around the upper arm 100 mmHg above systolic blood pressure (SBP) for 5 min. After cuff release, blood flow reaches its maximum rapidly and then falls in a logarithmic fashion (351). Blood flow was recorded sequentially after cuff release and plotted against time on a logarithmic scale. Linear regression analysis was used to determine blood flow 5 s after cuff release in each subject. The individual regression lines were linear (r=0.97-1.00). Unlike vasodilation induced by shear stress (flow-mediated vasodilation) (52), which is observed 0.5-1.5 min postocclusion and can be abolished by L-NMMA, postocclusion reactive hyperemic flow is not influenced by L-NMMA and provides a measure of vasodilatory capacity (352).

5.3 Measurement of antioxidant status

5.3.1 Total peroxyl radical-trapping capacity (TRAP)

The combined capacity of all antioxidants to neutralize free radicals in serum, TRAP, was determined spectrophotometrically using a validated technique (353). This method utilizes a free
radical probe, dichlorofluorescein-diacetate, which oxidation by radicals from the azo-compound 2,2'-diazobis (2-amidinopropane) dihydrochloride generates highly fluorescent dichlorofluorescein-diacetate. The latter compound also has absorbance at 504 nm thus enabling spectrophotometric quantitation. To estimate to what extent observed changes in circulating antioxidants (urate, ascorbate, SH groups, α-tocopherol) explained changes in observed TRAP (TRAP_{obs}), the percent contribution of each antioxidant to TRAP was calculated by multiplying the concentration of each antioxidant by its stoichiometric value (molar amount of free radicals trapped by mole of each antioxidant) (TRAP_{calc}) (353).

5.3.2 Circulating antioxidants
Plasma α-tocopherol, β-carotene and retinol were measured by reverse phase high performance liquid chromatography (HPLC), as described by Schäfer-Elinder and Walldius (354). We used a Hewlett-Packard reverse phase HPLC column (ODS Hypersil 5 mm, 200 mm x 2.1 mm) connected to a Waters HPLC system. The latter consisted of a M600 Controller, M486 tunable UV-absorbance detector, M717+ autosampler and Millennium 2010 single system chromatography manager (Waters, Milford, MA). The UV detector was set at 326 nm for retinol, 292 nm for α-tocopherol and 450 nm for β-carotene. Lipid-standardized α-tocopherol was calculated by dividing the α-tocopherol concentration by the sum of serum total cholesterol and triglyceride concentrations (355). Ascorbic acid was measured using the spectrophotometric method of Denson and Bowers (356). Serum sulfhydryl groups were determined as described by Ellman (357). Plasma uric acid concentrations were measured by an enzymatic colorimetric assay (Roche Unimate 5 UA, Roche).

5.4 Measurement of inflammation
Serum TNFα (studies III and IV) and IL-6 (study III) concentrations were measured using ELISA kits from R&D Systems (Minneapolis, Minnesota). CRP (studies III and IV) was immunochemically measured and ESR (studies III and IV) by Westergren’s method.

5.5 Assessment of disease activity in rheumatoid arthritis
A rheumatologist evaluated disease activity in the patients with RA. Joint inflammation was assessed by counting the number of joints that were swollen (44 joint count) and tender (51 joint count). Visual analog scale (VAS 0-10 cm) was used to assess pain.
5.6 Measurements of serum lipid and lipoprotein concentrations

Studies I and III:

Isolation of lipoprotein subfractions. Serum lipoproteins were isolated using sequential ultracentrifugation (Beckman L8-70, Beckman, Palo Alto, CA) according to the following densities: VLDL d<1.006 g/ml, IDL d=1.006-1.019 g/ml, LDL d=1.019-1.063 g/ml, HDL d=1.063-1.210 g/ml.

Cholesterol, triglyceride, phospholipid, free cholesterol and protein concentrations in lipoprotein subfractions. The concentrations of cholesterol and triglycerides in serum and lipoprotein subfractions were determined by enzymatic colorimetric assays (Hoffman-La Roche, Basel, Switzerland) in an autoanalyzer (Cobas Mira, F Hoffman la Roche). Commercial kits were also used to measure the concentration of phospholipids (WakoChemicals, Neuss, Germany) and free cholesterol (Boehringer Mannheim, Germany) in lipoprotein subfractions. Protein concentrations in the lipoprotein fractions were measured by the method of Kashyap et al. (359).

Serum apolipoprotein concentrations. Serum apolipoprotein A I was determined by an immunoturbidometric kit from Boehringer Mannheim. Serum apolipoprotein B concentrations were measured by an immunoturbidometric assay (Apolipoprotein B, Orion Diagnostica, Espoo, Finland).

Studies II and IV:

Serum total, high-density (HDL) cholesterol and triglyceride concentrations were measured with respective enzymatic kits from Roche Diagnostics using an autoanalyzer (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan). LDL cholestrol concentration was calculated using the formula of Friedewald (360).

5.7 LDL oxidation in vitro

Isolation of LDL. LDL was isolated by short-run density ultracentrifugation as follows. Plasma (up to 5 ml) was adjusted with solid NaBr to a density of 1.5 g/ml and layered on the bottom of a centrifuge-tube. This layer was then successively overlaid with 2.5 ml each of 1.12 g/ml and 1.063 g/ml NaCl solutions, and 2.0 ml distilled water. All solutions contained 1 mg /ml of EDTA. The tubes were centrifuged in a Beckman SW 40 Ti rotor (Beckman Instruments, Fullerton, CA) in a Beckman L8-70 centrifuge (Beckman inc., Palo Alto, CA) at 40 000 rpm at 4°C for 2.5
METHODS

Robert Bergholm

hours. After centrifugation the main lipoproteins were well separated and EDTA was removed using small dextran-sulfate affinity columns (Liposorber LA-15, Kaneka, Osaka, Japan).

In vitro LDL oxidation was performed using a modification of the procedure described by Esterbauer et al (361). LDL oxidation was initiated by adding freshly prepared CuSO$_4$ (final concentration of 10.64 µmol/l) to the LDL subfraction. The kinetics of LDL oxidation was followed by monitoring the change in absorbance at 234 nm in a motorized Schimadzu spectrophotometer (Schimadzu UV 1201, Schimadzu, Kyoto, Japan) equipped with a 6-cuvette cell connected to a microcomputer. Absorbance was recorded every 1.5 min. The change in absorbance at 234 nm over time can be divided into three consecutive phases: lag phase, propagation phase and decomposition phase (361). The lag phase (in minutes) was used as a measure of LDL oxidation.

5.8 Quantitation of LDL particle size

Non-denaturing polyacrylamide gel electrophoresis was performed on serum samples, stored at -80°C, using Pharmacia 2/16 gels (Pharmacia, Uppsala, Sweden) by the method of Nicholas et al (362) as previously described (363). Gels were stained with Sudan Black B lipid stain and scanned at 595 nm with a computer-assisted scanning densitometer (Cliniscan 2, Helena Laboratories, Beaumont, Texas). Mean particle diameter of the major LDL peak was determined by comparing the mobility of the sample with the mobility of a calibrated reference LDL preparation run on each gel. The particle diameters of the reference LDL preparations were evaluated by electron microscopy. Coefficients of variation for intergel and intragel precisions of the use control sample were 1.0 and 1.8%, respectively. Diameters >25.5 nm = LDL A, <25.5 nm = LDL B and the mean particle size of the major LDL peak = LDL peak.

5.9 Maximal aerobic capacity (VO$_2$max)

VO$_2$max was determined using a work-conducted upright exercise test with an electrically braked cycle ergometer (Bosch ERG 220, Robert Bosch, Berlin, Germany) combined with continuous analysis of expiratory gases and minute ventilation (EOS-Spint, Erich Jaeger, Würtzburg, Germany). Exercise was started at a workload of 50 W, and was then increased by 50 W at 3-min intervals until perceived exhaustion or until a respiratory quotient (RQ) of 1.10 was reached. The highest VO$_2$ observed during a 30 sec period was defined as VO$_2$max.
5.10 Body and forearm composition

5.10.1 Fat free mass and the % body fat (studies I-IV) were determined using bioelectrical impedance plethysmography (Bio-Electrical Impedance Analyzer System, model #BIA-101A, RJL Systems, Detroit, MI) (364).

5.10.2 Waist to hip ratio
Waist circumference was measured midway between spina iliaca superior and the lower rib margin, and hip circumference at the level of the greater trochanters (365).

5.10.3 Quantitation of forearm composition
Resting forearm blood flow is closely correlated with relative muscularity of the forearm, which varies between 20 and 80 % in normal men and women (276). Since weight loss changes body and forearm composition, we measured forearm composition by magnetic resonance imaging (MRI) using a 1.5 T whole body system (Siemens Magnetom Vision, Erlangen, Germany) (Fig. 5). The extremity transmitting and receiving coil (knee) was used for the determination of subcutaneous fat of the arm, and the elbow was placed in the center. A series of T1-weighted trans-axial scans (15 slices) were acquired with a 150 mm field of view, a matrix of 224x512, number of acquisitions 4, a 5 mm slice thickness, a repetition time of 570 ms and an echo time of 14 ms. Subcutaneous fat and forearm muscle areas were quantified using an image analysis program (Alice 3.0, Parexel, Waltham, MA). The muscle volume of the slice, which corresponded to the site of blood flow measurement was used as the denominator when normalizing blood flow to muscle mass.

5.11 Other measurements
Plasma glucose concentrations (studies I, III, IV) were measured in duplicate with the glucose oxidase method using Beckman Glucose analyzer II (Beckman Instruments, Fullerton, CA) (366). Serum free insulin concentrations (study I) were measured by radioimmunoassay (PhadesePh® Insulin RIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) after precipitation with polyethylene glycol (367). Serum FFA (study I) were measured using a fluorometric method.
(368). HbA1C (study I) was measured by high-pressure liquid chromatography using the fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA).

5.12 Statistical analyses

In all studies, *p*-value of less than 0.05 was considered statistically significant. The calculations were performed using the GraphPad Prism version 2.0 or 3.0 statistical program (GraphPad, San Diego, CA) or with Systat version 8 or 10 (SPSS, Evanston, IL). All data are shown as mean±standard error of mean.

Study I

The unpaired t-test was used to compare single measurements between subjects in the orlistat and placebo groups. Single measurements before and after therapy were compared using the Student’s paired t-test. Comparison of blood flow responses to the two doses of vasoactive drugs was performed using analysis of variance (ANOVA) for repeated measures. Correlation analyses were calculated using Spearman’s non-parametric rank correlation coefficient.

Study II

Changes in single variables measured before and after endurance training were examined using Student’s paired t-tests. Analysis of a training effect on the different doses of ACh and SNP were performed using two-way analysis of variance. Simple and multiple linear regression analysis were used to determine the relationship between training induced changes in forearm blood flow and changes of lipid and antioxidant parameters.

Study III & IV

Student’s unpaired t-test was used to compare single measurements between the patients with RA and the normal subjects. Single measurements before and after therapy were compared using

Student’s paired t-test. Comparison of blood flow responses to the two doses of vasoactive drugs was performed using analysis of variance (ANOVA) for repeated measures (369). Correlation analyses were calculated using Spearman’s non-parametric rank and Pearson’s correlation coefficient for non-normally and normally distributed data.
6. RESULTS

6.1 Effects of weight loss on endothelial function

Before weight loss the study groups were comparable with respect to all measured variables (Table 4).

Effects of weight loss on body composition and metabolic parameters (Table 4)

Weight loss averaged 7.3±0.2 kg (8.3±0.1%) and 7.4±0.2 kg (8.2±0.1%) of initial body weight in orlistat and placebo groups (Table 4). The mean time to achieve weight loss averaged 20±1 weeks in the orlistat and 18±1 weeks in the placebo group (NS). The percent whole body fat decreased similarly in both groups (Table 4). Waist circumference decreased by 3±1 cm (p<0.01) and hip circumference by 6±1 cm (p<0.001) in both the orlistat and placebo groups. Forearm muscle and subcutaneous fat volumes decreased similarly in both study groups (Fig. 6).

Fasting serum insulin concentrations decreased significantly and by ~30% in both groups. Systolic and diastolic blood pressures remained unchanged. Serum LDL cholesterol decreased significantly in the orlistat group by 11% or -0.48±0.15 mmol/l (p<0.01) but not in the placebo (-0.17±0.09 mmol/l, NS) group. Serum triglycerides decreased slightly in the placebo but not in the orlistat group. Concentrations of fasting plasma glucose and HbA1c remained unchanged (Table 4). In summary, weight loss changed body composition and metabolic parameters similarly in both groups except LDL, which decreased significantly more in the orlistat group.
Table 4. Effects of weight loss on clinical and biochemical characteristics of the study groups in study I.

<table>
<thead>
<tr>
<th></th>
<th>Orlistat before weight loss</th>
<th>Change</th>
<th>Placebo before weight loss</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>23</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>39±1</td>
<td></td>
<td>39±1</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88.8±1.5</td>
<td>-7.3±0.2***</td>
<td>90.6±1.4</td>
<td>-7.4±0.2***</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>32.3±0.4</td>
<td>-2.7±0.1***</td>
<td>32.3±0.4</td>
<td>-2.7±0.1***</td>
</tr>
<tr>
<td>WHR</td>
<td>0.94±0.02</td>
<td>0.02±0.01*</td>
<td>0.95±0.01</td>
<td>0.03±0.01**</td>
</tr>
<tr>
<td>% fat</td>
<td>35.6±0.4</td>
<td>-1.7±0.3***</td>
<td>36.8±0.5</td>
<td>-2.4±0.4***</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>5.7±0.1</td>
<td>-0.03±0.1</td>
<td>5.6±0.1</td>
<td>0.04±01</td>
</tr>
<tr>
<td>HbA₁C (%)</td>
<td>5.5±0.1</td>
<td>-0.08±0.04</td>
<td>5.5±0.1</td>
<td>0.01±0.05</td>
</tr>
<tr>
<td>Fasting serum insulin (mU/l)</td>
<td>9.8±1.1</td>
<td>-3.2±0.8***</td>
<td>9.7±1.1</td>
<td>-3.2±0.8***</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126±3</td>
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<td>123±2</td>
<td>-2±1.2</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>85±2</td>
<td>-2±1</td>
<td>80±2</td>
<td>-2±1</td>
</tr>
<tr>
<td>Serum HDL cholesterol (mmol/l)</td>
<td>1.31±0.05</td>
<td>-0.02±0.03</td>
<td>1.28±0.06</td>
<td>0.07±0.03</td>
</tr>
<tr>
<td>Serum LDL cholesterol (mmol/l)</td>
<td>3.5±0.16</td>
<td>-0.48±0.15**</td>
<td>3.14±0.11</td>
<td>-0.17±0.09</td>
</tr>
<tr>
<td>Serum triglycerides (mmol/l)</td>
<td>1.45±0.15</td>
<td>-0.07±0.17</td>
<td>1.37±0.13</td>
<td>-0.27±0.09**</td>
</tr>
<tr>
<td>Serum free fatty acids (µmol/l)</td>
<td>72±35</td>
<td>-56±41</td>
<td>704±34</td>
<td>-40±46</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01, ***p<0.001 for change by weight loss. WHR = waist to hip ratio; HbA₁C = glycosylated hemoglobin A₁C; SBP = systolic blood pressure; DBP = diastolic blood pressure.
RESULTS

Before weight loss both groups had similar blood flow responses to SNP and ACh (Fig. 7). After weight loss, responses to low and high doses of ACh had increased significantly in the orlistat group by 41% (5.9±0.6 vs. 8.3±0.3 for before vs. after weight loss for flow in experimental/control arm, p<0.01) and 33% (7.6±0.8 vs. 10.1±0.6, p<0.001). The blood flow responses to ACh remained unchanged in the placebo group (Fig. 7). The ACh response increased significantly more in the orlistat than the placebo group (p=0.005). When blood flow was expressed per muscle mass measured with MRI, basal blood flow was similar in the orlistat (3.5±0.2 ml/dl muscle-min) and placebo (3.4±0.2 ml/dl muscle-min) groups, and endothelium-dependent vasodilatation increased significantly by orlistat but not placebo (Fig. 8).

In the orlistat group, endothelium-independent blood flow responses to the low dose of SNP (3 μg/min) increased by 35% from 5.2±0.4 vs. 7.0±0.5 for before vs. after weight loss for flow in experimental/control arm (p<0.01), and to the high dose (10 μg/min) by 25% (7.3±0.5 vs. 9.1±0.6, p<0.02). In the placebo group, there was a marginally significant (ANOVA p=0.05) increase in the blood flow response to SNP (Fig. 7). The changes in blood flow at the two doses of SNP between the groups were not significantly different (p=0.31). When blood flow during SNP infusions was expressed per muscle mass in the forearm, it did not change significantly either in the orlistat or in the placebo group (Fig. 8).
Figure 7. Forearm blood flow responses to sodium nitroprusside (SNP) and acetylcholine (ACh) before (open circle) and after (black circle) weight loss expressed as experimental/control arm. *p<0.05, **p<0.01, ***p<0.001.

Figure 8. Forearm blood flow responses to sodium nitroprusside (SNP) and acetylcholine (ACh) before (open circle) and after (black circle) weight loss expressed per muscle mass. *p<0.05, **p<0.01, ***p<0.001.
Interrelationships between measures of body composition, metabolic characteristics and endothelial function

Table 5 depicts interrelationships between various measures of body composition, metabolic parameters and blood flow responses to ACh and SNP. The only parameter, which change correlated with the change in blood flow responses to ACh, was serum LDL cholesterol. The change in LDL cholesterol within the orlistat group also correlated closely with blood flow responses to both the low dose of SNP (r=-0.53, p=0.009), the low dose of ACh (r=-0.50, p=0.016, Fig. 9), the high dose of SNP (r=-0.37, p=0.08) and the high dose of ACh (r=-0.48, p=0.023) after weight loss. When the changes in blood flow responses were compared between the groups, the change in the response to ACh was significantly greater in the orlistat than the placebo group even when baseline LDL cholesterol (which was slightly although not significantly higher in the orlistat than the placebo group) was included as a covariate (p=0.012). The relationships were essentially similar when blood flow was expressed per muscle mass (data not shown), although there was also a marginally significant correlation amongst all women before weight loss, between serum LDL cholesterol and mean blood flow expressed as ml/dl muscle min during infusions of SNP (mean blood flow during low and high doses) (r=0.41, p=0.08), and between LDL cholesterol and mean blood flow during ACh infusions (r=0.48, p<0.002).

Figure 9. Interrelationships between change in low-density lipoprotein (LDL)-cholesterol and blood flow responses to sodium nitroprusside (SNP) and acetylcholine (ACh) after weight loss.
Table 5. Relationships (Spearman correlations) between measures of body weight and composition, biochemical parameters and blood flow responses to ACh and SNP. *p<0.05

<table>
<thead>
<tr>
<th></th>
<th>All subjects (n=47)</th>
<th>Orlistat (n=23)</th>
<th>Placebo (n=24)</th>
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<tr>
<td></td>
<td>ACh+</td>
<td>SNP+</td>
<td>ACh+</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before weight loss</td>
<td>-0.02</td>
<td>-0.18</td>
<td></td>
</tr>
<tr>
<td>after weight loss</td>
<td></td>
<td>-0.20</td>
<td>-0.07</td>
</tr>
<tr>
<td>change</td>
<td></td>
<td>0.05</td>
<td>0.11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before weight loss</td>
<td>-0.08</td>
<td>-0.07</td>
<td></td>
</tr>
<tr>
<td>after weight loss</td>
<td></td>
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<td>-0.10</td>
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<tr>
<td>change</td>
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<td>0.08</td>
<td>0.28</td>
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<tr>
<td>WHR</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>before weight loss</td>
<td>-0.12</td>
<td>0.02</td>
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<tr>
<td>after weight loss</td>
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<td>change</td>
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<td>-0.35</td>
<td>-0.43*</td>
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<tr>
<td>Whole body fat %</td>
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<tr>
<td>before weight loss</td>
<td>-0.13</td>
<td>-0.18</td>
<td></td>
</tr>
<tr>
<td>after weight loss</td>
<td></td>
<td>-0.51*</td>
<td>-0.21</td>
</tr>
<tr>
<td>change</td>
<td></td>
<td>-0.26</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before weight loss</td>
<td>0.21</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>after weight loss</td>
<td></td>
<td>-0.02</td>
<td>-0.14</td>
</tr>
<tr>
<td>change</td>
<td></td>
<td>-0.44*</td>
<td>-0.25</td>
</tr>
<tr>
<td>Serum FFA (µmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before weight loss</td>
<td>-0.32*</td>
<td>-0.32*</td>
<td></td>
</tr>
<tr>
<td>after weight loss</td>
<td></td>
<td>-0.36</td>
<td>-0.22</td>
</tr>
<tr>
<td>change</td>
<td></td>
<td>-0.06</td>
<td>-0.25</td>
</tr>
</tbody>
</table>

* Mean blood flow responses in the experimental vs. control arm low and high doses of ACh and SNP. ** The correlation coefficients in the table denote relationships before vs. before, after vs. after and change vs. change by weight loss. BMI=body mass index; WHR=waist to hip ratio; FFA=free fatty acids.
6.2 Effects of physical training on endothelial function

Three months of physical training (running) impaired endothelial-dependent vasodilatation in forearm vasculature in healthy young men (Fig. 10). Changes in body composition, hemodynamic parameters, and VO\textsubscript{max} are listed in Table 6. Physical training was accompanied by decreases in all circulating antioxidants except ascorbate, which increased significantly (Fig. 11 and Fig. 12). Observed TRAP (TRAP\textsubscript{obs}) and calculated TRAP (TRAP\textsubscript{calc}) did not change (Fig. 11). The decrease in uric acid, quantitatively the most important determinant of TRAP, correlated with the decrease in endothelial function. Small, albeit significant, changes were observed in LDL composition (Table 7), but neither LDL cholesterol nor LDL size changed significantly during the training period (Table 7). No other lipid changes were seen (Table 8).

Table 6. Body composition and hemodynamic parameters before and after training.

<table>
<thead>
<tr>
<th></th>
<th>Before training</th>
<th>After training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>68.2±2.0</td>
<td>67.5±1.5</td>
</tr>
<tr>
<td>Body mass index (kg/m\textsuperscript{2})</td>
<td>21.8±0.4</td>
<td>21.5±0.3</td>
</tr>
<tr>
<td>Body fat%</td>
<td>14.8±2.0</td>
<td>11.0±1.5**</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>57.8±1.3</td>
<td>60.0±0.8**</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>10.3±1.7</td>
<td>7.6±1.2**</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>113±3</td>
<td>108±2</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73±2</td>
<td>70±2</td>
</tr>
<tr>
<td>VO\textsubscript{max} (ml/kg·min)</td>
<td>53±2</td>
<td>58±2*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 for before vs. after training. Abbreviations: SBP = systolic blood pressure, DBP = diastolic blood pressure, VO\textsubscript{max} = maximal aerobic power. Data are shown as mean±SEM.
RESULTS

Figure 10. Forearm blood flow during intrabrachial infusions of sodium nitroprusside (SNP, 3 µg/min 12-18 min, 10 µg/min 18-24 min), acetylcholine 7.5 µg/min 42-48 min, 15 µg/min 48-54 min) and L-NMMA in the experimental arm (solid line) before (○) and after (♦) a 3 month training period. The hatched line denotes blood flow in the contralateral control arm. *P<0.05 for ACh effect before vs. after training by analysis of variance. The insert shows the % decrease in basal blood flow by L-NMMA before (B) and after (A) training. *P<0.05 before vs. after.

Figure 11. Concentrations of circulating antioxidants before and after training.
Table 7. Composition, size and oxidability of plasma LDL before and after training.

<table>
<thead>
<tr>
<th>Composition of LDL particles</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>2.40 ± 0.12</td>
<td>2.37 ± 0.14</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.18 ± 0.01</td>
<td>0.13 ± 0.02*</td>
</tr>
<tr>
<td>Free cholesterol (mmol/l)</td>
<td>0.78 ± 0.04</td>
<td>0.70 ± 0.04*</td>
</tr>
<tr>
<td>Phospholipids (mmol/l)</td>
<td>67 ± 4</td>
<td>55 ± 3b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LDL size</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL A (%&gt; 25.5 nm)</td>
<td>92.4 ± 4.2</td>
<td>95.7 ± 0.9</td>
</tr>
<tr>
<td>LDL B (%&lt; 25.5 nm)</td>
<td>7.6 ± 4.2</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>LDL peak (nm)</td>
<td>26.9 ± 0.2</td>
<td>27.1 ± 0.1</td>
</tr>
<tr>
<td>LDL lag time (min)</td>
<td>161 ± 11</td>
<td>159 ± 24</td>
</tr>
</tbody>
</table>

* P<0.05.
  b P<0.01 for differences 0 vs. 3 months

Table 8. Concentrations of plasma lipids and lipoproteins before and after training.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.73 ± 0.10</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>Total VLDL</td>
<td>0.34 ± 0.08</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.03 ± 0.20</td>
<td>4.03 ± 0.20</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.80 ± 0.05</td>
<td>0.86 ± 0.09</td>
</tr>
<tr>
<td>HDL2</td>
<td>0.56 ± 0.02</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>HDL2/HDL3</td>
<td>1.42 ± 0.09</td>
<td>1.66 ± 0.18</td>
</tr>
<tr>
<td>Apoprotein AI (mg/dl)</td>
<td>122 ± 4</td>
<td>126 ± 5</td>
</tr>
</tbody>
</table>
6.3 Endothelial function in patients with rheumatoid arthritis

Physical and biochemical characteristics (Table 9). The groups were similar with respect to age, weight, gender and body composition. The patients with RA had higher serum IL-6, CRP concentrations and an elevated ESR compared to the normal subjects. The patients with RA had slightly lower LDL cholesterol concentrations than the normal subjects.

Endothelial function. During infusion of the endothelium-independent vasodilator SNP, blood flow increased less (p<0.01, ANOVA) during the low (4.1±0.3 vs. 5.4±0.4) and high (5.4±0.5 vs. 6.9±0.5, flow in the experimental vs. control arm) doses in the patients with RA than in the normal subjects (Fig. 13). During infusion of the endothelium-dependent vasodilator ACh, the blood response was also significantly (p<0.05, ANOVA) blunted in the patients with RA compared to the normal subjects (Fig. 13. Serum TNFα (r=-0.67, p<0.002) and CRP (r=-0.48, p<0.05) but not IL-6 or ESR were inversely correlated to the vasodilatory response to the low dose of SNP. Serum TNFα (r=-0.64, p<0.005) but not CRP, IL-6 or ESR was inversely related to the blood flow response to the high dose of ACh. Examples of these relationships are depicted in Fig. 14.

Basal flow and effects of L-NMMA on basal flow. Basal blood flow was 40 % higher in the patients with RA (2.5±0.3 ml/dl·min) than the normal subjects (1.8±0.1 ml/dl·min, p<0.05). Both CRP (r=0.48, p<0.05), TNFα (r=0.61, p<0.01), and the ESR (r=0.68, p<0.002) but not IL-6 were significantly correlated with basal blood flow within the patients with RA (Fig. 14). Systolic blood pressures were comparable between patients with RA (137±5 mmHg) and normal subjects (135±4 mmHg), while diastolic blood pressure was slightly lower in the RA patients (76±2 mmHg) than in the normal subjects (81±1 mmHg, p=0.064). Consequently, peripheral vascular resistance (mean arterial pressure divided by blood flow) was lower in the patients with RA [47±5 mmHg/(ml/dl·min)] than in the normal subjects [66±6 mmHg/(ml/dl·min), p<0.05].

During inhibition of NO synthesis (by both iNOS and eNOS) by infusion of L-NMMA, blood flow decreased significantly more (-34±2 %) in the patients with RA than in the normal subjects (-24±3 %, p<0.02).
Table 9. Clinical and biochemical characteristics of the study groups.

<table>
<thead>
<tr>
<th></th>
<th>RA</th>
<th>Normal subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (females/males)</td>
<td>17/3</td>
<td>27/6</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>53±2</td>
<td>54±1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69±4</td>
<td>74±2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26±1</td>
<td>26±1</td>
</tr>
<tr>
<td>WHR</td>
<td>0.84±0.02</td>
<td>0.86±0.02</td>
</tr>
<tr>
<td>% fat</td>
<td>32±1</td>
<td>34±1</td>
</tr>
<tr>
<td>S-IL-6 (pg/ml)</td>
<td>12±1***</td>
<td>3±1</td>
</tr>
<tr>
<td>S-TNFα (pg/ml)</td>
<td>2.1±0.3***</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>41±6***</td>
<td>8±1</td>
</tr>
<tr>
<td>S-CRP (mg/l)</td>
<td>30±6**</td>
<td>4±1</td>
</tr>
<tr>
<td>S-LDL cholesterol (mmol/l)</td>
<td>3.16±0.21*</td>
<td>3.85±0.15</td>
</tr>
<tr>
<td>S-HDL cholesterol (mmol/l)</td>
<td>1.53±0.08</td>
<td>1.44±0.08</td>
</tr>
<tr>
<td>S-Triglycerides (mmol/l)</td>
<td>1.33±0.13</td>
<td>1.24±0.10</td>
</tr>
<tr>
<td>Number of tender joints</td>
<td>13±2</td>
<td>-</td>
</tr>
<tr>
<td>Number of swollen joints</td>
<td>11±2</td>
<td>-</td>
</tr>
</tbody>
</table>

***p<0.001, **p<0.01, *p<0.05 for RA patients vs. normal subjects. Abbreviations: CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, IL-6 = interleukin - 6, TNFα = tumor necrosis factor α. RA = rheumatoid arthritis, WHR = waist to hip ratio. Data are shown as mean ±SEM.
RESULTS

Figure 13. Blood flow responses (flow in experimental / control arm) to intra-brachial artery infusions of low (3 \( \mu \text{g/min} \)) and high (10 \( \mu \text{g/min} \)) doses of SNP (sodium nitroprusside) and low (7.5 \( \mu \text{g/min} \)) and high (15 \( \mu \text{g/min} \)) doses of acetylcholine (ACh) in patients with RA (closed bars) and the normal subjects (open bars). *\( p<0.05 \), **\( p<0.01 \) for difference between groups by ANOVA.

Figure 14. Interrelationships between markers of inflammatory activity and basal flow (panels on the left) and responses to ACh or SNP (panels on the right).
6.4 Effect of anti-inflammatory therapy on endothelial function in patients with newly-diagnosed rheumatoid arthritis

Before treatment patients with RA and the normal subjects were similar with respect to age, gender and body weight (Table 10). The % whole body fat was also similar (32±2, 33±2 and 34±1% before and after therapy in patients with RA and in normal subjects, NS). At baseline the patients with newly-diagnosed RA had a higher serum CRP and TNFα concentrations and ESR than the normal subjects (Table 10). Serum lipid and lipoprotein concentrations are shown in Table 11. The patients with RA had significantly lower LDL cholesterol concentrations than the normal subjects.

Before treatment basal blood flows were comparable between the patients with RA and the normal subjects (2.1±0.2 for RA vs. 1.7±0.2 ml/dl·min, NS, in normal subjects). The blood flow responses (fold increase in flow in experimental control arm) to SNP were 30% (low dose 4.1±0.4 vs. 5.9±0.5) and 34% (high dose SNP 5.1±0.6 vs. 7.7±0.7) lower in the RA patients than the normal subjects (p<0.001, ANOVA). The responses to ACh were 50% (low dose ACh 3.0±0.5 vs. 6.6±0.7) and 37% (high dose ACh 5.0±0.4 vs. 7.9±0.8) lower in the patients than the normal subjects (p<0.001, ANOVA, Fig. 15). Within the group of patients with RA, the flow response to ACh was inversely correlated with the ESR (r=-0.56, p<0.05).

Effect of therapy

Body composition did not change during therapy (Table 10). After 6 months of therapy, clinical and laboratory markers of inflammation had significantly decreased (Table 10). After therapy, the concentration of serum LDL cholesterol had increased slightly and was no longer significantly lower in the patients with RA than the normal subjects (Table 11). The concentrations of other lipids, lipo- and apoproteins were comparable between the groups.

Basal blood flow did not change during therapy (2.1±0.1 vs 2.0±0.1 ml/dl·min, NS, RA before vs. after therapy). After therapy, endothelial function improved significantly as judged from blood flow responses to both low and high doses of ACh (p=0.02 for repeated measures ANOVA) (Fig. 15). The blood flow responses to SNP increased slightly but not significantly (Fig. 15). After therapy, the responses to ACh (p=0.33) or SNP (p=0.062) were no longer significantly lower than those in the normal subjects (Fig. 15).
Table 10. Clinical and biochemical characteristics of the study groups.

<table>
<thead>
<tr>
<th></th>
<th>Newly-diagnosed Before treatment (n=10)</th>
<th>Newly-diagnosed After treatment (n=10)</th>
<th>Normal subjects (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (females/males)</td>
<td>8/2</td>
<td></td>
<td>27/6</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>52±3</td>
<td>74±4</td>
<td>54±1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74±4</td>
<td>74±4</td>
<td>74±2</td>
</tr>
<tr>
<td>Body mass index (kg/m^2)</td>
<td>27±1</td>
<td>27±1</td>
<td>26±1</td>
</tr>
<tr>
<td>WHR</td>
<td>0.87±0.02</td>
<td>0.87±0.02</td>
<td>0.86±0.02</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>133±7</td>
<td>132±4</td>
<td>135±4</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76±3*</td>
<td>77±2</td>
<td>81±2</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>128±5**</td>
<td>128±3††</td>
<td>140±1</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>40±7***</td>
<td>19±2 ††††</td>
<td>8±1</td>
</tr>
<tr>
<td>S-CRP (g/l)</td>
<td>29±10***</td>
<td>8±3 ††††</td>
<td>4±1</td>
</tr>
<tr>
<td>S-TNFα (ng/l)</td>
<td>3.1±0.5***</td>
<td>2.3±0.5††</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>Number of tender joints</td>
<td>11±2</td>
<td>4±1</td>
<td></td>
</tr>
<tr>
<td>Number of swollen joints</td>
<td>8±3</td>
<td>4±2</td>
<td></td>
</tr>
<tr>
<td>Pain (pain scale 0-10 cm)</td>
<td>4.4±0.8</td>
<td>2.3±1.0</td>
<td>11/33</td>
</tr>
<tr>
<td>Smokers</td>
<td>3/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01, ***p<0.001 for RA patients before treatment vs. normal subjects, †p<0.05, ††p<0.01, †††p<0.001 for RA patients after treatment vs. normal subjects, p<0.05 for RA before vs. after treatment. Abbreviations: CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, TNF-α = tumor necrosis factor α, SBP = systolic blood pressure, DBP = diastolic blood pressure, WHR = waist to hip ratio. Data are shown as mean±SEM.
Table 11. Serum lipid, lipo- and apoprotein concentrations in patients with newly-diagnosed rheumatoid arthritis and in normal subjects.

<table>
<thead>
<tr>
<th></th>
<th>RA patients</th>
<th>RA patients</th>
<th>Normal subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.20±0.23</td>
<td>1.30±0.21</td>
<td>1.24±0.10</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.67±0.20</td>
<td>0.80±0.21</td>
<td>0.71±0.09</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.10±0.46</td>
<td>5.00±0.69</td>
<td>5.80±0.17</td>
</tr>
<tr>
<td>LDL</td>
<td>3.24±0.34*</td>
<td>3.39±0.31</td>
<td>3.85±0.15</td>
</tr>
<tr>
<td>HDL</td>
<td>1.44±0.07</td>
<td>1.49±0.19</td>
<td>1.44±0.06</td>
</tr>
<tr>
<td>HDL₂</td>
<td>0.69±0.07</td>
<td>0.66±0.13</td>
<td>0.72±0.05</td>
</tr>
<tr>
<td>HDL₃</td>
<td>0.72±0.06</td>
<td>0.75±0.11</td>
<td>0.73±0.02</td>
</tr>
<tr>
<td>Apoprotein AI (mg/dl)</td>
<td>136±7</td>
<td>148±6</td>
<td>147±4</td>
</tr>
<tr>
<td>Apoprotein AII (mg/dl)</td>
<td>34±3</td>
<td>36±2</td>
<td>35±1</td>
</tr>
<tr>
<td>Apoprotein B (mg/dl)</td>
<td>94±9</td>
<td>96±11</td>
<td>98±4</td>
</tr>
</tbody>
</table>

*p<0.05 for patients with RA before treatment vs. normal subjects. Data are shown as mean±SEM. VLDL = very low-density lipoprotein; LDL = low-density lipoprotein; HDL = high-density lipoprotein.
Figure 15. Forearm blood flow responses to intra-arterial SNP (upper panel) and ACh (lower panel) infusions in patients with RA before (closed circles) and after (closed triangles) anti-inflammatory therapy and in normal subjects (open circles) xxx p<0.001 for patients with RA before therapy vs. normal subjects (ANOVA for repeated measures). **p<0.02 for blood flow responses to ACh before vs. after therapy in the patients with RA (ANOVA for repeated measures). *p=0.05, **p<0.01 for comparison of patients with RA before therapy vs. normal subjects at individual doses of ACh and SNP.
7. DISCUSSION

7.1 Evaluation of methods

FBF responses to vasoactive agents ACh, SNP and L-NMMA were used to assess vascular function in all four studies. This method, where venous occlusion plethysmography is combined with intra-arterial infusions of vasoactive agents is considered as perhaps one of the best methods to measure endothelial function in peripheral circulation, in vivo. This is because this method, in contrast to FMD, has been shown to predict coronary events in both the forearm (58,57) and coronary (3,59,60) vascular beds. There is some correlation between forearm intra-arterial infusion plethysmography technique and FMD but only the former has been shown to predict cardiovascular events in patients with hypertension (57) and in patients with CAD (58). Impaired response of intracoronary infusion of ACh has been shown to be predictive for cardiovascular events in patients with CAD (3,59,60) and interestingly also in subjects with angiographically normal coronary arteries (3). Measurements with the same vasoactive agents have shown endothelial dysfunction in both the coronary and the forearm vascular bed in patients with CAD as well as in patients with hypercholesterolemia when compared to normal subjects (48,100,129,370). In line with this, lipid lowering therapy has improved endothelial function both in coronary (79,132,260) and forearm vessels (371,372). A recent study by Halcox et al. (59) showed that impaired response of intracoronary infusion of ACh in subjects with no apparent CVD was predictive for cardiovascular events. Responses to ACh in the forearm and coronaries correlate significantly with each other (62) and atherosclerotic lesions in the brachial artery in an autopsy study were shown to significantly correlate with lesions in both coronary and carotid arteries (373).

The two doses of ACh (7.5 and 15 µg/min) and SNP (3 and 10 µg/min) used in our studies were chosen based on the results from previous studies where the lower and the higher doses increase the blood flow similarly. Furthermore, comparable doses have been used in a multiple studies making the comparison of results easier (89,137,140,143,263,264,374,375). This contrasts several studies using the non-invasive approach to measure endothelial function. FMD, a surrogate of endothelium-dependent vasodilatation, generally increases brachial artery diameter by 3-4% while the most commonly used dose of GTN increases the diameter by 12%. Use of such a high dose of GTN may decrease the sensitivity of this test to detect alterations in endothelium-independent vasodilatation. The dose of L-NMMA (4 µmol/min) was chosen because this dose induces maximal inhibition of basal NO synthesis in normal subjects (157).
Although the method we used has several advantages compared to other methods, it has its limitations. This invasive test is too cumbersome do be used in clinical practice and the arterial cannulation needs experience and is not riskless. It should be noted that ACh releases not only NO but also other vasoactive substances such as prostacyclin and EDHF (376). Only 30-60% of ACh-induced vasodilatation can be blocked with L-NMMA (36,39). The coefficient of variation in our laboratory for this method has been acceptable 9-15% (II)(282).

7.2 Obesity, weight loss and endothelial function

Obesity is associated with endothelial dysfunction (94) and an increased risk of CVD (95,96,99,377). Obesity (94) and associated metabolic abnormalities such as dyslipidemia (100) hypertension (97), insulin resistance (96) and accumulation of fat in intra-abdominal rather than subcutaneous depots (95,98,99) may underlie altered vascular function. The exact cause of endothelial dysfunction in obesity is, however, unclear.

In study I, we chose to study a high-risk group of obese women with a history of gestational diabetes, which has been shown to be associated with endothelial dysfunction compared to normal subjects (178). We found that in this group of women moderate (8%) weight loss improved endothelium-dependent vasodilatation when a hypocaloric diet was combined with orlistat, but no change was seen when a similar weight loss was achieved with a hypocaloric diet combined with placebo. LDL cholesterol decreased significantly more in the orlistat than the placebo group, and the change in LDL by weight loss was significantly correlated with improved endothelial function in the orlistat group. This finding is in line with studies were lipid lowering with statins have improved endothelium-dependent vasodilatation (79,132,260,261). Orlistat, which is a lipase inhibitor, decreases fat-absorption by 30% (378) and facilitates weight loss (242). It lowers LDL cholesterol more than expected from weight loss alone (247). In the placebo group the observed lipid changes were in line with earlier weight loss studies (229).

The lack of significant changes in vascular function in the placebo group should not be interpreted to imply that it is not possible to enhance vascular function by weight loss. If one assumes that changes in vascular function are at least in part mediated via changes in markers of cardiovascular risk, these markers changed little by the 8% (7.4 kg) weight loss. The concentration of LDL cholesterol decreased by 0.17 mmol/l in the placebo group. This small change is in line with predictions from a meta-analysis (229), which concluded that every kg of weight loss decreases LDL cholesterol by 0.02 mmol/l i.e. 0.15 mmol/l for the observed 7.4 kg
loss of body weight in the placebo group. Serum triglycerides decreased by 0.27 mmol/l in the placebo group, which is consistent with previous studies in obese women who have lost moderate amounts of weight (5-10%) (248,379). The small change in serum triglycerides is unlikely to have any significant impact on LDL size, which may influence endothelial function independent of LDL cholesterol concentrations (137,380). HDL cholesterol remained unchanged, in keeping with the suggestion that modest short term weight loss does not increase HDL cholesterol (231,379). Fasting serum insulin concentrations did decrease by weight loss suggesting enhanced insulin sensitivity. However, LDL size rather than the serum insulin concentration may link insulin sensitivity and endothelial function (96,137,381). Unless changes in (hepatic) insulin sensitivity of triglyceride metabolism are sufficient to decrease synthesis of VLDL to the extent that LDL size decreases (363), endothelial function remains unchanged. Taken together the changes in lipid parameters or insulin sensitivity were not sufficient to influence endothelial function.

The decrease in LDL cholesterol but not other parameters (Table 5) correlated with the improvement in endothelium-dependent vasodilatation in the orlistat group. Several previous studies have documented that lowering of LDL cholesterol by statins improves the blood flow response to ACh but not to SNP (76,78,382), although this was not found in one study in patients with established coronary artery disease and relatively normal initial LDL cholesterol concentrations (3.3 mmol/l) (383). In the studies, where ACh was infused into the brachial artery, the improvements in the vasodilatory response to ACh (doses in the present study 7.5-15 µg/min) were 33% (ACh dose 12 µg/min, LDL cholesterol decrease -1.9 mmol/l, baseline LDL cholesterol 5.6 mmol/l) (78), 30% (30 µg/min, -1.7 mmol/l, 5.0 mmol/l) (382), 119% (10 µg/min, -1.3 mmol/l, 4.5 mmol/l) (76). In the present study, the increases were 41% (ACh 7.5 µg/min) and 36% (15 µg/min) and the LDL cholesterol decrease 0.47 mmol/l (baseline 3.5 mmol/l) i.e. except for the study of O’Driscoll et al. (76), the magnitude of improvement in endothelial function was similar to that observed in statin trials although LDL cholesterol decreased much less than with statins. These data raise the possibility that factors other than LDL cholesterol contributed to improved endothelial function in the orlistat group. FFA have been shown to induce endothelial dysfunction in vivo (143,384) and inhibit eNOS activity in vitro (144) but in the present study serum total FFA remained unchanged. Data are contradictory regarding the possibility that changes in fatty acid composition, which is altered during orlistat treatment (385) influences endothelial function. In the study of Vidgren et al., the proportion of linoleic acid (a fatty acid exclusively derived from the diet), decreased in serum cholesterol
esters, triglycerides, and phospholipids (385). In vitro, linoleic acid stimulates VCAM-1 protein and mRNA expression in cultured human endothelial cells via NF-kappaB signaling, and has therefore been considered to be proinflammatory and atherogenic via activation of endothelial cells (386). On the other hand in an in vivo study, linoleic acid in serum cholesterol esters was positively correlated with endothelial function (144).

Although there was a slight increase in endothelium-independent vasodilatory responses, the changes in responses to SNP by weight loss were not significant between the two groups, and when expressed per muscle mass, there was no change in endothelium-independent blood flow responses. Within the orlistat group, the change in the blood flow response to ACh by weight loss and ACh stimulated blood flow after weight loss correlated with the change in LDL cholesterol, but not with changes in body composition or other metabolic parameters. The latter data suggest that even modest decreases in LDL cholesterol may favorably influence endothelial function in obese women, while modest weight loss is ineffective in this respect.

There is only one recent study, published after submission of the paper I, where the effect of weight loss on endothelium-dependent and –independent vasodilatation has been evaluated. In this study by Sasaki et al. (387) a modest weight loss (6%) improved endothelium-dependent vasodilatation in obese patients with hypertension. Both blood pressure and LDL decreased significantly by weight loss and were associated with improved vascular function. There are some data available on effects of weight loss on other measures of vascular function. A 12-week hypocaloric diet in viscerally obese men resulted in 5% weight loss and improved ischemia induced vascular reactivity and reduced total cholesterol (388). No correlation between the change in lipids and vascular reactivity were, however, observed in this study. Marked weight loss of 22 kg by gastroplasty was reported to reduce the progression rate of atherosclerotic changes in the carotid artery bulb as measured by B-mode ultrasound during a 4-year follow-up (389). A significant reduction in carotid bulb and mean overall intima-media thickness by weight loss has also been reported in 14 premenopausal women, surprisingly already after 5 months of follow-up (390). Similar to the correlation observed in the present study in the orlistat group, the change in intima-media thickness was significantly correlated with LDL cholesterol, which decreased by 0.7 mmol/l. Ziccardi et al measured blood pressure responses to i.v. arginine before and after 9.8 kg weight loss, and found arginine to decrease blood pressure more after than before weight loss (214).
Although obesity with its accompanying metabolic alterations clearly is a cardiovascular risk factor, there are as yet few studies to show that weight loss protects against CVD (391). This contrast the growing number of intervention studies such as the Heart Protection Study, confirming that LDL cholesterol lowering is cardioprotective irrespective of baseline cholesterol concentrations (265). In the present study, weight loss combined with significant LDL cholesterol lowering by orlistat improved endothelial function, and the changes in LDL cholesterol and endothelial function were significantly interrelated. Whether inhibition of fat absorption with orlistat has effects beyond simple LDL cholesterol lowering, which improves endothelial function, warrants further investigation. The lack of significant changes in vascular function in the placebo group should not be interpreted to imply that it is not possible to enhance vascular function by weight loss. If one assumes that changes in vascular function are at least in part mediated via changes in markers of cardiovascular risk, these markers changed little by the 8% (7.4 kg) weight loss. Even so, the present data suggest that weight loss has beneficial effects on endothelial function, if accompanied by a decrease in LDL cholesterol.

### 7.3 Physical training and endothelial function

Based on observational follow-up studies there seems to be an inverse relation between increasing physical activity and cardiovascular disease incidence and mortality in a dose-response fashion (5). Multiple mechanisms exist to explain protective effects of physical activity on cardiovascular health. Physical training increases insulin sensitivity (392), lowers blood pressure (393), serum triglycerides, and increases serum HDL cholesterol concentrations (394). Serum LDL cholesterol concentrations remain unchanged or decrease slightly by physical training (394). All mentioned metabolic effects of physical training have been associated with improved endothelial function. On the other hand, aerobic exercise increases oxygen consumption and free radical formation (395). The latter consumes antioxidants in plasma and lipoproteins (396). This increases the likelihood of lipid peroxidation (397) and oxidation of LDL particles. This also seems to occur in vivo, as we and others recently found a significant increase in the susceptibility of plasma LDL to oxidation after high-intensity exercise (396,398). Interestingly, there are indications that long-term physical training may lower the susceptibility of LDL cholesterol to oxidation. In the study by Vasankari et al. (399), a 10-month exercise program lowered oxidized LDL in the circulation and it has also been shown that veteran endurance athletes have lower LDL oxidation than sedentary control subjects (400).
In study II, an unexpected finding was a decrease in endothelial function in forearm vasculature in healthy young men by 3 months of physical training (running). Physical training was also accompanied by decreases in all measured circulating antioxidants except ascorbate, which increased significantly. The decrease in uric acid, the quantitatively most important determinant of TRAP, correlated with the decrease in endothelial function. Small, albeit significant, changes were observed in LDL composition, but neither LDL cholesterol nor LDL size changed significantly during the training period.

Regarding the effects of physical-training-induced oxidative stress on the concentrations of the circulating antioxidants measured in the present study, uric acid concentrations have been found to be lower in runners than in untrained subjects, and negatively correlated with VO2 max (401). This was confirmed in the present study, in which uric acid decreased by training and its change was inversely correlated with that of VO2 max. These data raise the possibility that the changes in uric acid concentrations could reflect its antioxidant function in endothelial cells, where destruction of excessive free radicals is required for maintenance of adequate NO synthesis (402). Of course, this possibility remains to be directly tested using other experimental models, as causality cannot be proven in in vivo studies in humans.

In study II, we determined the lag time for the susceptibility of plasma LDL to oxidation by copper in vitro, but found no significant change during the training period. LDL susceptibility to oxidation has been shown to increase dramatically during a marathon run (398) and after 4 h of intense aerobic exercise (396). On the other hand, there are studies showing that the susceptibility of LDL to oxidation is lower in endurance athletes (399,400). One may also wonder why the lag time for LDL oxidation in vitro was not shortened, despite significant decreases especially in lipid soluble antioxidants such as \( \alpha \)-tocopherol. One possibility is that the 17% decrease in \( \alpha \)-tocopherol concentration in the present study was too small to significantly affect LDL oxidation, as increases in lag time have been noted in humans after increasing \( \alpha \)-tocopherol concentrations by 60% by vitamin E supplementation (800 IU/day) (403). In the latter study, the significant increase in the lag time for LDL oxidation was not accompanied by improved endothelium-dependent vasodilatation (403).

We observed only minor training induced alterations in serum lipids and lipoproteins in the present study. Although endurance exercise generally lowers plasma triglyceride concentrations, this effect appears largely restricted to individuals with elevated baseline concentrations (404). In
our young physically fit subjects, baseline concentrations were in the low normal range. In cross-
sectional studies, analysis of LDL cholesterol concentrations have produced mixed results, with
both no differences and differences (404) being reported. In endurance training studies, a small
decrease has been inversely related to the distance run (404). In the present study, VO₂max
increased by 10%. This modest increase combined with a low baseline LDL cholesterol
concentration and unchanged body weight, a significant determinant of changes in LDL
cholesterol (404) is likely to explain the unchanged mean LDL cholesterol concentration. Only a
few studies have examined the effect of endurance training on LDL size. In the study by
Houmard et al. (405), 14 weeks of endurance training increased VO₂max by 20% but did not
change either LDL cholesterol concentrations or LDL size. On the other hand, a predominance of
large LDL particles has been reported after 7 months and 1 year of aerobic training (406,407).
Differences in training duration and intensity may thus have contributed to the different results.

Despite modest alterations in lipids and lipoproteins, a clear decrease in endothelium-dependent
but not endothelium-independent vasodilation was observed in the forearm vascular bed, which is
not exposed to exercise induced hyperemia. This effect appeared specific for NO-dependent
vasodilation, as both the % inhibition of basal flow by L-NMMA and ACh stimulated blood
flows were significantly decreased. This result was unexpected, and contradicts the finding of
unaltered endothelial function by Green et al. after a 4-week handgrip-training program (110),
and enhanced endothelium-dependent vasodilatation after a 4-week cycle exercise-training
program by Kingwell et al. (113). The present study subjects were fitter, as judged from maximal
aerobic power, and the training period was three times longer than in the studies by Green et al.
(113) and Kingwell et al. (110).

It has been suggested that strenuous exercise may overwhelm the capacity to detoxify reactive
oxygen and the resulting oxidative stress (408). The decrease in the concentration of the lipid
soluble antioxidant, α-tocopherol, which is actively consumed during exercise and which cannot
be synthesized endogenously, could reflect increased oxidative stress in the present study (409).
The concentrations of antioxidants have not been measured in previous studies addressing
training induced changes in vascular function. In the present study, despite extensive analysis of
plasma lipoproteins, their size and composition, the only significant, and expected (410),
relationship was that between the change in LDL cholesterol and endothelium-dependent
vasodilation. The mean concentration of LDL cholesterol did not, however, change by training,
implying that other factors were responsible for training-induced endothelial dysfunction.
Physical training has been shown to improve endothelial function in most conditions where endothelial dysfunction is present (6,7,8,9,114,115,253,254,255). But there is currently only a few studies where healthy subjects have trained and where endothelium-dependent vasodilatation have been measured. In elderly athletes physical activity prevents age-related impairment in NO availability (116) and endothelium-dependent dilatation (256). Twelve weeks of brisk walking 5 to 7 times/week has improved forearm endothelium-dependent vascular relaxation in both normotensive and hypertensive subjects (255). In this study both LDL and blood pressure decreased significantly by physical training. In the study by Clarkson et al. (411) daily aerobic and anaerobic training for 10 weeks improved FMD. In this study no changes were seen in blood pressure or lipids, but they did not report serum triglyceride or LDL concentrations. The difference between our results and the results from these two studies may be due to the difference in the intensity of training. Our men trained 4 times/week for 60 min with high intensity (70-80% of maximal aerobic capacity) while in these two other studies the intensity was lower but the training sessions almost daily. In the study by Clarkson et al. (411) ten subjects out of 25 were current smokers, whereas none of our subjects smoked.

One can only speculate why we saw impairment in vascular function after a 12-week training period. One possibility is that there was a too fast progression in training intensity. In our study the subjects, who were previously untrained, started already from the beginning with very intensive training. A gradual increase in training intensity could have been more favourable for the body the adapt to the training. Training intensity in our study exceeded clearly current exercise recommendations for previously untrained subjects (412). Changes in dietary habits and thus changes in antioxidant intake are known to occur during training programs. In our study the subjects were instructed not to change their diet during the training period and a professional dietician interviewed the subjects before and after the training period to ensure that they had followed the instructions and did not use any vitamins or dietary supplements. We also measured circulating antioxidants, which not was done in the studies by Higashi (255) and Clarkson (411). It is, therefore possible that increased intake of antioxidants protected vascular function from exercise induced oxidative stress in these two studies.

There is abundant evidence that physical training and especially endurance training improves cardiovascular health (413,414,415). Physical or cardiorespiratory fitness is strongly inversely associated with all-cause mortality (416). In our study, physical fitness improved by 10%, which
according to follow-up studies reduces all-cause mortality significantly (416). Endurance training seems to give better protection against coronary heart disease than resistance training (413,414). Master endurance athletes with a history of long-term training have a significantly lower risk of heart attacks than untrained subjects (417). Even former elite athletes with a high leisure-activity show a low prevalence of ischemic heart disease, hypertension and diabetes (413). Altogether, it seems reasonable to conclude that long-term physical activity is beneficial for our cardiovascular health, but the major problem at present is that there are no randomised controlled trials where this would have been documented. Another obvious problem is the genetic selection bias. There is evidence that natural selection to sports or physical activity at a young age predicts occurrence of future cardiovascular disease (414). Interestingly, one recent twin cohort study showed that differences in leisure physical activity between monozygotic twins did not predict all-cause mortality when adjusted for both genetic and familial factors (418).

In conclusion, the present study is the first to suggest that training induced oxidative stress may adversely affect vascular function. We found that moderate to high intensity training for 3 months decreased all measured circulating antioxidants except ascorbate and impaired endothelium dependent vasodilatation. The decrease in uric acid, an antioxidant, which is predominantly localized in endothelial cells, was significantly correlated with the degree of endothelial dysfunction. This association does not prove causality but raises the possibility that training induced changes in antioxidant defences may influence endothelial function. These data should not be generalized to reflect effects of less intense training on endothelial function in previously untrained subjects or prolonged training in top athletes.

7.4 Rheumatoid arthritis and endothelial function
We examined in study III the integrity of NO-dependent vasoregulation in patients with RA. We found basal blood flow and its decrease by L-NNMA to be increased compared to a group of healthy control subjects. In contrast, the vasodilatory responses to both SNP and ACh were blunted in the patients with RA. The increase in basal flow was positively and the responses to SNP and ACh inversely correlated with inflammatory markers. Together these data suggest, that iNOS activity was increased and the responsiveness to NO in the vessel wall impaired.

In study IV we tested the hypothesis that RA is characterized by in vivo vascular dysfunction by measuring blood flow responses to intra-arterial infusions of ACh and SNP. The measurements were performed in DMARD naive patients with newly-diagnosed RA and repeated 6 months later
when the patients were on anti-inflammatory and/or DMARD therapy. The novel finding of the study was that patients with early, untreated RA had blunted vasodilatory responses both to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator SNP compared to normal subjects. After 6 months of anti-inflammatory therapy, the vasodilatory responses to ACh had improved significantly. Although increases in the blood flow responses to SNP were not statistically significant, the responses increased and were no longer significantly lower than those in the normal subjects.

Recently one study, which was published 3 weeks after study IV, has confirmed our findings. Anti-TNFα treatment was shown to improve endothelial function in patients with RA (419) indicating that TNFα could be involved in endothelial dysfunction. Another study found that FMD was normal in patients with RA compared to normal subjects but that arterial compliance was markedly reduced when measured with pulse wave analysis (420). In a retrospective study where patients with RA were followed since diagnosis, high inflammatory activity predicted subsequent cardiovascular events (322). In a recent prospective study, patients with RA had elevated concentrations of markers of endothelial activation including von Willebrand factor, D-dimer and PAI-1 antigen. All of these markers correlated with the ESR (70), findings resembling those found in our studies. PAI-1 antigen and D-dimer concentrations also correlated with cumulative disease activity (70). Active use of disease modifying antirheumatic drugs, especially methotrexate, has been demonstrated to decrease especially cardiovascular mortality (303). On the other hand, there is a study showing that methotrexate may promote atherosclerosis in patients with RA who have already signs of atherosclerotic vascular disease (421). Data concerning changes in mortality during the last 30-40 years among patients with RA have been conflicting. In the study be Gabriel et al. (422) there has been no improvement in excess mortality whereas recent results from a large population based cohort in Sweden showed decreasing mortality in patients with RA (423). Furthermore, there seems to be no excess mortality during the first 10 years from disease onset (424).

Regarding the possible mechanisms underlying the blunted vasodilatory responses to ACh and SNP, we found serum concentrations of TNFα to be inversely correlated with responses to both agents. TNFα is thus one potential cause of endothelial dysfunction in patients with RA. However, this would not explain the blunted response to the exogenous NO donor SNP. Another possibility, which appears to characterize at least experimental arthritis is that excessive NO production by iNOS results in formation of excessive amounts of superoxide, and peroxynitrate
from superoxide and nitric oxide (425). The latter reaction could reduce the amount of NO reaching vascular smooth muscle cells during stimulation of endogenous NO production from endothelial cells by ACh and that liberated from SNP. Superoxide production from multiple cellular sources is increased in RA, correlated with serum concentrations of TNFα (344) and thought to perpetuate the chronic inflammatory state (425). Regardless of the sequence of events, an increase in superoxide could limit the ability of endogenous and exogenous NO to induce vasodilatation.

Since ACh doesn’t increase iNOS production (334), a blunted vasodilatory response suggests impaired synthesis of NO by eNOS or accelerated NO destruction. Since the vasodilatory response to SNP was also blunted, the latter possibility appears the correct one. Although accelerated destruction would also apply to iNOS-derived NO, its rate of production was sufficient to result in a net iNOS-dependent overproduction of NO. We found TNFα, CRP and the ESR to correlate with basal flow, in keeping with a previous study demonstrating a significant positive relationship between CRP and the number of iNOS positive mononuclear cells in synovial fluid samples (337).

In study IV, the patients received various antirheumatic and –inflammatory therapies including methotrexate, low doses of corticosteroids and non-steroidal anti-inflammatory agents. When restudied 6 months later on these treatments, restoration of the blood flow response to especially ACh was observed. Due to the small number of patients studied, it is not possible to determine which therapy mostly was responsible but this finding is reminiscent of a recent report of restoration of vascular endothelial function in primary systemic vasculitis by immunosuppressive therapy consisting of cyclophosphamide and methylprednisolone (171). The blood flow response to SNP increased slightly and was after treatment no longer significantly lower than in the normal subjects. The significant increase in the ACh but not SNP response suggests that treatment increased NO bioavailability but not the sensitivity of vascular smooth muscle to NO.

The patients with RA had lower LDL-cholesterol than the normal subjects, which is in line with previous data (346,347). Despite lower LDL-cholesterol concentrations, the patients with RA had blunted blood flow responses to ACh compared to normal subjects before therapy. The concentration of LDL-cholesterol is one of the major determinants of endothelial function (426,427) and its lowering by drugs such statins improves endothelium-dependent vasodilatation (428). In study IV, serum LDL-cholesterol if anything slightly increased by therapy (Table 11)
indicating that other factors are likely to explain the improvement in endothelial function. On the other hand, we did not determine whether LDL was normal with respect to its size and other characteristics such as oxidability. Recently Hurt-Camejo et al. reported that RA patients have higher levels of small dense LDL despite a lower concentration of total LDL cholesterol (347). LDL particles from RA patients also had significantly higher binding affinity to glycosaminoglycans, which suggests that LDL particles may become trapped in the vessel wall matrix and be prone to oxidation. Modified but not native LDL inhibits endothelium-dependent vascular relaxation (429).

Data have been lacking regarding vascular function in chronic inflammatory conditions other than those primarily affecting the vascular wall. The present data add RA to the list of diseases characterized by vascular dysfunction. Our findings support the concept of atherosclerosis as an inflammatory condition by suggesting that a chronic inflammatory condition may cause, possibly via cytokines or increased NO destruction vascular dysfunction. Furthermore it can be concluded that an early suppression of systemic inflammation in RA not only diminishes disease activity but also appears to improve vascular function and may therefore decrease the risk of cardiovascular complications of these patients.
8. SUMMARY AND CONCLUSIONS

1. Moderate weight loss without a reduction in LDL cholesterol does not improve endothelial function in obese women with previous gestational diabetes. A similar weight loss achieved with a hypocaloric diet and orlistat improves forearm endothelium-dependent vasodilatation and this improvement is associated with a lowering of LDL cholesterol by orlistat.

2. Intense physical training for 3 months decreased all studied circulating antioxidants except vitamin C and impaired endothelial function in forearm vessels. This study suggests that oxidative stress induced by intensive training may adversely affect vascular function.

3. Patients with RA have dual abnormalities in NO-dependent vascular function. Basal flow is increased in proportion to inflammatory activity and more inhibited by L-NMMA than in normal subjects. In addition, the ability of SNP and ACh to increase blood flow is blunted consistent with reduced responsiveness to NO.

4. Patients with newly-diagnosed RA have vascular dysfunction, which is reversible with anti-inflammatory therapy.
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